Universidade Estadual de Campinas
Instituto de Biologia

ANÁLISE DAS PROTEÍNAS KI-1/57 E PRMT1: IDENTIFICAÇÃO, MAPEAMENTO E CARACTERIZAÇÃO FUNCIONAL DA INTERAÇÃO COM OUTRAS PROTEÍNAS

Dario Oliveira dos Passos

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Orientador: Dr. Jörg Kobarg

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Banca Examinadora

Prof. Dr. Jörg Kobarg (Orientador)

Assinatura

Prof. Dr. Sandro Roberto Valentini

Assinatura

Profa. Dra Lucymara Fassarela Agnes Lima

Assinatura

Profa. Dra Iscia Teresinha Lopes Cendes

Assinatura

Prof. Dr. Gonçalo Amarante Guimarães Pereira

Assinatura

Prof. Dr. Sérgio Marangoni

Assinatura

Prof. Dr. João Alexandre Ribeiro G. Barbosa

Assinatura

Dr. Júlio César Borges

Assinatura

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"Turnover" a reciclagem da vida.

Das proteínas, passando pelos minerais, aos seres vivos nada escapa desta regra.

Na busca da estabilidade e menor dispêndio de energia a matéria encontra-se em constante mudança.

Mas muitas vezes, a destruição seguida da reconstrução pode se tornar bastante econômica e viável, a ponto de favorecer a completa reciclagem.

Desta forma, turbulências fazem parte desta extensa e imbricada rede de relacionamento entre as diferentes formas obtidas pela constituição desigual da matéria a fim de atingir um equilíbrio efêmero, porém necessário à manutenção de algo mais complexo…

Dario O. Passos 10.03.04

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LISTA DE ABREVIAÇÕES E SIGLAS

3AT	3-aminotriazol
AD	domínio de ativação transcricional da proteína Gal4 (*Activation domain*)
ANS	8-anilino naftaleno-1sulfonato
APS	Persulfato de Amônio (*Ammonium Persulfate*)
BD	domínio de ligação ao DNA da proteína Gal4 (*Binding domain*)
BSA	Albumina Sérica Bovina (*Bovine Serum Albumin*)
BTBD2	proteína contendo 2 domínios BTB(*POZ) (BTB (POZ) domain containing 2*)
CD	Dicroísmo Circular (Circular Dichroism)
CDNA	DNA complementar
CGI-55	proteína identificada por comparação genômica 55 (*Comparative Genome Identified 55*)
CHD3	proteína com domínios cromo-helicase e de ligação ao DNA 3 (*Chromo Helicase*
DNA	*binding domain protein 3*)
CIRBP	proteína que se liga ao RNA induzida por frio (*Cold Inducible RNA Binding Protein*)
DAXX	proteína associada à morte (*Death-associated Protein*)
DOTAP	N-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethylammonium methyl-sulfate
DTT	1,4 ditiotreitol
EDTA	ácido etilenodiaminotetraacético
EWSR1	Sarcoma de Ewing 1 (Ewing`s Sarcoma 1)
FPLC	Cromatografia Líquida de Alta Pressão (*Fast Performance Liquid Chromatography*)
FXR1P	proteína relacionada com retardamento mental X-frágil 1 (*Fragile X mental retardation-related protein 1*)
GSH	glutationa reduzida
GST	glutationa-S-transferase
HMGBCG	proteína similar ao grupo de alta mobilidade (*High-Mobility Group*)
hnRNP A3	Ribonucleoproteína Heterogênea nuclear A3 (Heterogeneous Nuclear Ribonucleoprotein A3)
HPC2	Proteína hereditária de câncer de próstata 2 (Hereditary prostate câncer protein 2)
IHABP4	proteína que se liga a hialuronato 4 (*hyaluronan-binding protein 4*);
ILF3	Fator 3 de ligação e amplificação da interleucina (Interleukin enhancer binding factor 3)
IPTG	Isopropil beta-D-galactosídeo;
kDa	quilo Dalton (k*ilo Dalton*)
Kg	Quilograma (*Kilo gram*)
Ki-1/57	antígeno Ki-1 de 57 kDa (*Ki-1 antigen of 57 KDa*);
LB	(meio) Luria Bertani
MCS	sítio de clonagem múltipla *(Multiple cloning site*)
mg	miligrama (*milli gram*)

mRNA RNA mensageiro

ng nanograma (*nono gram*)

Ni-NTA ácido níquel-nitrilotriacético

NSAP1 Proteína associada a NS1 (NS1-associated protein)

NSEP1 proteína de ligação ao elemento sensível a nuclease 1 (*nuclease sensitive element binding protein 1*)

ONPG o-nitrofenil-β-D-galactopiranosídeo (*o-nitrophenol-β-D-galactopyranoside*)

p100 Co-ativador do fator de transcrição do vírus Epstein-Barr 2 ou EBNA-2;

PAI inibidor do ativador do plasminogênio (*plasminogen activator inhibitor*);

PAI_RBP1 proteína que se liga ao mRNA de PAI-1 (*PAI-1 mRNA- binding protein*);

pb pares de bases (*base pairs*)

PCR reação da polimerase em cadeia (*Polimerase Chain Reaction*)

PDB banco de dados de estruturas protéicas (*Protein Data Bank*)

PEG polietilenoglicol

PIAS Proteína inibidora de STAT ativada (*Protein inhibitor of activated STAT*)

PMA forbol 12-miristato 13-acetato (*phorbol 12-myristate 13-acetate*)

PMSF fluoreto de fenil metil sulfonila

ppm partes por milhão

PRMT1 Proteína Arginina Metiltransferase 1 (Protein Arginine Methyltransferase 1)

RACK1 Receptor para quinase C ativada 1 (Receptor for activated C kinase 1)

rpm rotações por minuto

SDS dodecil sulfato de sódio

SDS-PAGE Eletroforese de Gel Poliacrilamida SDS (*SDS polyacrylamide gel electrophoresis*)

SF2/p32 fator de splicing 2/p32

SFRS9 fator de splicing rico em arginina/serina (*splicing factor argine/serine rich 9*)

SMN Neurônio motor de sobrevivência (*Survival Motor Neurons*)

STAT sinalizador e ativador da transcrição

TOPORS proteína que se liga a topoisomerase I (*topoisomerase I binding protein*);

Tris tris-hidroximetil aminometano

Tween 20 polyoxyethylene sorbitanmonolaurate

UV ultravioleta

WB Western blot

X-gal 5-bromo-4cloro-3-indol beta-D-galactopiranosídeo (*5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside*);

µg micrograma (*microgram*)

RRM Motivo de Reconhecimento a RNA (*RNA Recognition Motif*)

RGG-box Motivo rico em argininas e glicinas

snRNP Ribonucleoproteínas pequenas nucleares (*Small nuclear ribonucleoproteins*)

AdoMet (**SAM**) S-adenosilmetionina (*S-Adenosylmethionine*)

AdoHcy	S-Adenosilhomocisteína (*S-Adenosylhomocysteine*)
SDHL	Sistema de Duplo Híbrido em Levedura (*Yeast two Hybrid System*)
BCFH	Biblioteca de Cérebro Fetal Humano (*Human fetal Brain Library*)
GFP	Proteína de Fluorescência Verde (*Green Fluorescence Protein*)
BRAF	Proteína B-raf (*B-raf protein*)
ERK	Proteína quinase regulada por sinal extracelular (*Extracellular Signal-Regulated Protein Kinase*)
NER	Reparo por excisão de nucleotídeos (*Nucleotides excision repair*)
MMR	Reparo por despareamento (*Mismatch repair*)
ATM	(*Ataxia-Telangiectasia-Mutated*)
BRC1	(*Broad-complex core-protein*)
BLM	**S**índrome de Bloom **(***Bloom syndrome*)
BER	Reparo por excisão de base (*Base excision repair*)
CD	Grupo de diferenciação (*Cluster Differentiation*)
Rb	Ritonoblastoma (*Retinoblastoma*)
ACS	Sociedade Americana de Câncer (*American Câncer Society*)
EBV	Vírus Epstein-Barr (*Epstein-Barr Virus*)
CMV	Citomegalovírus (*Cytomegalovirus*)
H-RS	Hodgkin e Reed-Sternberg (*Hodgkin and Reed-Sternberg*)
NFkB	Fator nuclear kB (*Nuclear factor kB*)
HABP4	Proteína ligante a ácido hialurônico 4 (*Hyaluronan acid binding protein 4*)
ATP	Trifosfato de adenosina (*Adenosine tryphosphate*)
EMSA	Ensaio de Retardamento da Mobilidade Eletroforética (*Eletrophoretic Mobility Shift Assay*)
RBD	*Domínio de ligação a RNA (RNA binding domain*)
KH	Domínio Homólogo K (K homology domain)
MMA	Monometilarginina (*Monomethylarginine*)
aDMA	Dimetilarginina assimétrica (*Asymmetric Dimethylarginine*)
sDMA	Dimetilarginina simétrica (*Symmetric Dimethylarginine*)
AR	Autoradiograma (*Autoradiogram*)
SMA	Atrofia Muscular Espinhal (*Muscular Atrophy Spinal*)

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RESUMO

A proteína Ki-1/57 que é encontrada tanto no núcleo quanto no citoplasma está associada com atividade de proteína quinase serina/treonina e é fosforilada nestes resíduos após ativação celular. Neste trabalho verificamos que Ki-1/57 interage com a proteína Chromatin-Helicase-DNA-binding domain 3 (CHD3) e com a proteína adaptadora/sinalizadora RACK1 no núcleo. Pelo sistema do duplo híbrido de levedura (SDHL) a proteína arginina metiltransferase 1 (PRMT1) foi selecionada como outra proteína de interação. A PRMT1 integra uma família representada por nove enzimas humanas que catalisam reações de metilação em resíduos de arginina. Em seguida, usando agora a PRMT1 como isca - no SDHL - identificamos as proteínas Ki-1/57 e hnRNPQ, juntamente com outras 13. A maioria delas contêm motivos "RGG-box" em suas seqüências de aminoácidos, que são conhecidos alvos para metilação. Posteriormente verificamos que Ki-1/57 e seu provável parálogo CGI-55 conservam dois motivos "RGG/RXR-box" e que são substratos *in vitro* para a metilação de argininas pela PRMT1. Estudos de mapeamento mostraram que todos os fragmentos contendo o motivo "RGG/RXR-box" interagem com a PRMT1 e são alvos à metilação *in vitro*. Ki-1/57 endógena, imunoprecipitada de células L540, mostrou ser metilada *in vivo*, além de ser um alvo a metilação pela PRMT1 *in vitro*, somente quando as células são previamente tratadas com o inibidor da metilação Adox. Tratamento das células Hela com o inibidor da metilação (Adox) causa desaparecimento da imuno-marcação citoplasmática de Ki-1/57 e relativa redistribuição do parálogo CGI-55 para o citosol. Assim, pode ser especulado que a metilação destas proteínas deve ser um evento importante para suas localizações sub-celulares e conseqüentemente para suas funções. Em resumo, nossos dados sugerem que o SDHL é um método efetivo na identificação de novos substratos celulares para a PRMT1 e poderia ser estendido para a identificação e caracterização de novos substratos para os outros integrantes da família das PRMTs humanas.

SUMMARY

The protein Ki-1/57 that is found both in the cytoplasm and nucleus is associated with serine/threonine protein kinase activity and gets phosphorylated on serine and threonine residues upon cellular activation. We demonstrated that Ki-1/57 interacts with the Chromatin-Helicase-DNA-binding domain protein 3 (CHD3) and with the adaptor/signaling protein RACK1 in the nucleus. By utilizing the yeast two-hybrid system (YTHS), we were further able to find the protein arginine-methylatranseferase-1 (PRMT1) as another interacting protein. PRMT1 is a member of the family constituted by 9 human enzymes that catalyze methylation reactions on arginine residues. Afterwards, by using PRMT1 as bait in the YTHS we identified both Ki-1/57 and NSAP1 as interacting proteins, along with 13 other proteins. The majority of them present RGG-box clusters in their amino acid sequences, which are known to be targets for arginine methylation. We further found that Ki-1/57 and its putative paralogue CGI-55 have two RGG/RXR-box clusters conserved between them and that they are substrates for arginine-methylation by PRMT1 *in vitro*. In mapping studies, we observed that all Ki-1/57 protein fragments containing the RGG/RXR-box clusters interact with PRMT1 and are targets for methylation *in vitro*. Endogenous cellular Ki-1/57 seems to be methylated in vivo and is a target for methylation by PRMT1 in vitro, only when cells have been previously treated with the methylation inhibitor Adox. Treatment of Hela cells with the inhibitor of methylation (Adox) causes the disappearance of the immuno-staining of Ki-1/57 in the cytoplasm and a relative redistribution of the paralogue CGI-55 to the cytosol. It can therefore be speculated that the methylation of these proteins is important for their sub-cellular localization and in consequence for their function. In summary our data suggest that the YTHS is an effective method for the identification of novel cellular PRMT substrates and could be extended for the identification and characterization of novel substrates to the other components of the human PRMT1 family.

I. INTRODUÇÃO

1. Câncer

1.1 Histórico

Câncer (Do lat. *cancer*, 'caranguejo') é uma classe de doenças ou desordens caracterizada por um crescimento celular desordenado, associado à habilidade destas células de invadirem outros tecidos, tanto os adjacentes por uma forma direta quanto os distantes por implantação. Esta capacidade de migração para tecidos distantes é facilitada pelo transporte destas células através do sistema sangüíneo ou linfático e é conhecida por metástase (Do gr. *metastasis*, 'mudança de lugar').

As mais antigas descrições de cânceres em humanos foram encontradas em dois dos sete papiros egípcios escritos entre 3000-1500 antes de cristo (AC). Estes papiros, conhecidos como "Edwin Smith" e "George Ebers", contêm detalhes de condições que são consistentes com modernas descrições do câncer. O papiro de Edwin Smith descreve oito casos de tumores de mama. O documento reconhece que não existe cura para esta condição e recomenda cauterização como medida paliativa. A medicina egípcia antiga tipicamente misturava medicina e religião. Estes médicos tratavam pacientes por muitas formas de cânceres. Inscrições hieróglifas e manuscritos papíricos sugerem que estes médicos antigos eram capazes de distinguir entre tumores benignos e malignos. Já o mais antigo espécime de câncer humano foi encontrado em remanescentes de um crânio feminino que viveu na Época do Bronze (1900-1600 AC). Além disso, esqueletos mumificados de Incas peruanos, datados de 2400 anos atrás, contêm anormalidades sugestivas do envolvimento com melanoma maligno.

Na Grécia, Hippocrates (460-370 AC), considerado o "pai da medicina" e indicado como a primeira pessoa a diferenciar tumores malignos dos benignos, descreveu cânceres de muitas regiões do corpo. E devido ao espessamento dos vasos sangüíneos ao redor dos tumores malignos, que o fizeram lembrar das garras do caranguejo, é que o levou a chamar a doença de *karkinos* (nome grego para caranguejo). Em português este termo é traduzido para carcinoma, que são tumores derivados de células epiteliais.

O progresso na compreensão e tratamento do câncer foi lento. Ele não se deu até o século XVIII, período este que em Reims, França, houve a fundação do primeiro hospital

de câncer, embora devido ao equívoco de acreditar que o câncer era uma doença contagiosa. Já no século XIX, o ginecologista Joseph Récamier (1774-1852) descreveu a invasão da corrente sangüínea por células cancerígenas em 1839, cunhando a palavra metástase.

Em 1895, Wilhelm Röntgen (1845-1923) descobriu o raio-x, que mesmo após um século ainda é largamente utilizado tanto para o diagnóstico quanto para o tratamento (radioterapia) de muitos dos diferentes tipos de cânceres conhecidos atualmente.

Um dos avanços mais importantes e expressivos na compreensão da biologia celular veio em 1953, quando Francis Crick (1916-2004) e James Watson (1928-) decifraram a estrutura do DNA. Desde então se deu início o estudo e entendimento das causas do câncer em níveis moleculares, além de investimentos no desenvolvimento de tratamentos baseados neste novo conhecimento.

Assim, nos últimos 50 anos valiosas informações têm sido acrescidas à comunidade científica e novas descobertas estão ocorrendo diariamente.

1.2 Estatísticas

O primeiro caso de câncer – associado a um agente ocupacional - foi identificado em 1775 pelo médico inglês Percival Pott (1714-1788), o qual verificou ser uma doença comumente encontrada no escroto de limpadores de chaminés. Dois séculos e meio depois - segundo a Organização Mundial de Saúde (WHO) - em muitos países, mais de um quarto das mortes foram atribuídas a cânceres no ano de 2000. Neste mesmo ano, 5.3 milhões de homens e 4.7 milhões de mulheres desenvolveram tumores malignos e juntos 6.2 milhões morreram. Para 2006 - somente nos EUA - 1.399.790 novos casos e 564.830 mortes de câncer são esperados, significando mais do que 1.500 mortes por dia (Jemal et al 2006, Câncer Statistics 2006). Em 2003, nos EUA, a segunda maior causa de morte ocorreu devido ao câncer, perdendo apenas para doenças do coração (Tabela 1). Na América Latina, estudos recentes mostram que em 2000 as maiores taxas de mortalidade por câncer em homens foram observadas na Argentina e Chile, com taxas comparáveis com as dos EUA. Para mulheres, Chile e Cuba apresentaram as maiores taxas da América latina, também com alíquotas comparáveis aos EUA (Bosetti et al., 2005).

Tabela 1 Ranking das 15 causas que levam a morte, Estados Unidos, 2003

Rank	Causas de Morte	Números de Mortes	Porcentagem total de Mortes	Taxa de Morte †
	All Causes	**2,448,288**	**100.0**	**831.0**
1	Heart diseases	685,089	28.0	231.6
2	Cancer	556,902	22.7	190.1
3	Cerebrovascular diseases	157,689	6.4	53.3
4	Chronic lower respiratory diseases	126,382	5.2	43.3
5	Accidents (unintentional injuries)	109,277	4.5	37.2
6	Diabetes mellitus	74,219	3.0	25.3
7	Influenza & pneumonia	65,163	2.7	21.9
8	Alzheimer disease	63,457	2.6	21.3
9	Nephritis, nephrotic syndrome, & nephrosis	42,453	1.7	14.4
10	Septicemia	34,069	1.4	11.6
11	Intentional self-harm (suicide)	31,484	1.3	10.7
12	Chronic liver disease & cirrhosis	27,503	1.1	9.3
13	Hypertension and hypertensive renal disease	21,940	0.9	7.4
14	Parkinson disease	17,997	0.7	6.1
15	Assault (homicide)	17,732	0.7	6.0
	All other & ill-defined causes	416,932	17.0	

†Taxas são por 100.000 habitantes e idade ajustada para população dos EUA 2000.

Nota: Porcentagens podem não totalizar 100 devido aproximações. Sintomas, sinais e anormalidades, eventos de situações indeterminadas, e pneumonias devido a ´solidos e líquidos foram excluídos da ordem das causas de câncer.

Fonte: Dados publicos de mortalidade dos EUA, 2003. Centro Nacional para Estatistica da Saúde, Centros para Controle de Doenças e Prevenção, 2006 (**Jemal *et al.* 2006, Câncer Statistics 2006**)

No Brasil, segundo o Instituto Nacional do Câncer (INCA), são estimados 472 mil novos casos de câncer para 2006, significando quase 1.300 novos casos por dia. Em homens, o câncer de próstata é significativamente o mais comum, seguido dos de traquéia, brônquio e pulmão, representando 20.2% e 7.6%, respectivamente. Já no grupo das mulheres, o câncer de mama é largamente o mais comum, seguido do tumor de colo de útero, representando 20.6% e 8.11%, respectivamente. Vale ressaltar que embora os dois principais tipos de cânceres que acometem homens e mulheres no Brasil sejam distintos, o número total de casos de neoplasias esperado para 2006 entre estes dois grupos é muito próximo (Tabela 2).

Tabela 2 Local das Principais Neoplasias Malígnas, Brasil, 2006

Tipos de cânceres	Homens	Mulheres
Traquéia, Brônquio e Pulmão	17.850	9.320
Estômago	14.970	8.230
Cólon e Reto	11.390	13.970
Esôfago	7.970	2.610
Leucemias	5.330	4.220
Cavidade Oral	10.060	3.410
Pele melanoma	2.710	3.050
Outras localizações	61.530	63.320
Pele não melanoma	55.480	61.160
Próstata	47.280	-
Colo do Útero	-	19.260
Mama Feminina	-	48.930
Total	234.570	237.480

Tipos de câncer mais incidentes, estimados para 2006. Fonte: Instituto Nacional do cancer (INCA)

1.3 Genética do câncer

Mutações em pelo menos 291 genes humanos, codificantes de proteínas, estão casualmente implicados na oncogenese. Assumindo que existam aproximadamente 25.000 genes codificantes no genoma humano, parece que mutações em mais de 1% dos genes foram até agora conhecidos por estarem envolvidos em patologias cancerígenas (Futreal *et al*. 2004).

Um estudo detalhado realizado em 2004 por Futreal e colaboradores mostrou que em cânceres humanos as mutações somáticas representam 90% contra 20% das germinativas (Figura 1) (Futreal *et al*., 2006). Além disso, a mutação do tipo translocação mostrou ser responsável por cerca de 75% do total de mutações somáticas encontradas em cânceres humanos (Figura 2) (Futreal *et al*., 2006).

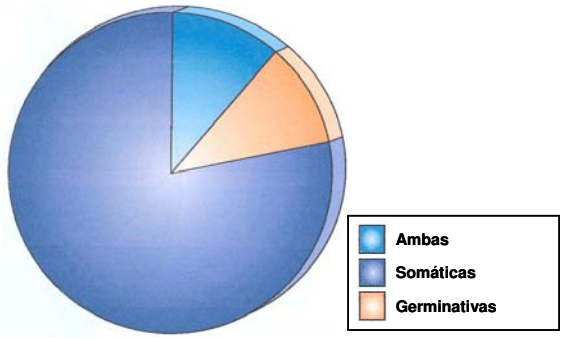


Figura 1 Tipos de mutações em cânceres humanos. Genes cancerígenos mostram mutações somáticas, mutações germinativas ou ambas mutações somática e germinativa em cânceres humanos.

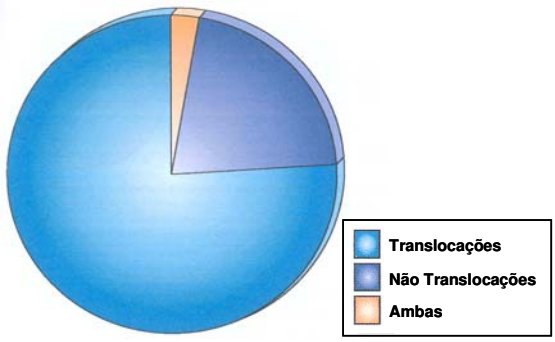


Figura 2 Proporção de genes translocados em cânceres humanos. Genes cancerígenos mutados somaticamente, com mutações outras além das translocações ou com ambas.

Três tipos de genes são responsáveis pela tumorogênese quando alterados: oncogenes, genes supressores de tumor e genes de estabilidade. Diferentemente de doenças como a fibrose cística (Guggino *et al*., 2006), ritonoblastoma (Harlow e Classon, 2002) ou atrofia muscular espinhal (SMA) (Emery HM, 1998), onde mutações em um único gene podem causar a doença, não é comum um único gene defeituoso sozinho ser responsabilizado por causar câncer. Isto porque células de mamíferos apresentam múltiplos "salva-vidas" para protegê-las contra os potenciais efeitos de mutações gênicas cancerígenas, e somente quando muitos genes estão defectivos um câncer invasivo se desenvolve. Assim, é mais correto pensar nos genes de pré-disposição mutados como contribuintes do que causadores de cânceres.

Oncogenes (Do gr. *ónkos*, 'tumor') são produtos da mutação de genes celulares, normalmente responsáveis pela codificação de proteínas regulatórias, que quando mutados – tornando-se constitutivamente ativos ou ativos sob condições em que o gene selvagem não era - levando a um crescimento rápido e descontrolado da célula. A passagem de um proto-oncogene a oncogene pode ser resultado de translocações cromossomais, amplificações gênicas ou mutações intragênicas afetando resíduos cruciais que regulam a atividade do produto gênico. Como exemplo, temos a forma mais comum de mutação por ativação do gene *BRAF* em cânceres humanos onde a valina é alterada para glutamato no códon 599, um resíduo dentro da alça de ativação do domínio quinase (Davies *et al*,. 2002). A alça de ativação é normalmente regulada pela fosforilação dos resíduos adjacentes (Thr598 e Ser601). Isto sugere que a substituição do glutamato no códon 599 mimetiza um grupo fosfato e ativa constitutivamente a enzima mesmo na ausência de sinais que poderiam normalmente resultar em fosforilação dos resíduos serina (Ser601) e treonina (Thr598) adjacentes. A quinase BRAF ativada fosforila alvos tais como a quinase regulada por sinal extracelular (ERK), levando a um crescimento celular aberrante (Fig. 3).

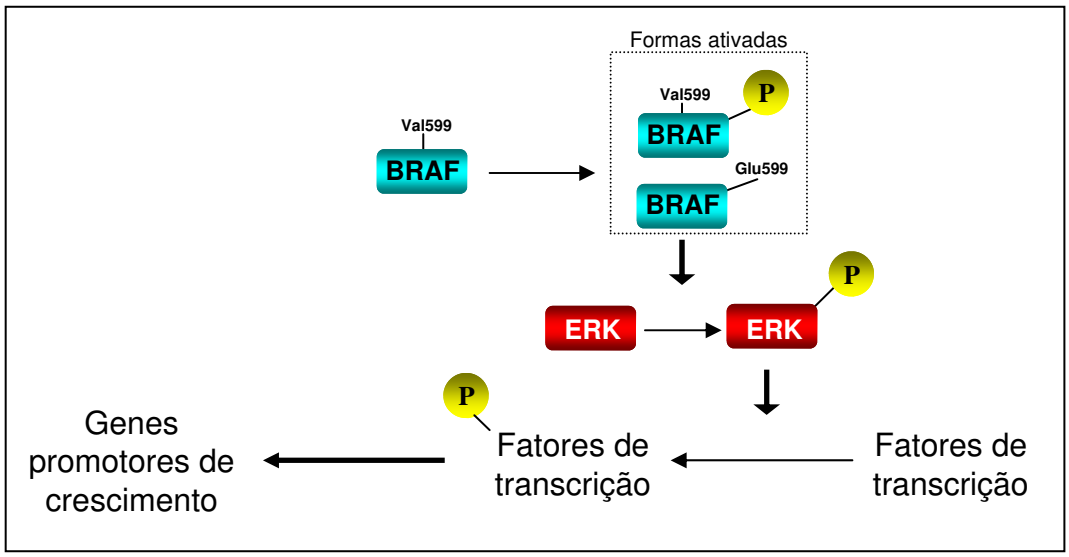


Figura 3 Esquema mostrando a atividade da proteína BRAF.

Genes supressores de tumor são alvos de alterações genéticas, que de maneira contrária aos oncogenes, acarretam em redução de atividade do produto gênico. Estes genes estão normalmente envolvidos na regulação negativa da divisão celular e positiva da apoptose. O principal e mais bem estudado gene supressor de tumor codifica a proteína P53. Atualmente, acredita-se que este gene possa encontrar-se mutado em mais de 50% de

todos os casos de cânceres humanos (Vousden *et al*., 2002). A inativação destes genes surge a partir de mutações em resíduos que são essenciais para sua atividade, por mutações que resultam em proteínas truncadas, por deleções ou inserções de vários tamanhos ou por silenciamento epigenético. Algumas recentes descrições de genes supressores de tumor têm sido especuladas para exercer vantagens seletivas a célula quando somente um alelo é inativado e o outro permanece funcional (Santarosa *et al*., 2004). No entanto, mutações em ambos alelos maternal e paternal dos genes supressores de tumor são geralmente necessários para conferir uma vantagem seletiva para a célula. Esta situação normalmente ocorre através da deleção de um alelo via eventos cromossomais grosseiros, como perda de um cromossomo inteiro ou braço do cromossomo, associado a mutações intragênicas do outro alelo (Knudson AG., 2002)).

Oncogenes e genes supressores de tumor mutados operam de maneira similar no nível fisiológico: ambos conduzem o processo neoplásico, aumentando o número de células tumorais através da estimulação do crescimento celular, ou inibição da morte celular e bloqueio do ciclo celular. O aumento pode ser causado por ativação de genes que controlam o ciclo celular, por inibição de processos apopitóticos normais ou por facilitação do provimento de nutrientes através da angiogênese.

A terceira classe de genes de pré-disposição, chamados genes de estabilidade, promovem tumorogênese de uma forma completamente diferente quando mutados. Esta classe inclui os genes responsáveis por reparar erros repentinos ocorridos durante a replicação normal do DNA, tais como reparo por excisão de nucleotídeos (*NER*), reparo por excisão de base (*BER*) e reparo por despareamento (*MMR*), bem como lesões ocasionadas por diferentes fatores, como radiações e produtos químicos considerados cancerígenos. Outros genes de estabilidade controlam processos envolvendo extensas porções de cromossomos, tais como aqueles responsáveis pela recombinação mitótica e segregação cromossomal (*BRCA1* (Tutt *et al*., 2002), *BLM* (Cheok *et al.*, 2005) e *ATM* (Löbrich *et al.*, 2005). Genes de estabilidade mantêm as alterações genéticas minimizadas, e por isso, quando eles estão inativados, mutações em outros genes ocorrem em altas taxas (Friedberg EC, 2003). Todos os genes são potencialmente afetados pelo resultante aumento na taxa de mutações, mas somente mutações em oncogenes e genes supressores de tumor afetam o

crescimento celular e podem com isso oferecer uma vantagem seletiva ao crescimento para as células mutantes.

Mutações nestas três classes de genes podem ocorrer em células germinativas, resultando em predisposição hereditária ao câncer, ou em uma única célula somática, resultando em tumores esporádicos. Vale a pena ressaltar que uma mutação é definida por qualquer alteração na seqüência do genoma. Estas alterações incluem aquelas que afetam um único par de bases, bem como aquelas que criam deleções curtas, extensas ou inserções, amplificações ou translocações. Em células germinativas, as mutações mais comuns são mutações pontuais ou pequenas deleções ou inserções, enquanto todos os tipos de mutações podem ser encontrados em células tumorais. De fato, cânceres representam um dos poucos tipos de doenças em que mutações somáticas ocorridas após o nascimento são patogênicas.

Como resultado das pesquisas desenvolvidas nas últimas décadas, atualmente está claro que existem muito mais genes do que caminhos de sinalização. Este conceito é muito familiar aos geneticistas que estudam leveduras, moscas, camundongos ou vermes – existem quase sempre umas variedades de genes que, quando alterados, levam a fenótipos similares (Dyke e Jacks, 2002; Horvitz HR, 2003). A aplicação deste conceito ao câncer foi solidificada pela elucidação das funções bioquímicas de genes de pré-disposição alterados, tanto em cultura de células quanto em camundongos e outros organismos. Os genes codificantes Rb (fator de transcrição) e p16 (interage e inibe cdk4) são genes supressores de tumor inativados por mutação, enquanto aqueles que codificam CDK4 (quinase que fosforila Rb) e Ciclina D1 (interage e ativa CDK4) são oncogenes ativados por mutação. Em estudos funcionais realizados em sistemas modelo, tipos de tumores individuais, detalhadamente estudados forneceram fortes evidencias de que estes quatro genes funcionam em um único caminho de sinalização em cânceres humanos. Este estudo mostrou que mutações ocorridas dentro deste caminho de sinalização obedecem a um "princípio de exclusividade": um e somente um dos quatro genes acima descritos apresenta-se mutado em um único tumor, exatamente como predito se o efeito funcional de cada mutação foi similar (Sherr CJ, 2000; Ortega *et al*., 2002; Classon e Halow, 2002; Ichimura *et al*., 2000).

Uma das mais importantes descobertas da última década foi que teoricamente todos os DNAs tumorais de vírus que causam tumor em animais de laboratório ou humanos codificam proteínas que inativam ambas proteínas P53 e Rb (Klein G, 2002; Munger e Howley, 2002; Hausen zurH, 2001). E com centenas de genes de pré-disposição, é interessante por que estes dois foram selecionados como alvos para inativação por todos os DNA tumorais virais. Talvez porque seja quase impossível para um tumor de origem epitelial se desenvolver sem que as vias de sinalização integradas por estes dois genes supressores de tumor estejam inativas. Esta conjectura é suportada por estudos mostrando que estes dois caminhos de sinalização são alterados em uma grande fração de muitos tipos de cânceres.

Em adição aos genes que são mutados em uma significante porção de cânceres, também há muitos outros genes que têm sido implicados em neoplasias, embora não tenham apresentado mutações. Estes genes têm mostrado serem expressos em níveis mais altos ou mais baixos do que em células normais (Brown e Botstein, 1999; Polyak e Riggins, 2001) e são freqüentemente associados a alterações epigenéticas – que são modificações covalentes do DNA ou cromatina, preservadas quando as células cancerígenas se dividem (Jones e Baylin, 2002; Feinberg e Tycko, 2004) (67-68). Diferentemente das alterações genéticas, mudanças epigenéticas idênticas àquelas encontradas em cânceres são freqüentemente encontradas em células normais em certos estágios de desenvolvimento.

Embora até o momento significativos avanços no estudo e compreensão do câncer tenha ocorrido, três pontos parecem essenciais para tornar este conhecimento mais aplicável. O primeiro seria a descoberta de novos genes que teriam um papel causal na neoplasia, particularmente aqueles que iniciam e concluem o processo. O segundo seria a delineação dos caminhos de sinalização através dos quais estes genes agem e a base para as variações de atividade em tipos específicos de células. O terceiro seria o desenvolvimento de novos meios de explorar este conhecimento para benefício dos pacientes.

Portanto, o estudo das proteínas e seus papéis funcionais são de grande relevância para um melhor entendimento a cerca dos eventos celulares, e pode, como mostrado acima, contribuir para o desenvolvimento de diferentes formas de tratamento a inúmeras patologias.

1.4 Classificações

Os tumores podem ser artificial e amplamente classificados como sólidos ou não-sólidos. Os primeiros são compostos por células epiteliais ou mesenquimais que normalmente são imóveis. Tumores não-sólidos incluem leucemias e linfomas, compostos por células neoplásicas, cujos precursores são normalmente móveis. Além disso, apenas uma ou duas mutações podem ser necessárias para o desenvolvimento de um tumor não-sólido maligno (Nowell ap, 2002), enquanto que pelo menos três mutações parecem ser necessárias para o sólido se desenvolver em adultos (Komarova *et al*., 2003). Contudo, por que mais do que 50% dos genes de pré-disposição com mutações somáticas estão associados aos tumores não-sólidos, já que estes tipos de tumores correspondem a menos do que 10% da incidência total de cânceres humanos? (Futreal *et al*., 2006). Segundo Futreal e colaboradores a explicação está no fato de os tumores não-sólidos apresentarem um elevado número de genes que sofrem translocações cromossomais, além de que no passado, parecia ser mais fácil a identificação de genes de pré-disposição pelo estudo de linfomas e leucemias do que pelo estudo de cânceres epiteliais. E se este raciocínio estiver correto, é possível que haja muitos outros genes de pré-disposição para serem identificados em associação com cânceres epiteliais comuns.

1.4.1 Linfomas

Os linfomas são cânceres que acometem o **sistema linfático** e são divididos em dois grandes grupos: os Hodgkin e os não Hodgkin. Neste trabalho daremos mais importância ao linfoma do tipo Hodgkin, já que uma das nossas proteínas foi identificada como antígeno neste tipo de linfoma. Para melhor compreendermos as características específicas das células malignas primeiro precisamos estudar mais aprofundado as células normais que as antecedem.

1.4.1.1 Sistema linfático

O sistema linfático é tradicionalmente dividido em tecidos linfóides primários e secundários (Fig. 4). Tecidos linfóides primários são os tecidos no qual linfócitos são gerados e diferenciados em linfócitos maduros: sendo medula óssea para linfócito B e o

timo para linfócito T. Já os tecidos linfóides secundários são os tecidos nos quais são iniciados as respostas imunes, e os vasos linfáticos que os conectam com os tecidos e corrente sangüínea.

O tecido linfóide secundário consiste do *baço*, o qual coleta antígenos da corrente sangüínea; dos *linfonodos* (gânglios linfáticos), os quais são agrupados em regiões como virilha, axila e pescoço, e coletam antígenos dos tecidos; dos *tecidos linfóides associados à mucosa*, os quais coletam antígenos dos tratos respiratórios, gastrintestinal e geniturinário, e são particularmente bem organizados no intestino, em estruturas conhecidas como *placas de Peyer*. Tudo isto fica conectado com os tecidos e a corrente sangüínea pelo sistema de vasos linfáticos. Os vasos linfáticos aferentes drenam o fluido extracelular (linfa) dos tecidos, incluindo mucosas, para dentro dos linfonodos; e os vasos eferentes carregam a linfa dos tecidos linfóides secundários para o ducto torácico e a partir daí para o interior da corrente sangüínea.

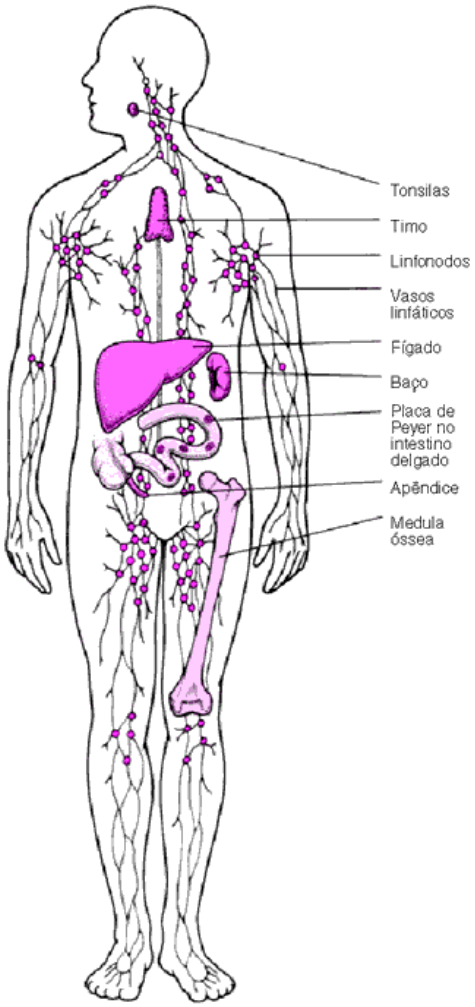


Figura 4 O sistema linfático.
Fonte:http://www.msd-brazil.com/msdbrazil/patients/manual_Merck/mm_sec16_167.html

Quando os linfócitos maduros saem dos tecidos linfóides primários eles entram na corrente sangüínea e então circulam continuamente através dos tecidos linfóides secundários, graças a sua migração através da parede dos vasos sangüíneos especializados, saindo via vasos linfáticos eferentes que por fim retornam a corrente sangüínea.

Existem três tipos celulares especializados através dos quais os tecidos linfóides secundários coletam antígenos. Nos linfonodos, esta função é exercida pela maior apresentadora de antígenos: as células dendríticas pegam antígenos nos tecidos e migram através de vasos linfáticos aferentes para o interior dos linfonodos onde eles ativam células T antígeno-específicas. Células dendríticas também prendem antígenos diretamente liberados do baço pela corrente sangüínea. Em tecidos mucosos, o antígeno é capturado a partir do lúmen por células especializadas conhecidas como *células M*, presentes nas placas de Peyer, que transportam partículas antigênicas que podem ser tão grandes como vírus e bactérias através do epitélio da mucosa e os entregam a camada adjacente de células dendríticas. O terceiro tipo especializado de coleta de antígenos é a *célula dendrítica folicular*. Estas células são bastante distintas tanto em linhagem quanto em função das células dendríticas que apresentam antígenos às células T, embora como elas, estendam longos processos e este é o motivo pelo quais ambas são chamadas células dendríticas. Células dendríticas foliculares atuam em uma parte central da maturação dos anticorpos de resposta dos linfócitos B.

1.4.2 Linfoma de Hodgkin

Embora o linfoma do tipo não Hodgkin seja quase oito vezes mais freqüente do que o do tipo Hodgkin, a Sociedade Americana de Câncer (ACS) estima que em 2006 haverá aproximadamente 7.800 novos casos da doença apenas nos Estados Unidos. Apesar de representar menos de 1% de todos os casos de cânceres - com uma incidência em torno de 1/25000 - é o terceiro tipo de câncer mais comum em crianças e adolescentes com idades entre 10-14 anos de idade.

O linfoma de Hodgkin foi descrito em 1832 pelo médico inglês Thomas Hodgkin (1798-1866), no artigo intitulado "*On Some Morbid Apperances of the Absorbent Glands and Spleen*". Thomas Hodgkin foi considerado um dos mais renomados patologistas da

época e pioneiro na medicina preventiva, tendo publicado "*On the Means of Promoting and Preserving health*" em forma de livro em 1841. Além disso, ele foi responsável pela primeira descrição da apendicite aguda, do formato bicôncavo das hemácias e das estrias nas fibras musculares.

A principal característica do linfoma do tipo Hodgkin está na presença de células B alteradas, conhecidas como células *Reed-Sternberg*. Este tipo de célula é encontrado pelo microscópio de luz em biópsias de indivíduos com linfoma de Hodgkin. Estes linfócitos B alterados receberam este nome após Dorothy Reed (1874-1964) e Carl Sternberg (1872-1935) terem feito as primeiras descrições definitivas da doença de Hodgkin por microscópio. Células *Reed-Sternberg* são grandes (20-50 micrometros), abundantes e podem ser multinucleadas ou terem um núcleo bilobado com proeminente eosinofilia do nucléolo, além de uma membrana nuclear espessa (Fig.5). Estas células são CD30 e CD15 positivas e normalmente CD20 e CD45 negativas. A nomenclatura para Grupo de Diferenciação (CD) foi desenvolvida empiricamente por imunologistas e é usada para definir um grupo de moléculas da superfície celular. Moléculas CD não são apenas marcadores da superfície celular e muitas delas trazem importantes características para as células que as carregam.

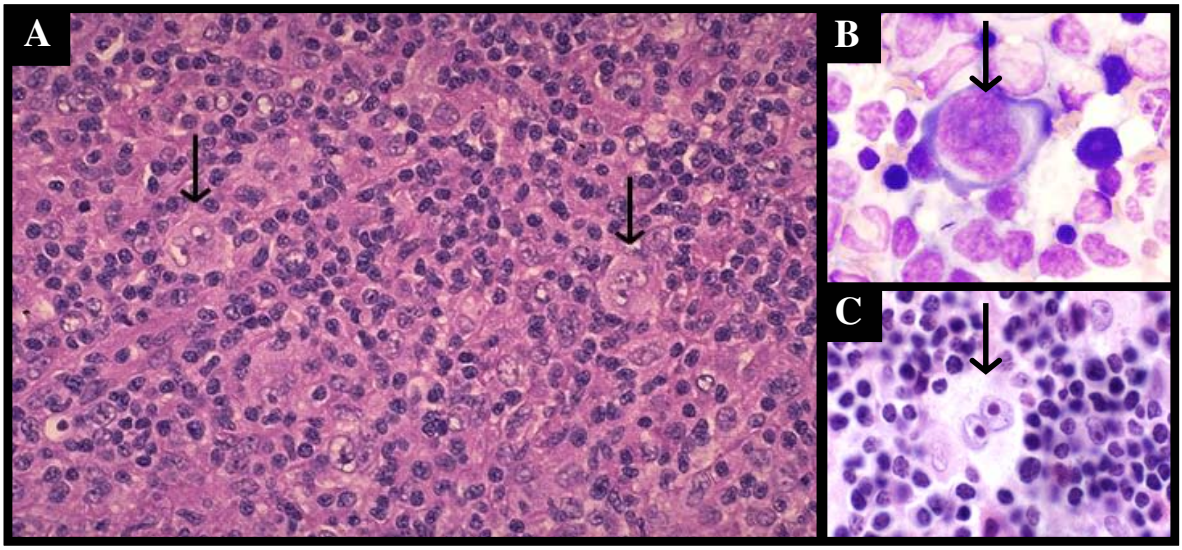


Figura 5 Microscopia de luz mostra células do tipo Reed-Sternberg (RS) em corte histológico de linfonodo.
As células indicadas pelas setas são as as células que caracterizam o linfoma de Hodgkin (RS).
Fontes: (A) http://www-medlib.med.utah.edu/WebPath/HEMEHTML/HEME044.html.
(B) http://www.ashimagebank.org/cgi/collection/hodgkins; (C) Kadin, M. ASH Image Bank 2002;2002:100484

Vale ressaltar que a presença destas células é necessária, mas não suficiente para o diagnóstico do linfoma de Hodgkin, uma vez, que estas células podem ser encontradas em outras linfoadenopatias.

As verdadeiras causas que conduzem os linfócitos B a adquirirem esta alteração, levando a doença de Hodgkin, ainda não são bem compreendidas, mas é sabido que em cerca de 50% dos casos houve infecção prévia pelo vírus Epstein-Barr (EBV). Também existem ligações entre a doença de Hodgkin e outros vírus causadores de mononucleose, como o citomegalovírus (CMV). Este termo mononucleose ou apenas mono foi adotado graças à preferência destes vírus em infectar leucócitos mononucleares (monócitos e leucócitos) levando a um expressivo aumento de 10000-20000 células por mm cúbico de sangue.

Nos últimos anos houve uma melhora significativa na forma de tratamento dos tumores, com uma maior precisão e cuidado nas doses radioterápiticas e quimioterpiticas, ainda consideradas as formas mais viáveis de tratamento para a grande maioria dos tipos de cânceres conhecidos. E é justamente devido a este avanço que a porcentagem de sobrevivência para portadores da doença de Hodgkin aumentou de 78% na década de 70 para 95% no ano de 2000 em crianças com menos de 15 anos de idade (Jemal *et al*., 2006).

2. CD30 e Ki-1/57 como antígenos em células de Linfoma de Hodgkin (L540)

O estabelecimento de linhagens celulares derivadas da doença de Hodgkin *in vitro* permitiu a produção de anticorpos monoclonais específicos para estas células. O anticorpo monoclonal Ki-1 desenvolvido em uma destas linhagens celulares reagiu com duas proteínas celulares distintas, uma de 120-kDa e outra de 57-kDa (Hansen *et al.*, 1989).

A glicoproteína de 120-kDa, conhecida como C*luster of Differentiation* 30 (CD30), Ki-1 ou Ber-H2 é um membro da superfamília dos fatores de necrose tumoral (TNF) de receptores de superfície celular, além de um antígeno ativador de linfócito (Falini B *et al*., 1995). Esta molécula CD30 foi originalmente descoberta como uma proteína marcadora, superexpressa na superfície de células Hodgkin e Reed-Sternberg (H-RS) (Schwab *et al*., 1982).

A superexpressão de CD30 é a marca de comprovação da autenticidade das células H-RS e conduz a uma constitutiva ativação de do fator –kB nuclear (NFkB) que é a base molecular para a patofisiologia do linfoma de Hodgkin (Watanabe et al., 2003).

O antígeno Ki-1 (CD30) provou ser um útil marcador no diagnóstico da doença de Hodgkin (Rohde *et al.*, 1992). Anticorpos anti-CD30 têm possibilidade de atuar no tratamento do linfoma de Hodgkin refratário (Schnell *et al*., 2005).

Estudos recentes mostram que em transplantes renais, os altos níveis sorológicos de CD30 solúvel no destinatário do enxerto prediz rejeição aguda e crônica, com perda do enxerto (Rajakariar *et al*., 2005) (Weimer *et al*., 2005) (Cinti *et al*., 2005).

3. A proteína Ki-1/57

A forma intracelular do antígeno reconhecido pelo anticorpo Ki-1, a proteína humana Ki-1/57, também conhecida como hyaluronan-binding protein 4 (HABP4), apresentou expressão em células tanto tumorais quanto em linfócitos ativados. (Hansen *et al*., 1989).

Os primeiros estudos da proteína Ki-1/57 mostraram-na em várias localizações nucleares, tais como envelope nuclear, na heterocromatina, em estruturas da eucromatina e no nucléolo (Hansen *et al*., 1989).

Hansen e colaboradores haviam verificado que o antígeno Ki-1/57 estava envolvido com atividade quinase, fosforilando ela mesma, além de outros substratos como CD30, cadeia pesada do anticorpo e histonas (Hansen *et al.*, 1990). Kobarg e colaboradores verificaram que a proteína Ki-1/57 quando imunoprecipitada de células e incubada com γ-^{32}P-ATP mostrava-se fortemente fosforilada, o que acreditava-se ser proveniente da auto-fosforilação (Kobarg *et al*., 1997).

O gene responsável pela proteína Ki-1/57 foi localizado por FISH na região 22 do braço longo do cromossomo 9 (9q22.3-q31). Nesta região ocorrem deleções secundárias em leucemia mielóide aguda dos tipos M2 [translocação t(8;21)] e M3 [translocação t(15;17)]. (Heim e mittelman, 1995).

A proteína Ki-1/57 foi observada ligando-se a RNA e hialuronato (um glicosaminoglicano) e com menor afinidade pelo RNA. (Huang *et al*., 2000).

Um trabalho recente também identificou a proteína Ki-1/57, dentre outras 10, como antígeno para possível candidato a marcador tumoral em carcinoma de células escamosas do esôfago (Shimada *et al.*, 2005).

Contudo, ainda não há registros científicos que provem o envolvimento da proteína Ki-1/57 no desenvolvimento de tumores, embora as proteínas descritas até o momento como interação corroborem com a possibilidade de se tratar de um proto-oncogene. Com isso, muitos estudos ainda deverão ser necessários para solucionar os verdadeiros eventos celulares nos quais ki-1/57 pode vir a estar envolvida.

4. A proteína CGI-55

CGI-55 apresenta cerca de 62% de similaridade em seqüência de aminoácidos com a proteína Ki-1/57, sendo que 39% dos resíduos são idênticos. Além disso, sua homóloga em *C. elegans* apresenta 52% de similaridade em resíduos de aminoácidos, o que nos leva a acreditar que esta proteína deva ter um importante papel funcional, já que se apresenta conservada em organismos tão distantes filogeneticamente.

A proteína CGI-55 é uma proteína de 55-kDa (SwissProt número de acesso Q8NC51), também conhecida como inibidor do ativador plasminogênico de RNA mensageiro (RNAm)-proteína 1 ligante a RNA (PAI-RBP1).

Heaton e colaboradores usando uma seqüência de 120nt do elemento cíclico de resposta-nucleotídica (CRS) do gene de rato, isolaram um complexo, no qual identificaram a proteína PAI-RBP1 a partir de células hepáticas (HTC) (Heaton *et al*., 2001).

No entanto, assim como para Ki-1/57, muito pouco ainda se conhece a respeito dos possíveis papéis funcionais destas proteínas. Usando ensaio de mobilidade eletroforética (EMSA) e experimentos de "cross-linking", foi verificado que CGI-55 se liga a estes elementos cíclicos de resposta-nucleotídica do gene PAIL em rato de maneira dependente da concentração. E por análise de mutações foi determinado que CGI-55 interage primariamente com regiões ricas em adenina do elemento de resposta (Heaton *et al*., 2001).

Ambas as proteínas Ki-1/57 (Huang *et al*., 2000) e CGI-55 (Heaton *et al*., 2001), interagiram com moléculas de RNA, embora nenhuma delas apresente um domínio característico para ligação a RNA. Por outro lado, ambas apresentam motivos "RGG-box", dois deles conservados entre as duas proteínas, e até mesmo na homóloga de CGI-55 em *C.*

elegans. Atualmente é sabido que estes motivos "RGG-box" – muito freqüentes em proteínas ligantes a RNA - também podem atuar como motivos de ligação a RNA (Gary *et al*., 1998).

5. A proteína RACK1

O receptor de proteína quinase ativada C 1 (RACK1) foi originalmente clonado a partir das bibliotecas de fígado de galinha e linhagens de células linfoblastóides-B humanas (Guillemot *et al*., 1989) e referida como C12.3 ou H12.3, respectivamente. O nome RACK1 foi adotado pelo grupo do Mochly-Rosen para descrever sua habilidade de ligação a PKC ativada (Ron *et al*., 1994).

Devido a sua habilidade de coordenar interações de moléculas sinalizadoras chave, RACK1 está se tornando amplamente aceita como central no papel a respostas biológicas críticas, como o crescimento celular (Mamidipudi *et al*., 2004).

RACK1 é uma proteína de 36-kDa (SwissProt número de acesso P25388) contendo sete repetições internas Trp-Asp 40 (WD40). Ela é homóloga a subunidade β da proteína G, tendo 42% de identidade com muitas substituições de aminoácidos conservados. As repetições WD de RACK1 podem ser preditas para formar uma estrutura hélice com sete lâminas (Sondek *et al*., 2001; Steele *et al*., 2001), com cada lâmina completando a folha β como mostrado em estudos cristalográficos para G_{β} (Wall et al. 1995; Sondek *et al*., 1996).

A seqüência WD de repetição de RACK1 é altamente conservada em *Drosophila melanogaster*, *Caenorhabditis elegans*, plantas (Kwak *et al*., 1997) e até mesmo em algas unicelulares (Schloss *et al*., 1990). Isto sugere que a função biológica de RACK1 foi estabelecida antes desta separação ocorrer (Neer *et al*., 1994). De fato, RACK1 é igualmente expressa em tecidos de mamíferos superiores e humanos (Guillemot *et al*., 1989), incluindo cérebro, fígado e baço, sugerindo que ela tem um importante papel funcional em muitas, se não em todas, as células (Chou *et al*., 1999).

Embora sua atividade como proteína ancoradoura de PKC tenha sido bastante explorada, vale ressaltar que todas as isoformas de PKC são expressas de forma muito específica em diferentes tecidos, e até em diferentes compartimentos celulares (Mellor *et al*., 1998). Como a distribuição tecidual de RACK1 não é sempre a mesma como favorecida para PCK (Chou et al., 1999), é muito provável que RACK1 esteja envolvida

em processos independentes da transdução sinal de PKC (Chou *et al*., 1999). De fato, dados acumulados de alguns laboratórios mostram que RACK1 interage com diferentes proteínas celulares e, como resultado, pode ter diversas funções, até mesmo célula-tipo-específicas (Tabela 3) (McCahill *et al*., 2002).

Tabela 3 Proteínas que interagem com RACK1.

RACK1 Interacting Proteins	References
PKCb: C2 and V5 domains	Ron et al., 1995; Stebbins and Mochly-Rosen, 2001
PDE4D5: RAID in unique N terminal region	Yarwood et al., 1999; Steele et al., 2001
Src family kinases: phosphotyrosine binding pocket of SH2 domain	Chang et al., 1998, 2001
PTPn catalytic region	Mourton et al., 2001
IL-3, IL-5, and GM-CSF receptors common β chain	Geijsen et al., 1999
PH domains of β-spectrin, β-dynamin, and p120GAP	Rodriguez et al., 1999; Koehler and Moran, 2001
GABA type A receptor: α1 and β1 subunits	Brandon et al., 1999
β-Integrin cytoplasmic domain	Liliental and Chang, 1998; Besson et al., 2002
Human type 1 interferon receptor: amino acids 300 to 346 of cytoplasmic domain	Croze et al., 2000
Pat1 Ran1 kinase with yeast RACK1 homologue Cpc2	McLeod et al., 2000
Pck2 with yeast RACK1 homologue Cpc2	Won et al., 2001
Epstein-Barr virus BZLF1 protein: transactivation domain	Baumann et al., 2000
Influenza virus M1 protein	Reinhardt and Wolff, 2000
P85 subunit of PI3 kinase and SHP-2	Kiely et al, 2002
HIV-1 Nef protein	Gallina et al., 2001
Adenovirus E1A protein	Sang et al., 2001
Type I interferon receptor and STAT1	Usacheva et al., 2001
Helicobacter pylori VacA cytotoxin	Hennig et al., 2001
NR2B subunit of the NMDA receptor	Yaka et al., 2002
NHERF1	Liedtke et al., 2002
IGF-1 receptor	Hermanto et al., 2002; Kiely et al., 2002

Outro papel funcional atribuído a RACK1, que vem ganhando força nos últimos anos, é o seu envolvimento com o ribossomo. Um estudo de espectrometria de massa mostrou que RACK1 pode ser encontrada associada a subunidade ribossomal menor (Yu *et al*., 2005).

6. A proteína NSAP1

A proteína associada a NS1 (NSAP1), também conhecida como GRY-RBP (Blanc *et al*., 2001; Lau *et al*., 2001) é uma integrante da família das hnRNPQ, que é composta por três proteínas, hnRNP: Q1 (62.4 kDa), Q2 (65.5 kDa) e Q3 (69.6kDa), ambas derivadas do mesmo gene por processamento alternativo.

As hnRNPQs são integrantes da superfamília das ribonucleoproteínas heterogêneas nucleares (hnRNP), atualmente composta por mais de 40 proteínas diferentes (Tabela 4) (Carpenter Brian *et al*., 2005). As hnRNPs foram primeiramente descritas como uma famíla de proteínas, que ligavam-se aos transcritos da RNA polimerase II para formar partículas

hnRNP. Inicialmente o complexo multi-subunidade foi acreditado de ser constituído por seis proteínas, embora, investigações subseqüentes envolvendo a imunoprecipitação dos complexos hnRNP identificaram fatores adicionais (Choi *et al*., 1984). Aproximadamente 30 pontos foram observados no gel bidimensional do complexo imunopurificado e isto foi usado como base para nomear os membros da família hnRNP, denominadas hnRNP A1 a hnRNP U (Pinol-Roma *et al*., 1988). Identificações subseqüentes e análises das clonagens gênicas mostraram que certos pontos de proteínas eram de fato diferentes isoformas geradas a partir do mesmo gene (Dreyfuss *et al*., 1993). Até o momento, apenas 19 genes foram identificados (Tabela 4).

Todas as hnRNPs compartilham alguns motivos estruturais, sendo os mais comuns o domínio de ligação a RNA (RBD) também chamado motivo de reconhecimento a RNA (RRM), que são geralmente localizados na porção N-terminal. Estudos de ligação têm mostrado que elementos seqüência-específicos de DNA ou RNA são ligados pelas hnRNPs através do RRM (Dreyfuss *et al*., 1993). Além de ligar a ácidos nucléicos, o RRM de certas proteínas, por exemplo, hnRNP A1, é envolvido na interação proteína-proteína (Hay *et al*., 2001).

As hnRNPs também têm regiões auxiliares como os motivos RGG and KH, embora não em todos os indivíduos. O motivo RGG nestas proteínas já foi verificado como participante da interação proteína-proteína, ativação transcricional e localização nuclear (Dreyfuss *et al*., 1993). Estes eventos funcionais, envolvendo este motivo, podem estar sendo regulados pela metilação de suas argininas. Já o domínio KH foi primeiramente descrito em hnRNP K e também é um motivo de ligação a RNA.

Tabela 4 Detalhes sobre os membros da extensa família hnRNP, localização cromossomal do gene e número de isoformas geradas por *splicing* alternativo.

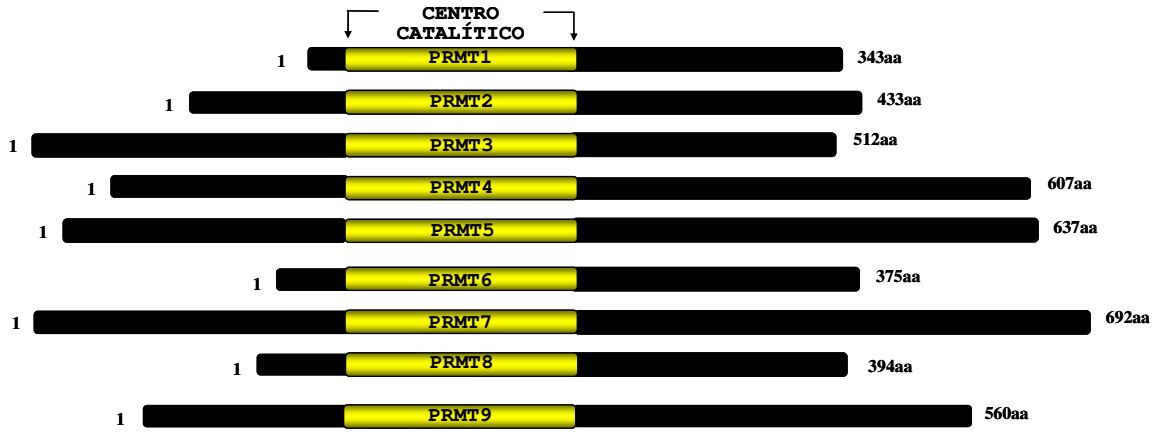
Factor	Chromosome locatio	Number of isoform	Differences between isoforms	References
HnRNP A1	12q13.1	A1 (320 aa) A1* (372 aa)	A1 is 52 aa shorter than A1* but maintains the same reading frame.	[4–6]
HnRNP A2/B1	7p15	A2 (341 aa) B1 (351 aa)	B1 contains an additional 10 amino acids at the N-terminus when compared with A2	[7]
HnRNP C1/C2	14q11.2	C1 (290 aa) C2 (303 aa)	C1 and C2 are identical except for an extra 13 aa near the middle of C2	[8,9]
HnRNP D (ARE/poly U binding factor-AUF 1)	4q21.1–q21.2	p37 (286 aa) p40 (305 aa) p42 (335 aa) p45 (354 aa)	All isoforms are the same except for differences at the N- and C-termini.	[10,11]
HnRNP E1 (poly (rc) binding protein 1-αCP1)	2p12–p13	(356 aa)		[12]
HnRNP E2 (poly (rc) binding protein 1-αCP2)	12q13.12–q13.3	(366 aa) (362 aa)	The 366 variant is exactly the same as the 362 variant expect for a 4 aa insert in the centre of the protein.	[12]
HnRNP F	10q11.2–q11.22	(415 aa)		[13]
HnRNP G (Glycoprotein p43; RNA binding motif protein, X chromosome)	Xq26.3	(436)		[14]
HnRNP H	5q35.3	(449 aa)		[13]
HnRNP H′	6q25.3–q26, or/and Xq22	(449 aa)		[13]
HnRNP I (Polypyrimidine tract binding protein 1-PTB 1)	19p13.3	A (557 aa) B (550 aa) C (531 aa) D (197 aa)	Isoform A is identical to B except A has a 7 aa insert midway through the protein. Isoform C lacks an 18 aa insert found mid way in A. Isoform D lacks an internal 360 aa stretch of A.	[15–17]
HnRNP 2H9	10q22	2H9 (346 aa) 2H9A (331 aa) 2H9B (297 aa) 2H9C (215 aa) 2H9D (145 aa) 2H9E (139 aa)	All have the same amino acid sequence but differ at the N- and C-terminii.	[18,19]
HnRNP K (Transformation Up Regulated Nuclear Protein-TUNP)	9q21.32–21.33	A (464 aa) B (463 aa) C (459 aa) D (458 aa)	All isoforms are the same except for short aa differences at the C-terminii	[20]
HnRNP L	19q13.2	1 (558 aa)		[21]
HnRNP M4	19p13.2–13.3	A (729 aa) B (690 aa)	Isoform A contains an additional 39 aa located near the N-terminus of B.	[22–24]
HnRNP P2 Translocated in Liposarcoma protein (LPS) FU	16p11.2	1 (526 aa)		[25]
HnRNP Q glycine and tyrosine-rich RNA binding protein (GRY-RBP)	6q14–q15	Q1 (561) Q2 (589) Q3 (623)	Differ at the C-terminii	[26]
HnRNP R	1p36.11	1 (633 aa)		[27]
HnRNP U (Scaffold Attachment Factor A-SAF A)	1q44	1 (824) 2 (806)	Variant 1 is the same as variant 2 except for a 18 aa insert in the middle of the N-terminal half of the protein	[28]

Fonte: Tabela retirada da *Bioch. Bioph. Acta* (BBA); Carpenter *et al*, 2006

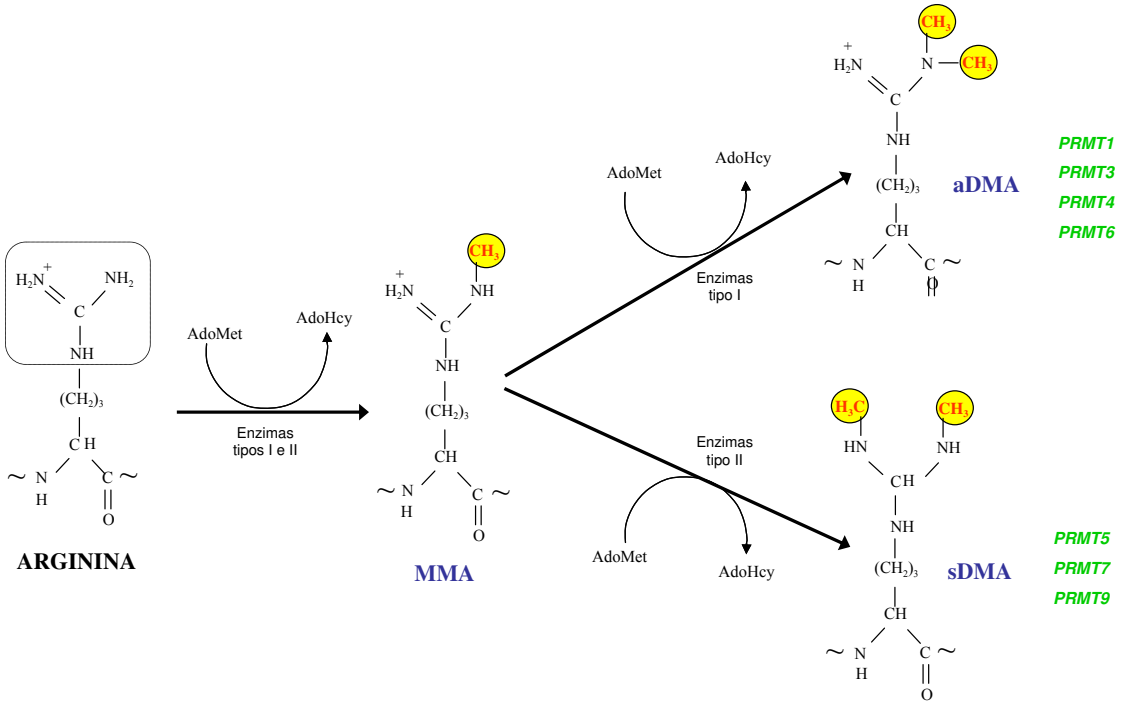
7. As características da família das PRMTs

Todos os integrantes desta família apresentam uma região central, centro catalítico, conservada, enquanto que suas regiões N- e C- terminal variam consideravelmente em extensão (Fig. 6). A PRMT1, embora seja a menor integrante da família, parece ser

responsável por cerca de 85% da atividade de metilação de argininas na célula (Gary *et al*., 1996; Pawlak *et al*., 2000).



A proteína arginina metiltransferase 1 (PRMT1) é uma integrante da família das PRMTs, atualmente composta por nove enzimas. Quanto ao produto da sua atividade, as PRMTs podem ser divididas em dois grandes grupos, que catalisam a reação na presença do doador metil S-adenosilmetionina (AdoMet): as do tipo I (PRMT1, PRMT3, PRMT4 e PRMT6), que fazem metilação do tipo N^G-monometilarginina (MMA) e N^G, N^G -dimetilarginina assimétrica (aDMA); as do tipo II (PRMT5, PRMT7 e PRMT9), que leva a metilações do tipo MMA e N^G, N^G -dimetilarginina simétrica (sDMA) (Fig. 7). Ambas PRMT2 e PRMT8 ainda não tiveram suas atividades enzimáticas determinadas.



A PRMT1 foi primeiramente descoberta por homologia com a metiltransferase de levedura Hmt1/Rmt1 e como proteína de interação – encontrada pelo sistema duplo híbrido de levedura (SDHL (ver anexo I – O Duplo Híbrido de Levedura)) – usando as proteínas TIS21 e BTG1 como iscas (Lin *et al*., 1996). A PRMT2 foi identificada pela homologia e a PRMT3 pela sua associação com a PRMT1 (Scott *et al*., 1998; Tange *et al*., 1998). A PRMT4, também conhecida como arginina metiltransferase coativadora-associada (CARM1), também foi descoberta por análise de SDHL, mas neste caso utilizando a proteína GRIP1 como isca (Chen *et al*., 1999). A PRMT5 foi inicialmente identificada em células humanas como proteína ligante a Jak2 também pelo SDHL (Pollack *et al*., 1999) e é uma homóloga de Skb1 em *Schizosaccharomyces pombe* e Hs17p em *Saccharomyces cerevisiae* (Gilbreth *et al*., 1998; Lee *et al*., 2005). A PRMT6 identificada por homologia é a única da família que apresenta atividade de autometilação e presença exclusiva nuclear após transfecção com fusão a GFP (Frankel *et al*., 2002). A PRMT7, que contem dois putativos centros catalíticos, foi isolada em uma busca por genes que controlam a resposta celular a agentes citotóxicos, sendo considerada um elemento supressor conferindo resistência ao inibidor da topoisomerase II (Gros *et al*., 2003). PRMT8, foi encontrada sendo expressa em cérebro, ancorada à membrana plasmática por sua porção N-terminal, que é meristilada na glicina seguinte a metionina (Lee *et al*., 2005). E recentemente a PRMT9 foi caracterizada como sendo a terceira metiltransferase do tipo II da família (Cook *et al*., 2006). A PRMT9 é também conhecida como FBXO11 ou "somente proteína 11 F-box". Proteínas F-box se associam com Skp1 e com o complexo ubiquitina ligase (Cardozo *et al*., 2004) e recrutam um grupo específico de substratos para o complexo. Há evidencias que levam a acreditar que a PRMT9 poderia estar atuando na regulação da degradação protéica no complexo de ubiquitinação (Cook *et al*., 2006).

Quase todas as proteínas alvos da metilação do tipo aDMA apresentam o motivo tri-peptídico "RGG-box" (Frankel e Clarke, 1999; Green *et al*., 2000; Lin *et al*., 2000; McBride e *et al*., 2000; Henry *et al*., 1996) e os resíduos metilados estão restritos a este domínio. O motivo "RGG-box" compreende seqüências RGG ou RXR, sendo X qualquer aminoácido (McBride e Silver, 2001). Um grupo de proteínas onde este motivo é freqüentemente encontrado são as proteínas ligantes a RNA, como a EWS, hnRNP A1,

hnRNP A2, fibrilarina, CIRP, entre outras (Belyanskaya *et al*., 2001; Shen *et al*., 1998; Nichols *et al*., 2000; Frankel *et al*., 1999; Aoki *et al*., 2002).

Metilação de argininas têm pouco efeito na carga, ou na extensão e direção do dipolo elétrico do grupo guanidino. Por outro lado, há intensa alteração no pKa da cadeia lateral. A adição de grupos metil aumenta a hidrofobicidade local e impedimento estérico. Talvez o mais importante efeito da metilação seja a mudança no potencial de ligação do hidrogênio da cadeia lateral da arginina devido ha remoção de hidrogênios aminos (Raman *et al*., 2001).

Embora esta modificação pós-traducional seja bem conhecida, o papel biológico da metilação de argininas bem como sua regulação, ainda permanecem pouco compreendidos. Antigamente, a metilação de argininas estava restrita a proteínas ligantes a RNA e histonas, que são proteínas abundantes na célula. Hoje, com advento de técnicas mais avançadas e eficientes como a espectrometria de massa e a ressonância plasmônica de superfície (Biacore), novos substratos para a metilação de argininas têm sido encontrados (Huang *et al*., 2002; Boisvert *et al*., 2003; Wu *et al*., 2004).

Nos últimos dez anos muitos substratos para as arginina metiltransferases foram identificados, e cada vez mais, estudos ampliam a compreensão a respeito dos diferentes papéis funcionais atribuídos a esta alteração pós-traducional até agora considerada irreversível. Metilação de argininas em proteínas pode levar a regulação da interação proteína-proteína (Mowen *et al*., 2001; Bedford *et al*., 2000), interação proteína-ácidos nucléicos (Rajpurohit *et al*., 1994), além de participarem da regulação de importantes eventos celulares como o processamento de RNA (Cote *et al*., 2003), transdução sinal (Pollack *et al*., 1999; Mowen et al. 2001), regulação transcricional (Zhang *et al*., 2001; Stallcup *et al*., 2001), reparo do DNA (Boisvert *et al*., 2005) e mesmo no desenvolvimento de doenças (Vallance *et al*., 2002; Kim *et al*., 2003; Carroll MC, 2004; Hong *et al*., 2004; Li *et al*., 2004; Boulanger *et al*., 2005). Em adição, devemos considerar que muitos substratos a metilação não mostraram mudança em suas atividades celulares após esta modificação, embora algumas tenham apresentado alteração de localização celular (Shen *et al*., 1998; Nichols *et al*., 2000). Portanto, é possível que o baixo conhecimento a respeito dos papéis funcionais atribuídos até o momento a metilação de argininas, se deva também ao baixo conhecimento quanto às funções conferidas aos substratos protéicos identificados

previamente. Assim, com o avanço no estudo de novas proteínas, associado a uma melhor compreensão dos eventos celulares regidos pelas proteínas, é possível que descubramos as ainda pouco compreendidas implicações da metilação de argininas, e a partir daí, entendermos porquê há tantas arginina metiltransferases em *Homos sapiens*.

A PRMT1 é a que apresenta maior conservação na seqüência de aminoácidos com a homóloga Hmt1/Rmt1, que parece ser a única arginina metiltransferase encontrada em levedura (Henry and Silver 1996; tange *et al*., 1998). Isto nos leva a pensar que as outras PRMTs poderiam ter atividades mais especializadas do que a PRMT1.

7.1 Metilação

De 3196 enzimas descritas na versão de 1992 da nomenclatura enzimática (Webb *et al*., 1992), aproximadamente 3% representam espécies que catalisam o ataque a uma variedade de nucleófilos, nitrogenio (N), oxigênio (O), carbono (C), enxofre (S), no grupo metil da S-adenosilmetionina (AdoMet ou SAM). Estas metiltransferases incluem enzimas que resultam na formação de ésteres metil, éteres metil, tioéteres metil, aminas metil, amidas metil e outros derivativos em proteínas, ácidos nucléicos, polissacarídeos, lipídeos, pectina, além de várias pequenas moléculas (Kagan *et al*., 1994; Cheng *et al*., 2005).

A transferência do grupamento CH_3 do AdoMet para estas moléculas é uma transformação química comum na biologia e o AdoMet é pretendido de ser o segundo substrato mais freqüentemente usado em reações biológicas após o ATP.

A ativação termodinâmica do SAM como um agente de metilação tem sido mensurado de -17 kcal/mol (Walsh *et al*., 1979). Comparado com os -7 kcal/mol da transferência do fosfato a partir do ATP, há uma enorme força termodinâmica direcionando a transferência do metil para o co-substrato nucleofílico.

A partir de análises estruturais e de informática, cinco famílias de proteínas metiltransferases têm sido propostas (Schubert *et al*., 2003a), sugerindo cinco independentes caminhos evolucionários para as metiltransferases. Classe I e classe V parecem conter a maioria das metiltransferases de proteínas, ficando as demais classes dedicadas a metilação de outras moléculas.

Historicamente, a metilação de proteínas foi detectada por hidrólise protéica e análise de aminoácido do N-metil aminoácido liberado. Os N-metils aminoácidos

sobrevivem a hidólise ácida, mas os O-carboximetils aminoácidos são lábeis a hidrólise, e, portanto não são detectados. Com o advento da radiomarcação [^{14}C]CH_3-SAM, se tornou possível a detecção estequiométrica da metilação em peptídeos fragmentados.

Em proteínas, as metilações mais comuns ocorrem nos átomos de oxigênio (O) e nitrogênio (N). Metilação nas cadeias laterais carboxiladas esconde a carga negativa e adiciona hidrofobicidade. Dimetilações e trimetilações da cadeia lateral de lisinas em proteínas levam a um aumento da hidrofobicidade e impedimento estérico, caso a interação proteína-proteína ou proteína-ácidos nucléicos envolva esta região.

7.2 Doenças relacionadas as arginina metiltransferases

Cânceres de mama e de próstata são tumores comumente hormônio-dependentes. O fato de que as PRMTs são conhecidos co-ativadores para receptores nucleares fazem deles prováveis candidatos para serem super-expressos nestes tipos de câncer. De fato foi verificado que o aumento da expressão da PRMT4 co-relaciona com a independência androgênica em carcinoma humano de próstata (Hong *et al*., 2004). Além disso, pequenas moléculas inibitórias das PRMT1 e 4 podem suprimir a ativação da transcrição mediada por estrogênio e androgênio (Cheng *et al*., 2004). Em adição, a habilidade da PRMT5 - quando superexpressa – de promover o crescimento celular independente de ancoragem reforça a provável implicação destas enzimas como candidatos para desregulação estados celulares transformados (Pal *et al*., 2004). A PRMT5 pode atingir levar a esta situação por inibir a expressão de supressores tumorais. Por fim, o envolvimento da metilação de arginina na resposta a danos do DNA pode significar exemplo de cânceres em que existe instabilidade genômica causada pela desregulação da metilação de argininas (Bedford e Richard, 2005).

A metilação de argininas também tem importante atuação em doenças cardiovasculares (Vallance e Leiper, 2002), onde análogos da arginina, MMA e aDMA, mas não sDMA (Fig. 7) inibem a atividade da enzima responsável pela formação de oxido nítrico (ON) a partir do aminoácido L-arginina, levando com isso a aterosclerose (Stuhlinger *et al*., 2001).

Em pacientes com atrofia muscular espinhal, doença autossômica recessiva resultante de deleções ou mutações com perda de função do gene SMN1 (Lefebvre *et al*., 1995), as proteínas contendo sDMA encontram-se fora de suas localizações celulares

comuns em indivíduos sadios (Boisvert et al., 2002). Além disso, tanto a proteína SMN, quanto a metilação de argininas são eventos fundamentais para a formação do complexo das ribonucleoproteins (RNP) (Meister *et al*., 2002 e Friesen *et al*., 2001b). O fato da perda de SMN levar à falha no controle do neurônio motor axonal sugere que SMN pode ser requerida para reunião, transporte e tradução de mRNP durante o desenvolvimento motor neural (Bassell e Kelic, 2004).

A proteína transativadora (Tat) do vírus humano da imunodeficiência (HIV) foi identificada como sendo a primeira proteína viral contendo argininas metiladas (Boulanger *et al*., 2005). Mais interessante ainda é que a inibição da proteína arginina metiltransferase leva a um aumento da expressão gênica do HIV (Boulanger *et al*., 2005). Estes achados sugerem que o aumento na metilação de argininas pode oferecer alguma proteção contra a infecção por HIV. Por outro lado, a inibição da metilação de argininas também parece oferecer proteção contra certos vírus, como acontece com o vírus da hepatite delta, onde a inibição da metilação leva ao bloqueio da replicação do vírus (Li *et al*., 2004).

Assim, a metilação de argininas tem mostrado, cada vez mais, estar implicada em eventos essenciais para um perfeito funcionamento das células e conseqüentemente dos organismos. Portanto, tendo em vista a indiscutível importância destas enzimas, assim como as extensas questões a serem respondidas acerca de sua regulação funcional é de extrema relevância o aprofundamento no estudo para melhor compreensão da sua regulação e das possíveis implicações funcionais atreladas a esta modificação pós-traducional.

II. OBJETIVO

O presente trabalho teve como objetivo geral o estudo funcional das proteínas Ki-1/57, CGI-55, NSAP1 e da PRMT1, considerando principalmente a influência da metilação.

III RESULTADOS

Artigo I

Characterization of a new family of proteins that interact with the C-terminal region of the chromatin-remodeling factor CHD-3

Taíla A. Lemos, Dario O. Passos, Flávia C. Nery and Jörg Kobarg

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Characterization of a new family of proteins that interact with the C-terminal region of the chromatin-remodeling factor CHD-3[1]

Taíla A. Lemos[a,b], Dario O. Passos[a,c], Flávia C. Nery[a,b], Jörg Kobarg[a,b,c,*]

[a] *Centro de Biologia Molecular Estrutural, Laboratório Nacional de Luz Síncrotron, Rua Giuseppe Máximo Scolfaro 10.000, C.P. 6192, 13084-971 Campinas, SP, Brazil*
[b] *Departamento de Genética e Evolução, Universidade Estadual de Campinas, Campinas, SP, Brazil*
[c] *Departamento de Bioquímica, Universidade Estadual de Campinas, Campinas, SP, Brazil*

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Abstract The two human proteins Ki-1/57 and CGI-55 have highly similar amino acid sequences but their functions are unknown. We analyzed them by yeast two-hybrid screens and found that they interact with the C-terminal region of the human chromatin-remodeling factor CHD-3 (chromo-helicase-DNA-binding domain protein-3). The interaction of CGI-55 and CHD-3 could be confirmed in vitro and in vivo by co-immunoprecipitations from *Sf*9 insect cells. Mapping showed that CGI-55 interacts with CHD-3 via two regions at its N- and C-terminals. The CGI-55 and Ki-1/57 mRNAs show highest expression in muscle, colon and kidney. A CGI55–GFP fusion protein was localized in the cytoplasm, nucleus and perinuclear regions of HeLa cells. These data suggest the possibility that CGI-55 and Ki-1/57 might be involved in nuclear functions like the remodeling of chromatin.

Key words: Chromatin remodeling; Protein–protein interaction; Two-hybrid; Domain mapping; Cellular localization

1. Introduction

Using the monoclonal antibody Ki-1, the first antibody described that specifically detects the malignant Hodgkin and Sternberg-Reed cells in Hodgkin lymphoma [1], we have previously identified the antigen Ki-1/57, a 57 kDa intracellular phospho-protein [2,3]. In vitro phosphorylation experiments performed with the Ki-1/57 antigen isolated from tumor cells suggested that it is associated with a serine/threonine protein kinase activity [4]. Electron microscopic analysis demonstrated that the Ki-1/57 antigen is not only located in the cytoplasm but also at the nuclear pores and in the nucleus where it is frequently found in association with the nucleolus [5]. Tryptic digestion of the Ki-1/57 antigen resulted in the cloning of a partial cDNA encoding Ki-1/57 [6]. The isolated contig of 1380 bp length, encoded the C-terminal 60% of the Ki-1/57 protein.

By searching for related proteins we discovered a cDNA sequence encoding the protein CGI-55, a possible human paralog of the Ki-1/57 protein of yet unknown function. Its cDNA is complete and encodes a hypothetical protein of 55 kDa. The protein sequence of CGI-55 shows 40.7% identity and 67.4% similarity with Ki-1/57. This suggests that the two human proteins might be paralogs and have similar functions.

In the present study, we wanted to obtain possible clues about the functional context of the proteins CGI-55 and Ki-1/57. We explored the yeast two-hybrid system to identify possible interacting proteins. We found that CGI-55 and Ki-1/57 interact with the C-terminal region of the human protein CHD-3 (chromo-helicase DNA-binding domain protein-3). The CHD proteins are members of the chromodomain family, a class of proteins that are involved in transcriptional regulation and chromatin remodeling [7–16]. Chromatin remodeling is likely to be an important step for the regulation of gene expression next to transcription factor based regulatory mechanisms [17,18]. Proteins that participate in the remodeling of chromatin might affect this process at levels as diverse as the nucleosome packing, the formation of DNA loops, its supercoiling or its attachment to the nuclear matrix [18]. The binding of the proteins CGI-55 and Ki-1/57 to CHD-3 can define them as new family of CHD-3 binding proteins and suggests the possibility that they might be involved in nuclear functions associated with the remodeling of chromatin.

2. Materials and methods

2.1. Plasmid construction

The full-length cDNA (DKFZp564M2423Q3) described in the database report was kindly provided by the Resource Center/Primary Database (Heubnerweg 6, D-14059 Berlin, Germany). This clone had been isolated from a human fetal brain cDNA library (DKFZhfb2) created by Stefan Wiemann (DKFZ, Heidelberg, Germany). Several sets of oligonucleotides were designed to allow sub-cloning of the complete CGI-55 coding region in different expression vectors. Insertion into pGEX-2TK (Amersham Biosciences) allowed to express CGI-55 (1–387) as a C-terminal fusion to GST (GST–CGI55). The cDNAs of CGI-55 and its deletion constructs were also inserted into the yeast two-hybrid expression vector pBTM-116 [19]. In a similar fashion the cDNA fragment encoding the C-terminal 60% of the Ki-1/57 antigen (122–413) was inserted in pBTM116. The cDNAs encoding CGI-55 (1–387), Ki-1/57 (1–413) and the C-terminal of CHD-3 (1839–2000) were inserted into the baculovirus (BV) transfervector pVL1392 vector (Pharmingen). This CHD-3 fusion protein contains N-terminal

*Corresponding author. Fax: (55) 19 3287 7110.
E-mail address: jkobarg@lnls.br (J. Kobarg).

[1] Nucleotide sequence accession numbers: huCGI55: AL080119, AF151813; huKi-1/57: U77327; hu-IHABP: AF241831; CeCGI-55, CGI-55 from *Caenorhabditis elegans*: AF016672.

Abbreviations: BV, baculovirus; CHD-3, chromobox helicase DNA-binding domain protein-3

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HA- and 6×His tags for immunodetection (6×His-HA-CHD3). The complete cDNA of CGI-55 was cloned into vector pEGFP-N1 (Clontech).

2.2. Northern blot analysis

A human 12-lane multiple tissue Northern RNA blot was obtained from Clontech and consisted of poly(A)-enriched RNA from the following tissues: brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, and peripheral blood leukocytes. A cDNA fragment of 1161 bp length encompassing the whole coding region of the CGI-55 protein was radiolabeled with α-[^{32}P]dATP using the random prime labeling kit (Roche). Hybridization and exposure of membranes to Kodak films were performed according to manufacturer of membrane (Clontech). The membrane was subsequently striped and reprobed first with a Ki-1/57 cDNA probe of 550 bp length and finally with the 2 kb control actin probe.

2.3. Yeast two-hybrid screening and interaction analysis

The pBTM116-CGI-55 [19] and pBTM116-Ki-1/57 (122–413) vectors were used to express the proteins CGI-55 and Ki-1/57 (C-terminal) linked to the C-terminus of LexA DNA-binding domain peptide in *Saccharomyces cerevisiae* strain L40. A human fetal brain cDNA library (Clontech) expressing GAL4 activation domain (AD) fusion proteins was co-transformed separately with both recombinant pBTM116 vectors. Selection of transformants, β-galactosidase activity test, plasmid DNA extraction and sequencing were performed as described [20].

2.4. Bacterial expression and protein purification

GST and GST–CGI55 proteins were expressed in *Escherichia coli* BL21-CodonPlus-RIL (Stratagene), and purified using glutathione–Sepharose 4B (Amersham) according to manufacturer.

2.5. Expression of 6×His-HA-CHD3, 6×His-CGI55 and Ki-1/57 in Sf9 insect cells

The recombinant transfer vectors pVL1393-HA-CHD3, pVL1392-CGI55 or pVL1392-Ki-1/57 were co-transfected with BV DNA (BaculoGold™, Pharmingen) in *Sf*9 insect cells by lipid transfection (DOTAP, Roche). Recombinant BV were separately amplified three to four times with fresh *Sf*9 cells. Cells were collected and sonicated in PBS, 0,1% Triton X-100 with protease inhibitors. 6×His-HA-CHD3 was purified by Ni-NTA Sepharose affinity chromatography.

2.6. Production of anti-CGI-55 monoclonal antibody

Monoclonal antibodies against CGI-55 were essentially generated as described [6,21]. Briefly, BALB/c mice were immunized four times with intervals of 2–4 weeks intraperitoneally with 100 μg of bacterial GST–CGI55 fusion protein. Spleen cells were fused with X63-Ag8.653 myeloma cells. Hybridoma supernatants were screened by ELISA for the presence of CGI-55 antibodies. The supernatant of re-cloned anti-CGI-55 hybridoma 10.5.6 was used for the experiments.

2.7. In vitro binding assay and Western blot analysis

14 μg of 6×His-HA-CHD3 (1839–2000) fusion protein was coupled to Ni-NTA Sepharose beads. Next 14 μg of GST–CGI55 or GST control protein were incubated for 2 h with the beads and then washed with buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl). Proteins bound to the beads were separated by SDS–PAGE, transferred to a PDVF membrane and visualized by immuno-chemiluminescence using a mouse anti-GST antibody and secondary anti-mouse IgG-HRP conjugate.

2.8. In vivo binding assay and Western blot analysis

1.0×10^7 *Sf*9 cells were infected with wild type BV (BV-WT) or recombinant BV (BV-6×His-HA-CHD3(1839–2000), BV-6×His-CGI55, BV-Ki-1/57) at a multiplicity of infection >20. Two days after infection, cells were lysed in 1 ml Tris–HCl (pH 8.5) containing protease inhibitors. Lysate was treated with DNase (Promega) and cleared at 14 000×*g* for 15 min. Next 20 μl protein A Sepharose beads (Pharmacia) were loaded with the indicated antibodies (anti-HA mAb: Clontech), washed (Tris–HCl, pH 8.5) and incubated with the indicated lysates for 2 h at 4°C. After further two washes with the same buffer the beads were resuspended in SDS–PAGE loading buffer, boiled and analyzed by SDS–PAGE and Western blot using different mAb.

Western blots were developed by chemiluminescence as described [20].

2.9. Analysis of CGI55–EGFP (enhanced green fluorescence protein) fusion protein by fluorescence microscopy

HeLa cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% heat inactivated fetal calf serum. Cells were cultured on glass coverslips for 24 h and were transfected with either wild type pEGFP vector (Clontech) or with recombinant vector CGI55–pEGFP-N1 using the lipid transfection method (Lipofectamine, Invitrogen). After 24 h coverslips were washed and mounted in 80% glycerol/10 mM Tris–HCl (pH 7.5) on coverglasses and analyzed with a fluorescence microscope (Eclipse E600, Nikon).

3. Results

3.1. Sequence analysis of CGI-55 and Ki-1/57

An alignment of the deduced amino acid sequences of human CGI-55, human Ki-1/57 and a possible *Caenorhabditis elegans* CGI-55 ortholog (Fig. 1) revealed possible ATP-binding motifs and nuclear localization signals. The central of the two ATP-binding motifs present in the CGI-55 sequence is conserved in the Ki-1/57 sequence and also in an ortholog protein sequence from *C. elegans*, whereas the possible C-terminal ATP-binding motif of CGI-55 is not conserved in the other two proteins. The putative nuclear localization sequence

A

```
huKi-1/57 MKGALGSPVAAAGAAMQESFGCVVANRFHQLLDDES---DPFDILREAERRQQQLQRKR  57
huCGI-55  MPGHL-----------QEGFGCVVTNRFDQLFDDES---DPFEVLKAAE------NKKKE  40
CeCGI-55  ----------------MSTEYGCQVTNKFGLPSDDDDEYDDPRELIQKVS----QIAAKKK  41

huKi-1/57 RDEAAAAAGAGPRGGRSPAGASGHRAGAGGRRESQKERKSLPAP---VAHRPD--SPGGG 112
huCGI-55  AGGGGVGGPGAKSAAQAAAQTNSNAAGKQLRKESQKDRKNPLPPSVGVVDKKEETQPPVA 100
CeCGI-55  EEKSVKPAQPVKPAAAPVAATKTDGAAGRGRGGRGRGRGRGAGRP---RDGERVSNENGDR  98

huKi-1/57 LQAPGQKRTPRRGEQQGWNDSRGPEGMLER-AERRSYREYRPYETERQADFTAEKFPDEK 171
huCGI-55  LKKEGIRRVGRRPDQQ----LQGEGKIIDRRPERRPPRERR-FEKPLEEKGEGGEFSVDR 155
CeCGI-55  PQGENRRGGPRRGGER------GAARPAGRGGRGGFTRENR----EGEEPKQEVSFEDGQ 148

huKi-1/57 PGDRFDRDRPLRGRGGPRGGMR-GRGRGGPGNRVFDAFDQRGKREFERYGGNDKIAVRTE 230
huCGI-55  P----IIDRPIRGRGGLGRGRG-GRGRGMGRG---DGFDSRGKREFDRHSGSDRSGLKHE 207
CeCGI-55  D------TRAPRRRGGFTLGGGPSGGRGGGRG----GRG----RQFDRQSGSDRTGVRSF 194

huKi-1/57 DNMGGCGVRTWGSGKDT-SDVEPTAPMEEPTVVEESQGTPEEESPAKVPELEVEEETQVQ 289
huCGI-55  DKRGGSGSHNWGTVKDELTDLDQSNVTEETPEGEEHHPVADTEN--KENEVEEVKEEGPK 265
CeCGI-55  DKKDGHGKGNWGDQKDE---LAGETENIAPEGAESTEPEVPREKTAEELAYEAELAVLAK 251

huKi-1/57 EMTLDEWKNLQEQTRPKPEFNIRKPESTVP---SKAVVIHKSKYRDDMVKDDYEDDSHVF 346
huCGI-55  EMTLDEWKAIQNKDRAKVEFNIRKPNEGADGQWKKGFVLHKSKSEEAHAEDSVMD--HHF 323
CeCGI-55  QKTLKEFKAAAKADAPK--FNTRKAGEGAADTFGKLVPIKKEVIP-DREEDEVVVIHKAP 308

huKi-1/57 RKPANDITSQLEINFGNLPRPGRGARGGTRGGRGRIRRAENNYPRAEVVMQDV-APNPDD 405
huCGI-55  RKPANDITSQLEINFGDLGRPGRGGRGG-RGGRGRGGRPNRGSRTDKSSAS---APDVDD 379
CeCGI-55  RKQVLDISITFRNDRPERERNDRSERPQRGGPRGGGRGGGRGGQRQGGHGGRNNTPFNAS 368

huKi-1/57 PEDFPALS--   413
huCGI-55  PEAFPALA--   387
CeCGI-55  DDAFPALGAK   378
```

B

	huCGI-55	huKi-1/57	CeCGI-55
huCGI-55	-	-	-
huKi-1/57	40,7* / 67,4:	-	-
CeCGI-55	28,8 / 55,3	23,9 / 53,2	-

Fig. 1. Protein sequence alignment of human Ki-1/57, human CGI-55 and *C. elegans* CGI-55. A: Comparison of huKi-1/57 (U77327 and AF241831), huCGI-55 (AL080119 and AF151813) and CeCGI-55 (CGI-55 from *C. elegans*: AF016672). Asterisks (*) indicate identical whereas colons (:) mark similar residues. Predicted ATP-binding motifs are boxed. Basic motif: G-X-G-X-X-G-$(X)_{13-22}$-K; [26,27] with white highlighting. Putative NLSs were predicted by the program PSORT II and are indicated by boxes and gray highlighting. B: Table comparing the identity (*) and similarity (:) values between the analyzed protein sequences.

(NLS) of CGI-55 begins at proline 132, whereas that of Ki-1/57 is located closer to the N-terminus at arginine 55. The amino acid sequence similarity between Ki-1/57 and CGI-55 is high: 40.7% of the residues are identical and 16.7% are similar. These data suggest that both human proteins might be paralogs with similar or overlapping functions. In the *C. elegans* amino acid sequence only the N-terminal of the ATP-binding motifs is conserved. Its NLS is found approximately 20 amino acids downstream of that found in the human CGI-55 (proline 153).

3.2. Comparison of the tissue expression of CGI-55 and Ki-1/57

To investigate the human CGI-55 and Ki-1/57 expression pattern in different human tissues we conducted a Northern blot analysis. As shown in Fig. 2 two major transcripts of human CGI-55, of ~5.5 kb and ~4 kb as well as a minor transcript of ~2.2 kb, were observed. The first two transcripts might be unprocessed pre-mRNA, whereas the transcript of ~2.2 kb is the mature CGI-55 transcript. The signal intensity decreases in the order: heart, skeletal muscle, kidney, placenta, liver and brain. The other tissues showed only very faint bands of the two larger CGI-55 transcripts, indicating low levels of CGI-55 expression in these tissues. In contrast, two transcripts of human Ki-1/57 of ~2.8 kb and ~2 kb were observed in the order of decreasing signal intensity in brain, kidney, heart, and skeletal muscle. All other tissues showed weak expression of predominantly the ~2 kb transcript. A control hybridization with the β-actin probe confirmed the uniform loading of the lanes with poly(A)$^+$ RNA.

3.3. Yeast two-hybrid screens

To gain functional insights via the identification of interacting proteins of CGI-55 and Ki-1/57, the yeast two-hybrid system [19,20,22,23] was employed, utilizing a human fetal brain cDNA library. For CGI-55 a screen of 0.6×10^6 co-transformants yielded 125 clones positive for both His3 and

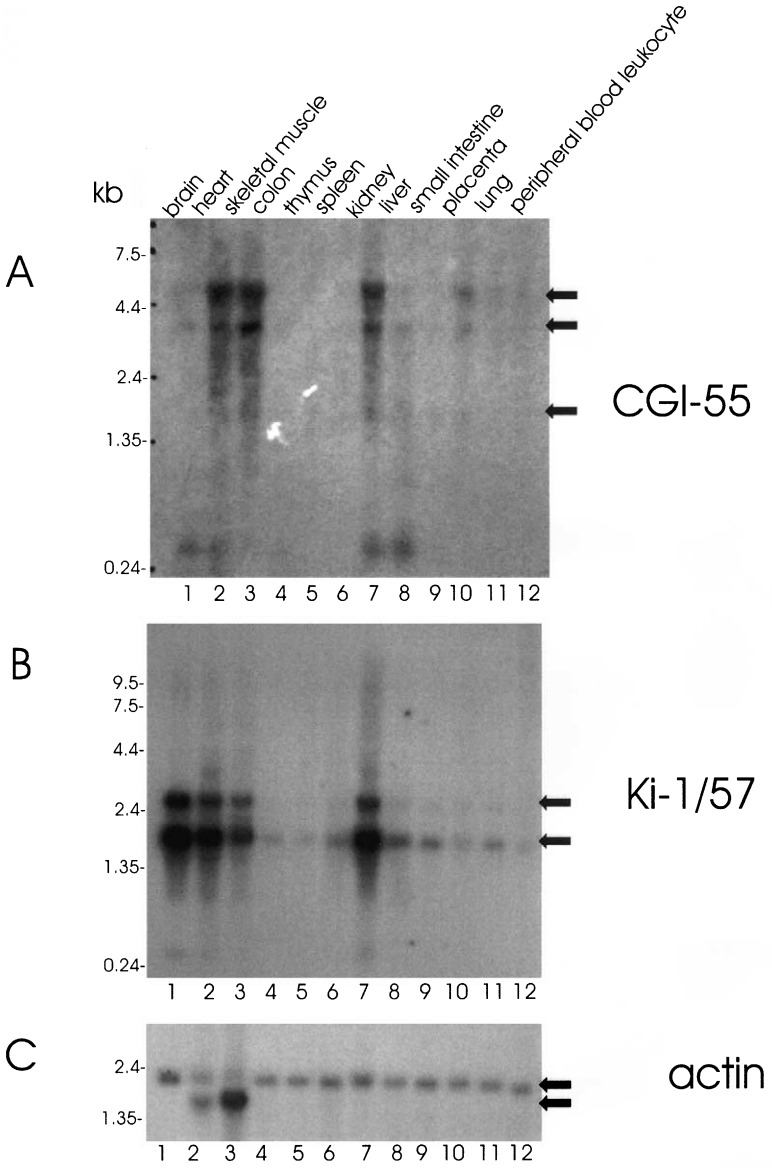

Fig. 2. Differential expression of CGI-55 and Ki-1/57 mRNAs in human tissues. A Northern blot of poly(A)$^+$ RNA isolated from several human tissues (Clonetech) was hybridized with human CGI-55 (A), Ki-1/57 (B) and β-actin (C) cDNA probes.

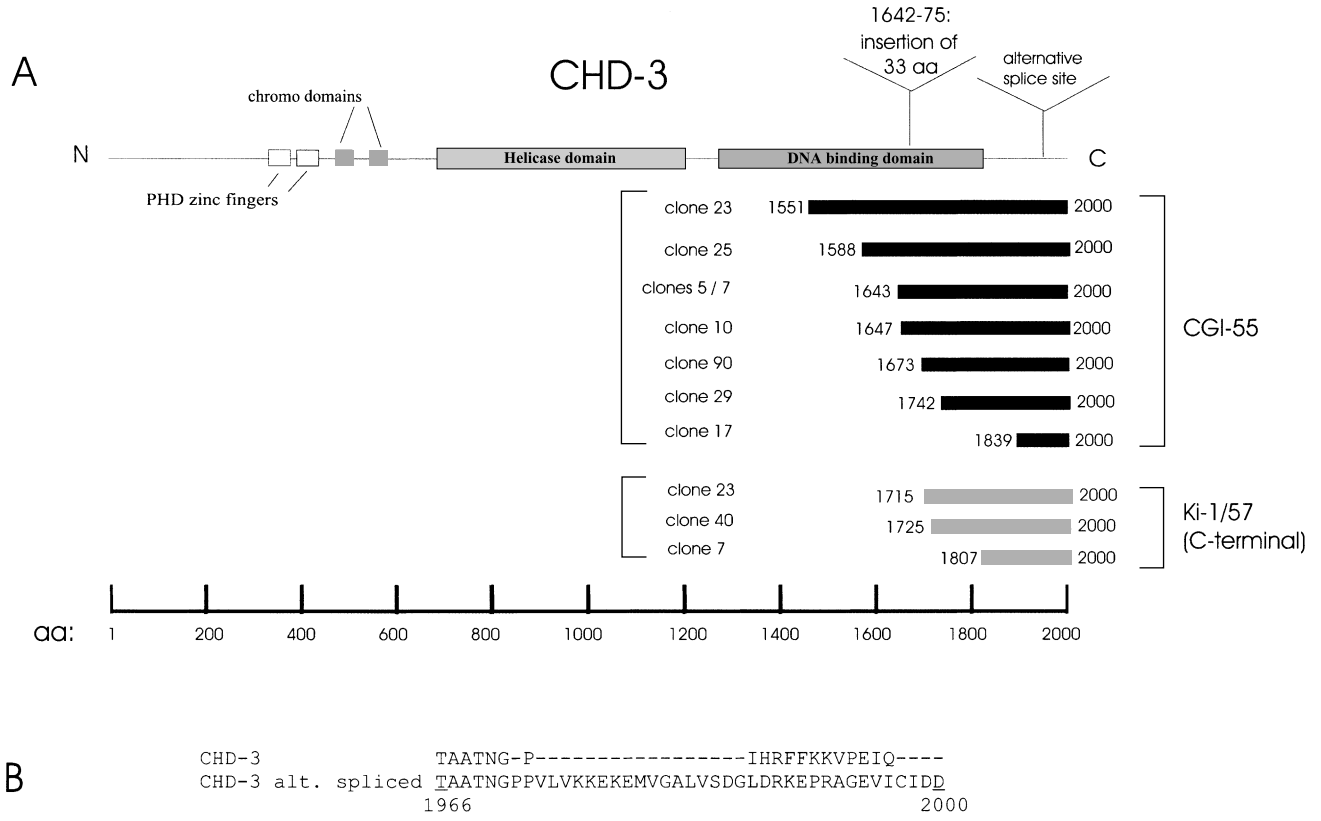

Fig. 3. CGI-55 and Ki-1/57 interact with the C-terminal region of CHD-3. A: Schematic representation of the domain organization of the human protein CHD-3. The position and of the identified CGI-55 interacting clones is shown along the full-length CHD-3 protein. The ruler indicates the length of CHD-3 in amino acids. B: Sequence alignment of the classical [13] and alternatively spliced C-terminal of CHD-3 [11].

LacZ reporter constructs. Library plasmids of 19 clones were sequenced. 42% of the sequenced clones encoded the C-terminal region of human chromatin remodeling factor CHD-3 (amino acid residues 1551–2000) (Fig. 3) [13]. All clones represent an alternative version of the CHD-3 protein that probably rises through alternative splicing and had been termed SNF2-like zinc-finger helicase [11,13] (Fig. 3B). For Ki-1/57 we performed a two-hybrid screen of the same cDNA library using the construct pBTM116-Ki-1/57(122–413). We isolated three independent alternatively spliced CHD-3 clones (Fig. 3).

3.4. Mapping the interaction site of CGI-55 with CHD-3

Next, we mapped the CGI-55 region required for the interaction CHD-3 using the yeast 2-hybrid method (Fig. 4). N- and C-terminal deletion constructs of the CGI-55 protein were fused to the lexA DNA-binding domain and tested for their ability to bind CHD-3. Only the construct 4 of CGI-55 (128–259), that contains the central region of CGI-55, failed to bind to CHD-3. The co-transformation of pBTM116-CHD3(1893–2000) with an unrelated 'bait' construction (pBTM116-AUF1) [24] showed no interaction. Our data suggest that CGI-55 interacts with CHD-3 via two independent binding sites that are located in its N- and C-terminal regions.

3.5. In vitro confirmation of the CGI55–CHD-3 interaction with purified proteins

We carried out pull down assay with purified recombinant proteins that had been expressed in *E. coli* (GST, GST–CGI55) or in the BV system (6×His-HA-CHD3) to confirm the interaction between CGI-55 and CHD-3 in vitro. As shown in Fig. 5B GST–CGI55 bound specifically to the C-terminal of CHD-3, while the control protein GST did not. The input controls identify the corresponding proteins in the blot. In Fig. 5A we controlled the equal loading of the Ni-NTA Sepharose beads with 6×His-HA-CHD3(1893–2000) by developing the Western blot with an anti-4×His mAb.

3.6. Co-immunoprecipitation from BV infected Sf9 insect cells

In order to test if CGI-55 (or Ki-1/57) and CHD-3 can form a stable complex when they are co-expressed in animal cells we employed the BV expression system for co-infection and co-immunoprecipitation studies. When *Sf*9 cells were co-infected with the two recombinant BVs BV-HA-CHD3(1893–2000) and BV-CGI55 and the recombinant protein HA-CHD3(1893–2000) was immunoprecipitated with an anti-HA tag mAb, the protein CGI-55 co-precipitated (Fig. 5C, lane 3) and could be detected by an immunoblot with anti-CGI55 mAb. This immunoprecipitation was specific, since no CGI-55 protein was detected when the *Sf*9 cells were infected with BV-WT or with the recombinant BV-HA-CHD3 alone (Fig. 5C, lanes 1 and 2). The lysate of the *Sf*9 cells infected with BV-CGI55 alone was used to identify the recombinant CGI55 protein in the anti-CGI55 immunoblot (Input lane; Fig. 5C, lane 4). In a similar fashion when CGI-55 was immunoprecipitated (using ant-CGI55 mAb 10.5.6) the protein HA-CHD3(1893–2000) co-precipitated only from the lysate of *Sf*9 cells that had been co-infected by both recombinant BVs: BV-HA-CHD3 and BV-CGI55 (Fig. 5D, lane 3). The immunoprecipitation of lysates of *Sf*9 cells that had been infected with BV-WT or BV-CGI55 alone, did not result in the

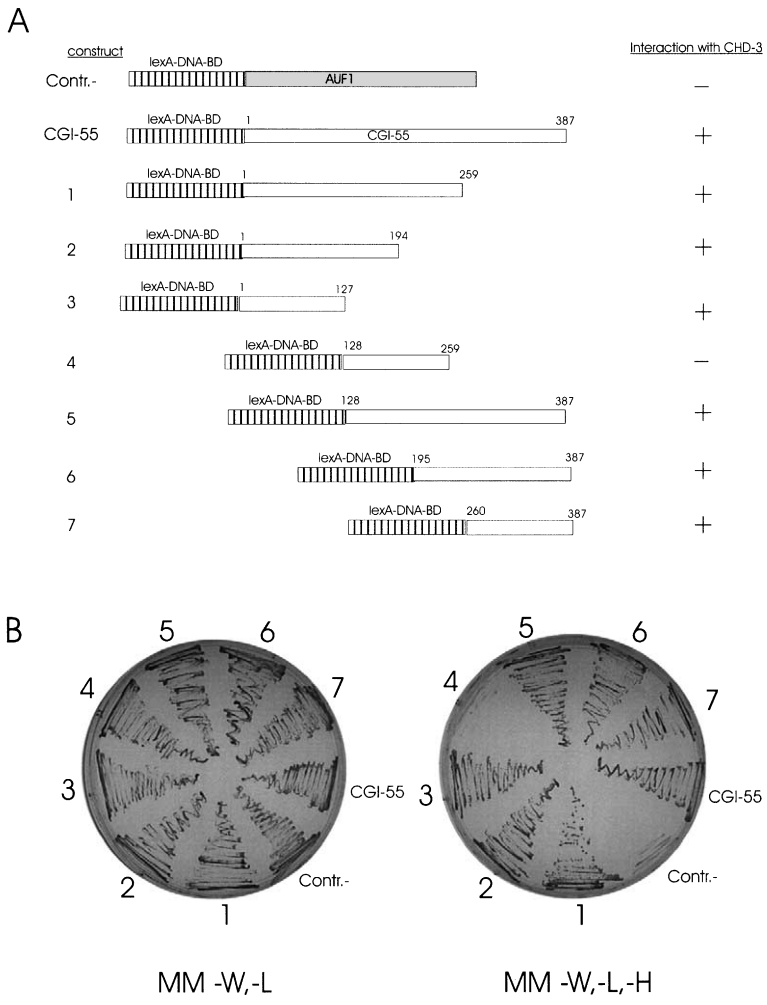

Fig. 4. Mapping of the regions of CGI-55 that interact with CHD-3. A: Various N- and C-terminal truncations of human CGI-55 were fused in frame to the DNA-binding domain of lexA in plasmid pACT2 and transformed into yeast L40 together with the fusion CHD3-Gal4-AD cloned in pBTM116. B: Interaction was determined by the ability of the co-transformant cells to grow on minimal medium (MM),-W,-L,-H (right). Presence of 'bait' and 'prey' plasmids in the co-transformed cells was controlled by growth on MM-W,-L (left).

detection of HA-CHD3 protein (Fig. 5D, lanes 1 and 2). The lysate of *Sf*9 cells infected only with BV-HA-CHD3 identifies the HA-CHD3 protein in the anti-HA immunoblot (Fig. 5D, lane 4, Input). In a parallel approach we were able to demonstrate that also Ki-1/57 specifically co-precipitates with HA-CHD3 protein (Fig. 5E).

3.7. Subcellular localization of CGI-55

Human HeLa cells were transfected with a vector containing GFP alone or with a vector that contains the cDNA encoding CGI-55 fused to the N-terminal of GFP (Fig. 6). In the control an even distribution of GFP was observed in the HeLa cells. In the case of the CGI55–GFP fusion construct we observed a less intense overall staining that appeared in a punctuated pattern throughout the cell. The speckled pattern was found in both the cytoplasm and to a lesser extend also in the nucleus. In addition we observed a marked perinuclear accumulation of the fluorescence in the transfected cells.

4. Discussion

Very few functional data exist about the proteins CGI-55 and Ki-1/57 [6,25,28]. The data that are available in the literature so far suggest, that CGI-55 (also named PAI-RBP1 for plasminogen activator inhibitor mRNA–RNA-binding protein 1) is a mRNA-binding protein [28]. These researchers studied proteins that bind to the 3′-terminal most 134 nt of the PAI-1 (plasminogen activator inhibitor 1) mRNA, that might be involved in the control of this mRNA's stability. They suggested that CGI-55 might be a protein that is involved in the regulation of the stability of the PAI-1 mRNA.

The only functional studies concerning Ki-1/57 come from our group [2–6] and from one other group [25]. The latter had described Ki-1/57 as a novel hyaluronan-binding protein and re-named it IHABP4 (for intracellular hyaluronan binding protein 4). They also found that IHABP4 (= Ki-1/57) binds to other negatively charged glycosaminoglycans like chondroitin sulfate, heparane sulfate, and also RNA, although with lower affinity. The binding of IHABP4/Ki-1/57 to a series of negatively charged macromolecules might be due to its relatively high content of positively charged amino acids, in particular Arg (12.8%). The biological meaning of the interaction of a protein localized in the cytoplasma and nucleus [2–5,25] with glycosaminoglycans, which are mainly found outside the cell [25], remains open.

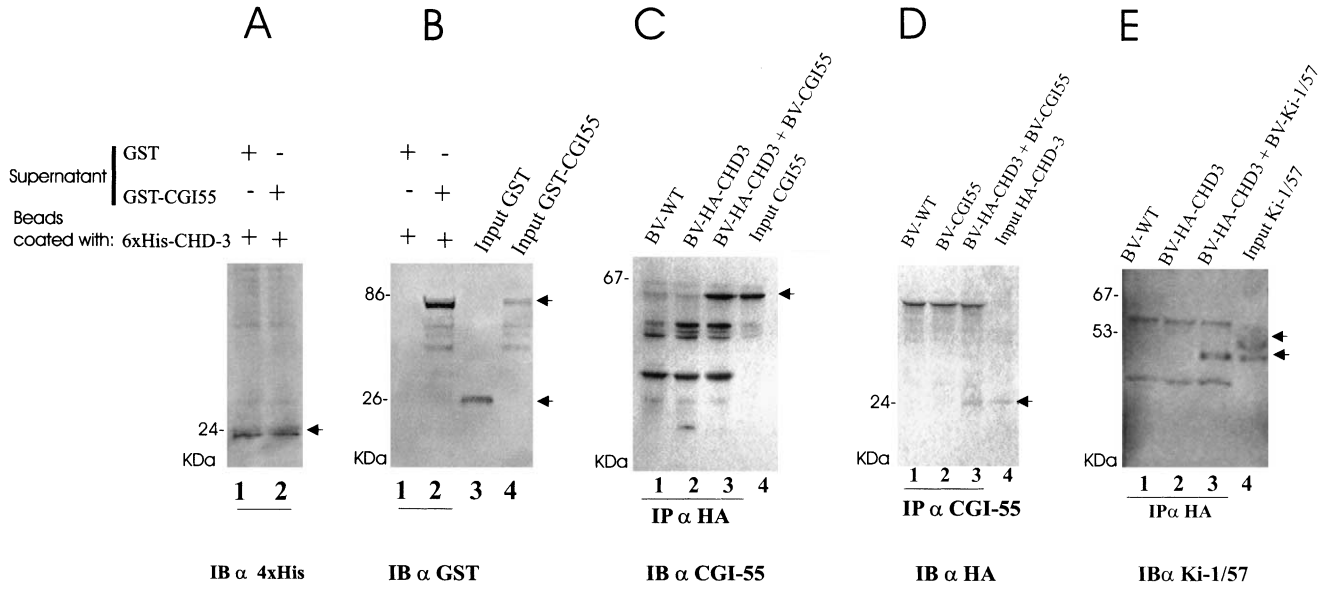

Fig. 5. In vitro binding assay and co-immunoprecipitation from *Sf*9 insect cells infected with different combinations of recombinant BV. A,B: In vitro binding assay. Ni-NTA Sepharose beads were loaded with purified 6×His-HA-CHD3(1983–2000) protein. Beads were then incubated with purified GST or GST–CGI55, washed three times, separated by SDS–PAGE, transferred to PVDF membrane and probed with anti GST antibody (B, lanes 1 and 2) or anti-4×His mAb (A, lanes 1 and 2). Purified GST and GST–CGI55 proteins were used as input controls (B, lanes 3 and 4). C,E: Co-immunoprecipitation of CGI-55 and Ki-1/57 with HA-CHD3. *Sf*9 cells were infected with the BV indicated on top of the panel. C: HA-CHD3(1983–2000) was immunoprecipitated (IP) with anti-HA antibody. The immunoprecipitates were probed (IB, immunoblot) with anti-CGI-55 antibody 10.5.6. Input: expression and identity of CGI-55 were controlled by applying lysate of *Sf*9 cells infected with BV-CGI55 (C, lane 4). D: CGI-55 was immunoprecipitated with antibody 10.5.6. Immunoprecipitates were probed with anti-HA antibody. Input: Lysate of *Sf*9 cells infected with BV-HA-CHD3 (D, lane 4). E: HA-CHD3(1983–2000) was immunoprecipitated with anti-HA antibody. The immunoprecipitates were probed with anti-Ki-1/57 antibody Ki-1. Input: Lysate of *Sf*9 cells infected with BV-Ki-1/57. Molecular mass markers are shown in kDa on left side of the panels. Arrows on the right indicate specific proteins identified by immunoblot.

In order to identify a functional context for the protein paralogs Ki-1/57 and CGI-55 we set out to perform yeast two-hybrid screens to identify possible interacting protein partners. Screens of a human fetal brain cDNA library with both CGI-55 and Ki-/57 identified an alternative spliced version of the DNA remodeling factor CHD-3. The longest CHD-3 clone encodes its C-terminal 412 amino acids and includes part of the predicted DNA-binding domain. The shortest of the interacting clones however, includes only the C-terminal 161 amino acids of the CHD-3 protein, suggesting that the interaction occurs in a very defined region at the C-terminal region of CHD-3. Ki-1/57 also interacted with clones that represent the C-terminal region (1807–2000) of CHD-3.

Our analysis of the sub-cellular localization of the EGFP–CGI55 fusion protein in human HeLa cells suggests that CGI-55 might have both cytoplasmic and nuclear functions and it is tempting to speculate that the distribution of CGI-55 between these two compartments might be regulated like that of other proteins shuttling between the nucleus and the cytoplasma.

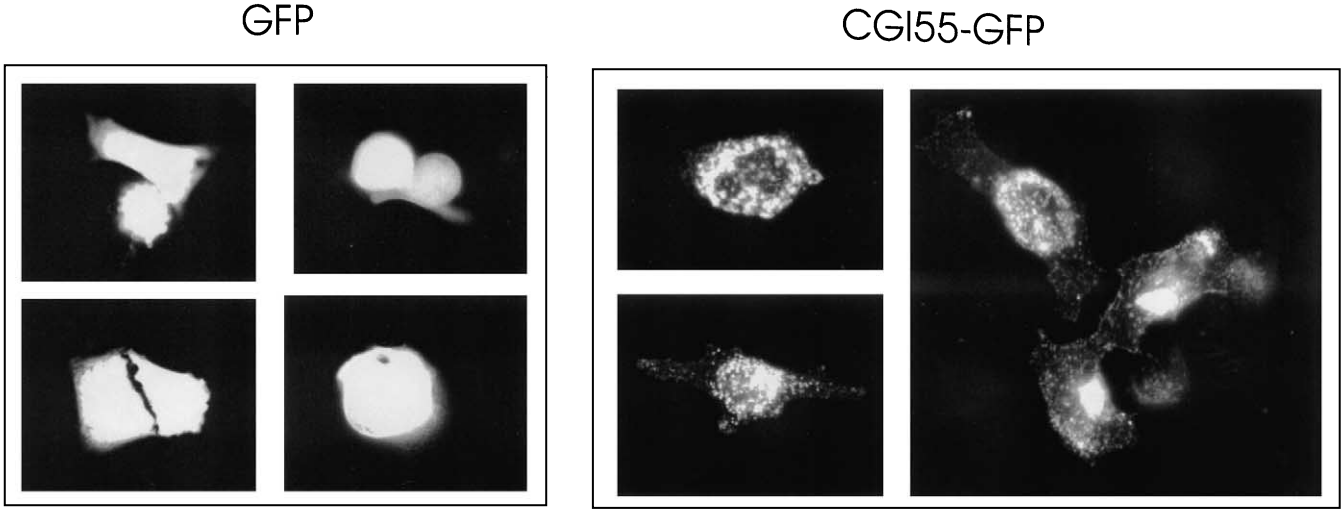

Fig. 6. Cellular Localization of CGI55–EGFP. HeLa cells were transiently transfected with expression vector pEGFP or recombinant expression vector CGI55–pEGFP-N1. After 24 h transfected cells were examined with a Nikon microscope.

In summary, the new functional data on the homologous proteins Ki-1/57 and CGI-55 that we described here, demonstrate that these proteins interact with the nuclear protein CHD-3, which is involved in chromatin-remodeling and transcriptional regulation. To our knowledge, this is the first report that describes a specific protein–protein interaction for CGI-55 and Ki-1/57 and might define them as a new family of proteins. It is tempting to speculate that these interactions might be relevant for the regulation of the CHD-3 mediated chromatin-remodeling.

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III RESULTADOS

Artigo II

Ki-1/57 interacts with RACK1 and is a substrate for the phosphorylation by phorbol 12-myristate 13-acetate-activated protein kinase C

Flávia C. Nery, Dario O. Passos, Vera S. Garcia and Jörg Kobarg

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Ki-1/57 Interacts with RACK1 and Is a Substrate for the Phosphorylation by Phorbol 12-Myristate 13-Acetate-activated Protein Kinase C*

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Flávia C. Nery‡§, Dario O. Passos‡¶, Vera S. Garcia‡, and Jörg Kobarg‡§¶‖

From the ‡Centro de Biologia Molecular Estrutural, Laboratório Nacional de Luz Síncrotron, Rua Giuseppe Máximo Scolfaro 10.000, C.P. 6192, 13084-971 Campinas, SP, Brasil and the Departamentos de §Genética e Evolução and ¶Bioquímica, Universidade Estadual de Campinas, 13083-970 Campinas, SP, Brasil

Ki-1/57, the 57-kDa human protein antigen recognized by the CD30 antibody Ki-1, is a cytoplasmic and nuclear protein that is phosphorylated on serine and threonine residues. When isolated from the Hodgkin's lymphoma analogous cell line L540 Ki-1/57 co-immunoprecipitated with a Thr/Ser protein kinase activity. It has been also found to interact with hyaluronic acid and has therefore been termed intracellular IHABP4 (hyaluronan-binding protein 4). Recent studies demonstrated, however, that Ki-1/57 engages in specific interaction with the chromo-helicase-DNA-binding domain protein 3, a nuclear protein involved in chromatin remodeling and transcription regulation. We used the yeast two-hybrid system to find proteins interacting with Ki-1/57 and identified the adaptor protein RACK1 (receptor of activated kinase 1). Next, we confirmed this interaction *in vitro* and *in vivo*, performed detailed mapping studies of the interaction sites of Ki-1/57 and RACK-1, and demonstrated that Ki-1/57 also co-precipitates with protein kinase C (PKC) when isolated from phorbol 12-myristate 13-acetate (PMA)-activated L540 tumor cells and is a substrate for PKC phosphorylation *in vitro* and *in vivo*. Interestingly, the interaction of Ki-1/57 with RACK1 is abolished upon activation of L540 cells with PMA, which results in the phosphorylation of Ki-1/57 and its exit from the nucleus. Taken together, our data suggest that Ki-1/57 forms a stable complex with RACK-1 in unstimulated cells and upon PMA stimulation gets phosphorylated on threonine residues located at its extreme C terminus. These events associate Ki-1/57 with the RACK1/PKC pathway and may be important for the regulation of its cellular functions.

The first monoclonal antibody that specifically detected the malignant Hodgkin's and Sternberg-Reed cells in Hodgkin's lymphoma was called Ki-1 and binds to the 120-kDa lymphocyte co-stimulatory molecule CD30 (Ki-1/120) on the surface of the Hodgkin's cells (1, 2). It has however been noticed early on that this antibody also cross-reacts with an intracellular phosphoprotein antigen of 57 kDa termed Ki-1/57 (3, 4). *In vitro* phosphorylation experiments performed with the Ki-1/57 antigen isolated from tumor cells demonstrated that it is associated with a serine/threonine protein kinase activity (5). Electron microscopic analysis showed that the Ki-1/57 antigen is located in the cytoplasm, at the nuclear pores, and in the nucleus, where it is frequently found in association with the nucleolus and other nuclear bodies (6). Tryptic digestion of the Ki-1/57 antigen resulted in the cloning of a partial cDNA encoding Ki-1/57 (7). The isolated contig[1] of 1380 bp length encodes the C-terminal 60% of the Ki-1/57 protein. Later, another group cloned the full-length Ki-1/57 cDNA (8). Huang *et al.* (8) found that Ki-1/57 has a hyaluronan binding activity and gave it the second name, intracellular hyaluronan-binding protein 4 (IHABP4). They also found that IHABP4/Ki-1/57 binds to other negatively charged glycosaminoglycans like chondroitin sulfate, heparane sulfate, and RNA, although with lower affinity. The functional meaning of Ki-1/57 interaction with these macromolecules remains open.

When we were searching the sequence data bank for Ki-1/57 related molecules, we found the human protein CGI-55, which amino acid sequence has 40.7% identity and 67.4% similarity with that of Ki-1/57 (9). This high degree of similarity suggests that both proteins might be paralogues and may have related functions. CGI-55 has also been described to bind to the 3′-region of the mRNA encoding the plasminogen activator inhibitor (PAI) type 1 (10). Heaton *et al.* (10) have therefore termed CGI-55 as PAI RNA-binding protein 1 and suggested that it could be involved in the regulation of the stability of the PAI mRNA, although they do not provide experimental data to support this hypothesis.

We explored the yeast two-hybrid system to identify possible interacting proteins for both Ki-1/57 and CGI-55 and in this way obtain clues for the functional context of these proteins. Our analysis resulted in the identification of the human protein chromo-helicase-DNA-binding domain protein 3 (CHD3) as a partner for both proteins (9). The CHD proteins are members of the chromo domain family, a class of proteins that are involved in transcriptional regulation and chromatin remodeling (11–17). The binding of the proteins Ki-1/57 and CGI-55 to CHD3 might define them as a family of CHD3-binding proteins and suggested the possibility that they could be involved in nuclear functions associated with the remodeling of chromatin and the regulation of transcription. Whereas in the case of the

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‖ To whom correspondence should be addressed. Tel.: 55-19-3287-4520 (ext. 516); Fax: 55-19-3287-7110; E-mail: jkobarg@lnls.br.

[1] The abbreviations used are: contig, group of overlapping clones; AR, autoradiography; CHD3, chromo-helicase-DNA-binding domain protein 3; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PDE, phosphodiesterase; IHABP4, intracellular hyaluronan-binding protein 4; PAI, plasminogen activator inhibitor; Ni-NTA, nickel-nitrilotriacetic acid; RACK1, receptor of activated kinase 1; Ki-1/57, the 57 kDa protein antigen detected by antibody Ki-1; WD repeat, denotes conserved tryptophan and aspartic acid residues within this domain.

CGI-55, 42% of the found interacting clones represented CHD3, only 4% of the clones interacting with Ki-1/57 represented CHD3 (9).

Here we report that the vast majority of clones (54%) found to interact with Ki-1/57 represent the scaffold and regulatory protein RACK-1 (receptor of activated kinase 1), a protein that we did not identify in the interaction screen of the putative Ki-1/57 paralogue CGI-55. RACK1 has a molecular mass of 36 kDa and is composed of seven WD repeats (18, 19). Its overall structure resembles that of the β-subunit of G proteins (20, 21). RACK1 has been reported to interact with PKCβ (22–24); Src (25); β-integrins (26); PDE4D5 (27); the β-subunit of the granulocyte-macrophage colony-stimulating factor, interleukin 3, and interleukin 5 receptors (28); type 1 interferon receptor (29); STAT1 (30); and a number of viral proteins (31–33). RACK1 is up-regulated in human carcinomas and during tissue regeneration after ischemic renal injury (34, 35). Furthermore, RACK1 has been functionally implicated in the development of cardiac hypertrophy (36), the regulation of cell adhesion (37), the increase of focal adhesion (38), and the protection from viral, E1A protein-induced apoptosis (32). On a molecular level the interaction of RACK1 with Src has been described to result in an inhibition of the kinase activity of Src (25). The activity of PDE4E5 on the other hand was unaffected by the binding of RACK1 (27). Although RACK1 has been reported to have a stimulatory effect on the substrate phosphorylation by PKC (22, 39), others found that RACK1 does not influence the kinase activity of serine/threonine kinases such as PKC, cAMP-dependent protein kinase, and casein kinase II (25), indicating that the RACK1 activity on PKC may be influenced by the kind of substrate involved.

Here we show that RACK1 interacts with Ki-1/57, confirm this interaction *in vitro* and *in vivo*, and map the interaction sites of Ki-1/57 and RACK-1 in detail. Furthermore, we found that Ki-1/57 is a substrate for PKC and that its interaction with RACK1 is abolished in the course of the PMA activation of the cells. Our data suggest that Ki-1/57 is involved in specific protein-protein interactions and provide a plausible explanation for the long known fact that Ki-1/57, which does not contain a kinase domain, in fact co-precipitates with kinase activity. The co-precipitated kinase activity appears to be PKC. This could be confirmed by the co-immunoprecipitation of Ki-1/57 with PKC, which associates with Ki-1/57 after PMA stimulation of the cells. Our results further suggest that the cellular functions of Ki-1/57 may be subject to regulation via a PKC/RACK1 pathway.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Several sets of oligonucleotides were designed to allow subcloning of the complete Ki-1/57 coding region in different expression vectors. Insertion into pGEX-2TK (Amersham Biosciences) allowed expression of Ki-1/57(1–413) as a C-terminal fusion to GST (GST-Ki-1/57). The cDNAs encoding full-length Ki-1/57, Ki-1/57(122–413), and the eight other indicated deletion constructs (numbered in the same way) were inserted into the yeast two-hybrid expression vector pBTM-116 (40–43). Other deletions were also subcloned into bacterial expression vectors peT28a, pProEx, or pGEX to allow their expression as His_6-tagged or GST-tagged fusion proteins. In a similar fashion the cDNAs encoding the indicated deletions of the protein RACK-1 were amplified and inserted into the yeast two-hybrid vector pGAD424 (Clontech), and full-length RACK1 was inserted into the bacterial expression vector pET-28a (Novagen) to allow expression of the His_6-RACK1 fusion protein.

Yeast Two-hybrid Screening and Interaction Analysis—The pBTM116-Ki-1/57(122–413) (40) vector was used to express a fragment spanning 60% of the C terminus of the protein Ki-1/57 linked to the C terminus of LexA DNA-binding domain peptide in *Saccharomyces cerevisiae* strain L40. A human fetal brain cDNA library (Clontech) expressing GAL4 activation domain fusion proteins was co-transformed with the recombinant pBTM116-Ki-1/57 vector. Selection of transformants, the β-galactosidase activity test, plasmid DNA extraction, and sequencing were performed as described previously (9).

Bacterial Expression and Protein Purification—GST, GST-Ki-1/57, and His_6-RACK-1 proteins were expressed in *Escherichia coli* BL21-CodonPlus-RIL (Stratagene) and purified using glutathione-Sepharose 4B (Amersham Biosciences) or Ni-NTA-Sepharose as described before (44).

In Vitro Binding Assay, Western Blot Analysis, Antibodies, and Cell Culture—GST or GST-Ki-1/57 fusion proteins were coupled to glutathione-Sepharose beads. After washing, the beads were incubated with His_6-RACK-1 for 2 h at 4 °C and then washed with buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl). The proteins bound to the beads were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and visualized by immunochemiluminescence using a mouse anti-GST antibody (for control of equal loading of beads) or anti-His_5 monoclonal antibody (Qiagen) and secondary anti-mouse IgG-horseradish peroxidase conjugate. The anti-RACK-1 monoclonal antibody was from Transduction Laboratories. The specific anti-Ki-1/57 monoclonal antibodies A26, E203 (7), and Ki-1 (1) have been described previously. Anti-Ki-1/67 control antibody had been provided by Prof. Dr. Hilmar Lemke (45). An anti-phospho-PKC antibody sampler kit was purchased from Cell Signaling Technology. L540 Hodgkin's analogous cells (46) were cultivated in RPMI 1640 medium, supplemented with 20% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C and 5% CO_2 (L540 standard medium). HeLa cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) under equal conditions.

In Vivo Binding Assay—5.0×10^7 L540 cells were or were not stimulated with PMA (100 ng/ml) for 4 h (33). The cells were lysed in 1 ml of buffer NaCl/Tris (25 mM Tris, pH 7.5, 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100, protease inhibitors). The lysates were treated with DNase (Promega) and cleared at $14.000 \times g$ for 30 min. Next 20 μl of protein A-Sepharose beads (Amersham Biosciences) were loaded with the indicated antibodies overnight at 4 °C, washed in buffer NaCl/Tris, and incubated with the L540 lysate overnight at 4 °C. After further three washes with the buffer Tris/EDTA (10 mM Tris, pH 7.5, 1 mM EDTA, 0.5 M NaCl), the beads were resuspended in SDS-PAGE loading buffer, boiled, and analyzed by SDS-PAGE and Western blot using the indicated antibodies. Western blots were developed by chemiluminescence as described previously (44). Loading controls consisted either of protein detection by SDS-PAGE or of Western blot development with control antibodies as indicated in the figures.

In Vitro Phosphorylation Assay and Phosphoamino Acid Analysis—5×10^7 L540 cells were treated or not with PMA, collected, and lysed as described above. Endogenous PKC was immunoprecipitated from these lysates with anti-phospho-PKC-Pan antibody (Cell Signaling) coupled to protein A-Sepharose beads. Next these beads were incubated with purified GST-Ki-1/57, His_6-RACK-1, both, or GST in kinase buffer (25 mM Tris, pH 7.5, 1.32 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM EDTA, 1.25 EGTA, 1 mM dithiothreitol) containing 10 nM PMA, 5 μM ATP, and 0.5 μCi of [γ-^{32}P]ATP, in a total volume of 25 μl for 30 min at 30 °C. Phosphorylated proteins were run out by SDS-PAGE. The gel was stained, dried, and exposed to x-ray film. In other experiments purified GST, GST-Ki-1/57, His_6-RACK1, and deletion constructs of Ki-1/57 were phosphorylated in complete kinase buffer in a final volume of 50 μl at 30 °C with purified PKC-Pan, PKCζ, or PKCθ for 15 min. The PKCζ or PKCθ are human recombinant His-tagged and affinity-purified proteins (Promega). PKC-Pan was purified from rat brain and consists predominantly of the PKC isoforms α, β, and γ (Promega). Radioactively labeled proteins were visualized as described above.

Phosphoamino acid analysis was basically performed as described in Machado *et al.* (47). Briefly, the ^{32}P-radiolabeled phosphorylated proteins were hydrolyzed with 6 N HCl for 60 min at 90 °C. The hydrolysate was lyophilized, dissolved in water, and spotted onto Sigma cell type 100 cellulose thin layer chromatography plates (Sigma). The solvent system was isobutyric acid, 0.5 M ammonium hydroxide (5:3). Phosphoserine, phosphothreonine, and phosphotyrosine standards (2 μg) (Sigma) were mixed with the radiolabeled protein hydrolysate and spotted together on the TLC plates. Amino acids were visualized with 0.2% ninhydrin in ethanol, and radiolabeled residues were detected by autoradiography (AR). Theoretical phosphorylation site prediction was performed by the software NetPhos 2.0 Prediction server available at the web site of the Center for Biological Sequence Analysis (www.cbs.dtu.dk/services/NetPhos).

Metabolic Labeling, in Vivo Phosphorylation Assay, and Kinase Inhibitors—5×10^6 L540 cells were preincubated or not for 1 h with

protein kinase inhibitors: Ro-32-0432 (28 nM), and staurosporine (0.7 nM) (Calbiochem). This inhibitor incubation was performed with phosphate-free L540 standard medium (the fetal calf serum in this medium had been dialyzed against a 150 mM NaCl solution). Next the cells were activated or not by the addition of 100 ng/ml of PMA for a second hour. In parallel to the PMA treatment, the cells were metabolically labeled by the addition of 0.4 mCi of radioactive ^{32}P-labeled inorganic phosphate (Amersham Biosciences). After lysis Ki-1/57 was immunoprecipitated from the lysates of the metabolically labeled L540 cells with anti-Ki-1 antibody A26 coupled to protein A-Sepharose beads and analyzed by autoradiography and SDS-PAGE.

Preparation of Cytoplasmic and Nuclear Cell Fractions—L540 cells were harvested and incubated with 300 μl of hypotonic buffer A (10 mM Tris, pH 7.5, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and a mixture of protease inhibitors) for 30 min on ice (33). The nuclei were recovered by centrifugation at 14,000 rpm for 10 min. The supernatant represents the cytoplasmic fraction. To obtain the nuclear fraction, the crude nuclear pellet was resuspended in 200 μl of hypertonic buffer B (20 mM Tris, pH 8.0, 0.4 M NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 25% v/v glycerol) followed by incubation on ice for 30 min. After centrifugation, the fractions were incubated with the antibodies at 4 °C overnight. On the next day 20 μl of protein A-Sepharose were added for 2 h.

Immunofluorescence Analysis—HeLa cells grown on glass coverslips were stimulated or not with PMA for 4 h at 37 °C. The cells were fixed with 100% methanol and immunostained with primary antibody monoclonal mouse Ki-1, mouse anti-RACK1, or rabbit anti-Phospho-PKC, and secondary antibody fluorescein anti-mouse or rhodamine anti-rabbit antibody. The cells were examined with a Nikon microscope. DAPI staining was used to show the positions of the nuclei. The cells were examined with Nikon fluorescence microscope. Immunolabeled proteins were presented with the respective color. Superimposing the two colors (merge) results in a yellow/orange signal.

RESULTS

Yeast Two-hybrid Screen—To identify Ki-1/57 interacting proteins, the yeast two-hybrid system (40–43) was employed, utilizing a human fetal brain cDNA library (Clontech). In a first screen we used a fragment of the Ki-1/57 cDNA that encodes its C-terminal 60% as a bait. 2.0×10^6 screened cotransformants yielded 250 clones positive for both His3 and LacZ reporter constructs. Library plasmids DNA of 80 clones were sequenced. 54% of the sequenced clones all encoded the full-length protein RACK1 (48). Another protein identified was CHD3, which had already been described previously elsewhere (9) and represented 4% of the interacting clones. Other nuclear proteins involved in the regulation of transcription have also been identified but will be described elsewhere.

Mapping the Interaction Sites of Ki-1/57 and RACK1—Next, we mapped the Ki-1/57 region required for the interaction with RACK1 using the yeast two-hybrid method (Fig. 1). N- and C-terminal deletion constructs of the Ki-1/57 protein were fused to the LexA DNA-binding domain (Fig. 1*A*) and tested for their ability to bind full-length RACK1 (Fig. 1*B*). The two constructs that encompass the N-terminal and central regions: Ki-1/57(1–150) and Ki-1/57(151–263) failed to bind to RACK1. Full-length Ki-1/57, the C-terminal construct used in the two hybrid screen Ki-1/57(122–413) as well as the C-terminal deletion Ki-1/57(264–413) all interacted with RACK1. This suggests that the RACK1-binding site is located at the Ki-1/57 C terminus. The co-transformation of pBTM116-Ki-1/57 with several unrelated "bait" constructions, including pACT2-AUF1 (44) (not shown) and with empty pBTM116 vector (Fig. 1*B*), showed no interaction.

Furthermore, we mapped the RACK1 regions that are required for the interaction with Ki-1/57. N- and C-terminal deletion constructs of the RACK1 protein were fused to the Gal4 activation domain (vector pACT2; Fig. 1*C*) and tested for their ability to bind to full-length Ki-1/57 (Fig. 1*D*). None of the four different deletion constructs of RACK1 interacted with Ki-1/57. This shows that full-length RACK1 is required for an interaction with Ki-1/57.

In Vitro Confirmation of the Ki-1/57-RACK1 Interaction with Purified Fusion Proteins—To confirm the interaction between Ki-1/57 and RACK1 *in vitro*, we next performed *in vitro* pull-down assays with purified recombinant proteins that had been expressed in *E. coli* (GST, GST-Ki-1/57, and His_6-RACK1) and purified by affinity chromatography. GST-Ki-1/57 bound specifically to RACK1, whereas the control protein GST did not (Fig. 2*A*, *left panel*). We controlled the equal loading of the glutathione beads with GST or GST-Ki-1/57 fusion protein by developing the same membrane with an anti-GST monoclonal antibody (Fig. 2*A*, *right panel*).

Co-precipitation of Ki-1/57 with His_6-RACK1 from a Lysate of L540 Cells—When a lysate of L540 cells was incubated with His_6-RACK1-loaded Ni-NTA-Sepharose beads, Ki-1/57 could be specifically pulled down (Fig. 2*B*, *left panel*). On the other hand, when Ni-NTA-Sepharose beads were loaded with control proteins such as the nonrelated protein His_6-FEZ1 (49) or the Ki-1/57 homologue protein CGI-55 (9), no co-precipitated Ki-1/57 band could be detected in the Western blot. We obtained a corresponding result when we used glutathione-Sepharose control beads loaded with GST-Ki-1/57 (Fig. 2*C*, *left panel*). RACK1 was only pulled down from the lysate of L540 cells with the GST-Ki-1/57 but not with the GST-loaded beads. Both Western blot experiments were checked by detecting the input proteins. These were run out on SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and visualized by anti-His_5 monoclonal antibody or anti-GST antibody, depending on the nature of the fusion part (Fig. 2, *right panels*).

In Vitro Phosphorylation of Ki-1/57 by PKC Isolated from PMA-stimulated L540 Cells—Early experiments with the Ki-1/57 antigen had demonstrated that the Ki-1 antibody immunoprecipitated an serine/threonine protein kinase activity that had been initially attributed to the Ki-1/57 antigen (5). The cloning of the cDNA encoding Ki-1/57 revealed, however, that the Ki-1/57 sequence does not encode a kinase domain (7, 8). Further experiments with the full-length recombinant Ki-1/57 protein also did not reveal a kinase activity of the Ki-1/57 protein toward itself or other proteins (data not shown). Our finding that Ki-1/57 strongly interacts with the protein RACK1 in the yeast two-hybrid system immediately suggested a hypothesis for an alternative explanation for the co-immunoprecipitation of the kinase activity with Ki-1/57. RACK1 is an receptor for activated protein kinase C and interacts *in vitro* and *in vivo* with activated PKC. Therefore, the observed kinase activity in the Ki-1 immunoprecipitate might be PKC associated to RACK1 and/or to Ki-1/57. To test this hypothesis, we first analyzed whether Ki-1/57 can be a substrate for the phosphorylation by PKC immunoprecipitated from the lysate of unstimulated and PMA-stimulated L540 cells (Fig. 3*A*, *left panel*). The GST-Ki-1/57 fusion protein used in this assay was only weakly phosphorylated by the PKC that had been immunoprecipitated from the lysate of nonactivated cells by an anti-Phospho-Pan-PKC antibody (Fig. 3*A*, *left panel*, *second lane*). It was, however, strongly phosphorylated by the PKC isolated from the lysate of PMA-activated L540 cells (Fig. 3*A*, *fifth lane*). RACK1 itself did not suffer phosphorylation by PKC under these conditions (Fig. 3*A*, *left panel*, *first* and *fourth lanes*), nor did its presence influence the extend of phosphorylation of Ki-1/57 (Fig. 3*A*, *left panel*, *third* and *sixth lanes*). The *right panel* of Fig. 3*A* demonstrates the equal protein loading of the different lanes. GST control protein was not phosphorylated by PKC (Fig. 3*B*, *upper panel*, *first lane*).

In a similar approach we next wanted to know whether PKC isoforms have a differential phosphorylation activity toward Ki-1/57 and tested therefore a panel of monoclonal antibodies against different PKC subtypes isolated from PMA-activated

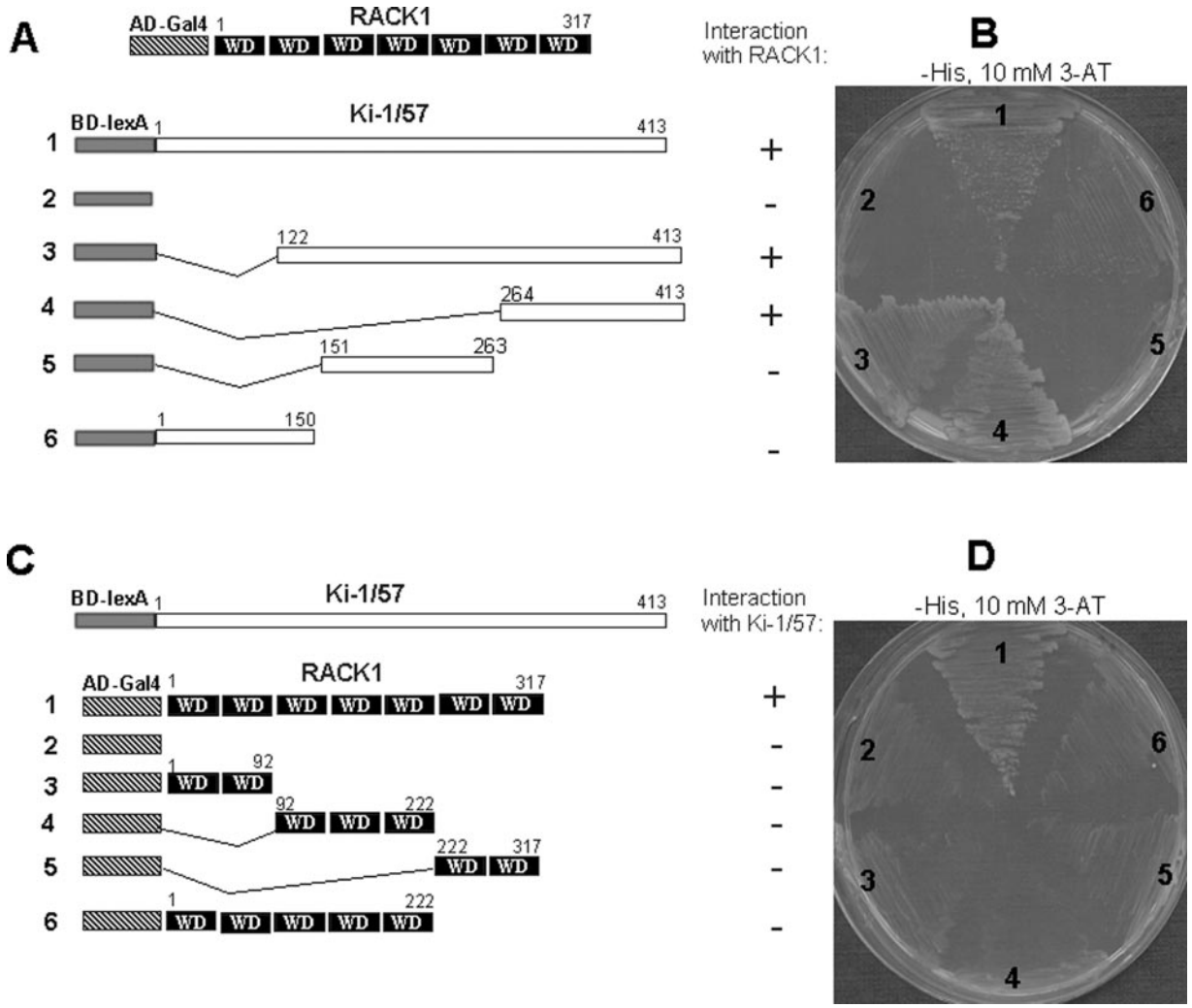

FIG. 1. **Mapping of the Ki-1/57 and RACK1 interaction sites.** *A*, schematic representation of Ki-1/57. Various N- and C-terminal truncations of human Ki-1/57 (*open boxes*) were fused in-frame to the DNA-binding domain of LexA (*filled boxes*) in plasmid pBTM116 and transformed into yeast L40 together with the fusion full-length RACK1-Gal4 activation domain construct in vector pACT2. *B*, interaction was determined by the ability of the co-transformant cells to grow on minimal medium,-Trp,-Leu,-His plates in the presence of 10 mM 3-amino-1,2,4-triazole inhibitor for suppression of unspecific reporter activation. Presence of bait and prey plasmids in the co-transformed cells was controlled by growth on minimal medium-Trp,-Leu (data not shown). *C*, schematic representation of RACK1. The positions of the seven WD motifs are indicated. Various N- and C-terminal truncations of human RACK1 (*dark boxes*, *WD*) were fused in-frame to the DNA-binding domain of Gal4 activation domain (*hatched boxes*) in plasmid pACT2 and transformed into yeast L40 together with the fusion full-length Ki-1/57 (*open box*) cloned in pACT2. *D*, interaction was determined as in *B* in the presence of 10 mM 3-amino-1,2,4-triazole.

L540 cells (Fig. 3*B*, *upper panel*). The PKCs had been immunoprecipitated by anti-phospho-PKC antibodies or as indicated in Fig. 3*B*. We found a strong phosphorylation of Ki-1/57 by PKC$\alpha\beta$, PKCδ, PKCλ/ζ, and especially by PKCθ, however not by PKCμ. These data show that Ki-1/57 can serve in principal as a substrate for a wide variety of different PKC isoforms but also that its phosphorylation is strongest with PKCθ. The equal loading of the different lanes is demonstrated by the control SDS-PAGE shown at the *bottom panel* of Fig. 3*B*.

We also performed phosphorylation experiments with commercial purified PKC-Pan and found a strong phosphorylation of Ki-1/57, which was neither promoted nor inhibited by the presence of His_6-RACK1 protein in equal amounts or slight excess (Fig. 3*C*).

Ki-1/57 Interaction with RACK1 Is Abrogated by Its Phosphorylation or by the PMA Activation of the Cell in Vivo—When we performed the pull-down experiments of RACK1 with a GST-Ki-1/57 protein that we had previously submitted to *in vitro* phosphorylation with PKC-Pan, we observed a complete abrogation of the interaction (Fig. 4*A*, *left panel*, *lane 3*). Such a down-regulation of the interaction by the phosphorylation of Ki-1/57 might be functionally relevant and could serve to down-regulate the adaptor functions of RACK1 once that PKC has phosphorylated Ki-1/57 *in vivo*. Therefore we tested whether this effect can also be observed *in vivo*. We found that His_6-RACK1 fusion protein coupled to Ni-NTA-Sepharose beads co-sediments an approximately three times smaller quantity of Ki-1/57 from lysates of PMA stimulated then from unstimulated L540 cells (not shown). This indicates that the phosphorylation of Ki-1/57 *in vivo* also diminishes its capacity to interact with external recombinant RACK1.

Ki-1/57 Is Only Phosphorylated on Its Extreme C Terminus (Residues 346–413)—We now expressed several deletion constructs of the Ki-1/57 protein and submitted the purified recombinant proteins to *in vitro* phosphorylation experiments to determine the regions of Ki-1/57 that are a target for the phosphorylation by PKC (Fig. 4*B*). From these studies it became clear that neither the N-terminal region (1–150) nor the middle region of Ki-1/57(151–263) but only its C-terminal region (both 264–413 and 122–413) are phosphorylated by PKC-Pan (Fig. 4*B*, *left panel*). Recombinant PKCθ and PKCζ gave the same results as purified PKC-Pan (not shown). The loading

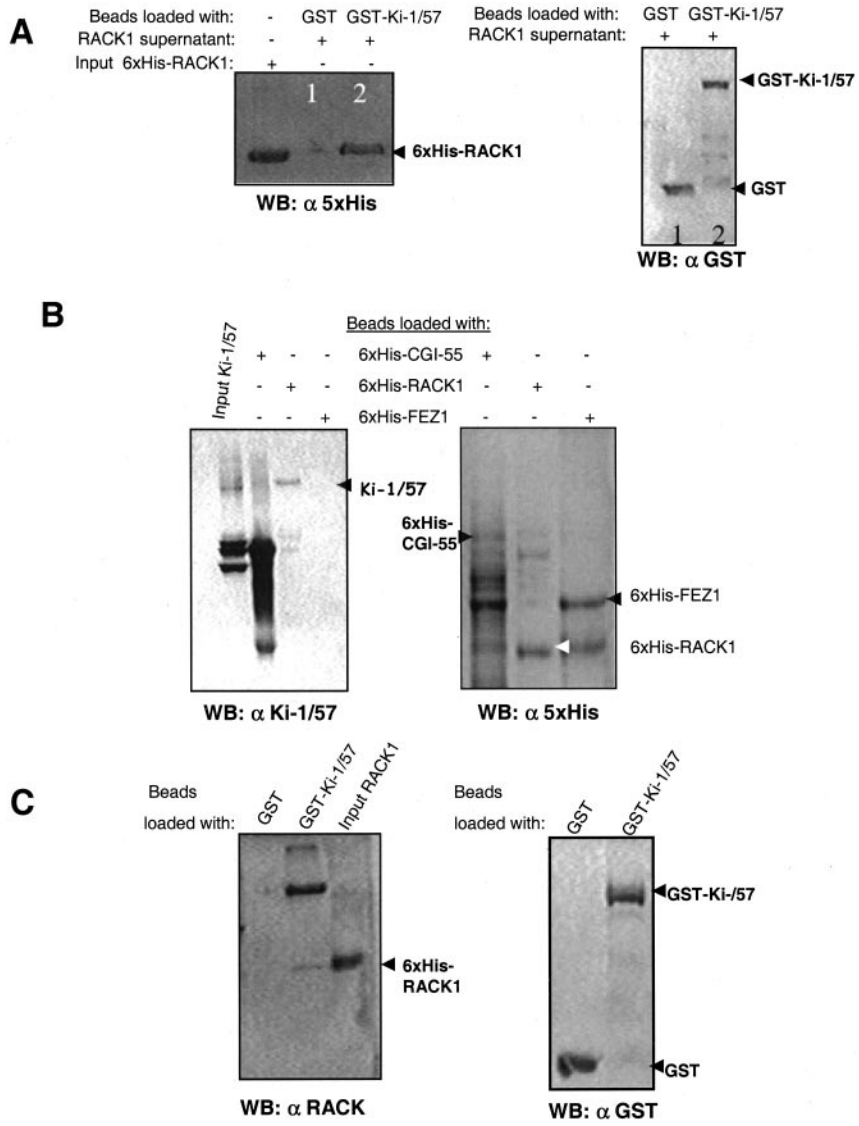

FIG. 2. ***In vitro* binding assays of RACK1 and Ki-1/57.** *A*, glutathione-Sepharose beads were loaded with purified GST or GST-Ki-1/57 proteins. The beads were then washed and incubated with purified His_6-RACK1, washed three times, separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with anti-His_5 antibody (*left panel*) or anti-GST monoclonal antibody (*right panel*). *B*, Ni-NTA-Sepharose beads were loaded with His_6-RACK1 or control proteins His_6-CGI-55 or His_6-FEZ1. Loaded beads were than incubated with the total cell lysate of 1×10^7 L540 cells and washed three times. The bound proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane and probed with Ki-1/57 antibody (A26). *C*, glutathione-Sepharose beads were loaded with GST or GST-Ki-1/57 proteins and incubated with lysate of L540 cells for 3 h at 4 °C. After washes and processing as above in *B*, RACK1 was probed with specific antibody. Purified GST, GST-Ki-1/57, and His_6-RACK1 proteins were used to identify the precipitated bands on the blot. The *arrows* indicate the detected proteins. *A* and *B*, the panels shown on the *right* represent membranes that were developed with control antibodies against the fusion part GST or His_6 of the used recombinant proteins to demonstrate equal loading. *WB*, Western blot.

of the *in vitro* phosphorylation reaction with equal amounts of recombinant protein fragments is shown in Fig. 4*B* (*right panel*). These results suggested performance of a more detailed deletional analysis of the C terminus of Ki-1/57, because this region contains 15 Ser/Thr residues (Fig. 5*A*), all of which could be target residues of phosphorylation by PKC. Therefore, we generated the indicated subdeletions (Fig. 5*A*) of the fragment Ki-1/57(264–413) and expressed them in bacteria as GST fusions proteins for *in vitro* studies and in the yeast as LexA fusion proteins to be able to assess their capacity to still interact with RACK1. Two of the constructs, Ki-1/57(294–413) and Ki-1/57(346–413) were still able to interact with RACK1 (Fig. 5*B*). Most interestingly, these same two constructs, when expressed in fusion with GST and used as substrates in the *in vitro* phosphorylation assays with PKC-Pan, were the only two of the five tested subdeletions that could be phosphorylated (Fig. 5*C*, *left panel*). *In vitro* phosphorylations of these five fragments with PKCθ and PKCζ gave the same result (not shown). Fig. 5*C* (*right panel*) shows equal loading of the reactions with proteins or control protein GST, which was not phosphorylated.

Ki-1/57 Phosphorylation Can Be Blocked by Protein Kinase Inhibitors in Vitro and in Vivo—To gather further evidence that the kinase that phosphorylates Ki-1/57 is PKC, we tested a series of protein kinase and PKC inhibitors for their potential to block Ki-1/57 phosphorylation *in vitro* (Fig. 4*C*) and *in vivo* (Fig. 4*D*). We found that the general kinase inhibitor staurosporine and the PKC-specific inhibitor Ro-32-0432 were the most effective inhibitors of the phosphorylation of His_6-Ki-1/57 (264–413) by PKC-Pan (Fig. 4*C*, *lanes 7* and *8*), PKCζ, and PKCθ (not shown) *in vitro*. There was no difference in the inhibition profile for the three PKCs tested. We then tested the best two inhibitors *in vivo* and found that only Ro-32-0432 but not staurosporine (at the tested relative low concentration) can inhibit the phosphorylation of Ki-1/57 *in vivo* (Fig. 4*D*, *lanes 3* and *4*). These data support the hypothesis that Ki-1/57 is also a substrate for PKC phosphorylation *in vivo*. A comparison of *lanes 1* and *2* of Fig. 4*D* demonstrates the increased phosphorylation of Ki-1/57 after the stimulation of the L540 cells with PMA and in the absence of inhibitor. The equal loading of the lanes with immunoprecipitated Ki-1/57 is shown in the *lower panel* of Fig. 4*D*.

Ki-1/57 Is Phosphorylated at Two Threonine Residues Located at Its Extreme C Terminus (Residues 346–413)—Our phosphoamino acid analysis confirmed previously published data (5) that Ki-1/57(264–413) is phosphorylated mainly on threonine residues. PKCζ phosphorylated Ki-1/57 (264–413) strongly on threonine but also on serine residues (Fig. 5*D*), whereas PKC-Pan phosphorylates this fragment on threonine only.

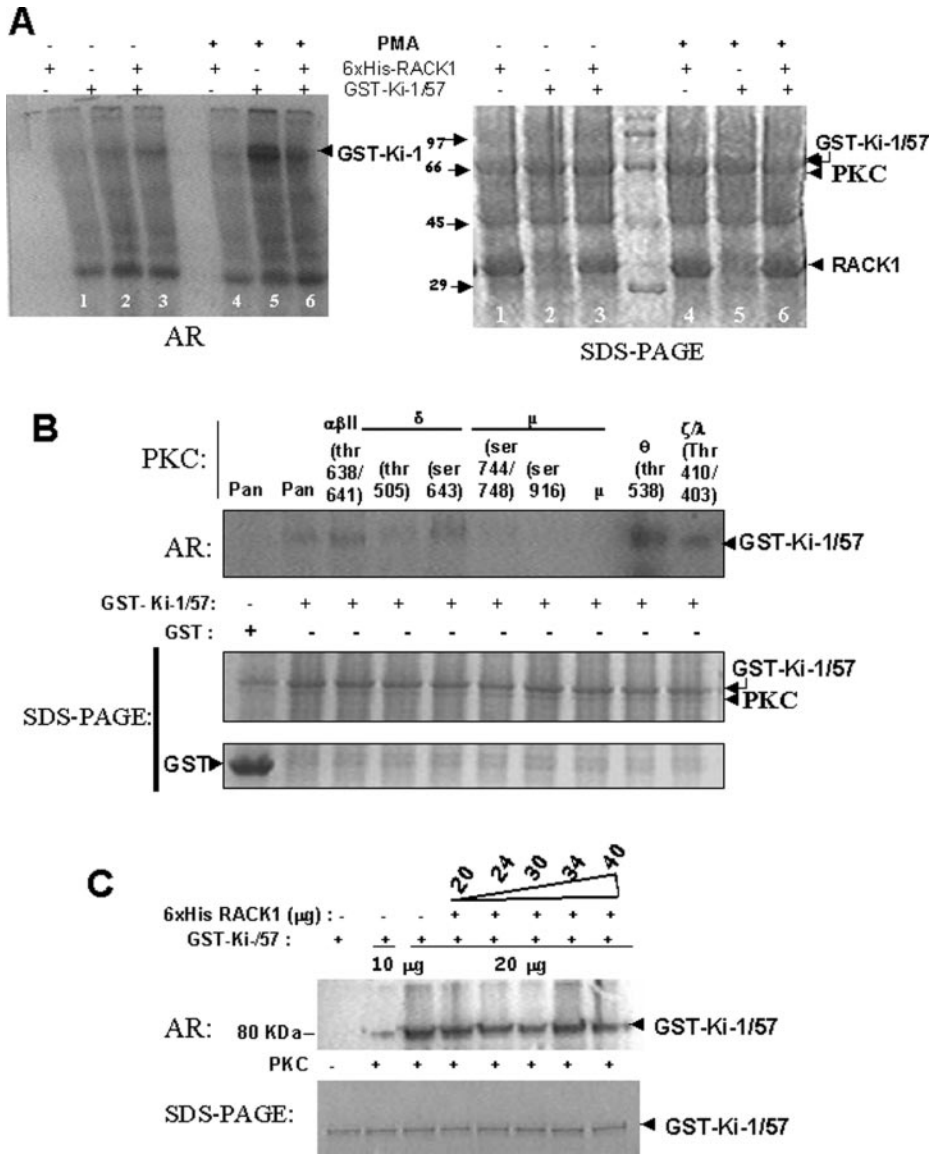

FIG. 3. ***In vitro* phosphorylation of Ki-1/57 by PKC.** *A*, L540 cells were or were not treated for 4 h with PMA. PKC was immunoprecipitated (*IP*) from the lysate of 10^7 L540 cells with anti-phospho-PKC-Pan antibody and then incubated with His_6-RACK1 or GST-Ki-1/57 or both in the presence of [γ-^{32}P]ATP, for 1 h at 37 °C. *Left panel*, radiolabeled proteins were detected by AR. The *right panel* demonstrates equal loading of lanes with tested proteins. *B*, immunoprecipitation of different isoforms of PKC from the lysate of equally PMA-treated L540 cells. The PKC was isolated with antibodies directed against the indicated phosphorylated amino acids in PKC, with the exception of PKC μ, which is recognized by its antibody in the nonphosphorylated form. GST or GST-Ki-1/57 were then *in vitro* phosphorylated with the indicated immunoprecipitated phospho-PKC isoforms. *Upper panel*, AR. *Lower panels*, show loaded proteins by SDS-PAGE. *C*, *in vitro* phosphorylation of GST-Ki-1/57 by purified PKC-Pan is neither inhibited nor stimulated by the presence the His_6-RACK1. Recombinant GST-Ki-1/57 was phosphorylated by PKC-Pan (0.02 unit) with or without increasing amounts of RACK1. *Upper panel*, AR. *Lower panel*, SDS-PAGE of loaded GST-Ki-1/57.

The extreme C-terminal fragment Ki-1/57(346–413) in fusion with GST is only phosphorylated on threonine using PKC-Pan. PKCθ and PKCζ also phosphorylated this fragment only on threonine, but the degree of phosphorylation was lower (not shown). This suggests that the two threonine residues present in this fragment (Thr^{354} and Thr^{375}) might be the main target residues for phosphorylation by PKC *in vitro*. This also demonstrates that the phosphorylation of Ki-1/57 by PKC is highly specific, considering that there are 34 Ser/Thr residues in the whole amino acid sequence of Ki-1/57, and apparently only the two most C-terminal threonines are targets of phosphorylation *in vitro*. The reaction was controlled with free GST protein, which itself does not suffer phosphorylation by the three PKCs tested (not shown; see also Fig. 5*C*).

Ki-1/57 Interacts with RACK1 in Vivo Only before PMA Activation and with PKC Afterward—Next we wanted to test whether Ki-1/57 engages also in interaction with RACK1 and PKC in human cells. To assess the subcellular localization of the co-immunoprecipitated proteins, we analyzed the cytoplasmic and nuclear compartments of the L540 cells separately. When we immunoprecipitated RACK1 from the lysate of L540 cells, we detected the 57-kDa band of co-immunoprecipitated Ki-1/57 in the Western blot developed with A26 antibody only in the nuclear compartment of the cell but both before and after cell stimulation with PMA (Fig. 6, *lanes 25* and *26*). There was a slight decrease in the co-precipitated amount of Ki-1/57 after the addition of PMA.

When we immunoprecipitated Ki-1/57 we also co-immunoprecipitated RACK1 but only from the nucleus and in the absence of PMA (Fig. 6, *lane 16*). These results suggest that Ki-1/57 and RACK1 form a stable complex in human L540 cells, until the cells are activated by PMA. Interestingly, we also detected co-immunoprecipitated Ki-1/57 when we used anti-phospho-PKCθ (and anti-phospho-PKCζ, not shown) antibody in the immunoprecipitation, in the nucleus and a little less in the cytoplasm, but only after PMA activation (Fig. 6, *lanes 30* and *32*). Anti-phospho-PKC$\alpha\beta$II did not co-immunoprecipitate Ki-1/57, neither with our without PMA treatment (not shown).

Ki-1/57 Exits the Nucleus upon PMA Activation—Ki-1/57 is located in the cytoplasm and the nucleus of cells (6) and interacts with nuclear proteins involved in the regulation of transcription and the remodeling of chromatin such as CHD3 (9). Therefore, we were interested to know whether its localization to the nucleus or that of its interacting proteins RACK1 and PKC is affected by the cell stimulation with the PKC activator PMA and whether the interaction of RACK1 and Ki-1/57 is affected by PMA. First, we stimulated L540 cells with PMA or not and then fractionated the cellular lysates into cytoplasmic

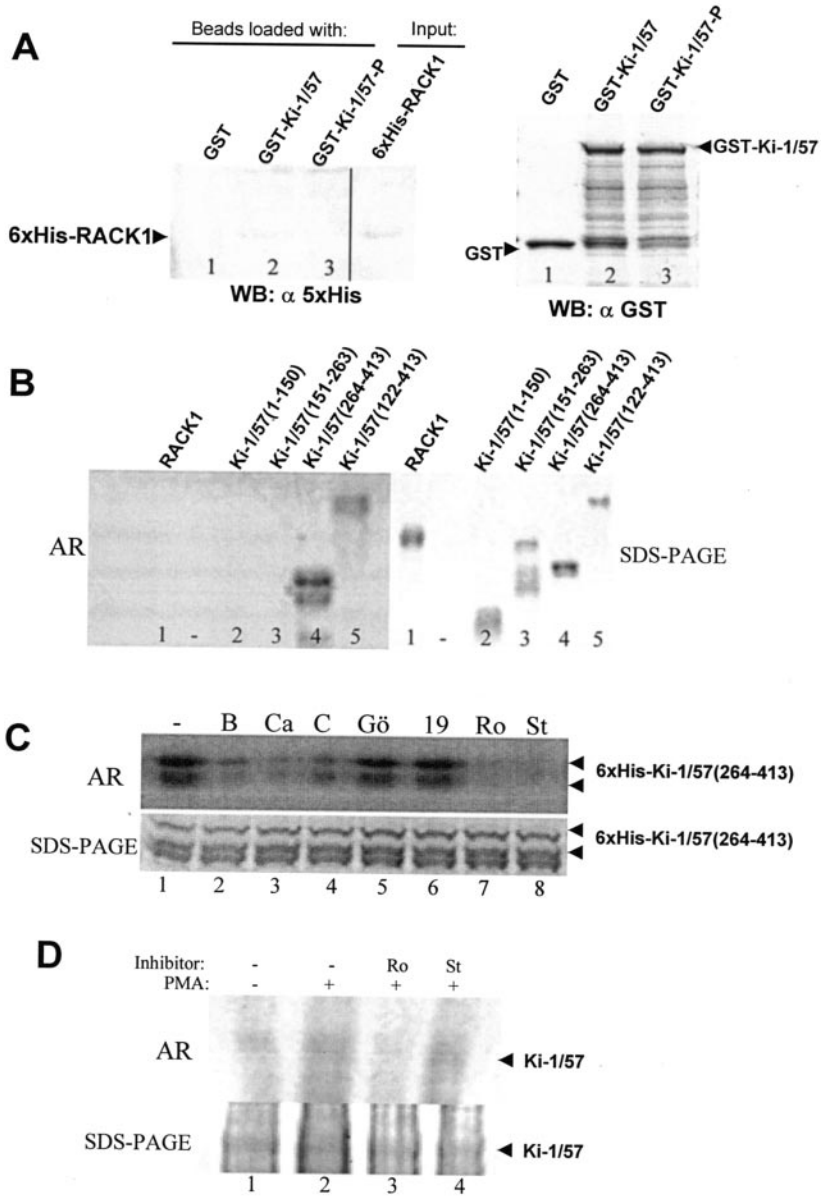

FIG. 4. **The interaction between Ki-1/57 and RACK1 *in vitro* is abolished by the phosphorylation of Ki-1/57.** *A*, *left panel*, GST-Ki-1/57 *in vitro* phosphorylated by purified PKC-Pan no longer co-precipitates RACK1 *in vitro*. *Right panel*, Western blot (*WB*) anti-GST for demonstration of equal protein loading of the lanes. *B*, *in vitro* phosphorylation of different deletion constructs of Ki-1/57 by recombinant PKCθ. All of the indicated fragments of Ki-1/57 were expressed as fusion proteins with an N-terminal His_6 tag and had been purified by affinity chromatography. *Left panel*, autoradiography of ^{32}P-labeled proteins (*AR*). *Right panel*, control SDS-PAGE of loaded proteins. *C*, *in vitro* inhibition of His_6-Ki-1/57 (264–413) phosphorylation by PKC-Pan by using a panel of different kinase inhibitors. *Upper panel*, autoradiography after incubation of PKC-Pan and GST-Ki-1/57 without or with the indicated protein kinase inhibitors: Bisindoylmaleimide I (*lane B*, 10 nM), calphostin C (*lane Ca*, 50 nM), chelerythrine chloride (*laneC*, 660 nM), Gö 6976 (*lane Gö*, 6.2 nM), inhibitor 19-27 (*lane19*, 8 μM), Ro-32-0432 (*lane Ro*, 28 nM), and staurosporine (*laneSt*, 0.7 nM). *Lower panel*, SDS-PAGE for demonstration of equal loading with protein. *D*, *in vivo* inhibition of the phosphorylation of Ki-1/57 in L540 cells treated or not with PMA for 1 h and incubated with or without the protein kinase inhibitors Ro-32-0432 (*Ro*) and staurosporine (*St*). Ki-1/57 was immunoprecipitated from the nuclear fraction. *Upper panel*, AR. *Lower panel*, control SDS-PAGE to demonstrate equal loading with immunoprecipitate.

and nuclear fractions, from which we then immunoprecipitated Ki-1/57, RACK1, and Phospho-PKC (Fig. 6). When immunoprecipitated by antibody A26, the nuclear fraction of Ki-1/57 is no longer detectable after 4 h of PMA stimulation of the cells (Fig. 6, *lanes 35* and *36*), whereas there was no alteration in the amounts of cytoplasmic Ki-1/57. When immunoprecipitated with antibody E203, this decrease of the amount of nuclear Ki-1/57 could also be noticed, albeit to a lesser degree in comparison with A26 (Fig. 6, *lanes 39* and *40*). There is slightly more RACK1 in the cytoplasmic fraction after PMA stimulation (Fig. 6, *lanes 1* and *2*), whereas the nuclear fraction is unaffected by the PMA stimulation (Fig. 6, *lanes 3* and *4*). The amount of RACK1 co-immunoprecipitated with phospho-PKC-Pan is increased both in the cytoplasm as well as in the nucleus, whereas RACK1 that co-immunoprecipitated with PKC$\alpha\beta$II was only detectable in the cytoplasm after PMA stimulation (Fig. 6, *lane 10*).

The fact that Ki-1/57 is only associated with RACK1 in the nucleus and in the absence of cell stimulator PMA (Fig. 6, *lanes 15* and *16*) suggests that the interaction of Ki-1/57 with RACK1 is only stable under unstimulated conditions but is abrogated after the activation with PMA. These experiments were controlled with an antibody against the nonrelated protein Ki-67 (46), which co-immunoprecipitates neither RACK1 (Fig. 6, *lanes 17–20*) nor Ki-1/57 (Fig. 6, *lanes 41–44*).

We next tested whether the observed disappearance of Ki-1/57 from the nucleus (Fig. 6, *lanes 36* and *40*) can be seen by immunofluorescence localization studies in human HeLa cells (Fig. 7). We observed that both RACK1 (Fig. 7*C*) and Ki-1/57 (Fig 7, *A* and *B*) exit the nucleus upon PMA activation. The exit of Ki-1/57 from the nucleus is accompanied by that of phospho-PKC$\alpha\beta$II and phospho-PKCζ/λ (Fig. 7), and that of RACK1 is accompanied by the exit of phospho-PKC$\alpha\beta$II. These experiments were repeated with L540 cells and essentially gave the same results (not shown).

DISCUSSION

To find a functional context for the protein Ki-1/57 we set out to perform a yeast two-hybrid screen to identify possible interacting protein partners. Screens of a human fetal brain cDNA library with both Ki-1/57 and with its homologue protein CGI-55 previously identified the chromatin remodeling factor CHD3 (9). This was the first report that described a specific protein-protein interaction for both CGI-55 and Ki-1/57 and could

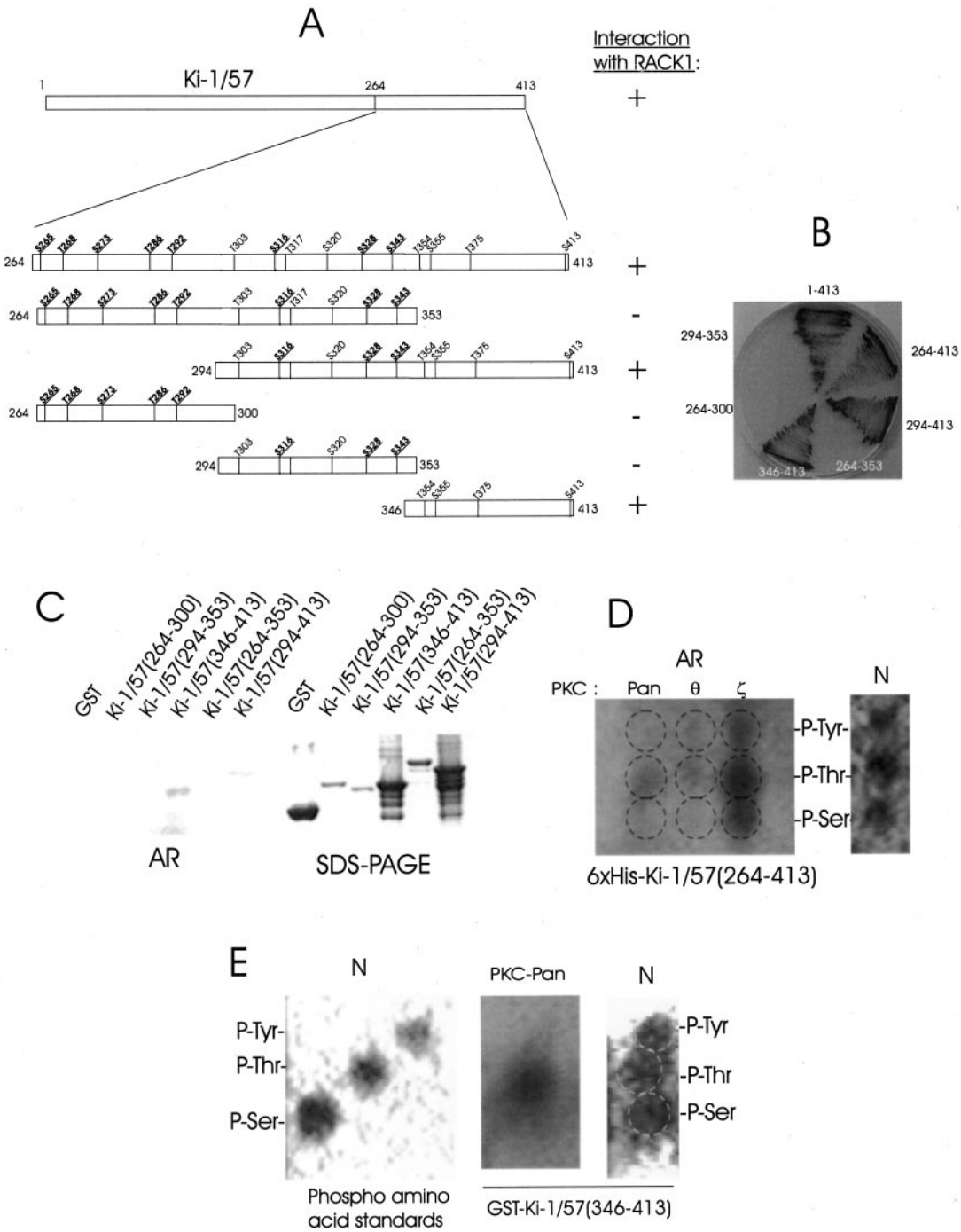

FIG. 5. **Mapping of the phosphorylation and interaction characteristics of Ki-1/57 C-terminal region (264–413) and phosphoaminoacid analysis.** *A*, schematic representation of the C-terminal region of Ki-1/57 with indication of the present Ser and Thr residues. *Underlined bold type* indicates residues with a predicted probability of >78% to be phosphorylated, whereas residues without underlining have a probability of <45% (prediction made by using the NetPhos 2.0 Prediction Server). *B*, interaction of the indicated Ki-1/57 fragments with RACK1 in the yeast two-hybrid system. The ability of the co-transformant cells to grow on minimal medium,-Trp,-Leu,-His was tested in the presence of 10 mM 3-amino-1,2,4-triazole inhibitor for suppression of unspecific reporter activation. Presence of bait and prey plasmids in the co-transformed cells was controlled by growth on minimal medium-Trp,-Leu (data not shown). *C*, *in vitro* phosphorylation of subfragments of the C-terminal region of Ki-1/57, which had been all expressed as GST fusion proteins in *E. coli*. *Left panel*, AR. *Right panel*, control SDS-PAGE to demonstrate equal loading with GST fusion proteins. *D*, phosphoamino acid analysis. His_6-Ki-1/57(264–413) was phosphorylated *in vitro* by the indicated PKC isoforms. After hydrolyzing the phosphoproteins, the phosphoamino acids were analyzed by TLC. *Left panel*, AR of the TLC plate. *Right panel*, staining of the standard phosphoamino acids with ninhydrin (*N*) for identification of the type of phosphoamino acid. *E*, phosphoamino acid analysis of GST-Ki-1/57(346–413) that had been phosphorylated with PKC-Pan *in vitro*. *Left panel*, demonstration of the separation of the three standard phosphoamino acids by the TLC method employed. *Middle panel*, AR of the radiolabeled phosphoamino acid threonine of GST-Ki-1/57(346–413). *Right panel*, identification of the radiolabeled amino acid shown in the *middle panel* as phosphothreonine.

define them as a new family of CHD3 interacting proteins. The majority (54%) of clones found to interact with Ki-1/57, however, represent the signaling adapter molecule RACK1.

The interactions between Ki-1/57 and RACK1 were confirmed *in vitro* and *in vivo* by co-precipitation assays from L540 Hodgkin's disease analogous cells. Because RACK1 has been described previously to be an adapter protein for activated protein kinases C and helps to maintain PKC in an activated

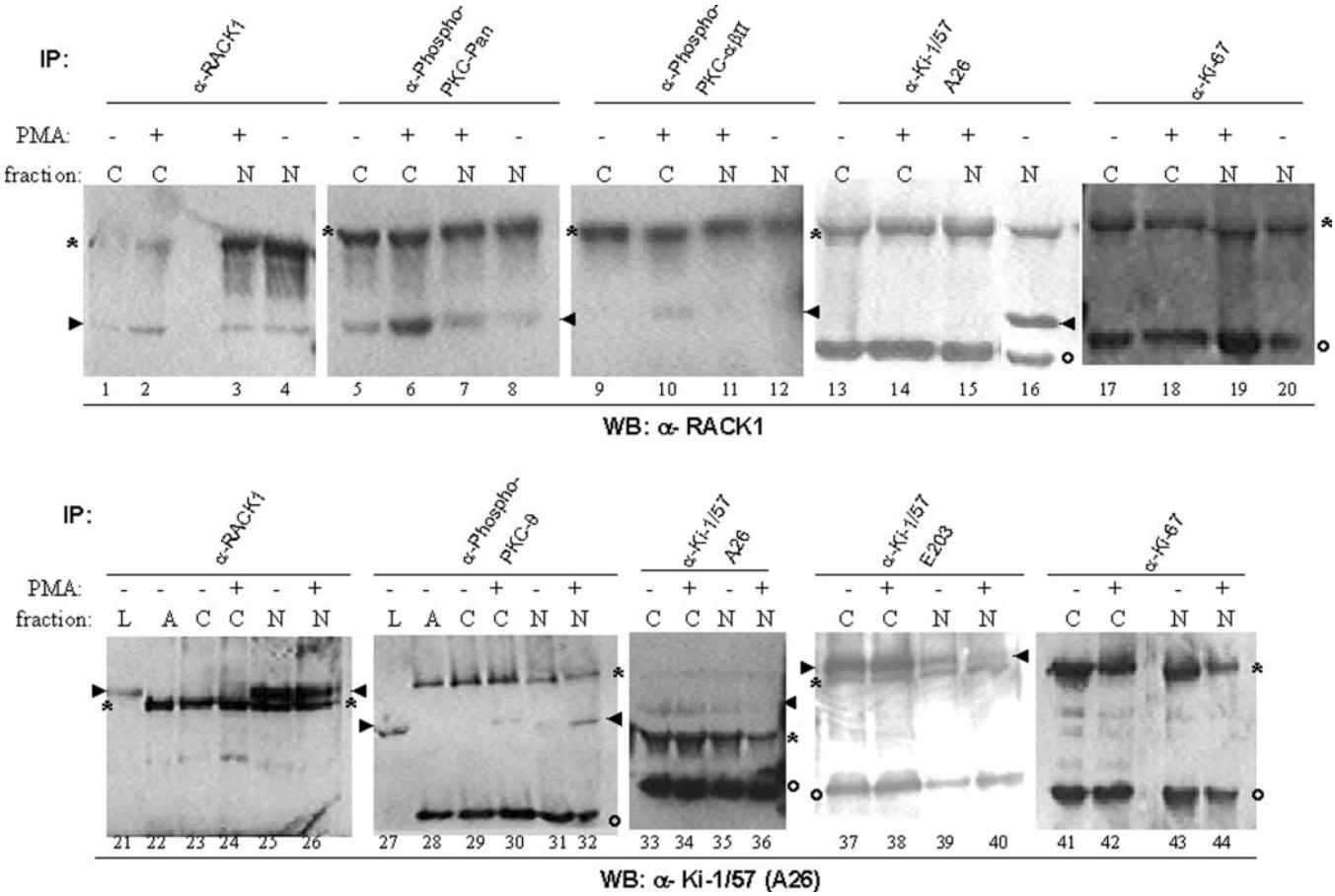

FIG. 6. **The nuclear association between Ki-1/57 and RACK1 is abolished upon PMA activation of the L540 cells.** L540 cells were or were not stimulated for 4 h with PMA. The cytoplasmic (*C*) or nuclear (*N*) fractions of their cell lysates were immunoprecipitated (*IP*) with antibodies anti-RACK1, anti-phospho-PKC-Pan, anti-phospho-PKCab_{II}, anti-phospho-PKCθ, anti-Ki-1/57(A26 or E203), or the unrelated control antibody anti-Ki-67 (46). Ki-1/67 co-immunoprecipitates some protein bands of a molecular mass between 30 and 50 kDa, the molecular mass of which corresponds neither to Ki-1/57 (57 kDa) nor to PKC (~80 kDa) (see *lanes 41–44*). Co-immunoprecipitation of RACK1 (36 kDa) or Ki-1/57 (using antibody A26) was detected by specific Western blots (*WB*), as indicated. *Black arrowheads* indicate specific proteins (*upper panels*, RACK1; *lower panels*, Ki-1/57) identified by Western blot. ***L*** in the *lower row* indicates the loading control of the lysate to indicate the position of the input Ki-1/57 protein. ***A*** in the *lower row* indicates lanes that have only been loaded with antibody to demonstrate the position of the heavy and light chains of the antibody. Control experiments demonstrated that the bands marked with *asterisks* represent the heavy chain antibodies (~55 kDa), and the bands marked by *open circles* represent the light chain antibodies (~25 kDa) (not all data shown).

state, we tested whether Ki-1/57 also interacts with cellular PKC and whether it represents a target molecule for PKC phosphorylation. Our phosphorylation assays with PKC show that Ki-1/57 is a substrate for PKC isolated from PMA-activated but not from nonactivated control L540 cells. Immunoprecipitation of PKC from PMA-stimulated but not from unstimulated L540 cells showed co-immunoprecipitated Ki-1/57 protein, thereby demonstrating that PKC interacts with Ki-1/57 after cell activation. The interaction of Ki-1/57 with RACK1, however, was abolished after PMA stimulation, suggesting that this interaction is regulated. Together these results might suggest a hypothesis for a sequential mode of interactions between the three molecules Ki-1/57, RACK1, and PKC: (i) before PMA activation Ki-1/57 is firmly attached to RACK1, this interaction occurs mainly in the nucleus; (ii) after PMA activation Ki-1/57 gets phosphorylated, this results in the abrogation of the interaction with RACK1; and (iii) the newly created phosphoamino acid groups in the C terminus of Ki-1/57 could now serve as docking sites for the interaction of kinases or other proteins with Ki-1/57.

It had been shown previously that different proteins that interact with RACK1 interact with different docking sites involving one or more of the seven individual blades of the putative propeller structure of RACK (18). In case of the protein Src, the smallest unit of RACK1 that was capable of an interaction consists of only a single WD repeat blade (50). The binding of the interferon receptor on the other hand involves five of the seven blades (30). Therefore, we set out to map the regions of RACK1 involved in the interaction with Ki-1/57 and found that none of our constructed deletions but only full-length RACK1 was able to engage in protein-protein interaction with Ki-1/57. This is corroborated by the fact that all of the clones we identified in the yeast two-hybrid screen contained the full-length RACK1 coding region. Ki-1/57 seems therefore to be the first protein found that only interacts with full-length RACK1. The mapping of the interaction site of Ki-1/57 on the other hand demonstrated that its extreme C terminus (amino acids 346–413) is fully capable to interact with RACK1. This suggests that Ki-1/57 might be a multi-domain protein with its C terminus containing a docking domain/motif for RACK1.

It was shown before that although RACK1 engages in protein interaction with several structurally and functionally different proteins (22–33), its interaction among proteins of the same family is highly specific (50, 27). Yarwood *et al.* (27) have for instance shown that RACK1 interacts with PDE4D5 but with none of the other PDE4 isoforms tested. This high degree of selectivity of the interaction holds true in the opposite direction, too. PDE4 does not interact with any of a series of WD repeat-containing proteins other than RACK1 (27). Our results confirm this trend, because none of the other proteins identified

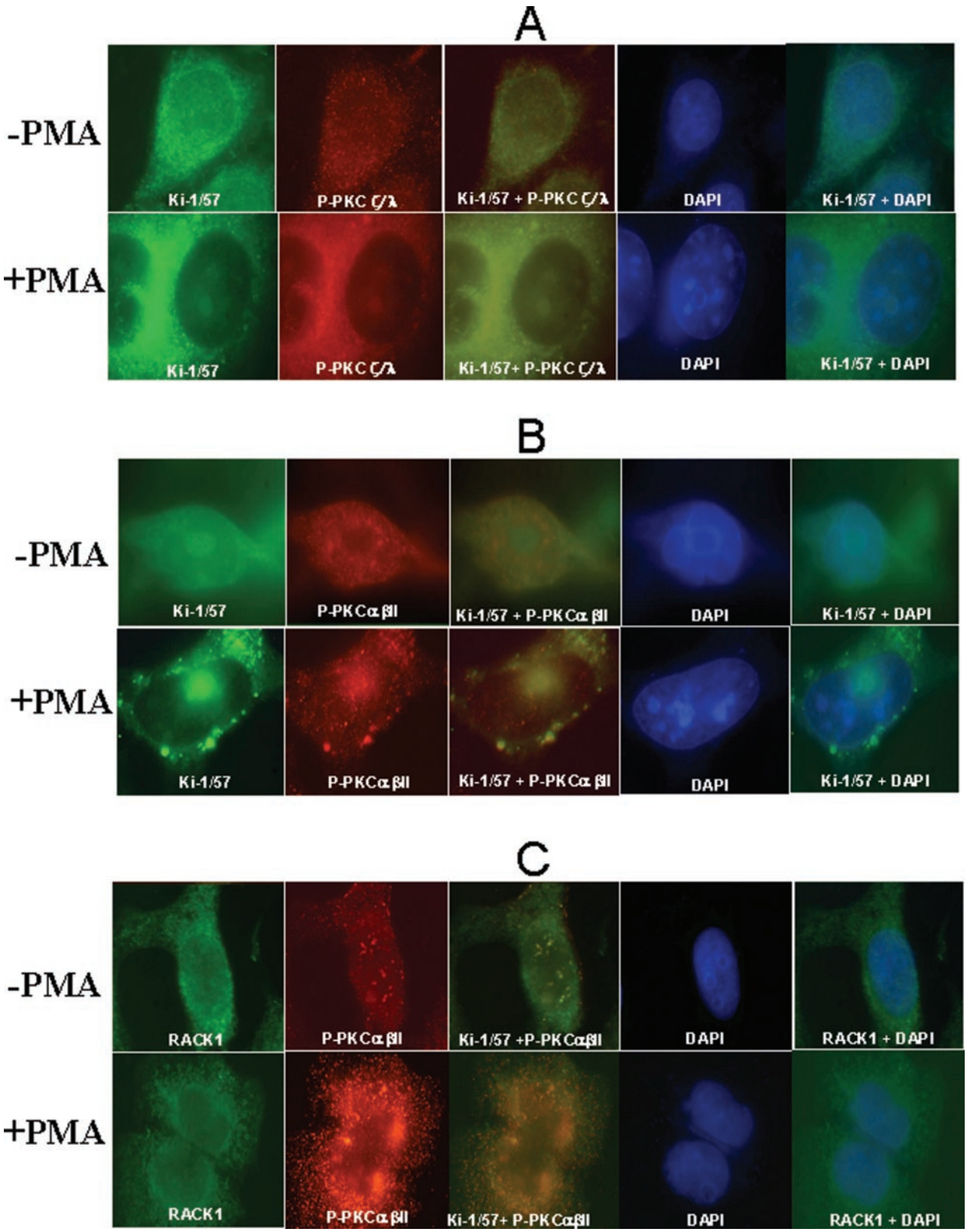

FIG. 7. **Subcellular localization of endogenous RACK1, Ki-1/57, phospho-PKC$\alpha\beta_{II}$, and phospho-PKCζ/λbefore and after treatment of cells with PMA.** HeLa cells grown on glass coverslips were stimulated or not with PMA for 4 h at 37 °C. The cells were fixed with 100% methanol, and the proteins were immunodetected with the following primary antibodies: Ki-1 monoclonal mouse antibody or mouse anti-RACK1 and the indicated rabbit anti-phospho-PKC antibodies. Fluorescein-coupled anti-mouse (*green*) or rhodamine-conjugated anti-rabbit antibodies (*red*) served as secondary antibodies. DAPI staining (*blue*) was used to show the positions of the nuclei. The cells were examined with a Nikon fluorescence microscope. Superimposing the two colors (merge) results in a *yellow/orange* signal.

so far in our yeast two-hybrid analysis of the Ki-1/57 protein belongs to the WD repeat-containing family of proteins (not shown). RACK1 interacts neither in the yeast two-hybrid system nor *in vitro* with the Ki-1/57 homologue protein CGI-55 (not shown).

RACK1 has been described to have an activating influence

on PKC activity and even a small peptide derived of amino acids 234–241 of WD blade 6 of RACK1 bound to PKC and activated it *in vitro* and *in vivo* (22, 46). However, in contradiction to this finding another report showed no influence of RACK1 on the kinase activity of PKC, cAMP-dependent protein kinase, or casein kinase II toward peptide substrates but an inhibitory effect of RACK1 on the autophosphorylation activity of Src and Yes and on the peptide phosphorylation activity of Src and Lck (25). These results demonstrate that it is not yet possible to conclude whether RACK1 has an overall stimulatory or inhibitory role on the kinase activity of different kinases but rather suggest that not only the type of kinase but most likely also the kind of substrate involved might be of importance. Our results with the phosphorylation of the PKC substrate Ki-1/57 in the presence of RACK1 did not show any influence of RACK1 on the outcome of the kinase reaction (Fig. 3*C*).

Ki-1/57 has been also described previously as an intracellular hyaluronan-binding protein (IHABP4), because of its capacity to interact with a series of negatively charged macromolecules, including hyaluronan, heparan sulfate, chondroitin sulfate, and RNA (8). According to Huang *et al.* (8), the binding of IHABP4/Ki-1/57 to hyaluronan depends on the presence of so called hyaluronan binding motifs of the structure (R/K)X_7(R/K). However, the majority of nuclear proteins are overproportionally rich in the positively charged amino acids Lys and Arg. Our analysis of several randomly selected nuclear proteins revealed that all of them contained several of such putative hyaluronan-binding motifs. However, some of them contained many of such putative hyaluronan-binding motifs: CHD3 (accession number NM_001272.1) contains 49; Topors (accession number AF098300) contains 36; human polycomb2 (accession number AF013956) contains 7; p53 (accession number AAH03596) contains 3; and c-Fos (accession number K00650) contains 2. This would suggest that the majority of Arg/Lys-rich nuclear proteins have the potential to interact with hyaluronan. Even Huang *et al.* (8) state that it remains open whether hyaluronan is indeed a natural ligand for IHABP4/Ki-1/57 (8). They speculate that because both hyaluronat and Ki-1/57 have been found in the nucleus and cytoplasm, Ki-1/57 might be involved in the regulation of hyaluronan functions (8).

Our recent studies point to other possible nuclear functions of Ki-1/57 as well as its homologue CGI-55 (9). We found that CGI-55 and Ki-1/57 interact with CHD3, a nuclear protein involved in the remodeling of chromatin and the regulation of transcription (9). Furthermore, both Ki-1/57 (6) and CGI-55 are localized in the nucleus, nucleolus, and other small nuclear bodies, and CGI-55 has been shown to co-localize to p80 coilin-positive nuclear coiled bodies, which have been functionally implicated in the regulation of transcription and the processing of RNA.[2] Furthermore, the other proteins identified to interact with CGI-55 in the yeast two-hybrid screen are in their majority nuclear proteins, and several of them, including the proteins Daxx (a Fas-binding protein), Topors (a topoisomerase-binding protein), and hPc2 (human polycomb 2), are like CHD3 involved in the regulation of transcription.[2] In fact we also had identified both Daxx and Topors as interacting partners of Ki-1/57 in our yeast two-hybrid screen (data not shown). These findings demonstrate that Ki-1/57 and CGI-55 have common interacting nuclear protein partners (CHD3, Daxx, and Topors) as well as specific interaction partners like RACK1 for Ki-1/57 and hPc2 for CGI-55. They further point to the possibility that both Ki-1/57 and CGI-55 might be involved in nuclear functions such as the remodeling of chromatin and the regulation of transcription, like several of its interacting nuclear protein partners.

In this context our observation of the nuclear exit of Ki-1/57 after stimulation of the cells with PMA may be of functional relevance. It has been shown recently that the activity of the chromatin-remodeling factor HDAC7 is regulated by its PMA-induced export from the nucleus (51, 52). The combined PMA/ionomycin treatment mimics the T cell receptor activation, and the PMA-induced nuclear export of HDAC7 was accompanied by a drop in a HDAC7-dependent Nur77 promotor activity, which controls a luciferase reporter gene. This demonstrates how the regulated nuclear export of a protein can affect the transcriptional regulation of genes. Because Ki-1/57 has been shown to interact with CHD3, another factor involved in chromatin remodeling and transcriptional regulation, it is tempting to speculate that the PMA-dependent nuclear export of Ki-1/57 could have functional consequences for CHD3s activity. While this manuscript was in the review process we became aware of a recent publication by Ozaki *et al.* (53). This group had found that RACK1 interacts with the C terminus of the p53 homologue protein p73. Most interestingly, Ozaki *et al.* were able to demonstrate that RACK1 inhibits both p73-mediated transcription from a test promoter as well as p73-mediated apoptosis. Future experiments will address whether and how Ki-1/57 and CGI-55 are involved in the regulation of transcription and what are the exact functions of these interesting novel proteins.

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III RESULTADOS

Artigo III

A spectroscopic analysis of the interaction between the human regulatory proteins RACK1 and Ki-1/57

Flávia C. Nery, Gustavo C. Bressan, Marcos R. Alborghetti, Dario O. Passos, Taís M. Kuniyoshi, , Carlos H. I. Ramos, Sérgio Oyama Jr and Jörg Kobarg

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Short Communication

A spectroscopic analysis of the interaction between the human regulatory proteins RACK1 and Ki-1/57

Flávia C. Nery[1,2], Gustavo C. Bressan[1,3], Marcos R. Alborghetti[1], Dario O. Passos[1,3], Taís M. Kuniyoshi[1], Carlos H.I. Ramos[1,3], Sergio Oyama Jr.[1] and Jörg Kobarg[1,2,3,*]

[1] Centro de Biologia Molecular Estrutural, Laboratório Nacional de Luz Síncrotron, Rua Giuseppe Máximo Scolfaro 10.000, C.P. 6192, 13084-971 Campinas SP, Brazil

[2] Departamento de Genética e Evolução,

[3] Departamento de Bioquímica, Universidade Estadual de Campinas, 13084-970 Campinas, SP, Brazil

* Corresponding author
e-mail: jkobarg@lnls.br

Abstract

Ki-1/57 is a 57-kDa cytoplasmic and nuclear protein associated with protein kinase activity and is hyper-phosphorylated on Ser/Thr residues upon cellular activation. In previous studies we identified the receptor of activated kinase-1 (RACK1), a signaling adaptor protein that binds activated PKC, as a protein that interacts with Ki-1/57. Here we demonstrate that the far-UV circular dichroism spectrum of the WD repeat-containing RACK1 protein shows an unusual positive ellipticity at 229 nm, which in other proteins of the WD family has been attributed to surface tryptophans that are quenchable by *N*-bromosuccinimide (NBS). As well as NBS, *in vitro* binding of 6×His-Ki-1/57(122–413) and 6×His-Ki-1/57(264–413) can also quench the positive ellipticity of the RACK1 spectrum. We generated a model of RACK1 by homology modeling using a G protein β subunit as template. Our model suggests the family-typical seven-bladed β-propeller, with an aromatic cluster around the central tunnel that contains four Trp residues (17, 83, 150, 170), which are likely involved in the interaction with Ki-1/57.

Keywords: circular dichroism; emission fluorescence; molecular modeling; protein-protein interaction; regulatory proteins; surface tryptophans.

Ki-1/57, the 57-kDa human protein antigen recognized by the CD30 antibody Ki-1 in Hodgkin and Sternberg-Reed cells in Hodgkin's lymphoma (Schwab et al., 1982), is a cytoplasmic and nuclear protein that is phosphorylated on serine and threonine residues (Hansen et al., 1989) and hyper-phosphorylated upon cellular activation with PMA (Nery et al., 2004). When isolated from the Hodgkin's lymphoma analogous cell line L540, Ki-1/57 co-immunoprecipitates with a Thr/Ser protein kinase activity (Hansen et al., 1990). Its expression in diverse cancer cells and association with kinase activity in mitogen-activated peripheral blood cells suggested a role in cellular activation and possibly in cell signaling (Kobarg et al., 1997).

In recent studies, in which the yeast two-hybrid system was used to identify proteins that interact with Ki-1/57, we found that Ki-1/57 engaged in specific interactions with the chromatin-helicase-DNA-binding domain protein 3 (CHD-3) (Lemos et al., 2003), a nuclear protein involved in chromatin remodeling and transcription regulation, and also with the specific transcription factors MEF2C (Kobarg et al., 2005) and p53 (Nery et al., 2006). Furthermore, we identified the receptor of activated kinase C-1 (RACK1) (Nery et al., 2004), an adaptor protein that interacts with activated protein kinase C (PKC) (Ron et al., 1994) as a Ki-1/57-interacting protein. Ki-1/57 has also been termed IHABP4 (intracellular hyaluronan binding protein 4) because it contains many positively charged amino acids and has been shown to interact with a series of negatively charged macromolecules, including RNA and hyaluronan (an extracellular glucosaminoglycan) *in vitro* (Huang et al., 2000).

PAI-1 mRNA-binding protein is an alternative splice variant of CGI-55, a putative paralog of Ki-1/57. It has been shown to bind specifically to the mRNA of type-1 plasminogen activator inhibitor (PAI-1) and is thought to be involved in the regulation of its stability (Heaton et al., 2001). CGI-55 showed a strikingly overlapping protein interaction profile with Ki-1/57, including several transcription regulatory proteins such as CHD-3 (Lemos et al., 2003), Topors, Daxx, and PIAS, but not RACK1 (Lemos and Kobarg, 2006; Nery et al., 2004, 2006). In summary, the new data available for Ki-1/57 suggest that it may be a regulatory protein involved in transcriptional control.

RACK1, which interacts with Ki-1/57 but not CGI-55, is an adaptor protein and member of the WD-repeat protein family (Garcia-Higuera et al., 1996). This repeat unit was first recognized in the G protein β subunit, where it occurs as a seven-bladed β-propeller (Fong et al., 1986, Sondek et al., 1996). G proteins transduce signals across the plasma membrane (Fong et al., 1986). The function of RACK1 has been predicted in diverse cellular functions, ranging from the regulation of signal transduction (Chen et al., 2004), via pre-mRNA processing (Angenstein et al., 2002), gene transcription and translation (Sang et al., 2001; Sengupta et al., 2004) to cell cycle progression (Mamidipudi et al., 2004). Furthermore, RACK1 has been described as an adaptor protein that is involved in inhibition of the transcription regulation mediated by the transcription factor p73, a member of the p53

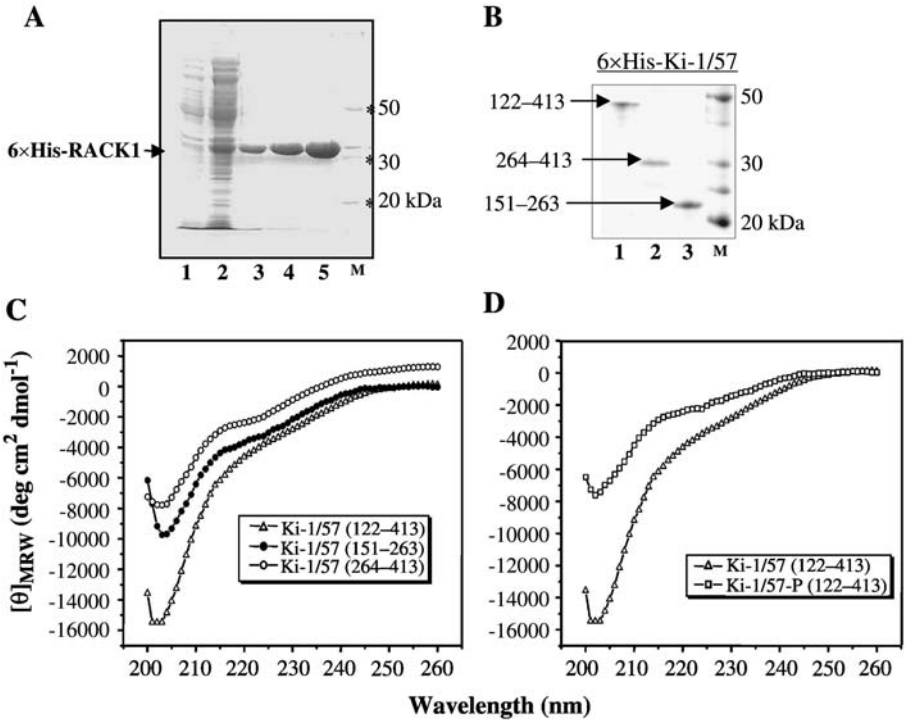

Figure 1 Expression and affinity purification of 6×His-RACK1 and 6×His-Ki-1/57 protein fragments and CD spectroscopic characterization of Ki-1/57 protein fragments.
cDNAs encoding human RACK1 or the indicated Ki-1/57 protein fragments were amplified by PCR and then directionally cloned into the bacterial expression vectors peT28a (Novagen, Madison, USA) or pPROEx (Invitrogen, Carlsbad, USA), respectively. After transformation of *E. coli* BL21-CodonPlus-RIL cells (Stratagene, La Jolla, USA), expression of the recombinant proteins was induced using 1.8 mM IPTG for 4 h. The resulting 6×His-tagged fusion proteins were purified by Ni-affinity chromatography as previously described (Surpili et al., 2003). (A) SDS-PAGE analysis of the expression and purification of full-length 6×His-RACK1. Lane 1, fraction not induced with IPTG; lane 2, fraction induced with IPTG; lanes 3–5, fractions after purification; lane M, molecular mass marker proteins. The marker protein ladder used was BenchMark™ Protein Ladder (Invitrogen), which consists of recombinant artificial proteins. The molecular masses of selected bands marked with an asterisk (*) are indicated. (B) SDS-PAGE of the expressed and affinity-purified Ki-1/57 protein constructs spanning the indicated amino acids. Aliquots of the peak fractions were loaded as indicated on the left. Lane M, BenchMark™ marker proteins with indicated selected molecular masses. (C) Circular dichroism (CD) spectra from 200 to 260 nm of the purified Ki-1/57 fragments indicated (all at 10 μM) at 25°C. All far-UV CD spectra for RACK1 and Ki-1/57 constructs were recorded on a Jasco J-810 spectropolarimeter with the temperature controlled by a Peltier Type Control System PFD 425S using a quartz cuvette of 0.1 cm optical path length. (D) CD spectra of phosphorylated C-terminal 6×His-Ki-1/57 fragment (122–413) (-P) and its unphosphorylated form. Phosphorylation of Ki-1/57 was performed with the 6×His fusion proteins coupled to Ni-NTA Sepharose beads for 10 min at 30°C with 20 mU of PKC (Promega, Madison, USA), 20 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.1 mg/ml PMA, 10 mM ATP, in a final volume of 20 μl (Nery et al., 2004). In the case of the non-phosphorylated control protein, no PKC was added to the reaction. Subsequently, samples were washed three times. The 6×His fusion proteins were eluted and dialyzed before CD analysis. All CD spectra shown were normalized to molar ellipticity values in deg cm^2/dmol.

family of proteins (Ozaki et al., 2003). Interestingly, Ki-1/57 also interacts with p53 and 11 other proteins that are functionally or physically associated with p53, thereby emphasizing the functional association of RACK1 and Ki-1/57 in the context of transcriptional regulation (Nery et al., 2006).

We expressed 6×His-RACK1 in mg amounts in *E. coli* and purified it to apparent purity (Figure 1A) of at least 95%, as evidenced by SDS-PAGE analysis. RACK1 has a molecular mass of ~36 kDa and is a monomer in solution, as confirmed by analytical gel filtration chromatography and mass spectrometry analyses (data not shown). The purification of 6×His-RACK1 from 1 liter of culture yielded approximately 3 mg of protein. We were able to obtain 6×His-RACK1 fusion protein in soluble form expressed in *E. coli,* although other researchers have described RACK1 as an insoluble protein (Miller et al., 2004) and explained their results by a possible association of RACK1 with the membrane or insoluble fraction of mammalian cells. We speculate that this *in vivo* association with the membrane and insoluble fraction could be promoted by interactions with other proteins (Saxena et al., 1996). Furthermore, self-aggregation of RACK1 at high concentrations may occur, partially due to the large hydrophobic patches on its surface that we describe in our model below. The latter, however, did not occur in our hands *in vitro* after purification, since we were able to maintain RACK1 in a stable and non-aggregated form in a glycerol-containing buffer up to high concentrations.

However, the full-length interacting protein 6×His-Ki-1/57 showed only negligible expression in *E. coli* and suffered proteolytic degradation and instability (data not shown). This forced us to work rather with the deletion constructs, which were well expressed and were sufficiently stable upon purification (Figure 1B). Purification from 1 liter of culture yielded approximately 2 mg of construct 6×His-Ki-1/57(122–413), 1 mg of protein for 6×His-Ki-1/57(264–413) and of 3 mg of protein for

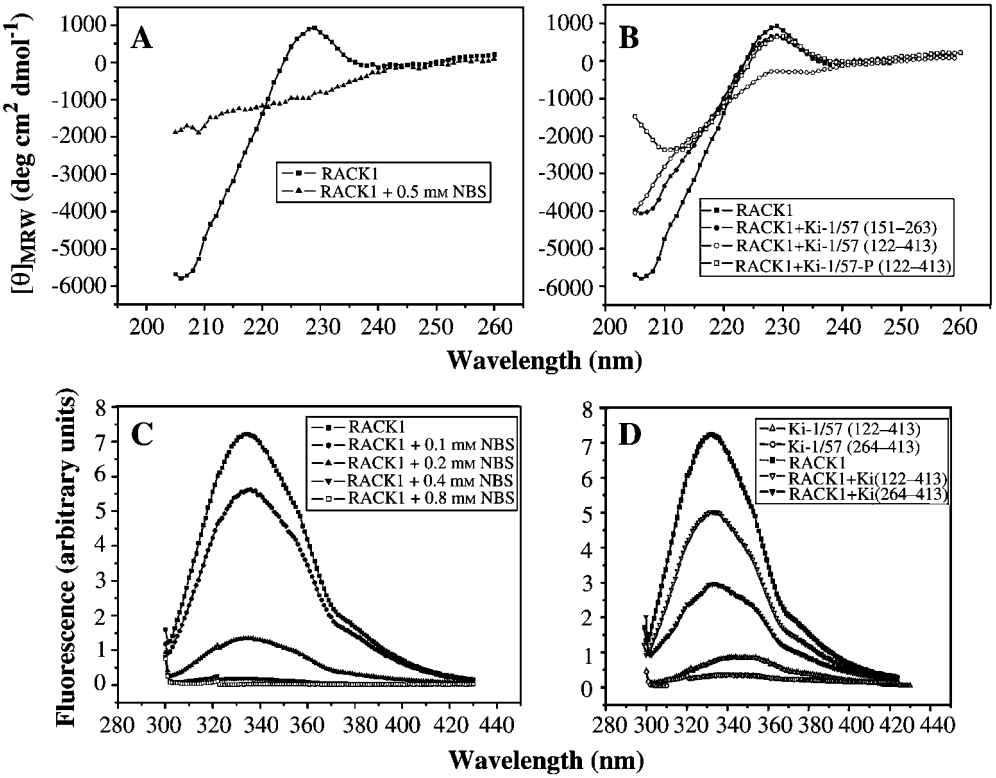

Figure 2 Circular dichroism (CD) and fluorescence emission spectra of 6×His-RACK1 in the presence and absence of NBS or different 6×His-Ki-1/57 fragments.

(A) CD spectra of 6×His-RACK1 (10 μM) in 0.1 M sodium phosphate buffer, pH 7.0 at 25°C, in the absence and presence of 0.5 mM of NBS (*N*-bromosuccinimide, immediate measurement). (B) Interference by the indicated 6×His-Ki-1/57 protein fragments (10 μM) in the positive band at 229 nm of the CD spectrum of 6×His-RACK1 (10 μM). A quartz cuvette with an optical path length of 0.1 cm was used. (C) Fluorescence emission spectra of 6×His-RACK1 (1 μM) treated or not with the indicated concentrations of NBS for 5 min. Next, the samples were excited at 295 nm and the emission was measured from 300 to 430 nm with a spectral band pass of 4 nm for excitation and emission. All fluorescence experiments were performed using an Aminco Bowman® Series 2 (SLM-AMINCO, Rochester, USA) spectrofluorimeter equipped with a 450-W lamp. (D) Fluorescence emission spectra of 6×His-RACK1 (1 μM) incubated or not with 6×His-Ki-1/57(122–413) or 6×His-Ki-1/57(264–413) and of 6×His-Ki-1/57(122–413) or 6×His-Ki-1/57(264–413) alone (all at 1 μM). The samples were excited at 295 nm and the emission was measured from 300 to 430 nm with a spectral bandpass of 4 nm for excitation and emission using a quartz cuvette with an optical path length of 1 cm.

6×His-Ki-1/57(151–263). The theoretical molecular masses calculated for these three fusion proteins are 36, 19, 15 and kDa, respectively. However, the apparent molecular masses of the three fusion proteins, as determined from SDS-PAGE, are significantly higher at ca. 47, 30 and 23 kDa, respectively. This anomalous electrophoretic mobility is already known for native and recombinant full-length Ki-1/57, which has a predicted molecular mass of 45.8 kDa and runs in SDS-PAGE with an apparent mobility of approximately 57 kDa. Most likely the observed retarded protein mobility is caused by the high content of charged, especially positively charged, amino acids in the Ki-1/57 protein sequence.

Next, we analyzed the purified recombinant proteins by circular dichroism (CD) and emission fluorescence spectroscopy. The shape and characteristic minima in the CD spectra of different protein fragments of Ki-1/57 (Figure 1C) suggests that the protein has rather low structural complexity and could belong to the group of native unfolded proteins (Dyson and Wright, 2002). Further biophysical studies on the stability of the protein must be conducted to address this issue.

To determine if phosphorylation interferes with the Ki-1/57 secondary structure, we phosphorylated Ki-1/57(122–413) *in vitro* with PKC as previously described (Nery et al., 2004) and analyzed it by circular dichroism spectroscopy. For phosphorylated Ki-1/57(122–413), we observed that the CD spectrum acquires a different shape and that the minimum at 208 nm in relation to unphosphorylated Ki-1/57(122–413) decreases from -10.000 to -5.000 molar ellipticity (Figure 1D). These data suggest that phosphorylation could alter the secondary structure of Ki-1/57 and thereby contribute to the observed inhibition of the interaction between RACK1 and Ki-1/57 upon phosphorylation of the latter (Nery et al., 2004). Another important factor is certainly the addition of negative charges by phosphorylation of the target Ser/Thr residues.

For RACK1 the overall percentage of α-helices was determined to be less than 5% from the molar ellipticity at 200–260 nm (Böhm et al., 1992). The far-UV CD spectrum of 6×His-RACK1, which contains 13 Trp residues, 8 Phe and 6 Tyr, shows a pronounced maximum around 230 nm at room temperature (Figure 2A). This maximum has been ascribed to the presence of disulfide bonds, interactions between aromatic residues, such as tryptophans, phenylalanines, and tyrosines (Hider et al., 1988), or proteins with predominantly β-sheet and β-turn sec-

ondary structures that contain aromatic amino acids. In a recent study, Bjorndal et al. (2003) showed CD spectra of their RACK1 preparation, which did not show positive ellipticity at 229 nm. Possibly, their RACK1 protein preparation was unstable under the buffer conditions used, since our CD data for RACK1 correspond very well to spectra of other members of the WD protein family (Saxena et al., 1996). Furthermore, this positive ellipticity starts to disappear at 45°C, at which some members of the WD and WW domain family already start to unfold, probably due to increased side-chain motions of the surface set of tryptophans (Saxena et al., 1996). We indeed observed pronounced thermal instability of RACK1, whereby the CD maximum disappeared just above 45°C (data not shown).

To determine the source of the positive ellipticity of 6×His-RACK1 at 229 nm, the tryptophan residues were specifically modified with 0.5 mM of *N*-bromosuccinimide (NBS). It is known that NBS can modify proteins through oxidation of primarily tryptophan residues (Saxena et al., 1996). Figure 2A shows the CD spectra of 6×His-RACK1 after treatment with 0.5 mM NBS. Treatment with NBS completely abolished the maximum at 229 nm, indicating that tryptophan residues may be responsible for the maximum at 229 nm. We speculated that some of these tryptophans may also be involved in the interaction with the regulatory protein Ki-1/57. Experiments involving circular dichroism of pre-incubated 6×His-RACK1 and 6×His-Ki-1/57 constructs (Figure 2B) showed that the positive ellipticity of RACK1 at 229 nm also disappeared in the presence of the Ki-1/57 fragments 122–413 or 264–413 (Figure 2B and data not shown), suggesting that the tryptophans are crucially involved in this interaction. We previously observed that phosphorylation of Ki-1/57 on its extreme C-terminus abolishes the interaction with RACK1 (Nery et al., 2004). Here, we found in additional experiments that phosphorylation of 6×His-Ki-1/57 (122–413) does not influence the unusual positive ellipticity of RACK1 at 229 nm (Figure 2B). This confirms that phosphorylation of Ki-1/57 blocks the interaction with RACK1. Furthermore, the addition of Ki-1/57 (151–263), a fragment of the protein that does not interact with RACK1 (Nery et al., 2004), did not diminish the positive molar ellipticity of RACK1 (Figure 2B).

Furthermore, the spectroscopic interference of NBS and both C-terminal Ki-1/57 constructs with RACK1 tryptophans was also confirmed by fluorescence emission studies. The RACK1 spectrum measured is typical of a tryptophan-rich protein. When excited at 295 nm, there is a strong emission maximum around 335 nm (Figure 2C). The effect of NBS modification on the fluorescence emission spectrum of RACK1 is also shown in Figure 2C. We observed progressive fluorescence quenching at gradually increasing concentrations of NBS, which corresponds to progressive oxidation of the tryptophans. At 0.1 mM, NBS begins to quench the tryptophan fluorescence of RACK1, which was completely quenched upon treatment with 0.4 mM NBS.

Another way to characterize the influence of 6×His-Ki-1/57 binding to 6×His-RACK1 is to measure the fluorescence emission upon titration of RACK1 with Ki-1/57(122–413) or (264–413) (Figure 2D), which themselves contain three or one Trp residues, respectively. In both cases, binding of the Ki-1/57 construct to RACK1 significantly quenched the relative fluorescence emission of RACK1. Depending on the Ki-1/57 fragment used, the fluorescence quenching varied from 30% to 60%. The shorter fragment Ki-1/57(264–413) was more efficient in quenching RACK1 fluorescence than the larger fragment Ki-1/57(122–413). This confirms that both Ki-1/57 constructs interact with the surface tryptophans of RACK1. The addition of the non-interacting protein fragment 6×His-Ki-1/57(151–263) to 6×His-RACK1 did not result in any quenching of the 6×His-RACK1 fluorescence (data not shown).

Since treatment with the Trp-specific reagent NBS caused complete quenching and interaction with Ki-1/57 protein partial quenching of the positive ellipticity of RACK1, we speculated that at least some of the 13 Trp residues may be exposed on the surface of RACK1 and may thus be accessible to modification by NBS. These exposed Trp residues may also be involved in the interaction with Ki-1/57. To see if we could identify any hydrophobic Trp-rich region on the surface of the RACK1 model, we next performed homology modeling of RACK1, using the β subunit of the G protein as a modeling template (Figure 3). According to the model, RACK1 assumes a compact β-propeller structure, in which the region prior to the strand of the first blade makes contact with the strand of the last blade, thus closing the ring. The blades of the propeller are organized around a central tunnel in such a way that the top surface is slightly narrower than the bottom surface. We identified an unusual extended apical hydrophobic region consisting of four Trp, one Phe and one Tyr residue. Trp is an amino acid that is generally rather localized in the hydrophobic core region of proteins and the nitrogen atom on the indole group usually forms hydrogen bonds with the main or other side chains (Bjorndal et al., 2003). We suggest that the tryptophans present in the aromatic region on the top surface of RACK1 are important for the interaction with Ki-1/57, and possibly other proteins, too. In support of this notion in the heterotrimeric G proteins, Gα sits on the narrow surface of the Gβ subunit, which we used as a modeling template. In our case we speculate that Ki-1/57 may also sit on the narrower top surface of RACK1, which is rich in tryptophan residues.

As expected, the model of RACK1 reveals a compact structure made up of seven WD repeats arranged in a propeller structure, as shown in Figure 3. From ten homology models for RACK1 generated by the MODELLER program, we chose the model in which 88.5% of residues were in most favored regions and no residues in disallowed regions in the Ramachandran plot. The blades of the propeller are formed by four twisted antiparallel β-stands organized around a central tunnel. The overall structure presents a wider radius at the base, as shown in Figure 3. It is worth noting that, in contrast to other members of WD domain family, RACK1 consists only of WD repeats with little or no amino- or carboxyl-terminal extensions, such as the protein SEC13 (Saxena et al., 1996). This indicates that in the case of these two proteins the secondary structure should be very similar. Our CD data for RACK1 are indeed very similar to those for the protein SEC13.

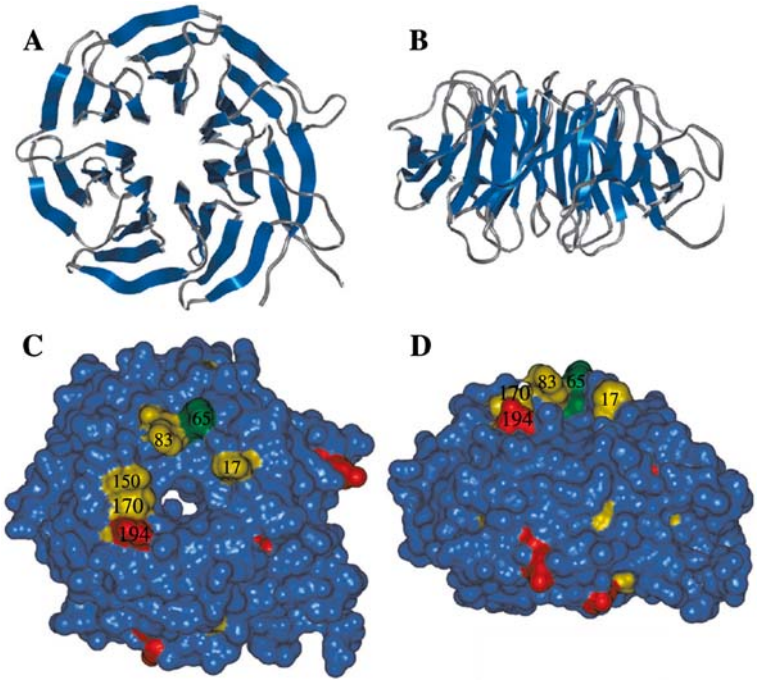

Figure 3 Homology modeling of human RACK1.
The three-dimensional model for human RACK1 was obtained using the MODELLER program (Sali and Blundell, 1993). The X-ray structure available for the Gβ protein (PDB: 1GOT) (Lambright et al., 1996) was used as a template. The model building and structural analyses were performed on a Compaq Alpha Server ES40 machine. Initially, prediction of the RACK1 secondary structure was obtained using the PSIPRED algorithm (Jones, 1999). After comparison of the target and template sequences, secondary structure patterns and conservation of key residue, a preliminary structural alignment was proposed. However, the N-terminal (residues 1–38) extension of Gβ was excluded from the alignment, since it contains an α-helical segment that is not present in human RACK1. The resulting alignment was manually adjusted in an iterative manner until good stereochemical quality was achieved and ten homology models for RACK1 protein were generated. The PROCHECK program (Laskowski et al., 1993) was used to monitor the structural quality of the final model. Molecular visualization and analysis were performed in the Insight II graphical environment (Accelrys Inc., San Diego, USA) running on an Octane2 workstation (Silicon Graphics Inc., Mountain View, USA). (A,B) Ribbon representations of modeled human RACK1 down the central tunnel (A) of the predicted β-propeller and in side view (B). (C,D) Predicted structure of RACK1 with indication of selected aromatic residues. Surface representation showing the exposed amino acids as observed down the central tunnel (C) and in side view (D). Note the aromatic region in the apical region of RACK1 around the central tunnel of the β-propeller. This region comprises four Trp (17, 83, 150 and 170) residues (in yellow), one Tyr (194, in red), and one Phe (65, in green).

Furthermore, the pattern of cysteine residue distribution in RACK1 (not shown), as suggested by our model, revealed the possibility to form two disulfide bonds between cysteines 153 and 168 or 240 and 249, since these residues pairs are close in space. The solvent-accessible localization of two impaired cysteine residues may explain why it is so important to utilize reducing compounds such as DTT or 2-mercaptoethanol in RACK1 preparations to avoid aggregation through the formation of putative unspecific intermolecular disulfide bridges in this protein.

In summary, our data demonstrate that Ki-1/57 interferes in the signals of CD and emission fluorescence of its interacting protein 6×His-RACK1, probably by interactions involving an apical group of exposed Trp residues on RACK. Furthermore, changes in the secondary structure of Ki-1/57 upon phosphorylation may contribute to the observed interruption of the interaction between the two proteins. Our data contribute to understanding of the regulatory mechanisms governing the interaction between Ki-1/57 and RACK1. Future studies must address the functional consequences of the interruption of this interaction in human cells and whether the Trp residues of RACK1 are also involved in interaction with other interacting proteins described.

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III RESULTADOS

Artigo IV

Ki-1/57 interacts with PRMT1 and is a substrate for arginine methylation

Dario O. Passos, Gustavo C. Bressan, Flavia C. Nery and Jörg Kobarg

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Ki-1/57 interacts with PRMT1 and is a substrate for arginine methylation

Dario O. Passos[1,2], Gustavo C. Bressan[1,2], Flavia C. Nery[1,3] and Jörg Kobarg[1,2,3]

1 Centro de Biologia Molecular Estrutural, Laboratório Nacional de Luz Síncrotron, Campinas, Brazil
2 Departamento de Bioquímica, Universidade Estadual de Campinas, Brazil
3 Departamento Genética e Evolução, Universidade Estadual de Campinas, Brazil

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Correspondence
J. Kobarg, Centro de Biologia Molecular Estrutural, Laboratório Nacional de Luz Síncrotron, Rua Giuseppe Máximo Scolfaro 10.000, C.P. 6192, 13084-971 Campinas – SP, Brazil
Fax: +55 19 3512 1006
Tel: +55 19 3512 1125
E-mail: jkobarg@lnls.br

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The human 57 kDa Ki-1 antigen (Ki-1/57) is a cytoplasmic and nuclear protein, associated with Ser/Thr protein kinase activity, and phosphorylated at the serine and threonine residues upon cellular activation. We have shown that Ki-1/57 interacts with chromo-helicase DNA-binding domain protein 3 and with the adaptor/signaling protein receptor of activated kinase 1 in the nucleus. Among the identified proteins that interacted with Ki-1/57 in a yeast two-hybrid system was the protein arginine-methyltransferase-1 (PRMT1). Most interestingly, when PRMT1 was used as bait in a yeast two-hybrid system we were able to identify Ki-1/57 as prey among 14 other interacting proteins, the majority of which are involved in RNA metabolism or in the regulation of transcription. We found that Ki-1/57 and its putative paralog CGI-55 have two conserved Gly/Arg-rich motif clusters (RGG/RXR box, where X is any amino acid) that may be substrates for arginine-methylation by PRMT1. We observed that all Ki-1/57 protein fragments containing RGG/RXR box clusters interact with PRMT1 and are targets for methylation *in vitro*. Furthermore, we found that Ki-1/57 is a target for methylation *in vivo*. Using immunofluorescence experiments we observed that treatment of HeLa cells with an inhibitor of methylation, adenosine-2′,3′-dialdehyde (Adox), led to a reduction in the cytoplasmic immunostaining of Ki-1/57, whereas its paralog CGI-55 was partially redistributed from the nucleus to the cytoplasm upon Adox treatment. In summary, our data show that the yeast two-hybrid assay is an effective system for identifying novel PRMT arginine-methylation substrates and may be successfully applied to other members of the growing family of PRMTs.

Ki-1/57 was initially identified by the cross-reactivity of the anti-CD30 mAb Ki-1 [1–5]. Initial studies on the Ki-1/57 protein antigen itself revealed that it is associated with Ser/Thr protein kinase activity [3] and that it is located in the cytoplasm, at the nuclear pores and in the nucleus, where it is frequently found in association with the nucleolus and other nuclear bodies [4]. Because Ki-1/57 was also found to bind to hyaluronan and other negatively charged glycosaminoglycans, such as chondroitin sulfate, heparan sulfate and RNA, although with lower affinity, it was also named intracellular hyaluronan binding protein 4 (IHABP4)

Abbreviations
Act D, actinomycin D; Adox, adenosine-2′,3′-dialdehyde; Daxx, Fas-binding protein; GST, glutathione *S*-transferase; IHABP4, intracellular hyaluronan binding protein 4; Ki-1/57, 57 kDa Ki-1 antigen; PKC, protein kinase C; PRMT, protein arginine methyl transferase; RACK1, receptor of activated kinase 1; RGG/RXR box, glycine/arginine-rich motif (where X is any amino acid); SAM, S-adenosyl-L-methionine; Topors, topoisomerase-binding protein.

[6]. Another human protein, CGI-55, has an amino acid sequence identity of 40.7% and a sequence similarity of 67.4% with Ki-1/57 [7], suggesting that both proteins could be paralogs. CGI-55 has also been shown to bind to the 3′-region of the mRNA encoding the type-1 plasminogen activator inhibitor (PAI-1) and was therefore also named PAI–RNA-binding protein 1 (PAI–RBP1) [8].

We have recently shown that both Ki-1/57 and CGI-55 interact with the chromatin-remodeling factor chromo-helicase DNA-binding domain protein 3 [7]. Furthermore, Ki-1/57, but not CGI-55, interacts with the transcription factor MEF2C [9], p53 [10] and the signaling adaptor protein receptor of activated protein C (RACK1) [11]. Recently, another group found that RACK1 interacts with p73, a paralog of p53, and that RACK1 reduces p73-mediated transcription by direct physical binding with it [12].

Arginine methylation is a post-translational modification of proteins in higher eukaryotes, the exact function of which is poorly understood. Several studies have pointed out that arginine methylation of proteins can regulate a wide range of protein functions, including nuclear export [13], nuclear import [14], and interaction with nucleic acids [15] or other proteins [16]. Functional outcomes of protein modification by methylation are the remodeling of chromatin [17] or the possible stabilization of specific mRNAs after cell activation-mediated methylation of mRNA-stabilizing proteins such as HuR [18]. The arginines can be mono- or dimethylated in a symmetrical or asymmetrical fashion. The target arginines of protein arginine methyl transferases are often embedded in typical Gly/Arg-rich motifs (RGG/RXR) [19]. These motifs can be found principally in proteins involved in RNA processing and transcriptional regulation. Protein arginine-methyltransferase-1 (PRMT1) is the major arginine methyltransferase in human cells, accounting for $> 85\%$ of the methylation of cellular protein substrates [20]. Although embryonic stem cells deficient for the PRMT1 gene are viable in culture, mice lacking the gene die during the embryonic phase [21], suggesting that protein methylation is crucial for development or differentiation.

Here, we report on the identification of an interaction between Ki-1/57 and PRMT1 in reciprocal yeast two-hybrid experiments and also confirm this interaction using *in vitro* pull-down experiments with recombinant purified proteins. Furthermore, we performed detailed mapping studies of the interaction and methylation sites and show that Ki-1/57 is a substrate for protein arginine methylation *in vivo*. Finally, we show that treatment of cells with the methylation inhibitor adenosine-2′,3′-dialdehyde (Adox) results in a reduction in the cytoplasmic labeling of Ki-1/57 in immunofluorescence microscopy. By contrast, CGI-55, the putative paralog of Ki-1/57, showed a partial redistribution from the nucleus to the cytoplasm, upon Adox treatment.

Results

Yeast two-hybrid screen with Ki-1/57 as bait

To identify Ki-1/57-interacting proteins, a yeast two-hybrid system [22] was employed, utilizing a human fetal brain cDNA library (Clontech, Palo Alto, CA). In a first screen we used a fragment of the Ki-1/57 cDNA encoding amino acids 122–413 as bait. We screened 2.0×10^6 cotransformants, which yielded 250 clones positive for both His3 and LacZ reporter constructs. We were able to obtain the sequences of 64 library plasmid DNA clones, two of which encoded PRMT1. In a second round of screening, we used a construction that encodes amino acids 1–150 of Ki-1/57 fused to the C-terminus of LexA (pBTM116) and tested it against the fetal brain cDNA library. Screening $\sim 2 \times 10^6$ cotransformants resulted in 66 DNA sequences, six of which encoded PRMT1. PRMT1 represented 6% of all the sequenced clones from both two-hybrid screens.

Yeast two-hybrid screen using PRMT1 as bait

We also performed a yeast two-hybrid screen with PRMT1(1–344) as bait to test if the two-hybrid system was suitable for screening a cDNA library for putative new substrates for PRMT1 arginine methylation and to test whether it would be possible to confirm the observed interaction of Ki-1/57 with PRMT1 by inverting bait–prey relations. We obtained 273 clones and isolated 36 recombinant bait plasmids to sequence their cDNA inserts. Table 1 lists all the proteins shown interact with PRMT1 [23–36]. We not only were able to confirm the interaction with Ki-1/57, which was found to be a PRMT1-interacting protein, but we did identify a further 14 PRMT1-interacting proteins.

Some of these proteins have previously been identified as substrates for arginine methylation, including CIRBP [29,37] and EWSR1 [31]. Others have been associated either functionally or physically with PRMT1, including tubulin [24] or ILF3 [36]. Most of these proteins contain one (86%) or more (66%) RGG/RXR boxes (Table 1). Two of the proteins are ribosomal proteins that do not contain any typical RGG/RXR box motifs in their sequences. It is known

Table 1. PRMT1-interacting proteins as identified by yeast two-hybrid system screen. ND, not determined.

Protein interacting with PRMT1 (synonym/s)	No. of RGG/RXR boxes	Insert length (bp)[a]	Coded protein residues (retrieved/complete sequence)	Domain composition[b]	Function[c]	Found clones[d]	Accession number	Ref.
Ki-1/57 (IHABP4)	14	1100	140–413/413	N-terminal Arg-rich region	Unknown, possibly involved in: signal transduction, transcriptional regulation, RNA metabolism, interacts with several other proteins (including RACK1, PKC, Daxx, Topors, CHD3	2	NM_014282	5–11
PRMT1 (HRMT1L2/ ANM1/HCP1/IR1B4)	1	1500	1–343/343	catalytic core	Methylates the guanidine nitrogens of arginyl residues in glycine and arginine-rich domains	5	NM_198318	23
Tubulin betapolypeptide	1	1500	291–445/445	–	Major constituent of microtubules	15	NM_178012	24
Ubiquitin-conjugating enzyme E21	1	1400	1–157/157	– –	catalyzes attachment of ubiquitin-like protein SUMO-1 to other proteins	1	NM_003345	25
hnRNP-A3 (FBRNP/ D10S102/ 2610510D13Rik)	11	1200	145–378/378	2 RNA recognition motifs (RRM) C-terminal Gly-rich region	Plays a role in cytoplasmic trafficking of RNA	1	NM_194247	26
Daxx (DAP6/BING2/ Fas-binding protein)	5	900	555–740/740	Acid-rich domain Ser/Pro/Thr-rich domain	Regulates JNK pathway, apoptosis and transcription in PML/POD/ND10 nuclear bodies in concert with PML	1	NM_001350	27
Ribosomal protein L37a	–	350	1–92/92	C4-type zinc finger-like domain	Component of the 60*S* ribosomal subunit	1	NM_000998	28
CIRBP (CIRP2/CIRP)	7	1250	31–172/172	1 RRM, C-terminal Gly-rich region	Cold-induced suppression of cell proliferation	1	NM_001280	29
NSAP1 (hnRNPQ/ SYNCRIP/pp68/ GRY-RBP/dJ3J17.2)	16	1800	390–623/623	3 RRM, C-terminal Tyr/Gly-rich region	Component of ribonucleosomes and heterogenous nuclear ribonucleoproteins, processing of precursor mRNA	1	NM_006372	30
EWSR1 (EWS)	25	ND0	1–713/713	1 RRM, zinc finger RanBP2-type, N-terminal Gln/Thr/Ser/ and C-terminal Gly-rich regions	Tumorigenesis	1	NM_013986	31
Ribosomal protein S29	–	800	1–56/56	C2–C2 zinc finger-like domain	Component of the 60*S* ribosomal subunit	2	NM_001032	32
SFRS1 (ASF/SF2/SRp30a)	15	ND	28–248/248	2 RRM, C-terminal Gly/Ser/Arg -rich regions	premRNA splicing factor	1	NM_006924	33
Topors (TP53BPL/LUN)	32	ND	873–1045/1045	Zinc finger RING-type, Ser/Arg/Lys-rich regions, a leucine zipper	PML association, ubiquitination, possible tumor suppressor	1	NM_005802	34

Table 1. (Continued).

Protein interacting with PRMT1 (synonym/s)	No. of RGG/RXR boxes	Insert length (bp)[a]	Coded protein residues (retrieved/complete sequence)	Domain composition[b]	Function[c]	Found clones[d]	Accession number	Ref.
ZCCHC12	7	ND	191–412/412	CCHC zinc finger domain (zinc-knuckle)	Nucleic acid binding, transcriptional regulation	1	NM_173798	35
ILF3 (MMP4/MPP4/ NF90/NFAR-1/ TCP80/DRBP76/ MPHOSPH4/NF-AT-90)	7	1100	546–894/894	2 double-stranded RNA-binding motifs (DSRM), C-terminal glycine-rich region	Transcription factor required for expression of interleukin-2 in T cells, binds RNA	2	NM_012218	36

[a] Approximate length of the sequences retrieved from the library. [b] Other domains may be present. [c] Other functions may be known. [d] Number of times the clone/protein was found among the 36 sequenced clones.

that other ribosomal proteins, such as yeast L12, are substrates of arginine methylation, although they do not contain RGG/RXR motifs [38]. Eight PRMT1-interacting proteins, including Ki-1/57, are likely candidate substrates for PRMT1 and have not been described as substrates previously.

This seems to indicate that yeast two-hybrid screens in general can be used to identify new PRMT substrates in different tissues or cells. Furthermore, it is worth noting that most of the proteins identified are nuclear proteins either characterized as RNA-interacting proteins (NSAP1, CIRBP, SFRS1) or implicated in the regulation of transcription, e.g. Fas-binding protein (Daxx) and topoisomerase-binding protein (Topors). In addition, we found PRMT1 itself to be a prey, confirming that PRMT1 forms dimers [39]. Finally, it is remarkable that many of the identified PRMT1-interacting proteins, including Daxx, Topors, CIRBP and SFRS1, also interacted with Ki-1/57 [10].

Prediction of putative methylation sites in Ki-1/57 and CGI-55

Analysis of the protein sequence of Ki-1/57 revealed that it possessed several clusters of RGG/RXR box motifs, which may be target sites for protein arginine methylation by PRMT1 (Fig. 1). These clusters are located at the N-terminus (amino acids 47, 55, 70), in the central region (178–199) and on the extreme C-terminus (369–383). Alignment with the putative Ki-1/57 paralog CGI-55 showed that the central and C-terminal clusters are conserved in both proteins (Fig. 1A,B). The central cluster (178–199) in Ki-1/57 contains seven RGG/RXR motifs, three of which are conserved in the corresponding cluster of CGI-55 (158–179), which contains five of such motifs. The C-terminal cluster in Ki-1/57 (369–383) contains four RGG/RXR motifs, all of which are conserved in CGI-55 (352–365), which contains an additional fifth motif (Fig. 1B).

Interaction and mapping of the interaction site of Ki-1/57 with PRMT1

Next, we wanted to map the Ki-1/57 region involved in the interaction with PRMT1 using the yeast two-hybrid method (Fig. 2). Nine N- and C-terminal deletion constructs of the Ki-1/57 protein were fused to the LexA–DNA-binding domain (Fig. 2A) and tested for their ability to bind full-length PRMT1 (Fig. 2B–E). Interestingly, the interactions of the N-terminus of Ki-1/57 (1–150), its C-terminus (122–413) and a fragment spanning its central region (151–260) with PRMT1 were each stronger than that of full-length Ki-1/57 (Fig. 2B,C).

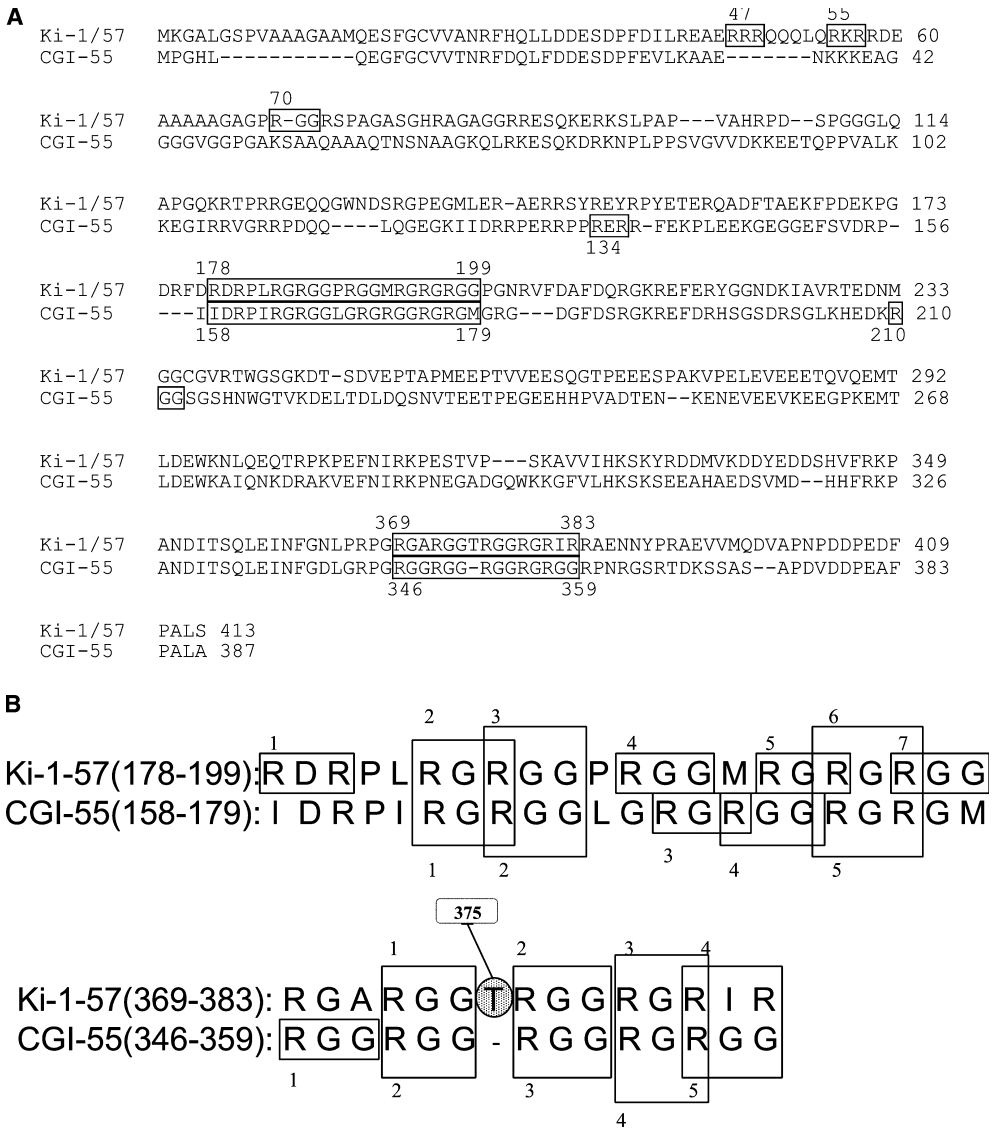

Fig. 1. Alignment of Ki-1/57 and CGI-55 and prediction of putative arginine methylation sites. (A) Protein sequence alignment of the putative homologs Ki-1/57 and CGI-55. Boxes indicate putative arginine methylation sites that could be targets for PRMT1 and their boundaries are marked with numbers. (B) Detailed representation of the two conserved multiple RGG/RXR boxes in the central region and at the C-terminus of Ki-1/57 and CGI-55. In the central region, three of the seven RGG/RXR targets are strictly conserved in CGI-55. For the C-terminal region, four of the five RGG/RXR motifs found in CGI-55 are conserved in Ki-1/57. The residue T375, located between two RGG motifs but not found in CGI-55, is pointed out because it is a target residue for phosphorylation by PKC *in vitro*.

The C-terminus (261–413) had approximately the same affinity as full-length Ki-1/57 (Fig. 2B,C). When we tested further subdeletions of this C-terminal fragment (Fig. 2D,E) we found that only the two subdeletions of Ki-1/57 containing the predicted RGG/RXR box cluster (369–383) interacted with PRMT1 (Fig. 2A,D,E). Empty vector or constructions containing subdeletions of Ki-1/57 lacking the C-terminal RGG/RXR box cluster did not interact with PRMT1.

Next, we performed an *in vitro* pull-down assay with the recombinant purified proteins 6xHis–K1/57 and GST–PRMT1 to confirm the interaction (Fig. 2F). The assay confirmed the specificity of the interaction, since glutathione–Sepharose beads coupled with GST–PRMT1 were able to coprecipitate 6xHis–Ki-1/57, but not the control protein 6xHis–RACK1. The figure also shows the equal loading and input controls of the tested proteins.

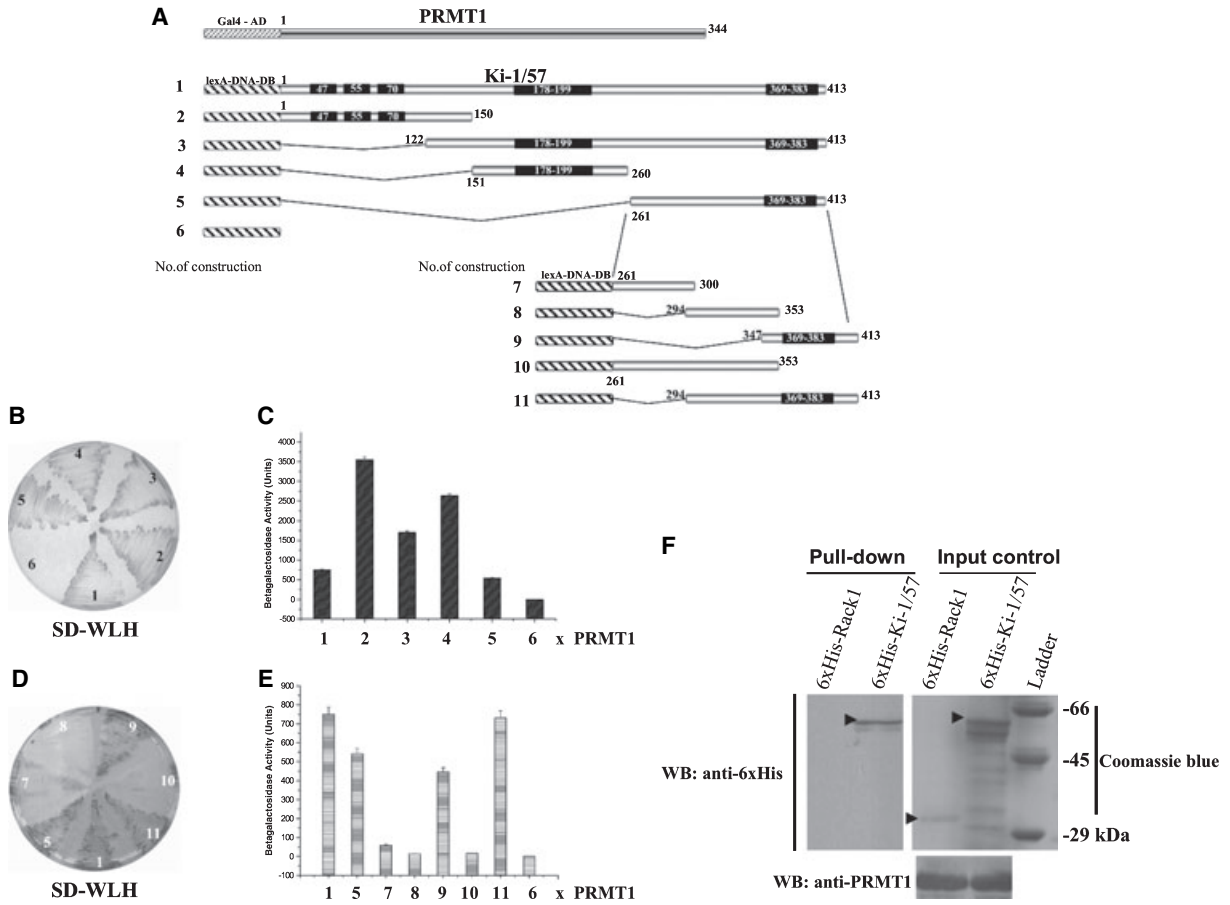

Fig. 2. PRMT1 interacts with all RGG/RXR box-containing protein regions of Ki-1/57. (A) Schematic representation of PRMT1 (cloned in pGAD424 in fusion with the Gal4-activation domain) and Ki-1/57 (1) and its deletion constructs 2–11 (cloned in pBTM116 in fusion with the LexA–DNA-binding domain) used in the yeast two-hybrid assay. Fusion proteins are indicated by striped boxes and the putative RGG/RXR boxes by black boxes, which indicate the involved amino acid regions. (B, D) The PRMT1 construct was transformed in L40 yeast cells. The indicated deletion constructs of Ki-1/57 were cotransformed and tested for interaction by assessing their ability to grow on the -Trp, -Leu, -His plates. The presence of plasmids was confirmed by growth of all cotransformants on -Trp, -Leu plates (data not shown). (C, E) Quantification of the strength of interaction by measurement of the β-galactosidase activity in a liquid ONPG assay (see Experimental procedures for details). The quantity of the produced yellow color is expressed in arbitrary units. (F) Pull-down assay for the confirmation of the interaction between PRMT1 and Ki-1/57 *in vitro*. Recombinant purified GST–PRMT1 protein was coupled to glutathione–Sepharose beads. After washing the beads were incubated with either bacterially expressed and purified 6xHis-Ki-1/57 or the control protein 6xHis–RACK1. After washing, coprecipitated proteins were analyzed by western blot against the 6xHis tag or PRMT1 (for control of equal loading). Equal loading with 6xHis fusion proteins was controlled by SDS/PAGE stained using Coomassie Brilliant Blue. Selected molecular masses of the protein ladder are indicated.

In vitro methylation of Ki-1/57 and CGI-55 by PRMT1

The interaction of Ki-1/57 with PRMT1 and the presence and conservation of the RGG/RXR box motifs in the amino acid sequences of Ki-1/57 and CGI-55 suggest that these two proteins are likely targets of arginine methylation by PRMT1. To test this hypothesis we incubated Ki-1/57 and its putative paralog CGI−55 as glutathione *S*-transferase (GST)-fusion proteins with GST–PRMT1 *in vitro* and performed a protein methylation assay. We found that Ki-1/57 and its putative paralog CGI-55 are good *in vitro* substrates for protein arginine methylation by PRMT1 (Fig. 3A), whereas control proteins like PRMT1 itself (which contains a RXR motif at its C-terminus), RACK1 and GST (as a fusion partner of GST–PRMT1) were not methylated.

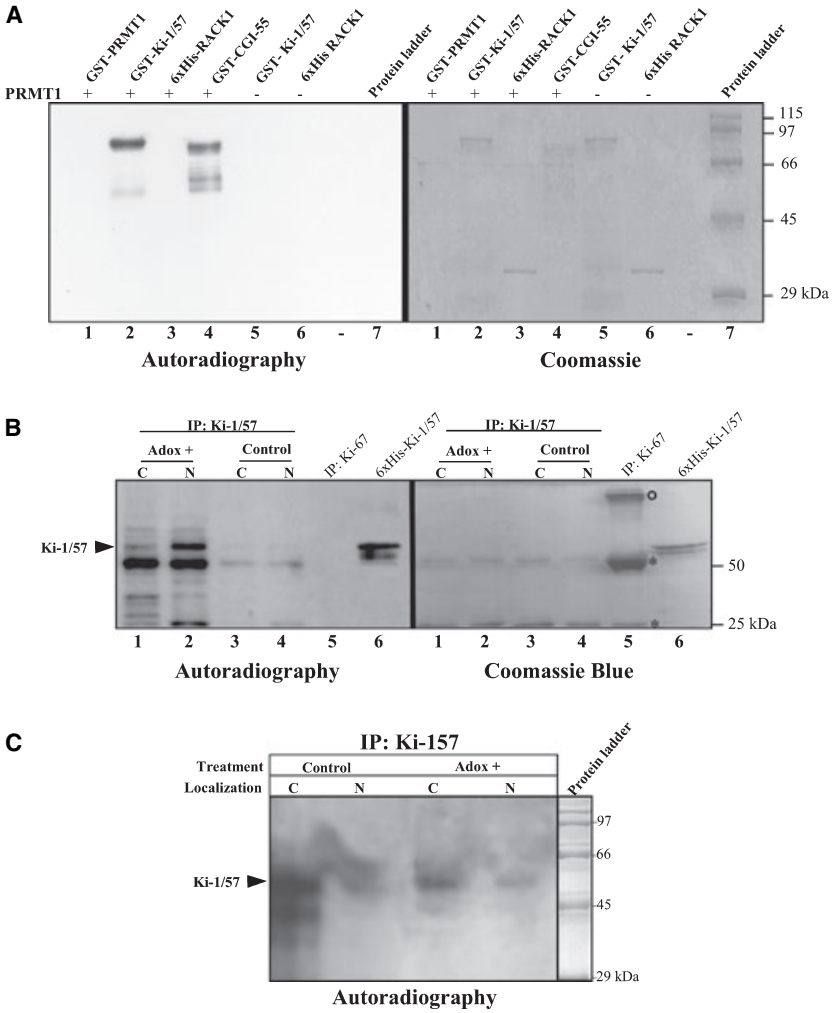

Fig. 3. Both Ki-1/57 and its putative paralog CGI-55 are substrates of arginine methylation by PRMT1 *in vitro* and Ki-1/57 is methylated *in vivo*. (A) *In vitro* methylation assay: PRMT1 was expressed and purified as a GST fusion protein in *E. coli* and incubated with the indicated recombinant proteins, all expressed in and purified from *E. coli*. An *in vitro* arginine-methylation assay was performed as described in Experimental procedures. Methylated proteins were run out on SDS/PAGE (right side) and the gel was exposed to a X-ray film. PRMT1 itself and RACK1 served as control proteins. (B) *In vivo* methylation assay: L540 Hodgkin-analogous cells were (lanes 1 and 2) or were not (lanes 3 and 4) incubated with the inhibitor of endogenous protein methylation Adox, lyzed and fractionated in nuclear (lanes 2 and 4) and cytoplasmic (lanes 1 and 3) fractions. Ki-1/57 was immunoprecipitated (lanes 1–4) and then submitted to methylation by PRMT1 *in vitro*. As a negative control we used mAb Ki-67 [44]. We immunoprecipitated its antigen (°), which was then submitted to *in vitro* methylation by PRMT1 (lanes 5). As expected it did not show any incorporation of radioactivity. The antigen recognized by Ki-67 is not known to be a substrate for methylation by PRMT1. Proteins were run out on SDS/PAGE and their methylation assessed by autoradiography. A parallel gel was analyzed by Coomassie Brilliant Blue staining. Lane 6: bacterial 6xHis-Ki-1/57 methylated *in vitro* was run out in order to facilitate localization of the cellular Ki-1/57 protein band. The heavy and light chains of the antibodies (*) served as molecular mass markers (50 and 25 kDa) (C) *In vivo* methylation of Ki-1/57. HeLa cells were or incubated or not with the inhibitor of endogenous protein methylation Adox, metabolically labeled with ^{3}H-SAM, lyzed and fractionated in nuclear and cytoplasmic fractions. After Ki-1/57 immunoprecipitation from both fractions, samples were assessed by autoradiography as described above. A parallel CGI-55 immunoprecipitation served as a control and did not result in the detection of any radioactively labeled bands (data not shown).

Endogenous Ki-1/57 can be methylated *in vitro* after Adox treatment of cells

When we isolated Ki-1/57 from the cytoplasmic and nuclear fractions of L540 Hodgkin analogous cells by immunoprecipitation and incubated it with recombinant GST–PRMT1, we observed that it cannot be methylated *in vitro* (Fig. 3B, lanes 3 and 4). We chose L540 cells for the following experiments, because they express a reasonable amount of Ki-1/57 protein,

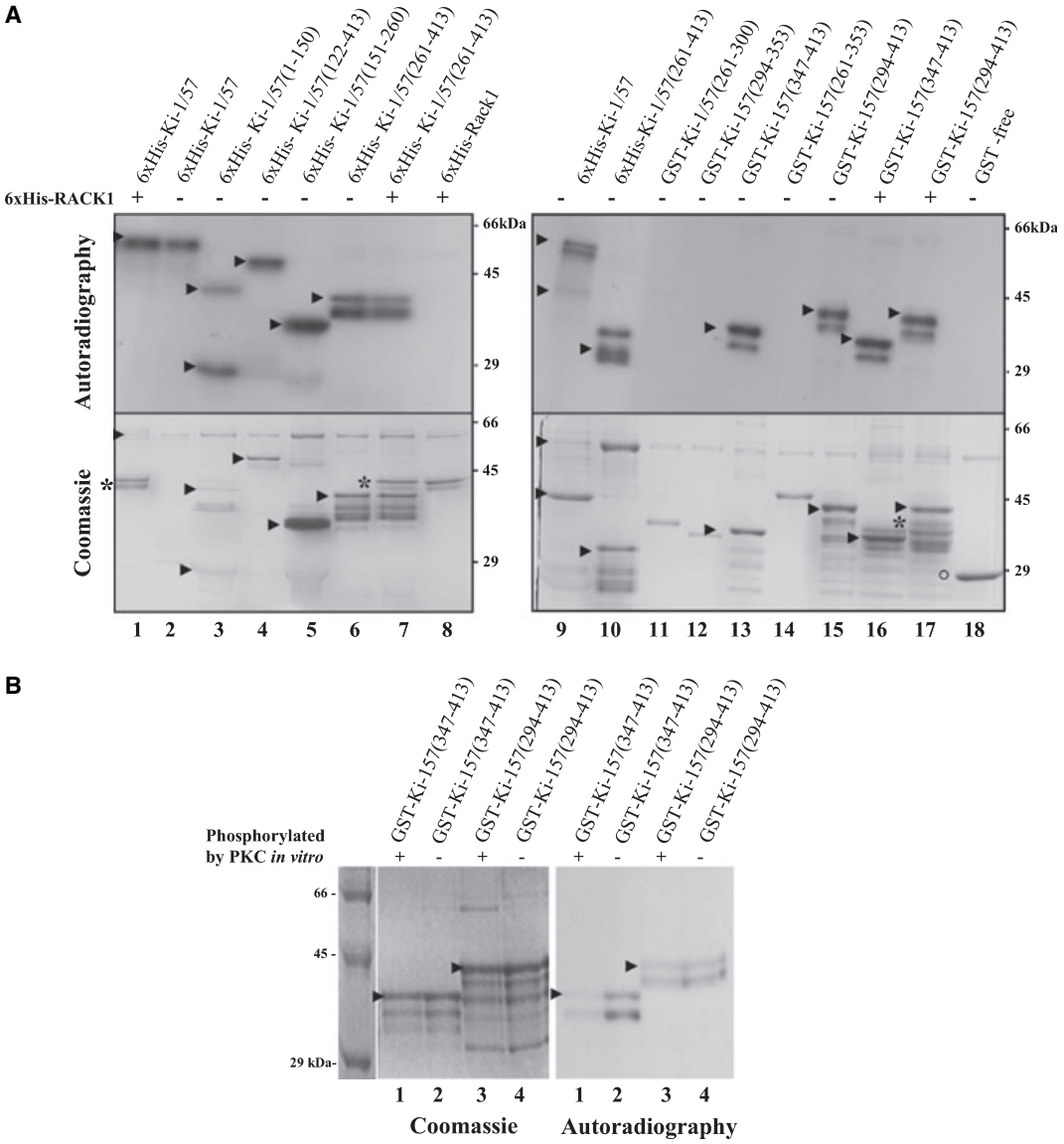

Fig. 4. Regions of Ki-1/57 containing RGG/RXR boxes are methylated by PRMT1 *in vitro* but methylation can be blocked by previous phosphorylation. (A) cDNAs encoding the Ki-1/57 protein fragments shown in schematic Fig. 2A were subcloned into the bacterial expression vectors, expressed as GST- or 6xHis fusions in *E. coli* and purified. The indicated protein fragments and control proteins were submitted to *in vitro* methylation using GST–PRMT1 and analyzed by autoradiography for incubated radioactive methyl groups. Loading of the reactions was controlled by SDS/PAGE (Coomassie Brilliant Blue). Molecular masses of selected marker proteins are indicated on the right of both left- and right-hand panels. Arrow-heads indicate the bands that correspond to the predicted molecular masses of the 6xHis- or GST-Ki-1/57 fragments. Asterisks indicate the position of 6xHis–RACK1 protein bands. The open circle indicates the GST protein band. (B) As (A) but with or without previous phosphorylation of the indicated GST–Ki-1/57-fusion proteins, by PKC-Pan, *in vitro*.

which was also isolated and identified by protein amino acid sequencing from these cells [5]. Methylation of Ki-1/57 isolated from L540 cells suggests that is already methylated *in vivo* in these cells. The *in vitro* methylation reaction is specific because the control antigen, immunoprecipitated by anti-(Ki-67) IgG, did not serve as a substrate for PRMT1 *in vitro* (lane 5).

When we pretreated the L540 cells with Adox, an inhibitor of the cellular synthesis of the methyl-group donor molecule *S*-adenosyl-L-methionine (SAM), we observed that Ki-1/57 was strongly methylated by PRMT1 (Fig. 3B, lanes 1 and 2) *in vitro*. These results show that Ki-1/57 already existed in a methylated form in L540 cells. Most interestingly, we observed that Ki-1/57 from the nucleus can be stronger methy-

lated by PRMT1 *in vitro*, than Ki-1/57 from the cytoplasm (Fig. 3B, lanes 1–2).

Metabolic labeling of HeLa cells *in vivo* with radioactive [^{3}H]-SAM showed stronger methylation of Ki-1/57 in the absence of the inhibitor Adox (Fig. 3C) than in its presence. This can be explained by the mode of action of the inhibitor Adox, which reduces the amount of the endogenous methyl group donor molecule SAM in the cells. As a consequence of this, the small amount of externally added radioactively labeled SAM may be suboptimal for an effective methylation of Ki-1/57 *in vivo*. Interestingly, we did not observe any radioactive labeling by methyl incorporation of the control immunoprecipitated protein CGI-55 (data not shown). This suggests that either the protein concentration of CGI-55 in HeLa cells is much lower than that of Ki-1/57 or that the degree of methylation of CGI-55 *in vivo* is much lower that of Ki-1/57 and not detectable under the conditions tested in Fig. 3C.

Mapping the protein regions of Ki-1/57 that are methylated by PRMT1 *in vitro*

To address which of the described RGG/RXR box clusters are possible targets for PRMT1 methylation, we submitted a series of deletion proteins of bacterially derived Ki-1/57 to an *in vitro* methylation assay with PRMT1 (Fig. 4A). We found that the N-terminal (1–150), central (151–260) and C-terminal (261–413) regions of Ki-1/57 are all strongly methylated by PRMT1 (Fig. 4A, lanes 3, 5 and 6) *in vitro*. This shows that all three major clusters of RGG/RXR boxes (Fig. 1B) are possible targets for arginine methylation by PRMT1. We also tested five subdeletions of the C-terminal region of Ki-1/57(261–413) (Fig. 2A). Only Ki-1/57(294–413) and Ki-1/57(347–413), both of which contain the predicted RGG/RXR box cluster, were methylated by PRMT1 (Fig. 4A, lanes 13 and 15), suggesting that the presence of this cluster is both necessary and sufficient for methylation of the C-terminal region of Ki-1/57.

To test whether the protein RACK1, which binds to the C-terminus of Ki-1/57 [11], influences the methylation reaction by PRMT1 it was added to the assay (Fig. 4A, lanes 1, 7, 16, 17). We found that the presence of RACK1, which is not itself methylated by PRMT1 (Fig. 4A, lane 8), had no influence on the outcome of the methylation reaction. This suggests that PRMT1 can still methylate the C-terminal domain of Ki-1/57, although RACK1 is bound to it.

Prior phosphorylation of Ki-1/57 can decrease its methylation by PRMT1 *in vitro*

We previously reported that the Ki-1/57 C-terminus is a target for phosphorylation by activated protein kinase C (PKC) *in vitro* and *in vivo* [11]. Therefore, we asked if there is an influence of the phosphorylation of Ki-1/57 on its methylation by PRMT1. First we used full-length protein 6xHis–Ki-1/57 previously phosphorylated or not *in vitro*. We did not observe any difference in the amount of subsequent methylation of the phosphorylated vs. nonphosphorylated form (data not shown). We speculate that it may not be possible to detect small local changes in the degree of methylation, because the overall Ki-1/57 sequence has many putative methylation sites.

We therefore also phosphorylated two C-terminal deletion constructs of the Ki-1/57 with 4β-phorbol 12-myristate 13-acetate-activated PKC–Pan *in vitro* and then methylated them with PRMT1 *in vitro*. We noted that methylation of the larger fragment Ki-1/57(294–413) is little influenced by prior phosphorylation, but methylation of the smaller fragment Ki-1/57(347–413) is significantly inhibited by previous phosphorylation (Fig. 4B). Both constructs contain the conserved C-terminal RGG/RXR box cluster 369–383, which contains, in the middle two RGG motifs, the target residue T375 for phosphorylation by PKC (Fig. 1C) [11]. Introduction of a negative charge in this region of the RGG box may lead to the observed inhibitory influence on protein methylation by PRMT1. The larger inhibitory effect on the smaller fragment in comparison with the larger fragment may be explained by a local effect of the phosphorylation and introduction of a negative charge, which may be expected to be relatively larger on a smaller protein fragment. Moreover, interaction of PRMT1 with the smaller fragment is weaker than with the larger one (compare Fig. 2A and E). Therefore, the inhibitory influence of phosphorylation on this weaker interaction with the smaller fragment may be more pronounced.

PRMT1 dimerization and its N-terminal domain are necessary for the methylation of full-length protein Ki-1/57

We also wanted to map the regions of PRMT1 that are important for both its dimerization and its interaction with Ki-1/57. Therefore, we generated a series of truncations of PRMT1 and cloned them into the yeast expression vector pGAD424 (Fig. 5A). We noted that only one of the five PRMT1 deletions, which contains both the catalytic core and the C-terminal domain,

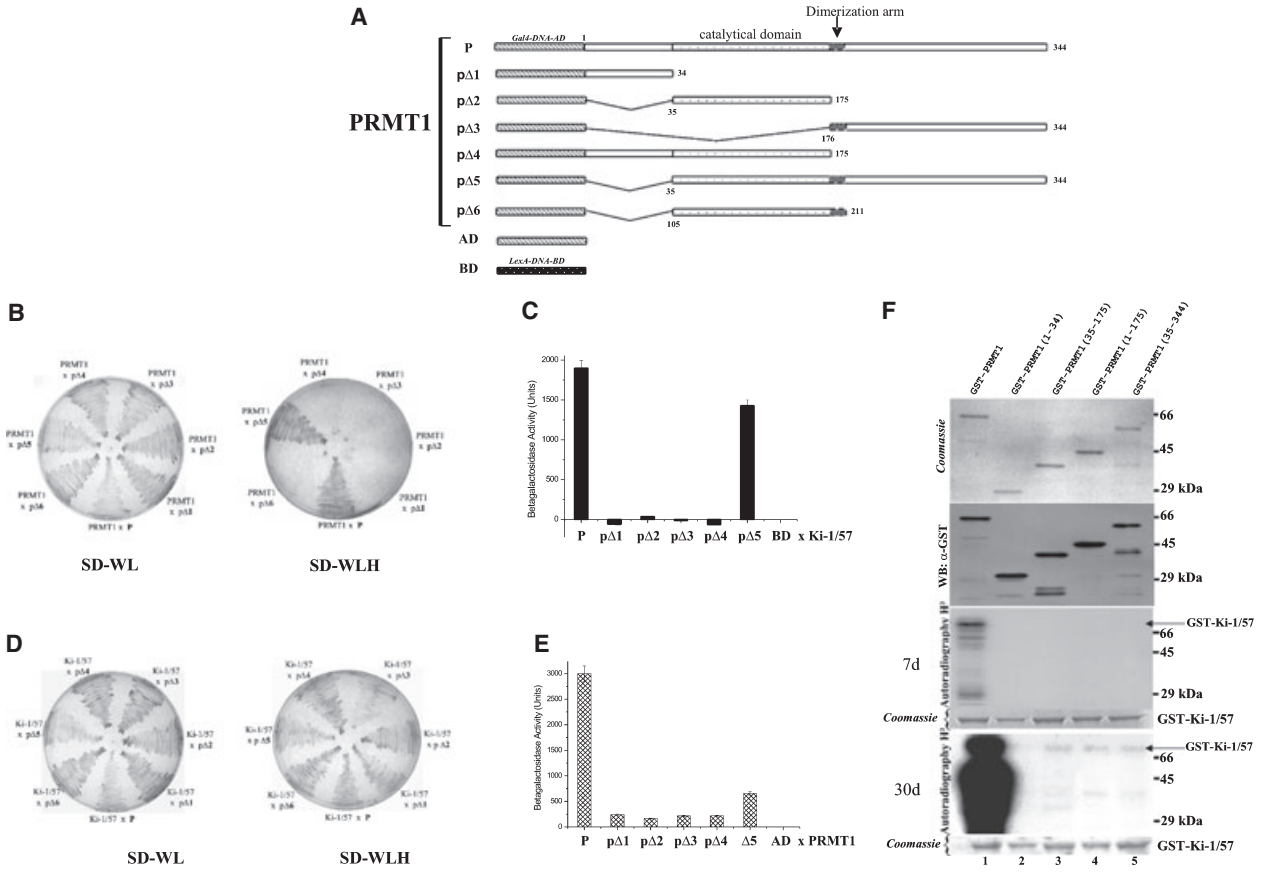

Fig. 5. PRMT1 deletion lacking the N-terminal first 34 amino acids dimerizes but shows strongly reduced recognition of the full-length protein substrate Ki-1/57 and residual methylation activity *in vitro*. (A) Schematic representation of full-length PRMT1 (P) and the six PRMT1 deletion constructs pΔ1–pΔ6 used in the yeast two-hybrid studies (B–E) and *in vitro* methylation assays of Ki-1/57 (F). The diagonal striped box indicates the Gal4 DNA-binding domain (AD), the vertical dotted box (35–175) in the middle of the PRMT1 protein represents the catalytic domain and the dark box (176–211) the dimerization arm. The black box below indicates the LexA–DNA-binding domain (BD). (B) Six PRMT1 deletion constructs (in vector pGAD424 fused to the Gal4 activation domain) were tested for their potential to dimerize with full-length PRMT1 (cloned in fusion with the LexA–DNA-binding domain in vector pBTM116). The indicated PRMT1 constructs were cotransformed into L40 yeast cells which were tested for interaction by assessing their ability to grow on the -Trp, -Leu, -His plates (right). Presence of plasmids was tested by growth on -Trp, -Leu plates (left). (C, E) Quantification of the strength of indicated interactions by measurement of the beta-galactosidase in a liquid ONPG assay (see Experimental procedures for details). The quantity of the produced yellow color is expressed in arbitrary units. (D) The full-length Ki-1/57 construct (cloned in pBTM116 in fusion with the LexA–DNA-binding domain) was transformed into L40 yeast cells. Full-length PRMT1 (P) or the indicated PRMT1 deletion construct (pΔ1–pΔ6) all cloned in fusion with the Gal4-AD in pGAD424, were cotransformed into L40 yeast cells which were tested for interaction as in (B) above. (F) *In vitro* methylation of GST–Ki-1/57 by different GST–PRMT1 deletion constructs (also see panel A). Methylation was assessed by autoradiography and exposition to X-ray film for 7 or 30 days. Protein loading was controlled by SDS/PAGE and anti-GST western blot as indicated. Molecular masses of selected marker proteins are indicated on the right of the panels.

PRMT1(35–344), was able to dimerize (Fig. 5B,C). This can be explained by the presence of the dimerization region of PRMT1 in the C-terminal domain. Previous studies have shown that this region is important for the dimerization of PRMT1 and that PRMT1 is catalytically active only in its dimerized form [39].

When the PRMT1 deletions were tested for interaction with Ki-1/57, only the PRMT1 deletion (35–344) showed significant interaction in a quantitative β-galactosidase assay (Fig. 5E), although all deletions showed residual growth in the plate assay (Fig. 5D). Nonetheless, the interaction of deletion PRMT1(35–344) decreased by ~ 75% (Fig. 5E) in comparison with full-length PRMT1. This suggests that the N-terminal region of PRMT1 is important for recognition of full-length protein substrates, and that PRMT1 dimerization is necessary but not sufficient for effective binding to a full-length protein substrate such as Ki-1/57.

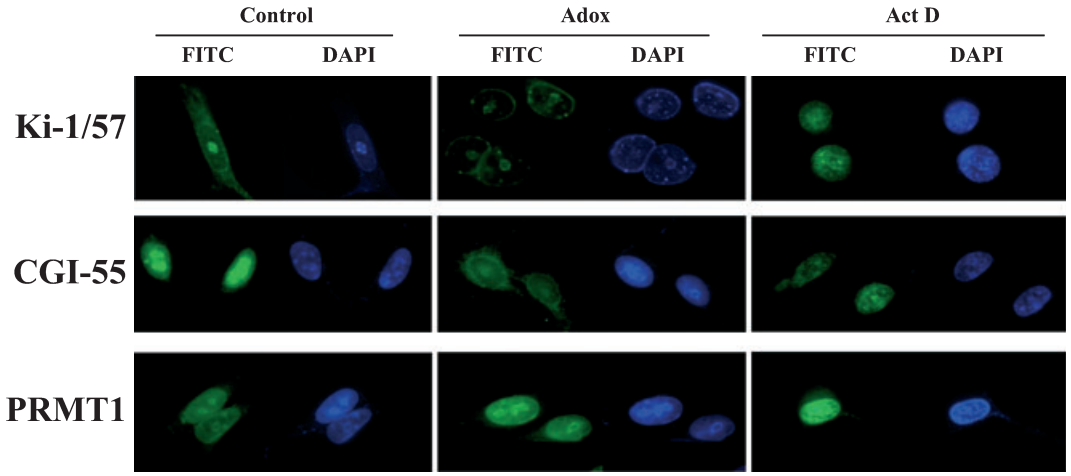

Fig. 6. Immunofluorescence analysis of the localization of the proteins Ki-1/57, CGI–55 and PRMT1. HeLa cells were grown on coverslips and incubated for 16 h with or without Adox or for 3 h with actinomycin D at 37 °C, with the addition of cycloheximide and chloramphenicol during the last 3 h in all conditions tested. Cells were fixed with 100% methanol and the indicated proteins were immunodetected with the following primary antibodies: mouse Ki-1 mAb, mouse anti-(human CGI-550) mAb 10.5.6., and mouse anti-(human PRMT1) IgG ab7027. Fluorescein-coupled antimouse (green) serum was used a secondary reagent. DAPI staining (blue) served to localize the position of the nucleus. Cells were examined with a Nikon fluorescence microscope.

Interestingly, the N-terminal portion of seven human PRMTs varies most substantially, supporting the hypothesis that these regions are somehow involved in the specific recognition of different protein substrates [40].

Next, we tested the *in vitro* methylation activity of four GST–PRMT1 protein deletion constructs expressed in *Escherichia coli*, using full-length GST–Ki-1/57 fusion protein as a substrate (Fig. 5F). In agreement with the interaction results described above, we found that only full-length PRMT1 effectively methylates Ki-1/57 *in vitro*, suggesting that both dimerization of PRMT1 and the presence of the N-terminal domain are required for effective methylation (Fig. 5F; autoradiography, 7 days exposure). Longer exposure of the gel (30 days) revealed that all three PRMT1-deletion constructs containing the catalytic core domain have a substantial residual methylation activity on the full-length Ki-1/57. By contrast, this was not observed with the RGG/RXR box region of the protein hnRNPQ/NSAP1 [41], which even after 30 days exposure did not show any signs of radioactive labeling (data not shown).

Immunofluorescence analysis of the localization of Ki-1/57, CGI-55 and PRMT1

It has been described previously that the methylation of proteins might be an important prerequisite for nuclear import/export or to address proteins to distinct cellular compartments [19]. Therefore, we performed immunolocalization studies of Ki-1/57, CGI-55 and PRMT1 in the absence and presence of the inhibitors Adox and actinomycin D (Act D) (Fig. 6). In untreated HeLa cells more Ki-1/57 is found in the cytoplasm than in the nucleus, but its cytoplasmic immunostaining is clearly reduced after treatment with the methylation inhibitor Adox. This suggests that the methylation status of Ki-1/57 can influence its distribution between the nuclear and cytoplasmic compartments. Treatment with Act D inhibits the synthesis and consequently the export of mRNA from the nucleus and we observed that it causes significant reduction in cytoplasmic imumunostaining for Ki-1/57. This suggests that the localization of Ki-1/57 may be also influenced by the localization and/or transport of mRNA. By contrast, CGI-55, the putative paralog of Ki-1/57, showed behavior contrary to that observed for Ki-1/57. In untreated cells CGI-55 is found predominantly in the nucleus and after Adox treatment it is partially redistributed to the cytoplasm. Act D has a similar effect to Adox on CGI-55. Interestingly, PRMT1 itself shows a predominantly nuclear staining that did not change much during the treatment with either Adox or Act D.

Discussion

When we found in yeast-two hybrid screens that a significant part of the identified Ki-1/57-interacting

clones represents PRMT1, we speculated that arginine methylation could be an important post-translational modification for this protein. We were able to confirm by other experiments that Ki-1/57 is a substrate for arginine methylation by PRMT1 *in vitro* and *in vivo*. Furthermore, we also performed a two-hybrid screen with the protein PRMT1 as bait and found Ki-1/57 among 12 RGG/RXR box-containing interacting proteins or putative substrates. This is not only a confirmative result for the finding that Ki-1/57 is a substrate for arginine methylation by PRMT1 but also indicates that the yeast two-hybrid system might serve as an effective method for identifying new substrates for PRMTs in general. We are aware of only one other study that used the yeast two-hybrid system to identify possible PRMT substrates [36]. The yeast two-hybrid system may prove very useful to identify specific and common substrates for the at least nine different human PRMTs [42].

Post-translational modifications of proteins are important to modify and regulate their functions. We previously found that Ki-1/57 is connected via the adaptor protein RACK1 to activated PKC and that PKC can phosphorylate it on distinct threonine residues located in its extreme C-terminus [11]. Because, in addition to this cell-activation-dependent phosphorylation, Ki-1/57 is also post-translationally modified by arginine methylation mediated by PRMT1, we wanted to see if there is an influence of the previous phosphorylation on the methylation by PRMT1, *in vitro*. In fact, T375, one of the putative target residues of PKC, is located in the center of the second conserved RGG/RXR box cluster at the C-terminus of Ki-1/57 (Fig. 1C) [11]. We found that phosphorylation of the protein fragment Ki-1/57(347–413) can affect its subsequent arginine methylation by PRMT1.

The structure resolution of various PRMTs from different species showed a structural conservation of PRMTs catalytic core and that the PRMTs must dimerize for catalytic activity [40]. Furthermore, the amino acid sequences of at least eight human PRMTs differ predominantly in their N-terminal regions [19]. We speculated that these regions might be important for differential recognition of target substrates. The structural requirements of PRMT1 for the recognition and methylation activity have so far, only been analyzed for small peptide substrates [39]. Recognition and methylation of an entire protein substrate may, however, be different to that described for small peptides. Hence, we performed interaction mapping and *in vitro* methylation assays of Ki-1/57 with a series of PRMT1 protein deletion constructs. We found that PRMT1 lacking its 33 amino acid N-terminal domain is capable of dimerizing to almost the same extend as wild-type PRMT1. The interaction of this same PRMT1 deletion with full-length Ki-1/57 was, however, inhibited by a significant 75%, suggesting that the N-terminal region may be involved in the recognition of this substrate. *In vitro* methylation experiments with PRMT1 deletion mutants expressed in *E. coli* initially demonstrated that only full-length PRMT1 is effectively methylating Ki-1/57. However, longer exposure of the same X-ray film revealed that all three mutant proteins that contained the catalytic core of the protein still show significant activity to methylate Ki-1/57, indicating that the catalytic domain alone, in the absence of dimerization or N-terminal domains, retains residual activity on a full-length protein substrate. This seems to depend on the nature of the protein substrate involved, because we did not observe any methylation of an isolated RGG/RXR box domain of the protein hnRNPQ/NSAP1 [41], even with longer exposure of the X-ray film, under the same conditions (data not shown).

One question that arises from the finding that Ki-1/57 is a substrate for PRMT1 arginine methylation is related to the functional consequence of methylation for the protein. Because Ki-1/57 function is not yet known, but methylation has been described as essential for regulation of the subcellular, principally nuclear, localization of a series of other proteins [19], we set out to test the localization of Ki-1/57 with and without methylation. We found that Ki-1/57, which is normally found more in the cytoplasm than in the nucleus, shows decreased cytoplasmic immunostaining when cells are treated with the methylation inhibitor Adox. This suggests that the methylation status of Ki-1/57 can influence its distribution between nuclear and cytoplamic compartments.

The fact that Act D, which inhibits the synthesis of nuclear mRNA, also causes a decrease in cytoplasmic Ki-1/57 immunolabeling, suggests a possible functional association with the export of RNA from the nucleus. Clearly, more experiments are necessary, however, these results together with other published data encourage further experiments to tests such a hypothesis: First, CGI-55 a putative homolog of Ki-1/57 has been described as an mRNA-interacting protein termed PAI–RBP1 [8]. Second, Ki-1/57 itself, also termed IHABP4, has been reported to interact with negatively charged extracellular macromolecules such as hyaluronan and RNA *in vitro* [6]. Finally, some early studies on Ki-1/57, using gold-labeled Ki-1 antibody in electron microscopy analysis [4], showed labeling of the nuclear envelope and of 'spiral-shaped' structures which were associated with

the nuclear pores and appeared to 'pass' through them.

The amino acid sequence of CGI-55 is very similar to that of Ki-1/57 (41% identity, 67% similarity), suggesting that the two proteins could be paralogs. In immunofluorescence microscopy studies CGI−55, which shows normally a predominan nuclear labeling, showed increased cytoplasmic staining after Adox treatment. This suggests that methylation has different effects on the distribution of the two proteins between the nucleus and cytoplasm. Future studies must address the detailed regulation of the cellular localization of these two proteins and if and how these proteins are involved in RNA binding and possibly metabolism.

Experimental procedures

Plasmid constructions

Several sets of oligonucleotides were designed to allow subcloning of cDNAs encoding the indicated amino acid sequences of the proteins studied. Cloning of the complete cDNA encoding Ki-1/57 and RACK1, or their deletions, in bacterial (pGEX, pET28a, or pProEx) and yeast expression vectors (pBTM116, pGAD424, pACT2) has been described previously [11]. Insertion of PRMT1 complete cDNA into pGEX-5X-2 (GE Healthcare, Waukesha, WI) allowed to express PRMT1(1–344) as a C-terminal fusion to GST (GST–PRMT1). The cDNA of full-length PRMT1(1–344) was inserted into pBTM116, and its indicated deletion constructs were inserted into vector pACT2. The deletion constructs of PRMT1 were also subcloned into bacterial expression vector pGEX to allow their expression as GST-tagged fusion proteins.

Yeast two–hybrid screenings and interaction analysis

pBTM116-Ki-1/57(122–413) [11], pBTM116-Ki-1/57(1–150) and pBTM116-PRMT1(1–344) vectors were used to express fragments spanning the C- or N-terminus of Ki-1/57 or full-length PRMT1, respectively, linked to the C-terminus of LexA DNA-binding domain. Recombinant plasmids were transfected in *Saccharomyces cerevisiae* strain L40. A human fetal brain cDNA library (Clontech, Palo Alto, CA) expressing GAL4 activation domain (AD) fusion proteins was cotransfected with each one of these three recombinant pBTM116 vector constructs in three separate screening assays. Selection of transformants, β-galactosidase activity test, plasmid DNA extraction and sequencing were performed as described previously [11,41]. The quantitative ONPG assay to assess the β-galactosidase activity was performed as described previously [43].

Bacterial expression and protein purification

GST, GST–Ki-1/57, GST–PRMT1, 6xHis–RACK1 and 6xHis–Ki-1/57 full-length proteins or indicated deletions were expressed in *E. coli* BL21-CodonPlus-RIL (Stratagene, La Jolla, CA) and purified using glutathione–Sepharose 4B (GE Healthcare, Waukesha, WI) or Ni-NTA Sepharose as described before [41].

Western blot analysis, antibodies and cell culture

Proteins were separated by SDS/PAGE, transferred to a poly(vinylidene difluoride) membrane and visualized by immunochemiluminescence using a mouse anti-GST IgG (to control equal loading of beads), mouse anti5xHis mAb (Qiagen, Hilden, Germany) or mouse anti-(Ki-1/57) mAbs A26 or Ki-1 and secondary anti-(mouse IgG)–HRP conjugate. The anti-(Ki-1/57) mAbs A26 [5], Ki-1 [1] and Ki-67 [44] have been described previously. Mouse anti-(human PRMT1) IgG ab7027 was purchased from Abcam, Inc. (Cambridge, MA). The anti-(CGI−55) mouse mAb has been described previously [7]. HeLa cells and L540 Hodgkin analogous cells were cultivated as described previously [11].

Pull-down assay

Recombinant purified GST–PRMT1 (3 μg) protein was coupled to glutathione–Sepharose beads. After washing beads were incubated with either bacterially expressed and purified 6xHis–Ki-1/57 (1 μg) or the control protein 6xHis–RACK1 (1 μg). After six washes coprecipitated proteins were analyzed by western blot against the 5xHis tag or PRMT1 (for control of equal loading) as described above. Equal loading with 6xHis fusion proteins was controlled by SDS/PAGE stained with Coomassie Brilliant Blue.

In vitro methylation and phosphorylation

Recombinant Ki-1/57, or the control proteins were incubated in NaCl/P_i containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 2 μL of radiolabeled SAM (2 μCi) (GE Healthcare) with or without GST–PRMT1 (bound to glutathione beads) for 1 h at 37 °C, as indicated in the figures. Reactions were stopped by heating to 100 °C for 5 min in sample buffer and then run on SDS/PAGE. After fixing the gel for 20 min in 10% v/v both methanol and acetic acid in water it was washed, and incubated in amplifying solution (GE Healthcare) for 1 h 30 min, washed again briefly, dried and exposed to Hyperfilm MP (GE Healthcare) for 2 days or for the indicated times. *In vitro* phosphorylation of Ki-1/57 was performed as described previously [11] utilizing commercial PKC-Pan (Promega, Madison, WI). PKC-Pan was purified from rat brain and consists predominantly of the PKC isoforms α,β and γ.

Preparation of cytoplasmic and nuclear extracts, methylation assays with cellular Ki-1/57 and metabolic labeling

L540 cells (5.0×10^7) incubated or not with Adox (20 μM) for 16 h, were lyzed for 1 h at 4 °C in 1 mL of modified cytoplasmic buffer (20 mM Tris pH 8.0, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothrietol, 2 mM phenylmethylsulfonyl fluoride and protease inhibitors) [45]. After centrifugation at 14 000 ***g***, the nuclear fraction was lyzed in 1 mL nuclear buffer (20 mM Tris pH 8.0, 0.4 M NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 25% v/v glycerol) at 4 °C for 1 h. The cytoplasmic and nuclear fractions were incubated for 2 h, at 4 °C, with 20 μL protein A–Sepharose beads (GE Healthcare), previously loaded with the indicated antibodies overnight at 4 °C, washed three times in cytoplasmic buffer and incubated with human recombinant protein GST–PRMT1 and 4 μL of radiolabeled SAM (4 μCi; GE Healthcare) in a final volume of 50 μL. Finally, the reaction was stopped by adding 10 μL of 6× SDS/PAGE sample buffer and boiling at 100 °C for 5 min. Western blots using the indicated antibodies were developed by chemiluminescence as described previously [41] (Fig. 3B).

For the *in vivo* metabolic labeling experiment (Fig. 3C), 5.0×10^7 HeLa cells were preincubated with 20 μM Adox as above for 16 h and subsequently labeled *in vivo* by incubation with 20 $\mu Ci \cdot mL^{-1}$ radiolabeled SAM, 10 mM cyclohexamide and 10 mM chloramphenicol, under constant agitation at 37 °C for 4 h, in the presence of freshly added Adox (20 μM). Lysis, fractionation of nucleus and cytoplasm, immunoprecipitation and SDS/PAGE were performed as above and autoradiography of the dried gel was performed on a Hyperfilm MP at 80 °C for 6 months.

Immunofluorescence analysis

HeLa cells grown on glass cover slips were incubated or not with Adox (100 $\mu g \cdot mL^{-1}$) for 16 h or with Act D (10 $\mu g \cdot mL^{-1}$) for 3 h at 37 °C. To inhibit protein synthesis we also added cycloheximide (100 $\mu g \cdot mL^{-1}$) and chloramphenicol (40 $\mu g \cdot mL^{-1}$). Cells were fixed with 100% methanol and immunostained with primary mouse mAbs Ki-1, anti-(CGI-55) 10.5.6 or anti-PRMT1, and secondary antibody fluorescein anti-mouse IgG. Cells were examined with a Nikon (Kanagawa, Japan) microscope. DAPI staining was used to show the positions of the nuclei. Cells were examined with a Nikon fluorescence microscope.

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III RESULTADOS

Artigo V

The methylation of the C-terminal region of hnRNPQ (NSAP1) is important for its nuclear localization

Dario O. Passos, Alexandre J. C. Quaresma , and Jörg Kobarg

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The methylation of the C-terminal region of hnRNPQ (NSAP1) is important for its nuclear localization ☆

Dario O. Passos [a,b], Alexandre J.C. Quaresma [a,b], Jörg Kobarg [a,b,*]

[a] *Centro de Biologia Molecular Estrutural, Laboratório Nacional de Luz Síncrotron, Rua Giuseppe Máximo Scolfaro 10.000, C.P. 6192, 13084-971 Campinas, SP, Brazil*
[b] *Departamento de Bioquímica, Universidade Estadual de Campinas, 13084-970 Campinas, SP, Brazil*

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Abstract

Protein arginine methylation is an irreversible post-translational protein modification catalyzed by a family of at least nine different enzymes entitled PRMTs (protein arginine methyl transferases). Although PRMT1 is responsible for 85% of the protein methylation in human cells, its substrate spectrum has not yet been fully characterized nor are the functional consequences of methylation for the protein substrates well understood. Therefore, we set out to employ the yeast two-hybrid system in order to identify new substrate proteins for human PRMT1. We were able to identify nine different PRMT1 interacting proteins involved in different aspects of RNA metabolism, five of which had been previously described either as substrates for PRMT1 or as functionally associated with PRMT1. Among the four new identified possible protein substrates was hnRNPQ3 (NSAP1), a protein whose function has been implicated in diverse steps of mRNA maturation, including splicing, editing, and degradation. By *in vitro* methylation assays we were able to show that hnRNPQ3 is a substrate for PRMT1 and that its C-terminal RGG box domain is the sole target for methylation. By further studies with the inhibitor of methylation Adox we provide evidence that hnRNPQ1-3 are methylated *in vivo*. Finally, we demonstrate by immunofluorescence analysis of HeLa cells that the methylation of hnRNPQ is important for its nuclear localization, since Adox treatment causes its re-distribution from the nucleus to the cytoplasm.

Keywords: Yeast two-hybrid system; Protein arginine methylation; Post-translational modification; Protein–protein interactions; Identification of methylated substrates; Sub-cellular localization

The hnRNPQ proteins are members of the large family of heterogeneous nuclear ribonucleoproteins (hnRNPs), which is composed by over 20 different proteins [1]. It has also been termed as GRY-RBP [2,3] or NSAP1 (non structural associated protein 1), since it has been described to interact with a non-structural protein from the minute virus of mice [4]. hnRNPQ appears in three protein isoforms called Q1–Q3, which are derived from alternative splicing of a single gene. Its smallest proteic isoform Q1 has an apparent molecular mass of ~62 kDa, whereas Q2 has a molecular mass of ~65 kDa, and Q3 of ~70 kDa. The exact molecular functions of each of these three isoforms are still not well understood [1].

Most members of the family of hnRNP proteins are known for their nuclear localization, nuclear-cytoplasmic shuttling, and their interaction with RNA or other RNA binding proteins, and are predicted to be functionally involved in diverse aspect of RNA metabolism [5–7]. Many of the hnRNP members contain so-called RGG-boxes

☆ *Abbreviations:* Ki-1/57, 57 kDa Ki-1 antigen; MM, minimal medium; PRMT, protein arginine methyl transferase; hnRNPQ, heterogeneous nuclear ribonucleoprotein type Q; SMN, survival of motor-neuron; NSAP1, NS1-associated protein 1; GRY-RBD, glycine-arginine-tyrosine-rich RNA-binding protein; RRM, RNA recognition motif; RGG/RXR-box, arginine-glycine rich box, where X is any amino acid; Act D, actinomycin D; Adox, adenosine-2′,3′-dialdehyde; IP, immuno-precipitation; SAM, *S*-Adenosyl-L-methionine; WB, Western blot; AP, alkaline phosphatase.

* Corresponding author. Fax: +55 19 3512 1006.
E-mail address: jkobarg@lnls.br (J. Kobarg).

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(Arg-Gly-rich regions) and most of them one to several RRMs (RNA recognition motifs) [1,8,9].

hnRNPQ has been functionally implicated in several different steps of mRNA maturation. One of the attributed functions for this protein was its association with the editosome complex, through the identification of its interaction with the mRNA editing cytidine deaminase Apobec1 [2,3]. The editing complex contains in addition to the catalytically active component Apobec1 also the essential cofactor ACF (Apobec1 complementation factor) [2,10]. hnRNPQ may be another regulatory component of the apoB RNA editing-complex, acting through binding to Apobec1, ACF, and the ApoB mRNA [2].

Furthermore, hnRNPQ has been identified to be a component of the SMN-complex (survival of motor neurons) [1] interacting with the wild-type form of the protein SMN, although not with its truncated form, which is found in spinal muscular atrophy [9]. Finally, hnRNPQ has been functionally associated to a multiprotein complex that regulates the translationally coupled process of mRNA degradation of specific mRNAs such as that of c-fos [11] or of mRNAs related to the circadian rhythm [12]. The importance of the association to the latter context is emphasized by the finding that hnRNPQ has been described to interact with the protein AUF1 [13], which is a key element for the destabilization of AU-rich mRNAs [14].

Protein arginine methylation is an irreversible post-translational modification found in eukaryotes. Only recently the functional relevance of this post-translational protein modification is being explored [15–19]. Until now, nine enzymes called PRMT1-9 were described [20–27], that catalyze the arginine methylation of proteins. PRMT1 seems to be responsible for ca. 85% of the total protein methylation in the cell [28,29]. Among the characterized protein substrates, the major group consists of the hnRNPs [30], which are normally methylated in their arginine- and glycine-rich regions (“RGG-boxes”) [31]. Protein arginine methylation has been implicated to be necessary for RNA processing [32,33], transcriptional regulation [34,35], signal transduction [36,37], DNA repair [17], and the regulation of the sub-cellular localization of proteins [32].

Here, we report the results of a yeast two-hybrid screen, where we used human PRMT1 as bait, in order to identify possible new *in vivo* protein substrates. Among the novel identified putative protein substrates was hnRNPQ, which interacts specifically with PRMT1 and is also its substrate *in vitro*. We also demonstrate that hnRNPQ is methylated on arginine residues *in vivo*. Its C-terminal region, which contains an RGG-box, is absolutely required for its localization to the nucleus. Furthermore, we report that the methylation of hnRNPQ *in vivo* seems to be important for its predominant nuclear localization, since the inhibition of protein methylation by treatment of HeLa cells with Adox results in the partial re-distribution of hnRNPQ from the nucleus to the cytoplasm.

Results and discussion

Yeast two-hybrid screen using PRMT1 as a bait

In order to identify new PRMT1 interacting proteins, the yeast two-hybrid system [38] was employed using the PRMT1 as bait against a human fetal brain cDNA library (Clontech). The 2.0×10^6 screened co-transformants yielded 273 positive clones for both His3 and LacZ reporter constructs. Among the 36 library plasmid DNA clones, we identified 26 clones that encode nine different proteins directly or indirectly involved in RNA metabolism. The other clones encoded proteins that will be described elsewhere.

Of the nine proteins involved in RNA metabolism five had been previously described as direct protein substrates or as functionally associated proteins for PRMT1. The latter consist of: CIRBP [39], ILF3 [40], β-tubulin [41], EWSR1 [42], and ribosomal protein S29 [43]. On the other hand, the screen resulted in the identification of four proteins involved in RNA metabolism, that may represent novel *in vivo* substrates for PRMT1: hnRNPQ (this study), Ki-1/57 (or IHABP4) [44–46], hnRNPA3 [47], and SFR1 [48]. All nine identified proteins except ribosomal protein S29 contain at least one (β-tubulin) or even up to 25 (EWSR1) RGG/RXR boxes, the typical target motif for protein methylation by PRMTs. This already seems to indicate that yeast two-hybrid screens, in general, can be used with success in order to identify new PRMT candidate substrate proteins. Here, we set out to test in more detail if the identified PRMT1 interacting protein hnRNPQ, which contains 16 RGG/RXR boxes in its C-terminal region, is a true substrate for PRMT1 and what are the possible functional consequences for the methylation of hnRNPQ *in vivo*.

PRMT1 interacts with the C-terminal region of hnRNPQ and methylates it in vitro

To test if hnRNPQ is a substrate for PRMT1, we performed an *in vitro* methylation assay (Fig. 1). The full-length 6× His-hnRNPQ (lane 3) as well as the fusion protein GST-hnRNPQ(389–623), which comprises only its C-terminal RGG/RXR-box region (lane 5), were both methylated by PRMT1 *in vitro*. As control, we used the RGG-box containing protein GST-Ki-1/57 [45–47], which was also methylated by PRMT1 (lane 6). The reaction was specific since neither the control protein GST nor the fusion construction lacking the C-terminal region GST-hnRNPQ(1–443) was methylated by PRMT1 *in vitro* (lanes 2 and 4, respectively).

In agreement with this *in vitro* methylation mapping, the prey-plasmid that showed interaction with lexA-PRMT1 in the yeast two-hybrid screen encodes the residues 390–623 located at the C-terminus of hnRNPQ. The C-terminal region of hnRNPQ 3 contains an extensive RGG/RXR-box motif, which includes 11 RGG/RXR boxes, seems to

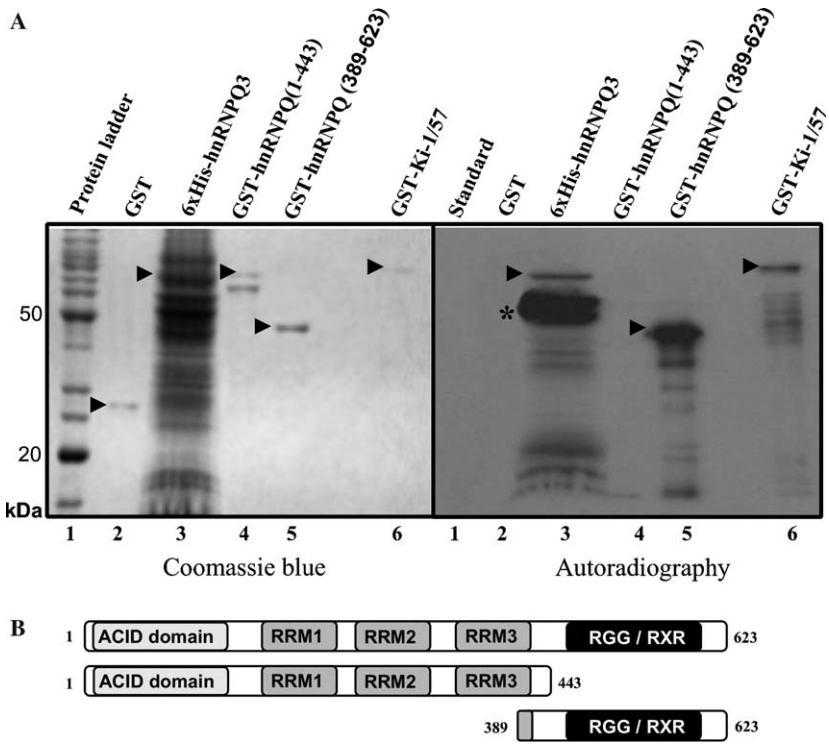

Fig. 1. hnRNPQ is a substrate for PRMT1 *in vitro*. (A) The indicated proteins were methylated by GST-PRMT1 *in vitro*. PRMT1 was expressed and purified as a GST fusion protein and incubated with the indicated recombinant proteins, all expressed in and purified from *E. coli*. Full-length hnRNPQ was expressed and purified from recombinant baculovirus-infected Sf9 insect cells. Its methylation reaction was carried out with whole cell lysate instead of with purified protein. Selected molecular masses of the protein standard in lane 1 are indicated at the side. The asterisk (*) points out degraded hnRNPQ protein. The arrows are indicating the protein bands that match with the predicted molecular masses. (B) Schematic representation of hnRNPQ3 constructs used in the *in vitro* methylation assay. The localization of the RGG/RXR boxes, three RRM domains, and the N-terminal acidic domain of hnRNPQ is indicated.

mediate binding to PRMT1, and is also the target for *in vitro* arginine methylation (Fig. 1).

hnRNPQ is methylated in vivo in HeLa cells

In order to test whether hnRNPQ is also methylated *in vivo*, we performed immuno-precipitation of hnRNPQ from whole cell lysates and then analyzed the immunoprecipitate by Western blot utilizing antibodies that specifically detect mono- and di-methylarginine, the two types of arginine methylation mediated by PRMT1 (Fig. 2). We found three protein bands of approximately 70, 67, and 62 kDa molecular mass (lane 2) that were labeled by the anti-mono/di-methylarginine antibodies. The bands likely represent the different protein isoforms of hnRNPQ. The labeling of the antibodies was specific since no such bands were detected when the anti-hnRNPQ antibody was not incubated with lysate (lane 1). Further confirmation, that the detected bands represent the *in vivo* methylated hnRNPQ proteins, comes from the observation that these bands disappeared, when the cells were pre-treated with the inhibitor of methylation Adox (lane 3). Together, these results demonstrate that the hnRNPQ proteins normally occur in methylated form *in vivo*, in HeLa cells.

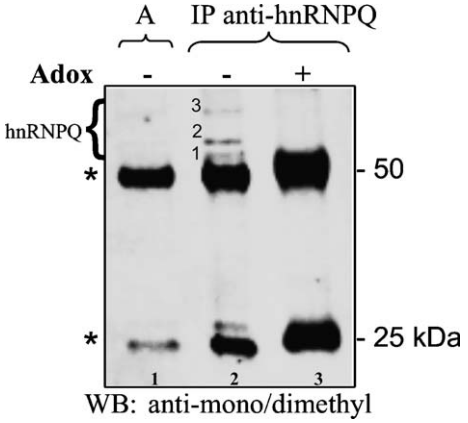

Fig. 2. hnRNPQ isolated from human cells shows methylation *in vivo*. hnRNPQ was immunoprecipitated (IP) from the whole cell lysate of HeLa cells treated (+) or non-treated (−) by methylation inhibitor Adox. Then the immunoprecipitated proteins were run out on by SDS–PAGE and transferred to a PVDF-membrane. Next we used a mixture of antibodies anti-mono and—dimethylarginine to probe the membrane by Western blotting. As a control anti-hnRNPQ antibody (A) (lane 1) was not incubated with cell lysate in order to be able to identify antibody bands. The asterisks (*) correspond to the heavy (above) and light (below) chains of the antibody, which also served as protein molecular mass markers of 50 and 25 kDa, respectively. The bracket indicates the specific methylated hnRNPQ 1–3 bands.

hnRNPQ2 and 3 are the main isoforms found in HeLa cells and are located predominantly in the nucleus

When we detected the three bands in Fig. 2, that likely represent the methylated isoforms of hnRNPQ, we were interested to confirm if they are indeed the three isoforms of hnRNPQ and how they may be distributed in the cytoplasmic and nuclear compartments. Therefore, we performed immuno-precipitations from both the nuclear and cytoplasmic fractions of the lysate of HeLa cells with anti-hnRNPQ antibody. We then tried to identify the predominantly observed protein bands by peptide sequencing using mass spectrometry analysis (Fig. 3).

We were only able to observe two predominant protein bands of ca. 67 and 70 kDa in the nuclear compartment of the HeLa cells (Fig. 3B). These bands were cut out of the gel and submitted to in gel tryptic digestion. After a mass fingerprint analysis of the tryptic peptide fragments by LC–MS/MS, it was possible to identify both protein bands as hnRNPQ (Fig. 3C and D). The sequenced peptides represented ca. 13% or 15% of the amino acid sequence of the corresponding 67 or 70 kDa proteins, respectively. It has been previously described that the antibody used in the immunoprecipitation can detect up to 4 bands that represent the four proteins [1]. These are: hnRNPR (80 kDa), and the three isoforms 1–3 of hnRNPQ, with respective molecular masses of ca. 62, 67, and 70 kDa. From the apparent molecular masses of ca. 70 and 67 kDa as detected by the SDS–PAGE (Fig. 3A), we were able to assign the two nuclear bands to the isoforms hnRNPQ3 and 2. Although the peptides identified by mass spectrometry did not represent regions that allow differentiating between these isoforms, we could rule out that the upper band of 70 kDa may be hnRNPR. The residues that differ between hnRNPR and hnRNPQ in the sequenced peptides are underlined in Fig. 3C. Together these data suggest that the two identified nuclear protein bands represent hnRNPQ3 (70 kDa) and hnRNPQ2 (67 kDa). The bands

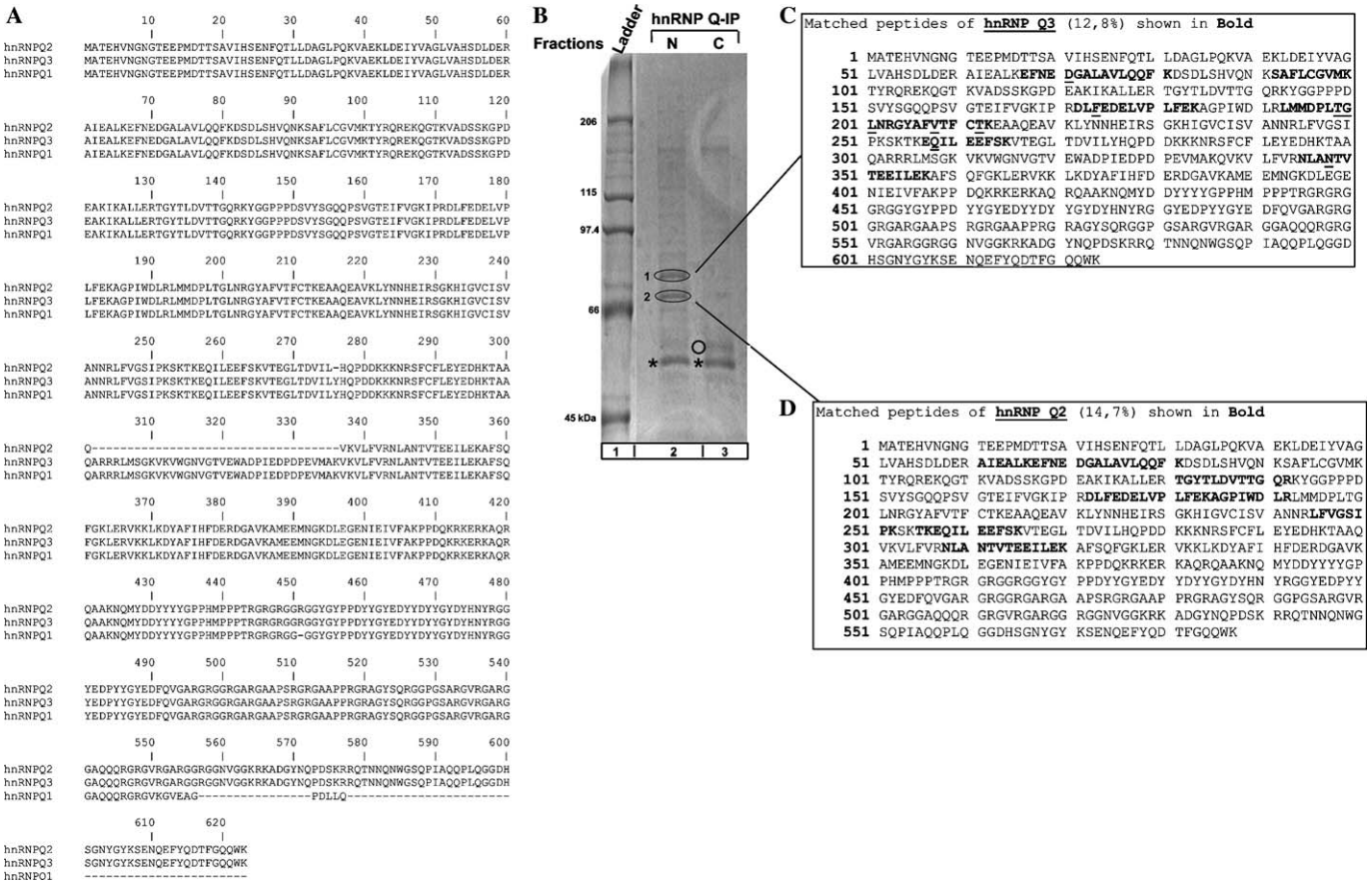

Fig. 3. Identification of the nuclear isoforms hnRNPQ2 and 3 by mass spectrometry. (A) Protein sequence alignment of the three hnRNPQ isoforms 1–3. (B) HeLa cells' nuclear and cytoplasmic fractions were separately immunoprecipitated with anti-hnRNPQ/R antibody and co-precipitated proteins were run out by SDS–PAGE. The two predominant nuclear bands of ca. 67 and 70 kDa were excised from the SDS–PAGE gel and digested by trypsin. The generated peptides were analyzed by Q-TOF mass spectrometry for the amino acid sequence determination. The asterisks (*) correspond to the heavy chain of the antibody. (°) indicates a protein band that has also been excised and analyzed by mass spectrometry. Through amino acid sequences obtained from sequencing tryptic peptides, it was possible to identify that this band consists of the proteins β-actin and β-tubulin. (C,D) The two bands that corresponded to the molecular mass predicted for hnRNPQ2 (67 kDa) and Q3 (70 kDa) were confirmed by finger print peptides specific for hnRNPQ. Bold labeled stretches of amino acids represent peptide sequences as identified by mass spectrometry in the NCBI data bank, using the Mascot Software. The underlined amino acids are those that are different in hnRNPR, which has a verified molecular mass of 80 kDa. hnRNPQ1 would have a significantly lower molecular mass of approximately 62 kDa.

also cannot represent hnRNPQ1, which has a molecular mass of 62 kDa. This conclusion can be drawn, since a band of 62 kDa would have a higher electrophoretic mobility than that of the marker protein of 66 kDa, and both observed bands had a lower electrophoretic mobility, which corresponded to proteins of 70 and 67 kDa, respectively.

In vitro methylation of endogenous cellular hnRNPQ by PRMT1

Next, we wanted to analyze the *in vitro* methylation of hnRNPQ, isolated from HeLa cells, as well as the importance of its methylation for its nuclear or cytoplasmic distribution. For this, HeLa cells were treated or not with the inhibitor of methylation Adox, and its nuclear and cytoplasmic fractions prepared and analyzed separately. The hnRNPQ proteins were immunoprecipitated using specific antibody and protein A Sepharose beads. These beads were then submitted to an *in vitro* methylation reaction in the presence of recombinant GST-PRMT1 (Fig. 4).

We found two bands of ca. 67 and 70 kDa, corresponding to hnRNPQ2 and 3, in nucleus and cytoplasm of untreated HeLa cells (lanes 3 and 4). However, the nuclear hnRNPQ bands (lane 3) present significantly more methyl-H^3 incorporation than the cytoplasmic bands (lane 4). On the other hand, we only were able to observe the band corresponding to hnRNPQ3 in the Adox-treated cells. In this case the cytoplasmic protein fraction (lane 2) was methylated stronger *in vitro* by GST-PRMT1 than the nuclear fraction (lane 1). This could reflect that hnRNPQ3 in presence of inhibitor Adox is re-distributed to the cytoplasm. This hypothesis obtains further support from the *in vivo* immunofluorescence experiment reported below in Fig. 5. At this point we do not yet understand why the hnRNPQ proteins from the nucleus of Adox-treated cells are less well methylated *in vitro* than those of untreated cells, since the contrary may be expected. We speculate that the inhibition of hnRNPQ methylation by Adox may lead to an increase in its association with other proteins, to conformational change or even to a decrease in protein stability. So the fraction corresponding to the nucleus of treated cells showed a significant reduction in its *in vitro* methylation by GST-PRMT1.

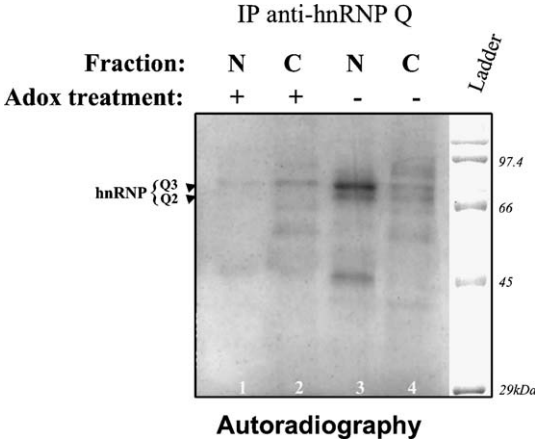

Fig. 4. *In vitro* methylation assay. HeLa cells were (+) or were not (−) incubated with the inhibitor of endogenous protein methylation, Adox. Next nuclear (N) and cytoplasmic (C) fractions of these cells were prepared and submitted to immunoprecipitation (IP) with anti-hnRNPQ antibody. Immunoprecipitated proteins were then methylated by GST-PRMT1 *in vitro*. Proteins were run out on SDS–PAGE and methylation assessed by autoradiography. The arrows in the autoradiography indicate the two isoforms hnRNPQ2 and 3 that could be identified due to their approximate molecular masses. Molecular masses of selected ladder proteins have been indicated on the right.

Inhibition of the methylation leads to a re-distribution of hnRNPQ to the cytoplasm

Our immunoprecipitation assays of hnRNPQ protein had revealed that the isoforms hnRNPQ2 and 3 seem to be localized predominantly in the nuclear compartment (Fig. 3A). When we performed immunofluorescence localization studies in HeLa cells (Fig. 5A–F), we were able to confirm the almost exclusive nuclear localization of hnRNPQ-specific immunofluorescence (A–C). However, we observed in a reproducible manner that a significant fraction of hnRNPQ immunofluorescence has been re-distributed to the cytoplasm after 16 h of treatment with Adox (D–F). This may suggest that methylation of these proteins is required for their nuclear localization and is in agreement with the findings reported in Fig. 4 (lanes 1 and 2), where we also observed a possible re-distribution of hnRNPQ3 from the nucleus to the cytoplasm.

The C-terminal RGG/RXR-box containing region of NSAP1 is essential for its nuclear localization

Since, hnRNPQ seems to be methylated on arginine residues and our results suggest that its methylation may be important for the regulation of its nuclear/cytoplasmic distribution, we wanted lastly to address the importance of the predicted target region of arginine methylation, the C-terminally located RGG/RXR-box (residues 444–559).

We therefore generated a hnRNPQ construction spanning the amino acid residues 1–443 fused C-terminally to GFP and transfected it transiently into HeLa cells. We observed a strictly cytoplasmic localization of this construction (Fig. 5G–I), indicating the importance of the RGG/RXR box region of hnRNPQ for its localization.

In conclusion, our data show that hnRNPQ is a substrate for PRMT1 and a target of arginine methylation *in vivo*. Furthermore, hnRNPQ is essentially methylated in its RGG/RXR box containing C-terminal region, which is required for its nuclear localization. Finally, the inhibition of its *in vivo* methylation by Adox causes a change of its localization from strictly nuclear to partially cytoplasmic. This suggests that hnRNPQ methylation in its C-terminal region is important for its nuclear localization. By demonstrating that hnRNPQ is a PRMT1 substrate

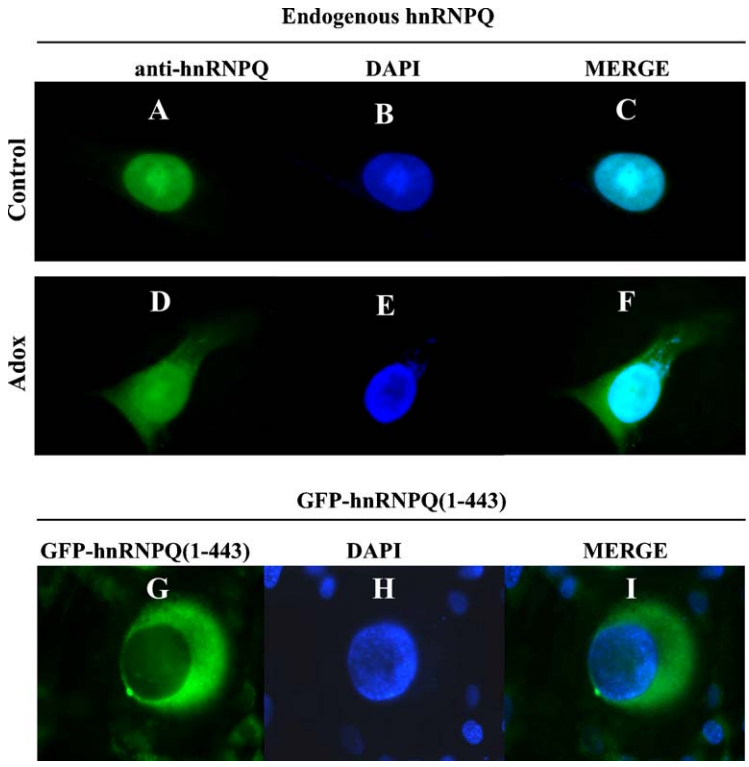

Fig. 5. Inhibition of the methylation of hnRNPQ leads to its re-distribution to the cytoplasm, and hnRNPQ requires its C-terminal region for nuclear localization. HeLa cells were grown on glass coverslips and incubated for 16 h with (D–F) or without Adox (A–C) at 37 °C. Cells were fixed with 100% methanol and endogenous hnRNPQ was detected by immunofluorescence using the monoclonal antibody anti-hnRNPQ and FITC-coupled anti-mouse (green) antibody as the secondary antibody (A,D). DAPI counter-staining (blue) was used to localize the position of the nucleus (B,E). MERGE shows the fusion of the images from the left two columns (C,F). GFP-hnRNPQ(1–443) lacks the C-terminal RGG box-containing region and shows a strictly cytoplasmic localization (G–I). A construction lacking the C-terminal (residues 444–623) of hnRNPQ(1–443) was fused to the C-terminal of GFP. After transfection of HeLa with this recombinant construct, the sub-cellular distribution of GFP fusion protein was analyzed by fluorescence microscopy. (G) Cell shows the restricted cytoplasmic localization for the mutant protein (GFP-NSAP1 (1–443)). (H) DAPI counter-staining of the nucleus. (I) MERGE: superimposition of the nucleus colored by DAPI (blue) and the cytoplasm with GFP-protein (green). (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)

and that its methylation has functional consequences we can further conclude that the yeast two-hybrid system is an efficient method for identifying new substrates for PRMTs, that may be applied in a larger scale to all PRMTs in order to understand the differences in the target protein spectra of these enzymes. In the future we may hopefully understand the specific functions of the intriguingly complex machinery of protein arginine methylation for each sub-set of protein substrates.

Materials and methods

Plasmid constructions. The full-length PRMT1 (1–344) and NSAP1 (1–623) cDNAs were obtained from human fetal brain cDNA library (Clontech), using the following forward and reverse primers, respectively; PRMT1: 5′-GGGAATTCATGGAGGTGTCCTGTGGCCAG-3′ and 5′-GCGGATCCTCGAGTCAGCGCATCCGGTAGTCGGTGG. PRMT1 cDNA was cloned via *Bam*HI and *Eco*RI sites into PBTM116 [38] and via *Eco*RI and *Xho*I sites into pGEX5X2 (Amersham Biosciences); hnRNPQ: 5′-CAGCGGCCGCATGGCTACAGAACATGTTAATGG-3′ and 5′-CGATCTCGAGCTACTTCCACTTGGGCCCAAAAG-3′, hnRNPQ was cloned via *Not*I into pFastBac-HTC (Invitrogen). Next, the following deletion mutant constructs were derived: hnRNPQ(1–443): 5′-GATT GGTACCGCATGGCTACAGAACATGTTAATG-3′ and 5′-GATCTC TAGAGTCGACTTACCTTTTCTGATCTGGTGGCTTGGC-3′, *Kpn*I and *Xba*I sites were used to sub-clone the fragment into pEGFP-N2 vector; hnRNPQ(1–443): 5′-CGATCTCGAGATGGCTACAGAACA TGTTAATGGAAATGG-3′, 5′-CTTTGCGGCCGCCTACTTCCACTG TTGCCCAAAAGTATC-3′, *Xho*I and *Not*I sites were used to sub-clone the fragment into pGEX5X1 (Amersham Biosciences) vector; hnRNPQ(389–623), 5′GATCTCTAGAGTCGACTTACCTTTTCTGAT CTGGTGGCTTGG-3′, 5′-GATTGGTACCGCATGGCTACAGAACA TGTTAATG-3′, *Xba*I and *Kpn*I sites were used to sub-clone this fragment into pGEX4T2.

Yeast two-hybrid assay. The yeast two-hybrid system (Y2H) screen was performed in the yeast strain L40 (Clontech), using a construction with the full-length cDNA of PRMT1 as bait. The cDNA of PRMT1 was cloned into the plasmid PBTM116 in-frame with the DNA-binding domain of LexA. After the co-transformation of the bait construction and a human fetal brain library (Clontech), cloned in vector pACT2, in-frame with the Gal4-activation domain, approximately 2×10^6 co-transformants were plated on synthetic minimal medium (MM) lacking tryptophan, leucine, and histidine but supplemented with adenine. The selected transformants, which expressed LexA-PRMT1 protein and its interaction partner fused to the activation domain Gal4, were re-streaked on MM plates and re-tested by a β-galactosidase filter-assay [13]. From 273 positive blue clones, 36 were sequenced, identifying 9 different proteins involved in RNA metabolism.

Expression and purification of recombinant proteins. To generate the proteins GST, GST-hnRNPQ(1–443), GST-hnRNPQ(389–623), GST-

PRMT1, and GST-Ki-1/57, the cDNAs were amplified by PCR and inserted into pGEX bacterial expression vector as described above. The recombinant plasmids were transformed into *Escherichia coli* strain BL21 (Stratagene), except the recombinant pGEX-plasmids encoding GST-hnRNPQ(1–443) and GST-hnRNPQ(389–623), which were co-transformed with pRARE vector into the BL21 strain. The protein expression was induced by 1 mM IPTG for 4 h at 37 °C. Protein affinity purification was performed using glutathione–Sepharose 4B (Amersham) using either a column or the batch technique, in the case of GST-PRMT1. cDNA encoding 6× His-hnRNPQ was sub-cloned into the baculovirus transfer vector pFastBac-HTC. After *in vitro* recombination and generation of a recombinant baculovirus DNA, in agreement with the manufacturer's instructions (Invitrogen), Sf9 cells were transfected to express full-length of 6× His-hnRNPQ in Sf9 insect cells.

In vitro methylation assays. The lysate of recombinant full-length 6× His-hnRNPQ protein and its purified deletion mutants as well as the controls, GST and GST-Ki-1/57, were incubated in PBS buffer containing 1 mM EDTA, 1 mM PMSF, and 2 μl of radiolabeled SAM [(methyl-^{3}H) *S*-adenosyl-L-methionine (2 μCi) (Amersham Pharmacia Biotech)] in the presence of recombinant GST-PRMT1 (bound to glutathione beads) for 1 h at 37 °C in a final volume of 50 μl. The reactions were stopped by heating to 100 °C for 5 min in SDS–PAGE sample loading buffer and then run out by 10% polyacrylamide SDS–PAGE. After fixing the gel for 20 min in water containing 10% methanol and 10% acetic acid, it was washed with water, and then incubated in amplifying solution (Amersham Pharmacia Biotech) for 1 h 30 min. After further washes, the gel was dried and exposed to Hyperfilm MP (Amersham Pharmacia Biotech) for 2 days.

For the analysis of the methylation of endogenous hnRNPQ *in vivo*, hnRNPQ was immunoprecipitated from HeLa cell fractions as described below. Then the immunoprecipitates were submitted *in vitro* to methylation by adding recombinant GST-PRMT1 as described above.

Cell culture and preparation of the cytoplasmic and nuclear extracts. 5×10^7 HeLa cells were incubated or not with methylation inhibitor adenosine-2′,3′-dialdehyde (Adox) (20 μM) for 16 h and lysed for 1 h at 4 °C in 1 ml modified cytoplasmic buffer (20 mM Tris, pH 8.0, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 2 mM PMSF, and protease inhibitors) [49]. After centrifugation at 14,000*g*, the nuclear fraction was separated and then lysed in 1 ml of nuclear extraction buffer (20 mM Tris, pH 8.0, 0.4 M NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 25% v/v glycerol) at 4 °C for 1 h.

Mass spectrometry analysis. The hnRNPQ protein was immunoprecipitated from 5×10^7 HeLa cells after cytoplasmic and nuclear separation and run out by SDS–PAGE. The gel was stained with Coomassie brilliant-blue R-250 in 50% (v/v) ethanol and 10% (v/v) acetic acid for 1 h and destained by over night incubation with 1 ml of 50 mM ammonium bicarbonate-50% methanol at 37 °C. Protein bands were excised and the gel sections were incubated in 100 μl of a solution containing 50 mM iodoacetamide/50 mM ammonium bicarbonate for 30 min in the dark at room temperature. After washing with water, the gel bands were submitted to digestion in a final volume of 50 μl in a solution of 1 pmol of trypsin (Sigma) in 50 mM ammonium bicarbonate buffer containing 10% acetonitrile, for 24 h at 37 °C. The resulting peptides were eluted in a solution containing 50% acetonitrile, 50 mM ammonium bicarbonate, and 0.1% TFA. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis was performed on a Q-Tof ultima API mass spectrometer (Micromass, Manchester, UK) coupled to a capillary liquid chromatography system (CapLC, Waters, Milford). A nanoflow ESI source was used with a lockspray source for mass measurement during the entire chromatographic run. The digested protein was desalted online using a waters Opti-Pak C18 trap column. The mixture of trapped peptides was then separated by elution with an gradient of 20%–50% (water/acetonitrile) 0.1% formic acid gradient through a Nanoease C18 capillary column. Data were acquired in data-dependent mode (DDA), and multiple charged peptide ions (+2 and +3) were automatically mass selected and dissociated in the MS/MS experiments. Typical LC and ESI conditions were: a 200 nl/min flow, a nanoflow capillary voltage of 3 kV, a block temperature of 100 °C, and 100 V cone voltage. The MS/MS spectra were processed using Proteinlynx 2.0 software (Waters, Milford) and the PKL file generated was used to perform database searches using the Mascot Software (Matrix Science, London, UK).

Immunoprecipitation and immunoblotting. The cytoplasmic (C) and nuclear (N) fractions were incubated for 4 h at 4 °C with 4 μl (1 mg/ml) of anti-hnRNPQ monoclonal antibody (Abcam-18E4). Then 20 μl of protein A Sepharose beads (Pharmacia) was added for a further incubation of 1 h and the beads were washed three times in the cytoplasmic buffer. Samples were heated to 100 °C for 5 min in the presence of SDS–PAGE sample buffer and proteins were separated on a 10% polyacrylamide SDS-gel. After SDS–PAGE, proteins were transferred to a nitrocellulose membrane. The membrane was blocked for 1 h with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20, washed, and incubated for 1 h with a solution of 1:5000 of both mouse monoclonal antibody anti-mono- and di-methylarginine (Abcam). The membranes were washed and bound primary antibody was detected by alkaline phosphatase-conjugated anti-mouse IgG antibody using the chromogenic substrates BCIP/NBT (Sigma) for visualization.

Immunofluorescence analysis. HeLa cells grown on glass coverslips were incubated or not with Adox (100 μg/ml) for 16 h at 37 °C. To inhibit de novo protein synthesis, we also added cycloheximide (100 μg/ml) and chloramphenicol (40 μg/ml) during the last 4 h of the experiment. Next, cells were fixed with 100% methanol and immunostained with primary monoclonal mouse antibody anti-hnRNPQ (1:1000) and secondary FITC-coupled anti-mouse antibody (1:100). DAPI staining was used for counterstaining nuclei. Cells were examined with a Nikon fluorescence microscope.

Transfection of HeLa cells. A construct of hnRNPQ was generated that lacks the C-terminal region spanning amino acids 444–623. In this construct, the cDNA encoding the N-terminal region of hnRNPQ(1–443) was fused to the 3′-region of the DNA encoding Green fluorescent protein (GFP) in vector pEGFP/C2 (Clontech). The recombinant vector construct, encoding the fusion protein GFP-hnRNPQ(1–443), was then transiently transfected in HeLa cells by lipid transfection using DOTAP (Sigma), following the manufacturer's instructions. Cells were counterstained with DAPI and analyzed 12 h post-transfection.

Acknowledgments

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IV. CONSIDERAÇÕES FINAIS

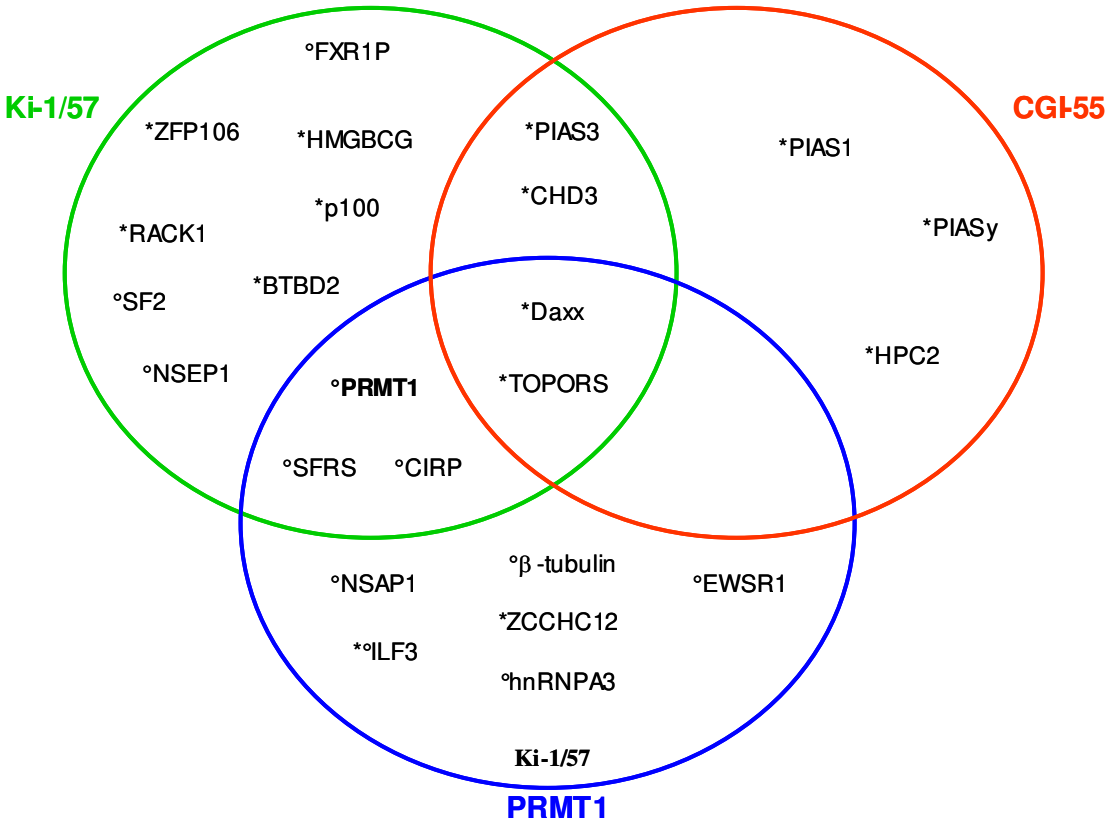

Figura 8. Duplo híbrido de levedura reforça envolvimento de Ki-1/57 com o metabolismo de RNAs ou regulação transcricional. Esquema de proteínas identificadas em estudos de duplo-híbridos de levedura realizados independentemente por diferentes alunos do grupo com sobreposição de proteínas que interagem. O *screen* de Ki-1/57 como isca foi realizado pela Dr. Flavia Cristina Nery e o de CGI-55 pela Dr. Taíla Andrade Lemos. Vale notar que grande parte das proteínas selecionadas pela PRMT1 e Ki-1/57 estão envolvidas com o metabolismo de RNA (°) ou na regulação da transcrição (*).

Ki-1/57, encontrada tanto em regiões nucleares quanto citoplasmáticas, é uma proteína ainda pouco compreendida funcionalmente. Estudos recentes têm sugerido o seu envolvimento no controle transcricional (Lemos e Kobarg, 2003; Nery *et al*., 2006), que é fortemente reforçado pelos resultados obtidos no SDHL quando a proteína Ki-1/57 é usada com isca (Fig. 19). Como podemos verificar acima, diversas proteínas que interagiram com

Ki-1/57 são proteínas que apresentam atividade envolvendo o controle ou regulação transcricional (*).

Também vale ressaltar, que duas proteínas (Daxx e Topors), ambas envolvidas com a transcrição, foram selecionadas nos três SDHL mostrados acima. Além disso, a proteína CHD3, que também tem envolvimento com a transcrição, não só foi selecionada nos SDHL de Ki-1/57 e CGI-55, como co-precipitada em ensaios *in vitro*, que sugerem um possível papel funcional em complexos de remodelamento da cromatina para estas proteínas (Lemos et al. 2003).

Em adição, a proteína Ki-1/57 também mostrou extensa interação com proteínas envolvidas no processamento de RNA mensageiro (RNAm) ou com proteínas que compreendem algum domínio de reconhecimento ou ligação a RNA, tais como RRM, RBD e KH (°) (Fig. 19). Além disso, grande parte das proteínas conhecidas como substratos para a PRMT1 – descritas até agora – têm envolvimento com o metabolismo de RNAs. Logo, como verificado no *screen* de SDHL, usando a PRMT1 como isca, uma grande parte das proteínas pescadas têm atividade funcional relacionada com metabolismo de RNAs e a outra grande parte é envolvida com a transcrição. Assim, Ki-1/57 parece ser mais um substrato para a PRMT1 a ampliar o grupo das proteínas arginina metiladas com envolvimento no processamento de RNA. Além disso, outras duas proteínas (CIRP e SFRS), ambas envolvidas com RNAs, foram selecionadas nos dois SDHL realizados (PRMT1 e Ki-1/57), o que nos leva a acreditar que a PRMT1 parece metilar complexos de proteínas, principalmente quando estas estão envolvidas com o processamento do RNA.

Ainda temos poucos dados para que possamos compreender melhor o papel da metilação destas proteínas envolvidas com RNA, já que algumas delas além de além de apresentarem outros motivos de reconhecimento a RNAs (RRM), não apresentaram alteração de sua afinidade pelo RNA após metilação (Valentini et al., 2001). No entanto, é possível que em certos casos a metilação esteja regulando apenas a interação entre as proteínas ligantes a RNA, e que em outros, esteja atuando na redução ou ampliação da afinidade da proteína ao RNA, o que *in vivo* poderia determinar um aumento de afinidade da proteína "X" e redução da "Y", ou redução da "X" e aumento da "Y" pelo RNA. Em outras palavras, é provável que a metilação possa não estar desligando completamente a interação da proteína pelo RNA, mas sim reduzindo ou ampliando-a de maneira tão sutil

que apenas ensaios *in vivo* poderiam demonstrar isso com clareza. Por outro lado, ensaios de EMSA com competidores específicos poderiam também contribuir para entendermos melhor o possível papel regulatório das interações proteína-RNA dependentes da metilação.

Portanto, os dados obtidos até o momento corroboram e reforçam a idéia de que Ki-1/57 tem como um dos seus papéis funcionais o processamento ou metabolismo de RNAm, além de estar envolvido em eventos transcricionais.

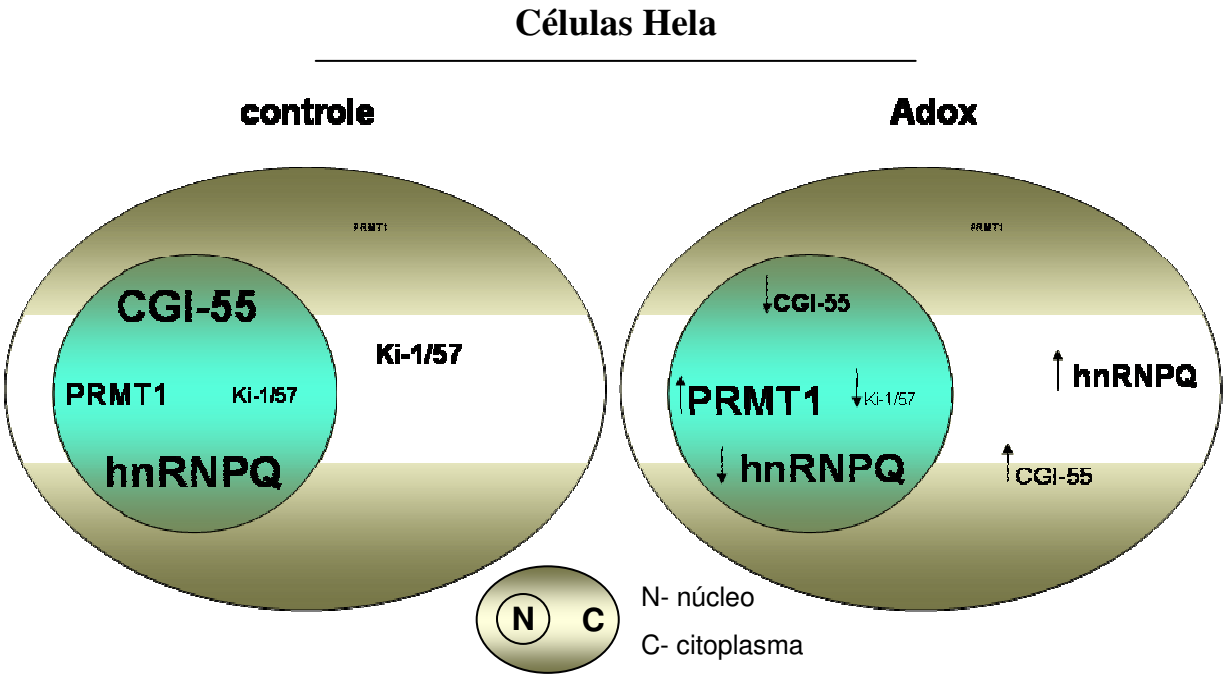

Figura 9. A metilação das proteínas Ki-1/57, CGI-55 e hnRNPQ3 parece ter relevância na regulação de suas localizações celulares. Nos últimos anos, a metilação de proteínas tem sido associada funcionalmente a diversos eventos celulares, embora muitas proteínas não tenham seus conhecidos papéis funcionais na célula alterados após a metilação. Desta forma, um número crescente de proteínas têm sido identificadas como substrato as argininas metiltransferases, mas nenhuma função especifica tem sido atribuída a metilação. Por outro lado, algumas destas proteínas têm suas localizações celulares dependentes da metilação, como podemos verificar acima. As setas voltadas para cima (↑) sinalizam aumento da intensidade relativa de fluorescência emitida pelo anticorpo (Imunolocalização) da proteína ao lado após tratamento das células com o inibidor da metilação Adox. As setas voltadas para baixo (↓) sinalizam redução da imunomarcação das proteínas próximas.

Nos últimos anos, a metilação de proteínas tem sido associada funcionalmente a diversos eventos celulares, apesar de muitas proteínas não apresentarem seus conhecidos papéis celulares alterados após a metilação. Da mesma forma, muitas das proteínas conhecidas até o momento, como substratos a arginina metiltransferases, são proteínas ainda pouco compreendidas funcionalmente, o que aumenta consideravelmente a dificuldade na caracterização funcional deste fenômeno. De maneira geral, motivos "RGG-box" já foram descritos mediando interações proteína-proteína e proteínas-RNAs, além de outras moléculas. Além disso, como mostrado acima, estes motivos parecem ter também importante papel no controle do transporte núcleo-citoplasma de algumas proteínas envolvidas no metabolismo de RNAs (Boisvert et al. 2002; Xu e Henry, 2004).

Uma forma bem explorada de verificar a importância da metilação na localização celular das proteínas se dá pela utilização de inibidores de metilação, sendo o Adox um dos mais usados. Tratando células com o inibidor, podemos comparar com o controle e verificarmos por imunolocalização se houve alteração de localização celular da proteína em estudo.

No esquema acima (Fig.19), células controle comparadas a células tratadas com Adox apresentaram alterações de localização celular para os substratos da PRMT1 encontrados no SDHL. Estes dados ampliam a "família" de proteínas identificadas como substrato a PRMT1 com alteração da função não caracterizada. Contudo, vale ressaltar, que a alteração da localização celular implica indiretamente em alteração funcional da proteína em questão, quer seja por ativação, inibição ou apenas mudança.

Assim, analisando com mais detalhe os efeitos nas proteínas apresentadas no esquema acima, podemos concluir que:

1) a proteína Ki-1/57 não demonstra uma migração entre os compartimentos nuclear e citoplasmático, mas sim uma redução drástica da sua imunomarcação nestes compartimentos, principalmente da citoplasmática, mantendo apenas uma imunomarcação na membrana nuclear, nucléolo e pontos nucleares. Sendo assim, é possível que a metilação seja uma modificação constitutiva que assegure a estabilidade da proteína na célula. Quanto à permanência da imunomarcação na membrana nuclear, nucléolo e pontos nucleares após o tratamento com o inibidor, é provável que o "*turnover*" destas proteínas seja mais lento ou que nestas regiões se encontrem estabilizadas por associação com alguma molécula ou

complexo. Este resultado, juntamente com dados recentemente verificados em nosso grupo (dados não mostrados) corroboram com os resultados de dicroísmo circular e expressão de mutantes tanto em bactérias quanto células de mamífero, no qual a metilação do motivo "RGG-box" central parece ser essencial para a estabilidade da proteína. Vale lembrar que embora a metilação de argininas seja um fenômeno irreversível, sugerindo uma alteração do tipo constitutiva, não há até o momento nenhum relato da sua importância na sinalização de proteínas para degradação ou mesmo para a sua preservação contra proteases.

2) no caso da proteína CGI-55, verificamos uma migração núcleo-citoplasma. CGI-55 que se apresenta quase exclusivamente nuclear nas células controle, quando tratadas com Adox desloca-se para o citoplasma. A proteína CGI-55, além de ter sido caracterizada como proteína de interação a proteína funcionalmente nuclear CHD3 (Lemos et al. 2003), também foi recentemente associada a corpúsculos nucleares, interagindo com coilina-p80, marcador molecular de corpúsculos Cajal (Lemos e Kobarg, 2006). Nenhum relato funcional foi atribuído a esta proteína no citoplasma até agora, o que é reforçado pelo ensaio de imunolocalização esquematizado acima, mostrando ser esta quase exclusivamente nuclear. No entanto, quando inibimos a metilação CGI-55 parece se deslocar do núcleo em direção ao citoplasma. É sabido que outras proteínas envolvidas a corpúsculos nucleares são conhecidos substratos a metilação de argininas. Logo, é possível que a metilação destas proteínas esteja regulando funcionalmente estas estruturas. E CGI-55 deve ter alguma função citoplasmática quando não metilada, pois caso contrário, era esperado que fosse degradada, ou seja, regulada negativamente.

3) no caso das hnRNPQ, que demonstra ser exclusivamente nuclear (controle), tem forte regulação dependente da metilação. Assim, parece que a metilação é fundamental para a exclusiva localização nuclear das hnRNPQ, de modo que ao inibirmos a metilação há um deslocamento parcial da hnRNPQ para o citoplasma. As hnRNPQ foram primeiramente descritas como ligantes ao complexo nuclear SMN (S*urvival Motor Neuron*) e indicados como participantes da biogênese das "*small nuclear Ribonucleoproteins"* (snRNP) e processamento de RNA (Mourelatos et al. 2001). As hnRNPs são numericamente as mais descritas na literatura como substratos a argininas metiltransferases. Mas mesmo assim, pouco ainda se sabe a respeito das implicações da metilação na regulação de suas funções. Assim, é possível que estas hnRNPQ tenham algum modulador citoplasmático, de modo

que quando metiladas o modulador as mantenham no citoplasma e após metilação este modulador deixaria de interagir com elas e as hnRNPQ poderiam dessa forma migrar para o núcleo, onde exerceria suas funções envolvendo o complexo SMN. Uma regulação parecida foi verificada com a proteína STAT1 (Mowen et al. 2001).

4) a PRMT1 não apresentou alterações consideráveis de localização celular, mostrando que o inibidor não parece influenciar sua localização celular. No entanto, um pequeno aumento de imunomarcação nuclear pode ser visto nas células tratadas, talvez por ter havido um aumento da expressão da proteína na célula. Este pode ser um indício de que a expressão da PRMT1 é regulada de acordo com a disponibilidade de substrato livre S-adenosilmetionina (AdoMet) ou co-substratos (proteínas alvo). Também não podemos descartar uma atividade regulatória por parte do S-adonosinilhomocisteína (AdoHcy), que é favorecido em concentração pelo inibidor.

V. CONCLUSÕES

Artigo I:

- Análises por "*Northern Blot*" mostraram que Ki-1/57 e seu putativo parálogo CGI-55 são largamente expressas em rins, músculo esquelético e coração. No cérebro humano, apenas Ki-1/57 apresentou forte expressão;

- Ambas as proteínas Ki-1/57 e CGI-55 interagiram com a proteína CHD-3 (c*hromo helicase DNA binding domain protein-3*);

- Tanto o N- quanto o C- terminal de CGI-55 interagem a proteína CHD-3 em ensaios do sistema de duplo híbrido de levedura (SDHL);

- CGI-55-GFP mostrou estar presente no núcleo e citoplasma de células Hela após transfecção com o vetor recombinante CGI-55-pEGFP-N1;

- Estes resultados sugerem a possibilidade de CGI-55 e Ki-1/57 terem um papel funcional junto a CHD-3 no remodelamento da cromatina no núcleo.

Artigo II:

- O ensaio de duplo híbrido de levedura, contra a biblioteca de cDNA de cérebro fetal humano (BCFH), utilizando a proteína Ki-1/57 como isca encontrou a proteína arginina metiltransferase (PRMT1), além do receptor de quinase ativada (RACK1);

- Esta interação foi confirmada por ensaios de *pull down*, co-precipitação e co-imunoprecipitação;

- Ki-1/57 mostrou ser um substrato para a proteína quinase C (PKC), sendo fosforilada *in vitro* e *in vivo* após ativação por PMA;

- Análise de fosfo-aminoácidos mostra que a proteína Ki-1/57 é fosforilada por PKC *in vitro*, predominantemente nas treoninas T354 e T375;

- RACK1, apesar de interagir com a região C-terminal de Ki-1/57, não interfere na sua fosforilação em ensaios *in vitro*;

- Ki-1/57 poderia estar participando de um complexo com atividade quinase, juntamente com as proteínas PKC e RACK1;

- Ki-1/57 após fosforilação deixa de interagir com RACK1 *in vitro* e *in vivo*;

- Células Hela e L540 após ativação com PMA mostram que a proteína Ki-1/57 migra do núcleo para o citoplasma;

- O mapeamento da interação entre as proteínas Ki-1/57 e RACK1, por SDHL, mostra que o extremo C-terminal de Ki-1/57 (347-413) é capaz de interagir com RACK1. Por outro lado, nenhuma das deleções de RACK1 foram capazes de interagir com Ki-1/57, apenas ela inteira;

Artigo III:

- Análise por dicroísmo circular (CD) dos fragmentos C-terminais de Ki-1/57 mostrou elevada presença de regiões de baixa complexidade estrutural, comum em proteínas consideradas "*native unfolded*";

- Fosforilação do fragmento de Ki-1/57 (122-413) reduz a elipcidade molar de -10000 para -5000. Isto poderia explicar, juntamente com a adição das cargas negativas, o desligamento de RACK1 após fosforilação, já que provavelmente esta modificação pós traducional acarreta uma alteração de estrutura secundária;

- Análises de CD mostram que RACK1 apresenta um máximo em 229nm, que é atribuído a triptofanos (Trp). Tratamento com N-bromosuccinamida (NBS) modifica triptofanos (Trp), abolindo o máximo em 229nm. Como na presença de Ki-1/57 o sinal de CD em 229nm também é abolido podemos inferir que a interação de RACK1 com Ki-1/57 envolve os Trp residuais de RACK1.

- O modelo de RACK1 baseado na homologia com a proteína G_{β} mostrou uma estrutura compacta formada por sete repetições WD arranjada em uma estrutura em hélice, onde 4 dos 13 Trp 1 Tyr e 1Phe formam uma região conjunta ("patch") hidrofóbica na superfície apical da estrutura.

Artigo IV:

- SDHL utilizando a PRMT1 como isca – contra uma BCFH – foi capaz de identificar 15 novas proteínas como possíveis substratos, tendo a maioria domínios de ligação a RNA, além de motivos "RGG-box";

- Entre as proteínas selecionadas pelo SDHL, temos a Ki-1/57, proteína que também havia pescado a PRMT1 em estudo anterior;

- Experimentos de *pull down* confirmaram a interação entre estas duas proteínas;

- Ensaios de metilação *in vitro* mostraram que Ki-1/57 e seu putativo parálogo CGI-55 são substratos para a metilação de argininas pela PRMT1;

- Análise da seqüência de aminoácidos das proteínas Ki-1/57 e CGI-55 revelaram a conservação de dois motivos "RGG-box" entre as duas proteínas; Da mesma forma, todas as regiões contendo "RGG-box" são metiladas pela PRMT1. No entanto, corroborando com o SDHL as construções sem este motivo não são metiladas; Estudo com mutantes da PRMT1 revelam que a porção N-terminal (1-34) tem intensa participação na sua dimerização e atividade enzimática;

- Imunoprecipitação (IP) de Ki-1/57 a partir das células L540, para ensaio de metilação *in vitro*, mostra que ela já se encontra metilada na célula, uma vez que apenas as proteínas imunoprecipitadas das células tratadas com o inibidor da metilação (Adox) são passíveis de serem metiladas *in vitro* pela PRMT1;

- Ki-1/57 imunoprecipitada de células Hela, tratadas ou não com Adox, incubadas com S-Adenosilmetionina marcada (methyl-^{3}H) apresentou incorporação do metil radiomarcado. Isto corrobora com nossos dados e reforça a idéia de que Ki-1/57 é um substrato para a metilação também *in vivo*;

- Ki-1/57 proveniente da fração nuclear é preferencialmente metilada;

- Mapeamento – por SDHL - das regiões de Ki-1/57 que interagem com a PRMT1 revelou que todas as regiões que contêm o motivo "RGG-box" são capazes de interagir;

- O mutante sem a porção N-terminal (35-344) teve sua habilidade de dimerização reduzida cerca de 25%;

- O mesmo mutante teve sua atividade enzimática praticamente eliminada, o que significa que a dimerização é importante para sua atividade, mas não é suficiente;

- Ensaio de metilação *in vitro* com estes mutantes mostrou que nenhuma das construções da PRMT1 é capaz de apresentar uma atividade significativa. No entanto, vale ressaltar que as tênues metilações verificadas com estes mutantes após 30 dias de exposição só foram visualizadas quando utilizamos Ki-1/57 como substrato. Quando utilizamos outro substrato (hnRNPQ3) não fomos capazes de verificar nenhuma marcação mesmo após os 30 dias;

- Inibição da síntese de RNA em células Hela revela retenção de Ki-1/57 no núcleo; Este resultado reforça dados anteriores que indicam que Ki-1/57 pode estar envolvida com o transporte ou metabolismo de RNAs;

Artigo V:

- O ensaio de SDHL com a PRMT1 como isca também identificou a proteína hnRNPQ3;

- Ensaio de metilação *in vitro* confirmou que a hnRNPQ3 é mais um novo substrato a metilação de argininas, e que esta metilação é restrita a sua porção C-terminal, onde encontramos um extenso motivo "RGG-box";

- Por estudo de espectrometria de massas conseguimos identificar e diferenciar as isoformas da hnRNPQs (1-3);

- Metilação *in vitro* das hnRNPQs, imunoprecipitadas a partir de Hela, revela que ambas as isoformas das hnRNPs Q2 e Q3 são substratos para a PRMT1.

- Ao contrário do verificado com Ki-1/57, apenas as proteínas nucleares provenientes de células não tratadas previamente com Adox apresentam uma incorporação do metil marcado. Este resultado sugere que a proteína não é completamente metilada *in vivo*;

- *Western Blot* anti-mono/dimetilarginina do Imunoprecipitado de hnRNPQs de células Hela, tratadas ou não com o inibidor da metilação, mostra que as isoformas Q2 e Q3 são metiladas hnRNPQs são metiladas *in vivo*;

- A exclusiva localização nuclear da hnRNPQ parece depender da metilação;

- Mutante sem o C-terminal "RGG-box" hnRNPQ (1-443) em fusão a GFP mostrou ser exclusivamente citoplasmático;

- Estes resultados nos levam a acreditar que o C-terminal "RGG-box" desta proteína é fundamental para sua localização nuclear, e que a metilação de suas argininas poderia estar modulando sua localização.

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VII. ANEXO I (O Sistema Duplo Híbrido em Levedura)

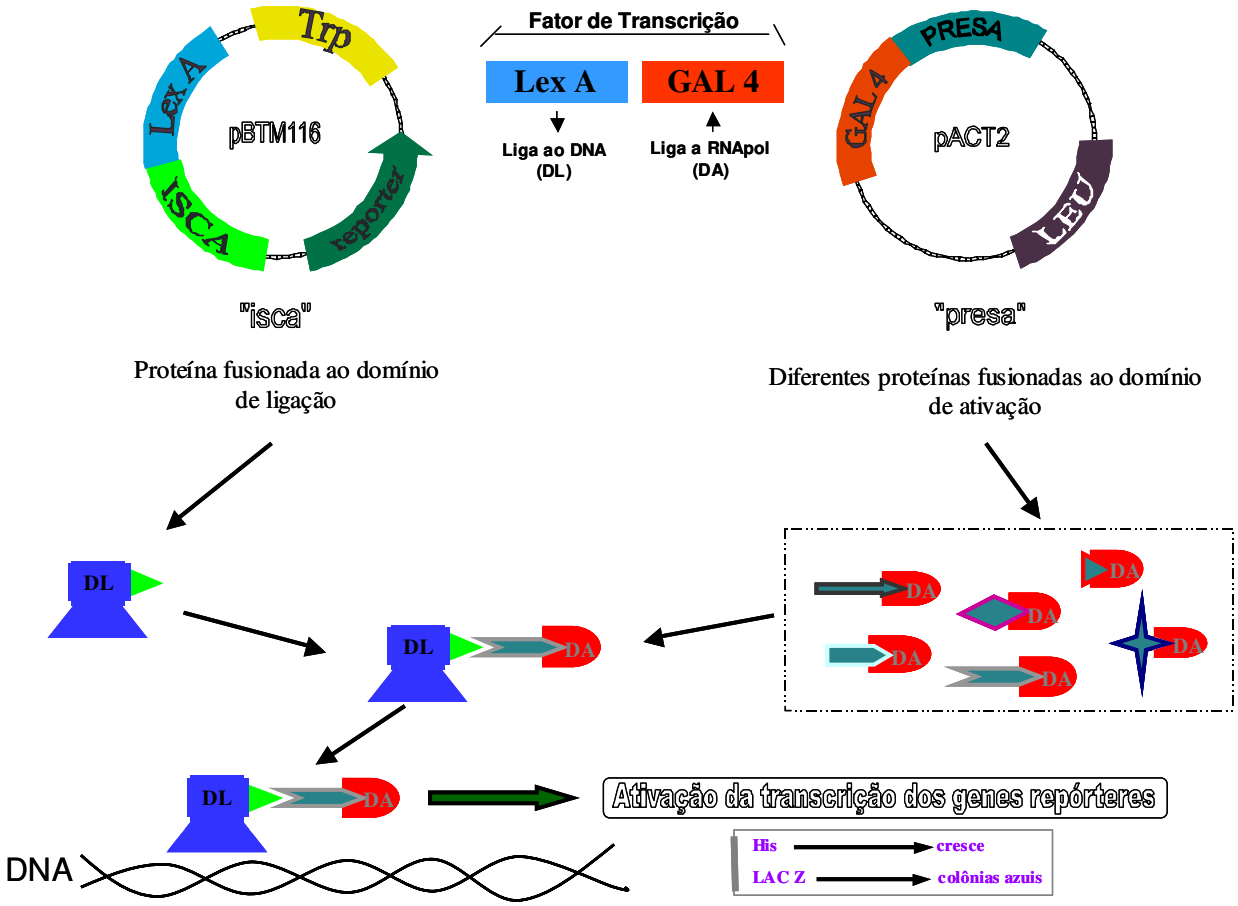

Figura 10. O esquema acima apresenta o princípio de funcionamento do Sistema do Duplo Híbrido em Levedura (SDHL).

O Sistema Duplo Híbrido em Levedura (*Yeast Two Hybrid System*) foi primeiramente descrito em 1989 por Stanley Fields e O-K Song (Fields e Song, 1989), e é uma poderosa técnica capaz de verificar a interação entre duas proteínas, mapear regiões de interação entre estas, e até mesmo identificar novas proteínas ("presas") que interagem com uma dada proteína ("isca") utilizando para isto uma biblioteca de cDNA. Assim, este sistema pode ser de grande valia na identificação de um possível papel funcional a uma dada proteína ainda desconhecida ou pouco estudada. A concepção básica surgiu a partir de estudos com fatores de transcrição, que normalmente apresentam dois domínios separados

fisicamente, o de ligação ao DNA (DL) e o de ativação da transcrição (DA). A primeira proteína a ser usada para isto foi a proteína GAL4 de levedura, que apresenta tanto o DL quanto o DA. Em seguida, estudos foram realizados com a proteína bacteriana de ligação ao DNA LexA, e a partir daí, a técnica foi otimizada.

O nome utilizado "duplo híbrido" se deve ao princípio básico do método em que duas proteínas, um domínio ou uma proteína de ligação ao DNA (Gal4-DL, LexA) e o domínio de ativação da transcrição (Gal4-DA), são cada uma delas fusionadas a uma determinada proteína de "teste". Assim são produzidos duas proteínas híbridas (LexA-"isca" e GAL4-DL-"presa") dentro da célula de levedura e caso haja interação entre as duas proteínas de fusão em estudo, as funções de ligação ao DNA (DL) e de transativação (DA) se complementam e um fator de transcrição funcional híbrido será reconstituído, permitindo a interação com a maquinaria de transcrição e a partir daí resultando na ativação da transcrição dos genes repórteres dados na célula de levedura repórter. Normalmente os genes repórteres utilizados são: B-galactosidase (LAC Z) e Histidina (His). O primeiro ao metabolizar o 5-bromo-4cloro-3-indol beta-D-galactosídeo (X-gal) libera como produto um cromóforo de coloração azul. O segundo promoverá a síntese de Histidina o que permitirá que a levedura cresça em meio seletivo (-H). No esquema da aplicação da técnica em forma de varredura de uma biblioteca de cDNA ("library screen"), como utilizado na maioria das vezes nesta tese de doutorado, as células de levedura que cresceram e que ficaram azuis ao mesmo tempo são amplificadas. O DNA do plasmídeo que codifica a proteína híbrida contendo a "presa" é em seguida isolado da célula e submetido a um seqüenciamento de DNA, que permite então a identificação da seqüência do cDNA que codifica a proteína "presa".