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ESTUDOS FUNCIONAIS E ESTRUTURAIS DA PROTEÍNA REGULADORA HUMANA Ki-1/57

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas, para obtenção do título de Doutor em Genética e Biologia Molecular na área de Genética Humana e Médica.

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Viver vale a pena!

Que a felicidade não dependa do tempo, nem da paisagem, nem da sorte, nem do dinheiro.

Que ela possa vir com toda a simplicidade, de dentro para fora, de cada um para todos.

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Viver é colocar seu sonho à prova. Os brotos da esperança crescem alimentados pelo sonho.

É preciso reviver o sonho e a certeza de que tudo vai mudar.

É necessário abrir os olhos e perceber que as coisas boas estão dentro de nós, onde os sentimentos não precisam de motivos nem os desejos de razão.

Mas é preciso ver as coisas com o coração e nem sempre com a razão.

Não se esqueça de que o aprendizado dura a vida toda.

Eleve suas expectativas. Pessoas com sonhos grandes obtêm energia para crescer.

Os vencedores pensam em como realizar seu objetivo. Por isso ...

Tenha metas claras. Ter objetivos evita desperdícios de tempo, energia e dinheiro.

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(Flávia C. Nery)

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Lista de abreviações e siglas

ACTB: Proteína actina beta;

AD: Domínio de ativação transcricional da proteína Gal4 (*Activation domain);*

ALEX2: Proteína com repetição Armadillo 2 *(Armadillo repeat protein X chromosome, 2);*

APLP1: Proteína similar a proteína amilóide precursora 1 (*Amyloid-precursor-like protein 1)*;

BD: Domínio de ligação a DNA da proteína Gal4 (*Binding domain*);

BTBD2: Proteína contendo 2 domínios BTB(POZ) (*BTB (POZ) domain containing 2*);

CD: Dicroísmo dircular (*Circular Dicroism*);

CD30: Receptor de citocina 30 (*Cytokine receptor 30*)

CGI-55: Proteína identificada por comparação genômica 3 (*Comparative Genome Identified* 55);

CHD3: Proteína com domínios cromo-helicase e de ligação ao DNA 3 (*Chromo-domain Helicase DNA binding domain protein* 3);

CIRBP: Proteína que se liga ao RNA induzida por frio (*Cold Inducible RNA Binding Protein*);

CTGF: Fator de crescimento de tecido conectivo (connective tissue growth factor);

DAXX: Proteína associada a morte *(Death-associated Protein);*

EB-1: Proteína associada a E2a-Pbx1 *(E2a-Pbx1 associated proteína);*

EPS8: Substrato 8 do receptor de fator de crescimento epidermal (*Epidermal growth factor receptor pathway substrate 8*) ;

Fas: Fator de apoptose (*Factor of apoptosis);*

FISH: Fluorescência de hibridação in situ *(fluorescence in situ hybridization);*

FPLC: Cromatografia líquida de proteínas por pressão;

FXR1P:Proteína relacionada com retardamento mental X-frágil (*Fragile X mental retardation-related protein 1*);

HMGBCG: Proteína similar ao grupo de alta mobilidade (*High-Mobility Group)*;

IHABP4: Proteína que se liga a hialuronato 4 *(hyaluronan-binding protein 4);*

IPTG: Isopropil beta-D-galactosídeo;

Ki-1/57: Antígeno Ki-1/ de 57 kDa *(Ki-1 antigen of 57 KDa);*

LB: Meio Luria Bertani;

ND10: Corpúsculos nucleares 10 *(nuclear bodies 10);*

NDUFAB1: Proteína NADH ubiquinona desidrogenase, sucomplexo alfa/beta 1 *(NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1);*

NSEP1 (YB-1): Proteína de ligação ao elemento sensível a nuclease 1 *(nuclease sensitive element binding protein 1*);

p53: Proteína de tumor p53 de 53 kDa *(tumor protein p53);*

p73: Proteína de tumor p73 de 73 kDa *(tumor protein p73);*

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

PAI: Inibidor do ativador do plasminogeno (*plasminogen activator inhibitor);*

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

PCR: Reação em cadeia da Polimerase (*Polymerase Chain Reaction*);

PDB: Banco de dados de estrutura de proteínas (Protein Data Bank);

PEG: Polietilenoglicol;

PKC: Proteina quinase C (Protein Kinase C);

PMA: 4 α -forbol 12-miristato 13-acetato (*4α-phorbol 12-myristate 13-acetate*)

PML: Proteína da leucemia promielocítica *(Protein of Promielocytic Leukemia);*

PML NBS: Corpúsculos nucleares PML (*PML nuclear bodies);*

RACK-1: Proteína adaptadora para quinase C-1 (*Receptor for C Kinase-1*)

RPL38: Proteína ribossomal l L38 (*ribosomal protein L38*);

SDS-PAGE: Eletroforese em gel de poliacrilamida em presença de duodecil-sulfato de sódio *(sodium duodecyl-sulphate polyacrylamide gel electrophoresis);*

SF2/p32: Fator de splicing 2/p32;

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

Sp100: Antígeno nuclear Sp100 *(nuclear antigen Sp100);*

STAT: Sinalizador e ativador da transcrição;

SUMO-1: Pequeno modificador relacionado a ubiquitina (*small ubiquitin-related modifier*);

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

UBC9: Enzima conjugada a ubiquitina E2I (*ubiquitin-conjugating enzyme E2I);*

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

ZFP: Proteina com dedo de zinco *(Zinc finger protein);*

3AT: 3-aminotriazol;

RESUMO

Ki-1/57 é um antígeno humano de 57kDa reconhecido pelo anticorpo Ki-1, o qual também reconhece CD30. Ki-1/57 se encontra no núcleo e no citoplasma sendo fosforilado nos resíduos de serina e de treonina após a ativação das células. Quando Ki-1/57 foi isolado da linhagem L540 de células de linfoma de Hodgkin, ela co-imunoprecipitou com atividade quinase em resíduos de Ser/Thr. Além disso, foi relatado que Ki-1/57 interage com o ácido hialurônico e conseqüentemente foi denominada de "proteína intracelular que se liga a hialuronato 4" (IHABP4). Nós usamos o sistema de duplo híbrido em levedura e encontramos que Ki-1/57 interage especificamente com a proteína com domínios cromo-helicase e de ligação ao DNA 3 (CHD3), uma proteína nuclear envolvida em remodelagem da cromatina e regulação da transcrição, e com RACK1 ("proteína adaptadora para quinase C ativada"). A interação entre Ki-1/57 e seus ligantes foi confirmada por outros experimentos *in vitro* e *in vivo*. Interessantemente, a interação entre Ki-1/57 e RACK1 foi abolida após fosforilação da Ki-1/57 e observamos que o estímulo de células L540 e HeLa com 4 α -forbol 12-miristato 13-acetato (PMA) resulta na saída de Ki-1/57 do núcleo. Ki-1/57 também demonstrou ser um substrato para proteína quinase C (PKC) quando ativada com PMA, e sua fosforilação foi confirmada *in vitro* e *in vivo*. Esses dados sugerem que Ki-1/57 está associada com a via de sinalização celular de RACK1/PKC e isto pode ser importante para a regulação de suas funções nucleares. Sua interação com CHD3 e com outras proteínas envolvidas na regulação transcricional, tais como: Topors, Daxx e Tip60, entre outras, sugere que Ki-1/57 pode ter uma função neste contexto funcional. RACK1 interage com p73, um parálogo de p53, inibindo sua ativação transcricional. Nós ainda encontramos que Ki-1/57 também interage com p53 não fosforilada e pode inibir sua ativação transcricional. A estrutura tridimensional da proteína Ki-1/57 é desconhecida, mas nossos estudos espectroscópicos mostraram que a proteína Ki-1/57 é predominantemente constituída por folhas β-pregueadas. Além disso, Ki-1/57(122-413) apagou o sinal do espectro de CD de RACK1 entre 229-300 nm, que é característico de proteínas ricas em triptofanos, e também diminuiu a intensidade de emissão de fluorescência de RACK1. Isso sugere que Ki-1/57 interage com os triptofanos na superfície de RACK1.

ABSTRACT

Ki-1/57, the 57-kDa human protein antigen recognized by the CD30 antibody Ki-1, is a cytoplasmic and nuclear protein, which is phosphorylated on serine and threonine residues upon cell activation. When isolated from the Hodgkin's lymphoma analogous cell line L540 Ki-1/57 was 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 hyaluronan binding protein 4 (IHABP4). We used the yeast-two-hybrid system to identify proteins interacting with Ki-1/57 and found that Ki-1/57 engages in specific interactions with the Chromatin-Helicase-DNA-binding domain protein 3 (CHD3), a nuclear protein involved in chromatin remodeling and transcription regulation, and with the adaptor protein Receptor of Activated Kinase-1 (RACK1). Next, we confirmed these interactions by in vitro and in vivo experiments. Interestingly, the interaction of Ki-1/57 with RACK1 is abolished upon activation of L540 cells with 4α-phorbol 12-myristate 13-acetate (PMA), which results in the phosphorylation of Ki-1/57 and its exit from the nucleus. We demonstrated that Ki-1/57 also co-precipitates with protein kinase C (PKC) when isolated from PMA activated L540 tumor cells and is a substrate for PKC phosphorylation in vitro and in vivo. These events associate Ki-1/57 with the RACK1/PKC pathway and may be important for the regulation of its nuclear functions. Its interaction with chromatin remodeling factors such as CHD3 and other proteins involved in transcriptional regulation including Topors, Daxx and Tip 60 may suggest that Ki-1/57 has also a function in this context and that this function is subject to regulatory events involving the PKC/RACK1 signaling pathway. RACK1 also interacts with the p53 paralogue p73. In that case, the physical binding of RACK1 to p73 can inhibit its transcription activation function. We found out that Ki-1/57 interacts with unphosphorylated p53 and that this binding inhibits p53 transcription activation function. The three-dimensional structure of Ki-1/57 is still unknown but our spectroscopic studies demonstrated that Ki-1/57 consists predominantly of β-sheets. Binding of Ki-1/57(122-413) to RACK1 abolishes its positive ellipticity at 229-300 nm, which is characteristic for tryptophan-rich proteins, and decreases its emission fluorescence. This suggests that surface tryptophans of RACK1 are involved in the interaction with Ki-1/57.

1 INTRODUÇÃO

1.1 O linfoma de Hodgkin

O linfoma de Hodgkin teve sua descrição anatômica feita em 1832 por Thomas Hodgkin (Hodgkin, 1832). Esta enfermidade é uma forma de câncer que se origina nos linfonodos do sistema linfático (Levine, 2002). O sistema linfático é um conjunto composto por órgãos, tecidos que produzem células responsáveis pela imunidade e vasos que conduzem estas células através do corpo (figura 1) (para melhor compreensão ler: Abbas *et al.,* 2000). Os tecidos e órgãos que compõem o sistema linfático incluem linfonodos, timo, baço, amídalas, medula óssea e rede de vasos linfáticos, e a linfa, um líquido claro que banha estes tecidos, e que contém proteínas e células linfóides. Os linfonodos são encontrados em todos as partes do corpo, principalmente no pescoço, virilha, axilas, pelve, abdômen e tórax; produzem e armazenam leucócitos denominados linfócitos. Nos linfonodos existem três tipos de células denominadas linfócitos, sendo elas: os linfócitos B (ou células B), os linfócitos T (ou células T), e as células NK ("*natural killer*"). Cada célula realiza uma função específica no combate a infecções, e também tem importância no combate ao câncer (Hall, 1998). Os linfócitos B produzem anticorpos, que se ligam na superfície de certos tipos de antígenos produzidos por bactérias, vírus e outros, e atraem e ativam células específicas do sistema imune. Os linfócitos T ajudam a proteger o organismo contra vírus, fungos e algumas bactérias. Também desempenham importante papel regulatório nas funções das células B. As células NK têm como alvo as células tumorais e protegem contra uma larga variedade de agentes infecciosos.

O Linfoma de Hodgkin (LH) pode ocorrer em qualquer faixa etária, no entanto a incidência da doença tem dois picos: o primeiro em torno de 15-35 anos e segundo acima dos 45 anos (MacMahon, 1957). O LH costuma atingir mais meninos e, com maior freqüência, nos adolescentes de 15 a 19 anos de idade do que qualquer outro câncer (Yahalom e Stratus, 2004). Sabe-se que esta doença tem maior freqüência em pessoas com doenças imunológicas e em famílias com casamentos consangüíneos (Yahalom e Stratus, 2004).

Estima-se que aproximadamente 38.000 a 50.000 brasileiros contrairão linfoma por ano, no entanto, a maioria dos pacientes com linfoma de Hodgkin pode ser curada com o tratamento atual (ABRALE-www.abrale.org.br). Dentre todos os tipos de linfomas existentes, este é o que apresenta maior chance de cura.

O tratamento clássico para o linfoma de Hodgkin, em geral, consiste de poliquimioterapia, com ou sem radioterapia (Hunger *et al*., 1994). Dependendo do estágio da doença no momento do diagnóstico, pode-se estimar o prognóstico do paciente com o tratamento. Para os pacientes que sofrem recaídas (retorno) da doença, estão disponíveis alternativas, dependendo da forma do tratamento inicial empregado. As formas empregadas usualmente, e com indicações relativamente precisas, são a poliquimioterapia e o transplante de medula óssea (Yaholom e Straus, 2004). A mortalidade foi reduzida em mais de 60% desde o início dos anos 70 devido aos avanços no tratamento (ABRALE-www.abrale.org.br).

O linfoma de Hodgkin surge quando um linfócito normal, freqüentemente um linfócito B (com aproximadamete 95% de chance de ser afetado) se transforma em uma célula maligna, capaz de crescer descontroladamente e disseminar-se (Braeuninger *et al*., 1997; Küppers *et al*., 2003; Berglund *et al*., 2003). A célula maligna começa a proliferar nos linfonodos, e com o passar do tempo, estas células malignas podem se disseminar para tecidos adjacentes, e, se não tratadas, podem atingir outras partes do corpo. No linfoma de Hodgkin, os tumores disseminam-se de um grupo de linfonodos para outros grupos de linfonodos através dos vasos linfáticos.

Os fatores de risco que contribuem para desenvolver o linfoma de Hodgkin são: sistema imune comprometido, doenças genéticas hereditárias, infecção pelo HIV, uso de drogas imunossupressoras e membros de famílias nas quais uma ou mais pessoas tiveram diagnóstico da doença (Forbes e Morbis, 1970; Chakravarti *et al*., 1986). Um outro importante fator de risco, o qual vem sendo bastante estudado, é a associação de pessoas infectadas pelo vírus Epstein-Barr e a presença de alguns subtipos do linfoma de Hodgkin nestes indivíduos (Kuze *et al*., 1996; Garcia *et al*., 2003).

O linfoma de Hodgkin pode surgir em qualquer parte do corpo, e os sintomas da doença dependem da sua localização (Hudson e Donaldson, 2002). Caso a doença se desenvolva-se em linfonodos que estão próximos à pele, no pescoço, axilas e virilhas, os sintomas provavelmente incluirão a apresentação de linfonodos aumentados e indolores nestes locais (figura 2).

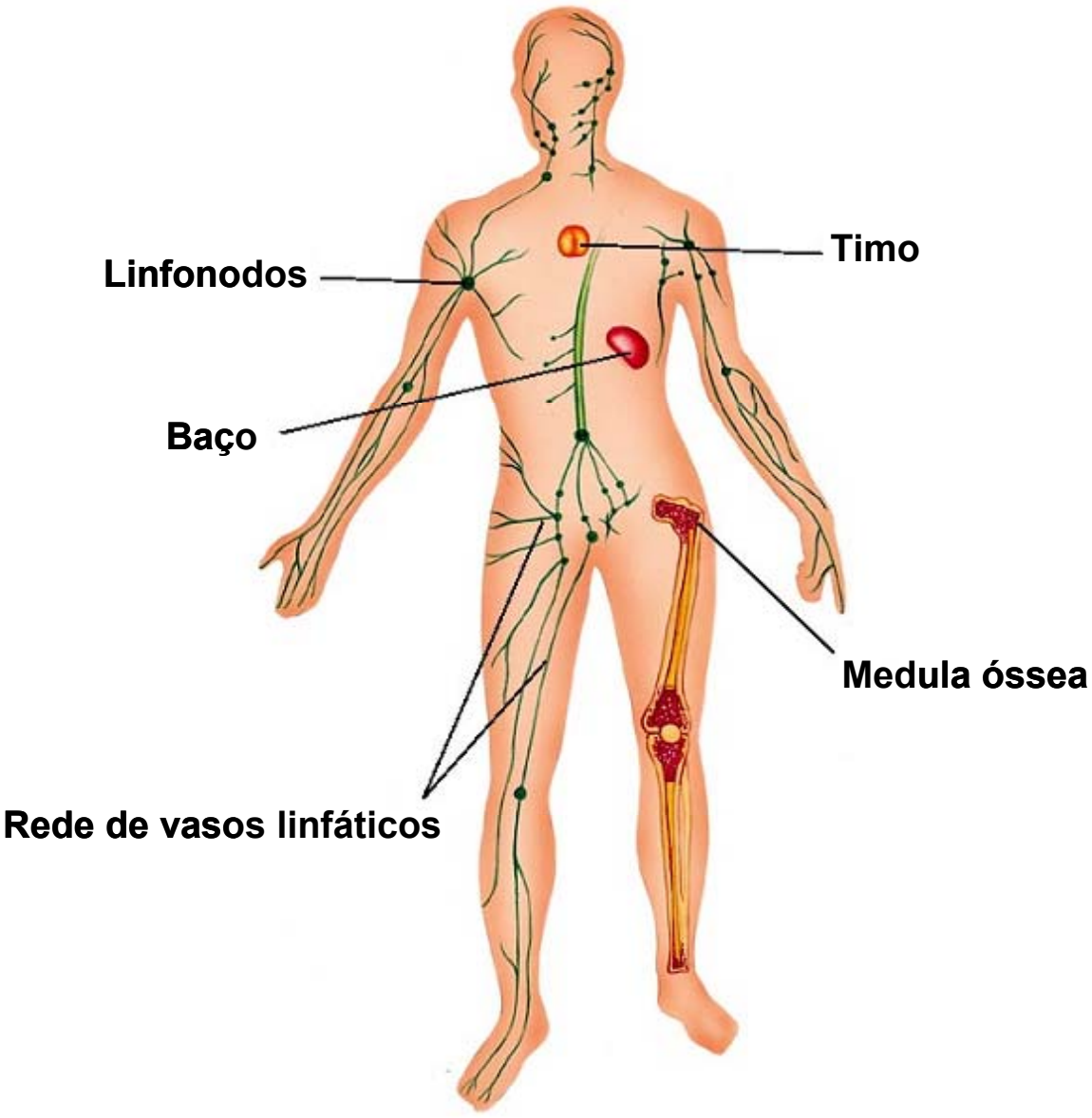

Figura 1: O sistema linfático humano.

(figura adaptada do site: http://www.escolavesper.com.br/defesasdocorpo.htm)

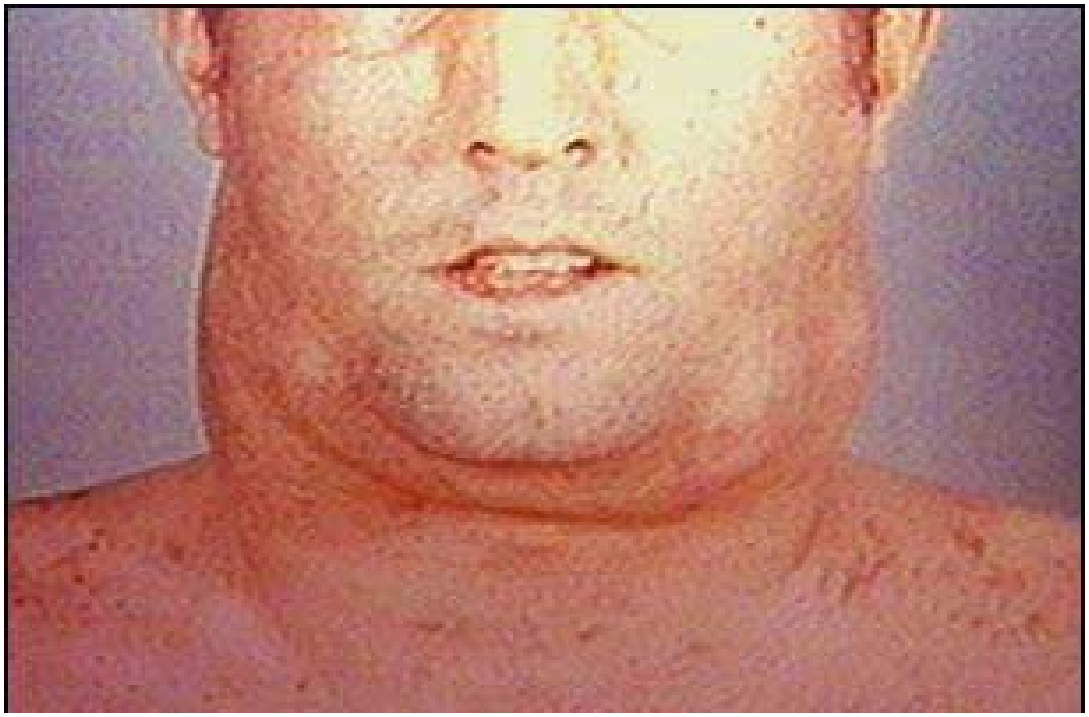

Figura 2: Uma pessoa que apresenta um aumento significativo dos linfonodos, na região do pescoço, devido ao avanço do linfoma de Hodgkin. (figura retirada do site: http://www.healthcentral.com/mhc/img/img1225.cfm)

Quando o linfoma de Hodgkin ocorre na região do tórax, os sintomas podem ser de tosse, "falta de ar" (dispnéia) e dor torácica. E quando se apresenta na pelve e no abdomem, os sintomas podem ser de plenitude e distensão abdominal (Toledo, 2004). Outros sintomas do linfoma de Hodgkin incluem febre, fadiga, sudorese noturna, perda de peso, e prurido ("coceira na pele") (Yaholom e Straus, 2004). No entanto, o local mais comum de envolvimento é o tórax, região também denominada mediastino.

Pode-se distinguir o linfoma de Hodgkin de outros tipos de linfoma em parte através do exame de amostras sob microscopia. O tecido obtido por biópsia de pacientes com linfoma de Hodgkin apresenta células mononucleadas e pequenas, denominadas de células Hodgkin malignas e células bi- ou polinucleares, denominadas de células Reed-Sternberg (RS) (Reed, 1902; Irsch *et al*., 1998) (figura 3).

As células Reed-Sternberg produzem pelo menos 12 citocinas diferentes, incluindo interleucina-1, e Fator de Necrose tumoral (TNF) (Toledo, 2004). Estas células também expressam constitutivamente altos níveis de Fator Nuclear Ativado Kappa Beta (NF-kB), que mostra um importante papel na sobrevivência do tumor (Hinz *et al.,* 2001). Esta aberrante ativação do NF-kB está relacionada a defeitos genéticos nos genes inibidores de NF-kB, infecção viral por Epstein Barr vírus (EBV) e estímulo por citocinas. EBV está presente em 90% das células RS, reforçando seu importante papel no desenvolvimento do linfoma de Hodgkin (Khanna *et al.*, 1995).

1.2 O papel da molécula CD30 no linfoma de Hodgkin

As células malignas de Hodgkin (H) e as células Reed-Sternberg (RS) no linfoma de Hodgkin são caracterizadas pela expressão abundante de uma proteína de membrana do tipo I, CD30, um membro da superfamília de receptores do fator de necrose tumoral (TNFR) que possui 120 KDa e contem seis motivos ricos em cisteínas que se repetem no seu domínio extracelular (Younes e Kadin, 2003; Pro e Younes, 2004; Hsu SM e Hsu PL, 1994; Dürkop *et al.,* 1992). Assim como os receptores TNF, CD30 pode apresentar uma forma solúvel (sCD30). A molécula sCD30 possui 85 KDa e é encontrada no sobrenadante de cultura de linhagens celulares CD30+ e em soro de pacientes com tumores CD30+ (Stein *et al.*, 2000; Younes e Kadin, 2003). Interessantemente, ao contrário dos outros membros da família de receptores TNF que disparam a sinalização para a apoptose, CD30 não apresenta o domínio de morte ("death domain") na cauda citoplasmática (figura 4). A cauda citoplasmática de CD30 associa-se com os fatores associados a TNFR (TNF Receptor-associated factor): TRAF1 e TRAF2, que sinalizam a ativação de NF-kB (De Young *et al.,* 2000).

A proteína CD30 é raramente expressa em outros linfomas do tipo T ou B ou na maioria de tecidos normais (Gruss *et al.,* 1994). O perfil restrito da expressão de CD30 em células H-RS explica porque esta é usada como um marcador tumoral do linfoma de Hodgkin, e sugere a possível participação de CD30 no mecanismo que conduz ao crescimento e à sobrevivência do tumor (Su *et al.,* 2004). A molécula CD30 se liga à molécula CD30L (ou CD153), expressa como uma glicoproteína de membrana do tipo II em linfócitos T ativados, linfócitos B reativos, macrófagos ativados, neutrófilos e eosinófilos ativados (Smith *et al.,* 1993; Nicod *et al.,* 1997; Pinto *et al.*, 1996; Shanebeck *et al.,* 1995; Wiley *et al.,* 1996). A via de sinalização de CD30L e CD30 leva à ativação do fator nuclear NF-kB e conduz à expressão elevada de citocinas. A interação com estes ligantes fazem com que a célula tumoral produza citocinas e quimiocinas que irão inibir a função do Linfócito T citotóxico. CD30 regula a expressão de CD28, Fas ligante, perforina e granzima B, sugerindo que CD30 pode ser um modulador da função do linfócito T citotóxico em linhagens de células deste linfoma (Bowen *et al.,* 1993; Muta *et al.,* 2000) (figura 5).

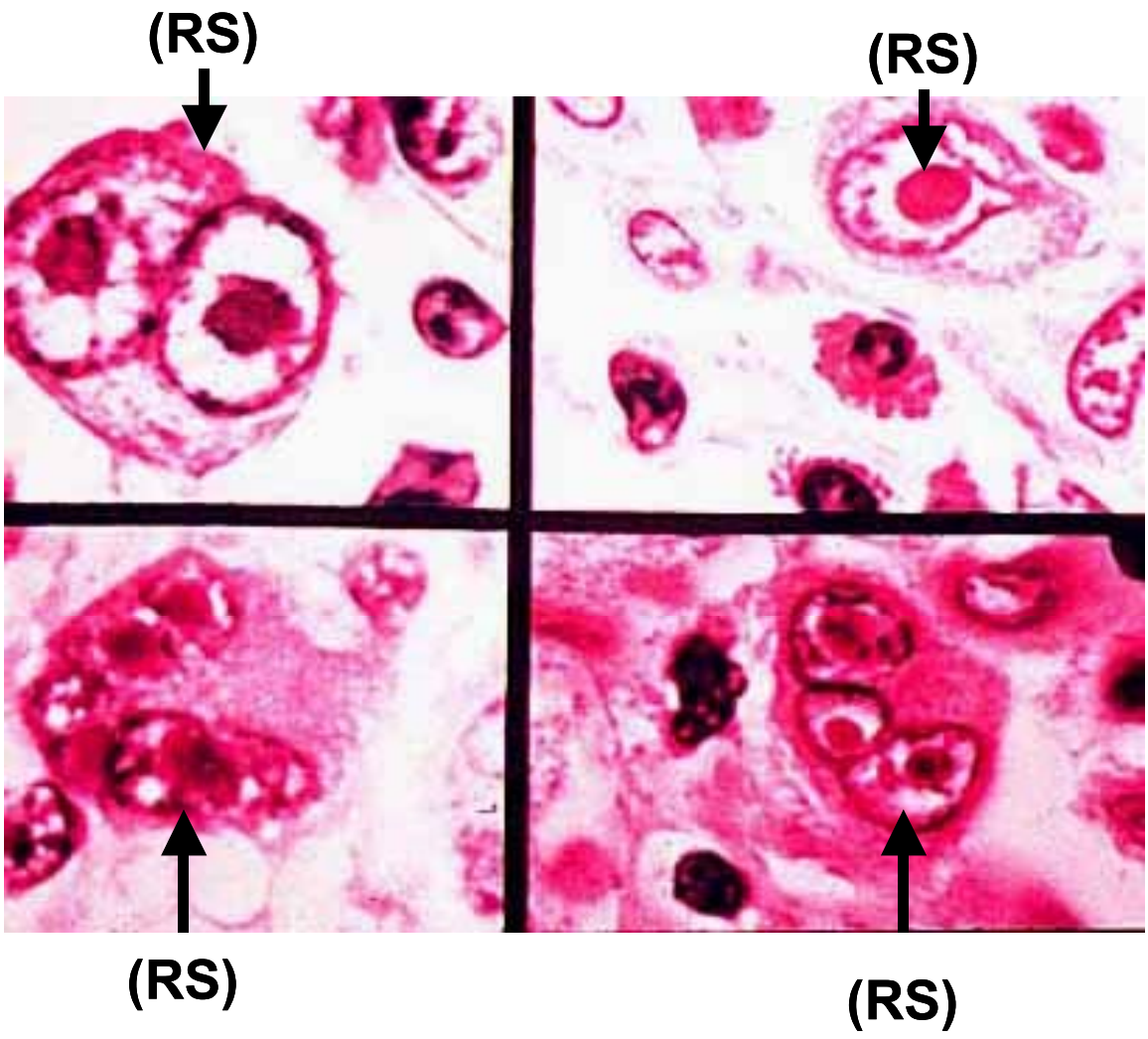

Figura 3: Corte histológico de um linfonodo de um paciente com linfoma de Hodgkin. A presença de células de Reed-Sternberg (RS) é indicada pelas setas. (figura retirada do site: http://researchpath.hitchcock.org/levylecture/selfasspln.html)

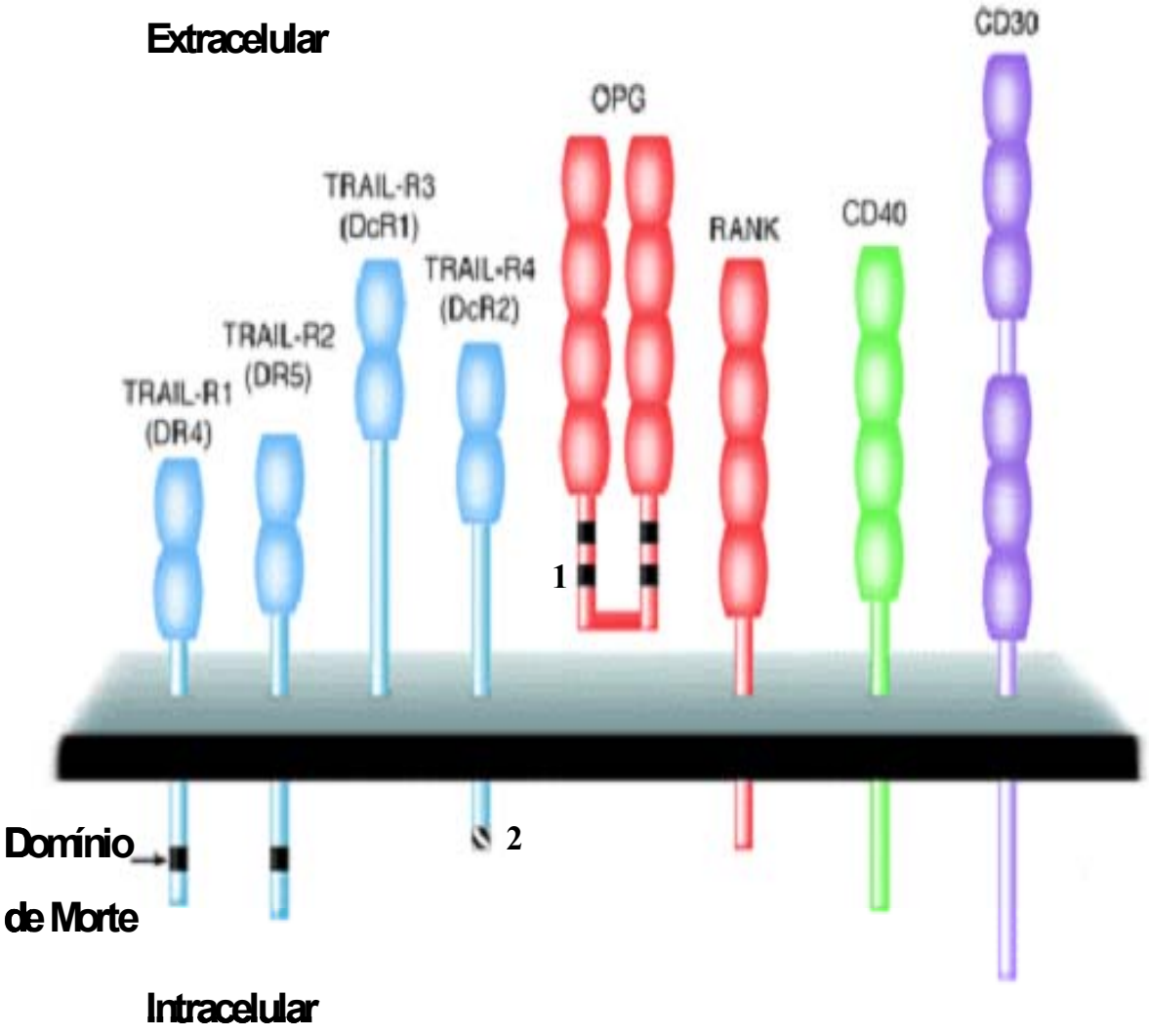

Figura 4: A família de receptores TNF. Estes receptores apresentam grande homologia em seus domínios extracelulares onde se encontra uma região variável rica em cisteínas repetidas e muitos deles apresentam um domínio de morte que induz à morte celular. O receptor de osteoprotegerin (OPG) é um receptor solúvel que também apresenta o domínio de morte (1). A cauda intracelular de CD30, assim como TRAIL-R3, RANK e CD40, não apresenta o domínio intracelular de morte, diferenciando-o dos outros membros da família TNF os quais apresentam este domínio, com exceção de TRAIL-R4 que apresenta um domínio de morte incompleto (2). (figura do artigo de Younes e Kadin, 2003).

Os níveis aumentados de CD30 solúvel são também observados em outras condições, tais como na artrite reumatóide, no câncer de colo retal, e na infecção viral (HIV e EBV) (Wang et al., 1997; Gerli *et al.*, 2001), e parecem também ser correlacionados com as respostas imunes Th1 deficiente e as respostas imunes Th2 dominantes. Um nível aumentado de CD30 solúvel pode ser um marcador em que o tratamento com IL-2 pode ajudar a restaurar o sistema imune comprometido (Gerli *et al.,* 2000). Ratos deficientes para o gene CD30 mostram uma seleção negativa defeituosa no timo (Amakawa *et al.,* 1996), e ratos transgênicos que super expressam CD30 em células T confirmaram o envolvimento de CD30 na seleção negativa do timo (Chiarlie *et al.,* 1999). Isso sugere um papel de CD30 na seleção ou na apoptose de células T no timo (Kadin, 2000).

As células H-RS produzem quimiocinas e citocinas que podem explicar a inflamação característica de tecidos afetados pelo linfoma de Hodgkin (Hsu *et al.*, 1995). Várias quimiocinas e citocinas que são produzidas conduzem a um influxo preferencial de células-Th2 e suprimem respostas imunes do tipo Th1 (Poppema,1989). Com isto, a inflamação causada pelo tumor não promove uma resposta anti-tumoral citotóxica. Um outro fator importante é que a molécula CD30 pode estar associada ao estado imunodeprimido no linfoma de Hodgkin, limitando o potencial e a ativação proliferativa das células T (Hsu *et al.* 1993). Por outro lado, CD30 pode não só inibir a proliferação de células T, mas também inibir a produção de interleucina-2 (IL-2) e a expressão de CD25 e de CD26 pelas células T (Su *et al.,* 2004).

A molécula CD30 é um bom alvo para a terapia com anticorpo monoclonal, uma vez que a expressão desta molécula é restrita a um pequeno número de linfócitos T e B ativados e saudáveis (Schnell *et al.*, 2002; Borchmann *et al*., 2004; Heuser *et al*., 2004). Interessantemente, estas células saudáveis contribuem para a propagação do linfoma de Hodgkin, uma vez que também expressam o CD30L. Se esta população de células normais for eliminada com as células tumorais, isto pode ser benéfico para o paciente (Pro e Yones, 2004).

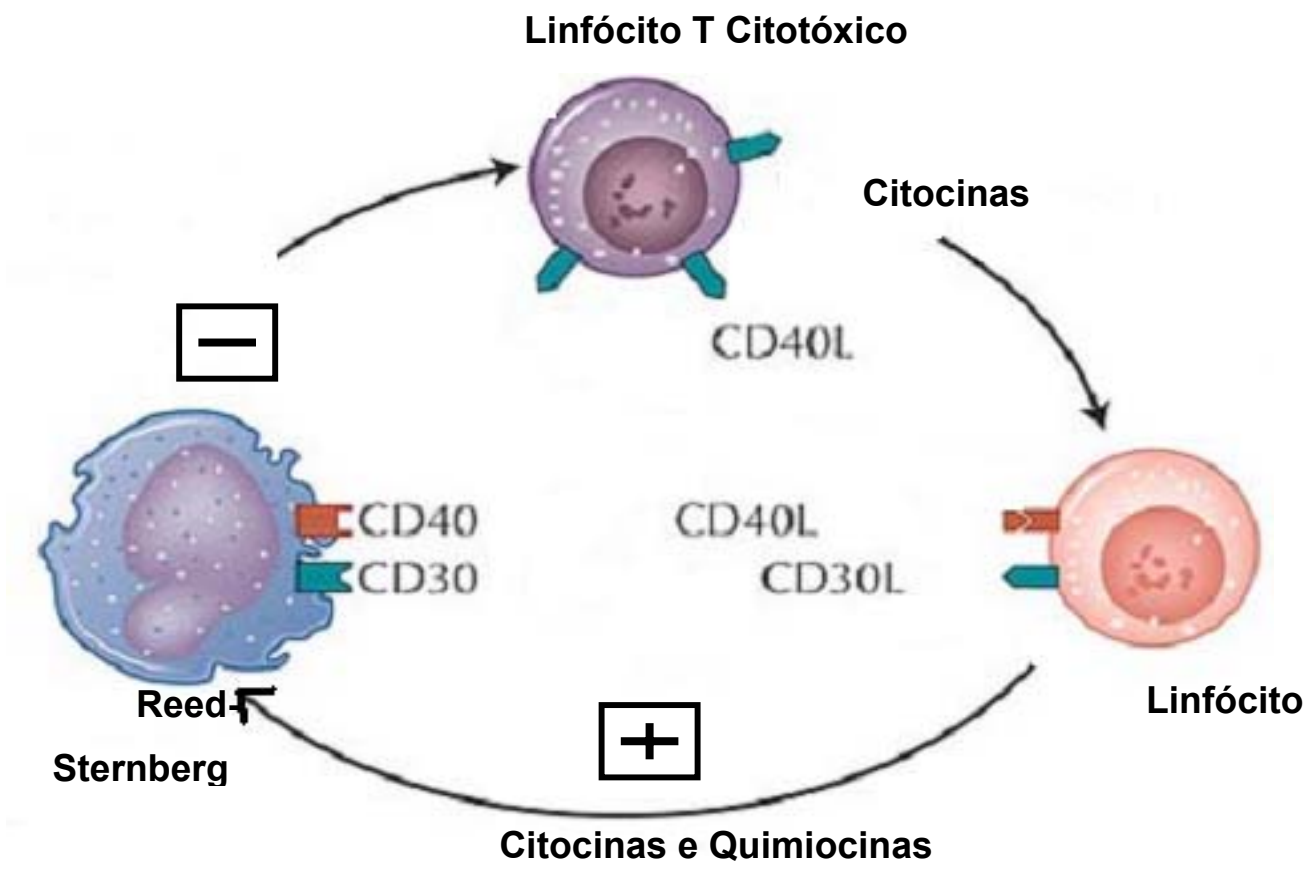

Figura 5: CD30 modula a função do linfócito T citotóxico. As células Reed Sternberg do linfoma de Hodgkin não expressam CD30L ou CD40L, que são expressas por linfócitos B reativos e normais. A interação entre estes ligantes faz com que a célula tumoral produza citocinas e quimiocinas que inibem a função do linfócito T citotóxico. Uma vez inibido, o linfócito T não atua sobre a célula tumoral e esta continua a se proliferar descontroladamente sem sofrer a ação citotóxica. (figura adaptada do artigo de Pro e Younes, 2004).

1.3 O anticorpo Ki-1 detecta CD30 e uma proteína de 57 KDa : Ki-1/57

O anticorpo monoclonal Ki-1 foi o primeiro anticorpo usado na detecção de células de Hodgkin, reagindo com células SR e H em todos os tipos histológicos de linfoma de Hodgkin e com uma pequena população de células grandes em tecido linfóide normal (Schwab *et al*., 1982). Análises bioquímicas mostraram que o anticorpo Ki-1 reage com CD30, uma glicoproteína de membrana de 120 kDa também conhecida como Ki-1/120. Antes de ser glicosilada CD30 apresenta uma forma intermediária de 85 kDa. No entanto, Hansen e colaboradores observaram que este anticorpo reage com uma outra proteína intracelular de 57 kDa que é sintetizada independentemente de CD30 (Hansen *et al.* 1990). Para chegar a esta conclusão, estes pesquisadores marcaram a linhagem L540 de células de Hodgkin *in vivo* com metionina radioativa (^{35}S) durante um curto período de tempo e as moléculas que reagiram com o anticorpo Ki-1 foram recuperadas por intervalos de tempo diferentes ("pulse chase experiment"). Eles observaram que após 3 minutos, a molécula intracelular de 57 kDa e a molécula precursora de CD30 de 85 kDa foram detectadas. Eles observaram que a intensidade da banda de 57 kDa não mudou durante todos os 270 minutos do experimento, no entanto, a banda de 85 kDa desaparece após 90 minutos, sendo substituída por uma banda de 120 kDa. Esses resultados sugerem que estes dois antígenos Ki-1/120 (CD30) e Ki-1/57 são sintetizados independentemente e que o anticorpo Ki-1 reconhece o Ki-1/57 por reação cruzada.

Usando a porção C-terminal de Ki-1/57 foram produzidos novos anticorpos monoclonais (A26 e E230) que interagem especificamente com Ki-1/57 pelas técnicas de "*Western-blot*" e imunoprecipitação (Kobarg *et al.,* 1997). Para testar o potencial de Ki-1/57 funcionar como marcador para doenças neoplásicas, Kobarg e colaboradores testaram a expressão desta proteína em vários tecidos neoplásicos por imunohistoquímica. Eles observaram que o anticorpo monoclonal A26 reage com células tumorais em linfoma de Hodgkin, leucemia linfática de célula B, linfoma de célula T- não-Hodgkin , carcinoma de bexiga e de próstata (Kobarg *et al.,* 1997).

1.4 Características da Proteína Ki-1/57

A proteína Ki-1/57, mas não Ki-1/120 ou CD30, é associada com atividade quinase e mostrou-se fosforilada em resíduos de serina e treonina quando isolada de células L540 do linfoma de Hodgkin (Hansen *et al*, 1989 e Hansen *et al.*,1990). No entanto, não foi encontrado nenhum domínio catalítico de quinase na seqüência de Ki-1/57.

Análises de microscopia eletrônica demonstraram que a proteína Ki-1/57 não está localizada apenas no citoplasma, mas também nos poros nucleares e no núcleo, associada ao nucléolo (Rhode *et al.*, 1992). Experimentos de marcação *in vivo* e de "*pulse-chase*" revelaram que somente a forma citoplasmática da proteína Ki-1/57 é fosforilada em resíduos de serina-treonina, enquanto que a forma nuclear não é fosforilada (professor Dr. H. Lemke, comunicação pessoal).

Peptídeos derivados da digestão tríptica de Ki-1/57 foram seqüenciados e as seqüências peptídicas resultantes foram usadas para a clonagem do cDNA parcial de Ki-1/57, a partir da biblioteca de cDNA derivada de células L540 (Kobarg *et al.*, 1997). O seqüenciamento de vários clones revelou que o cDNA contém 1380 bp e compreende aproximadamente 60% do C-terminal da proteína Ki-1/57. A tentativa de clonar o início 5´ da região do cDNA de Ki-1/57 por RT-PCR e 5´RACE inicialmente não teve sucesso porque nesta região o cDNA de Ki-1/57 contém uma seqüência de 100 pb rica em GC (82%) que dificulta a sua amplificação. A seqüência parcial do Ki-1/57 não revelou nenhuma homologia significativa com outras proteínas no banco de dados.

O cDNA parcial do Ki-1/57 foi usado para mapear a localização cromossômica do gene Ki-1/57 por FISH ("*Fluorescence In Situ Hybridization*"). O gene foi mapeado nas bandas 9q22.3-q31 no braço longo do cromossomo 9 humano (Kobarg *et al.*, 1997). Esta região é freqüentemente afetada por deleções secundárias na leucemia aguda mielóide do tipo M2 [translocação t(8;21)] e M3 [translocação t(15;17)] (Heim and Mitelman, 1986).

Outros pesquisadores obtiveram o cDNA inteiro que codifica a protéina Ki-1/57 (Huang *et al.*, 2000). Estes caracterizaram este antígeno como uma proteína intracelular que se liga a hialuronato, devido à presença de possíveis motivos de ligação a hialuronato (R/K-X_7-R/K) na sua seqüência, onde R corresponde a arginina, K a lisina e X a qualquer aminoácido. O glicosaminoglicano hialuronato possui uma função bem definida na matriz extracelular e na superfície das células, no entanto, há algumas

descrições de que este pode ser encontrado no citoplasma e também no núcleo da célula, mas com função desconhecida (Furukawa e Terayama, 1979; Ripellino *et al.*, 1988, Grammatikakis *et al.*, 1995; Evanko e Wight, 1999). Baseando-se neste motivo de ligação a hialuronato, nós observamos que em uma análise aleatória de 10 proteínas nucleares todas possuem esse motivo e, curiosamente, cinco entre estas são extremamente ricas nesses motivos. Entre estas cinco proteínas estão: CHD3 (NM_001272.1) com 49 repetições; Topors (NM_AF098300) com 36 repetições; Policomb2 Humana (AF013956) com 7 repetições; p53 (AAH03596) com 3 repetições e c-Fos (K00650) com 2 repetições. Isto sugere que a maioria das proteínas nucleares ricas em arginina e lisina são capazes de interagir com hialuronato? Esta questão ainda está em aberta uma vez que o papel intracelular e nuclear do hialuronato ainda não foi estudado.

Quando esse projeto foi iniciado, sabia-se muito pouco sobre as possíveis funções da proteína Ki-1/57. Além disso, nada se sabia a respeito da estrutura da proteína Ki-1/57.

2 OBJETIVOS

O objetivo geral deste trabalho foi estudar os aspectos funcionais e estruturais da proteína Ki-1/57 e de seus possíveis ligantes protéicos.

Para a caracterização do contexto funcional e aspectos estruturais desta proteína os objetivos específicos foram:

1- Analisar a expressão tecido específica do mRNA que codifica a proteína Ki-1/57;

2- Identificar proteínas com as quais a proteína Ki-1/57 interage, utilizando o sistema de duplo híbrido em levedura para;

3- Analisar a fosforilação de proteína Ki-1/57 e sua associação com a atividade de proteína quinase quando Ki-1/57 é isolada de células humanas;

4- Analisar a localização subcelular da proteína Ki-1/57;

5- Análises espectroscópicas da proteína Ki-1/57 por dicroísmo circular (CD) e fluorescência na presença e na ausência de possíveis ligantes.

3 RESULTADOS

3.1 Artigo I :

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

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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]

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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

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[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

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

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          * * *           :  :** *:*:*    **::   ** :::: ::        :*:
huKi-1/57 MKGALGSPVAAAGAAMQESFGCVVANRFHQLLDDES---DPFDILREAERRRQQQLGRKR  57
huCGI-55  MPGHL-----------QEGPGCVVTNRFDQLFDDES---DPFEVLKAAE------NKKKE  40
CeCGI-55  ----------------MSTEYGCQVTNKFGLPSDDDDEYDDPRELIQKVS----QIAAKKK  41

            :   :     :: : * :: : *:   :    : * :   *       :    :

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

           :  : :   **  ::       *       *  :    ** *       :      :*   :

huKi-1/57 LQAPGQKRTPRRGECQGWNDSRGPEGMLER-AERRSYREYRPYETERQADPTAEKFPDEK 171
huCGI-55  LKKEGIRRVGRRPDQQ----LQGEGKIIDRRPERRPPRERR-PEKPLEEKGEGGEFSVDR 155
CeCGI-55  PQGENRRGGPRRGGER------GAARPAGRGGRGGFTRENR----EGEEPKQEVSFEDGQ 148

                *: * ***   *   : ***   :     : ::   *:*:* :*:*: :::

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

          *: :* *  :**  **     :        :   *: :     *:  :    *       :

huKi-1/57 DNMGGCGVRTWGSGKDT-SDVEPTAPMEEPTVVEESQGTPEEESPAKVPELEVEEETQVQ 289
huCGI-55  DKRGGSGSHNWGTVKDELTDLDQSNVTEETPEGEEHHPVADTEN--KENEVEEVKEGPK 265
CeCGI-55  DRKDGHGKGNWGDQKDE---LAGETENIAPEGAESTEPEVPREKTAEELAYEAELAVLAK 251

          : **:*:*   :   :*  ** **: : :     * : ::*:       :*:     :

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

          **:: **:  :: :  :  * :*: *    * **      ::              :*   :

huKi-1/57 RKPANDITSQLEINFGNLPRPGRGARGGTRGGRGRIRRAENNYFRAEVVMQDV-APNPDD 405
huCGI-55  RKPANDITSQLEINFGDLGRPGRGGRGG-RGGRGRGGRFNRGSRTDRSSAS---APDVDD 379
CeCGI-55  RKQVLDISITFRNDRPERERNDRSERPQRGGPRGGGRGGGRGGQRQGGHGGRNNTPFNAS 368

           : ****:

huKi-1/57 PEDFFALS--   413
huCGI-55  PEAFFALA--   387
CeCGI-55  DDAFFALGAK   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

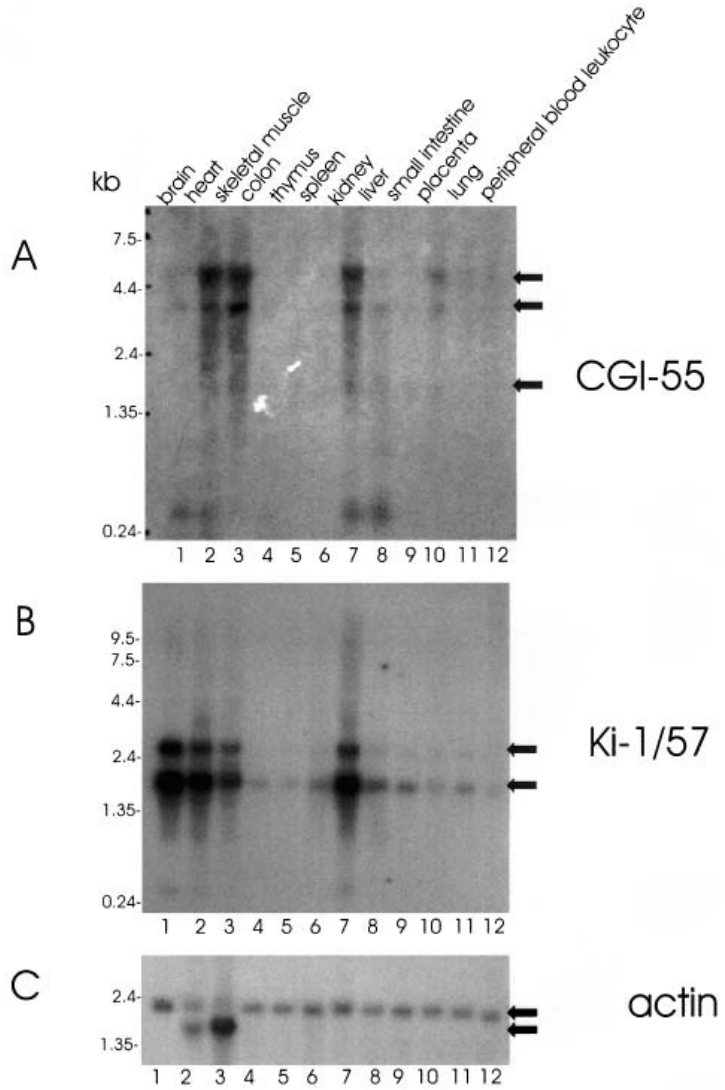
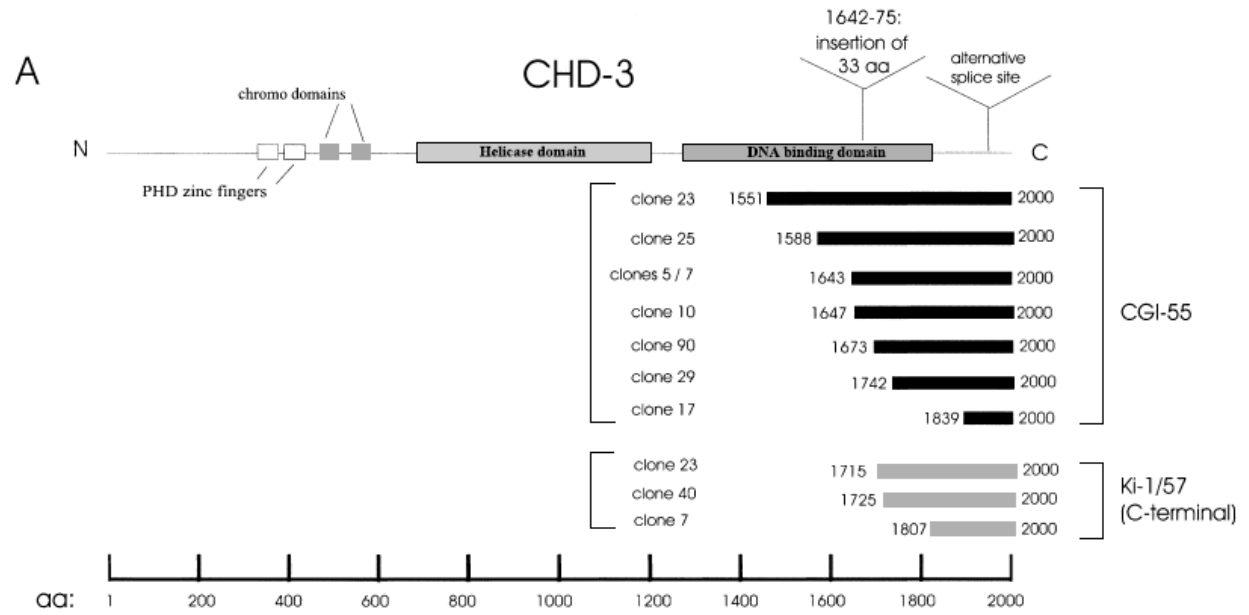


Fig. 2. Differential expression of CGI-55 and Ki-1/57 mRNAs in human tissues. A Northern blot of poly(A)$^+$ RNA isolated from several human tissues (Clonetech) was hybridized with human CGI-55 (A), Ki-1/57 (B) and β-actin (C) cDNA probes.



B

```
CHD-3              TAATNG-P----------------IHRFFKKVPEIQ----
CHD-3 alt. spliced TAATNGPPVLVKKEKEMVGALVSDGLDRKEPRAGEVICIDD
                   1966                                  2000
```

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

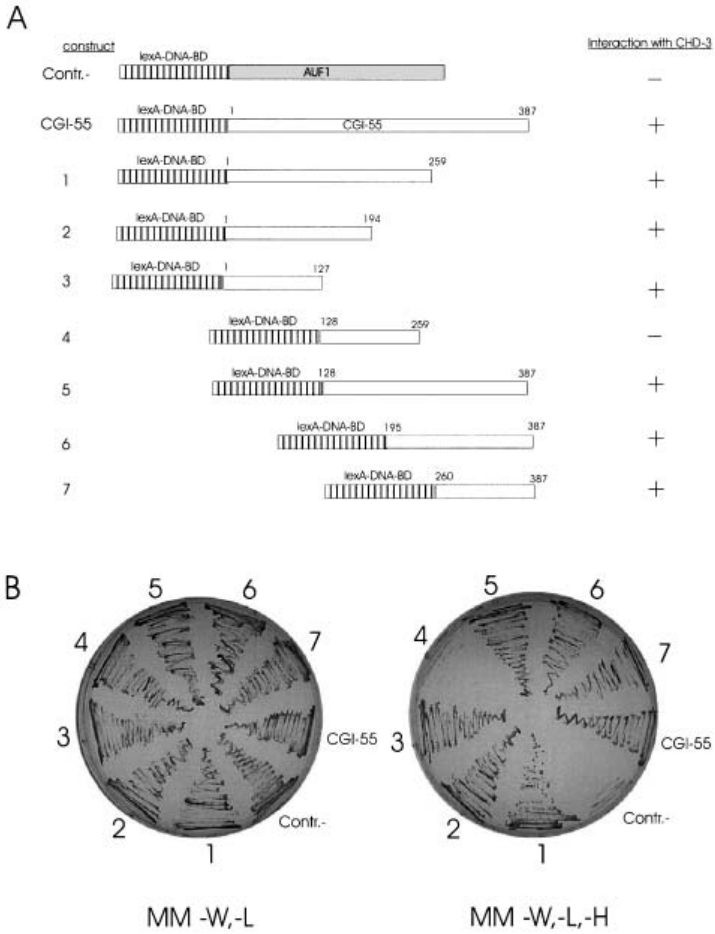


Fig. 4. Mapping of the regions of CGI-55 that interact with CHD-3. A: Various N- and C-terminal truncations of human CGI-55 were fused in frame to the DNA-binding domain of lexA in plasmid pACT2 and transformed into yeast L40 together with the fusion CHD3-Gal4-AD cloned in pBTM116. B: Interaction was 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.

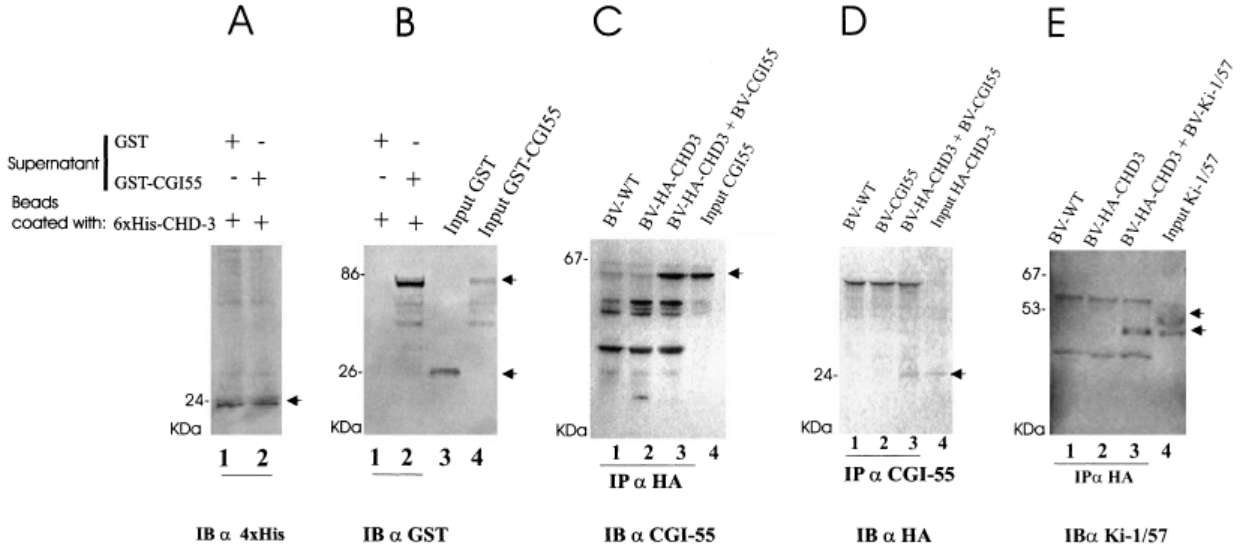


Fig. 5. In vitro binding assay and co-immunoprecipitation from *Sf*9 insect cells infected with different combinations of recombinant BV. A,B: In vitro binding assay. Ni-NTA Sepharose beads were loaded with purified 6×His-HA-CHD3(1983–2000) protein. Beads were then incubated with purified GST or GST–CGI55, 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.

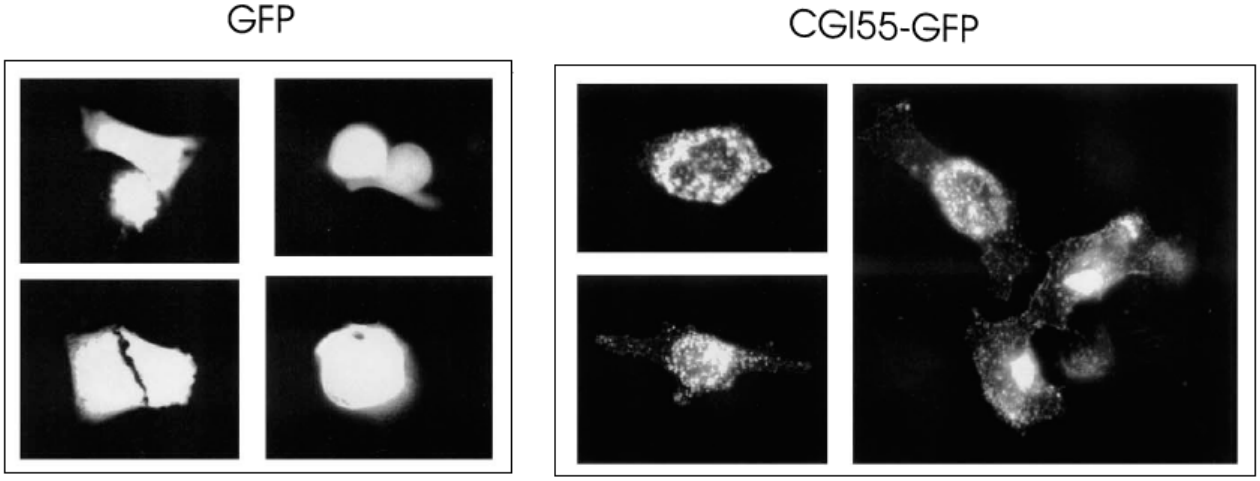


Fig. 6. Cellular Localization of CGI55–EGFP. HeLa cells were transiently transfected with expression vector pEGFP or recombinant expression vector CGI55–pEGFP-N1. After 24 h transfected cells were examined with a Nikon microscope.

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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3 RESULTADOS

3.2 Artigo II:

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

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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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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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[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_6 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 co-transformants 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

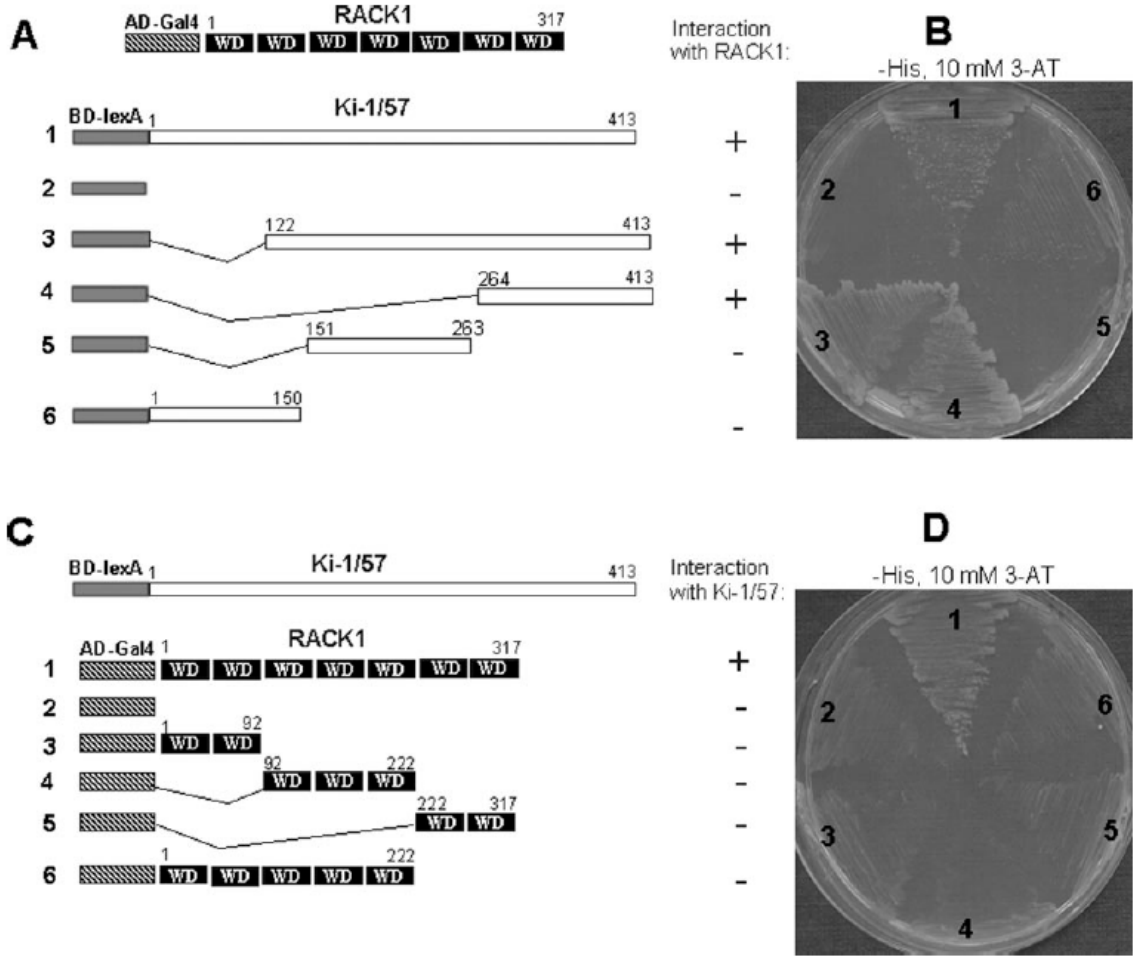

FIG. 1. **Mapping of the Ki-1/57 and RACK1 interaction sites.** *A*, schematic representation of Ki-1/57. Various N- and C-terminal truncations of human Ki-1/57 (*open boxes*) were fused in-frame to the DNA-binding domain of LexA (*filled boxes*) in plasmid pBTM116 and transformed into yeast L40 together 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

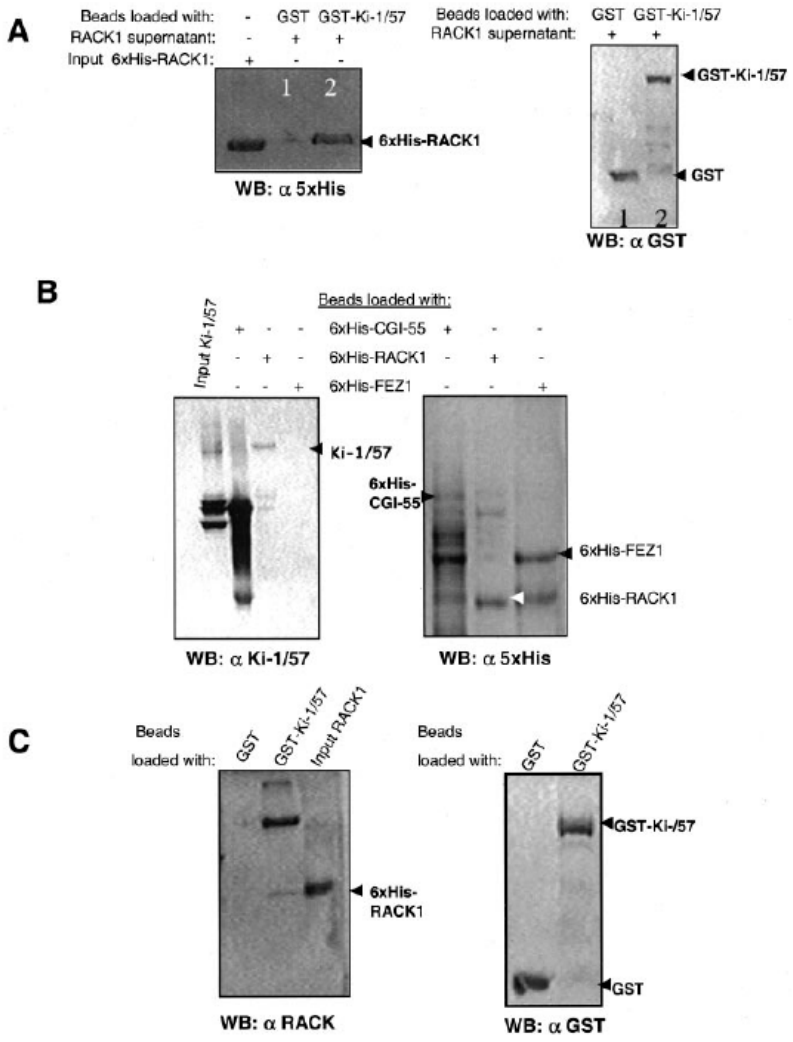

FIG. 2. ***In vitro* binding assays of RACK1 and Ki-1/57.** *A*, glutathione-Sepharose beads were loaded with purified GST or GST-Ki-1/57 proteins. The beads were then washed and incubated with purified His_6-RACK1, washed three times, separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with anti-His_6 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.

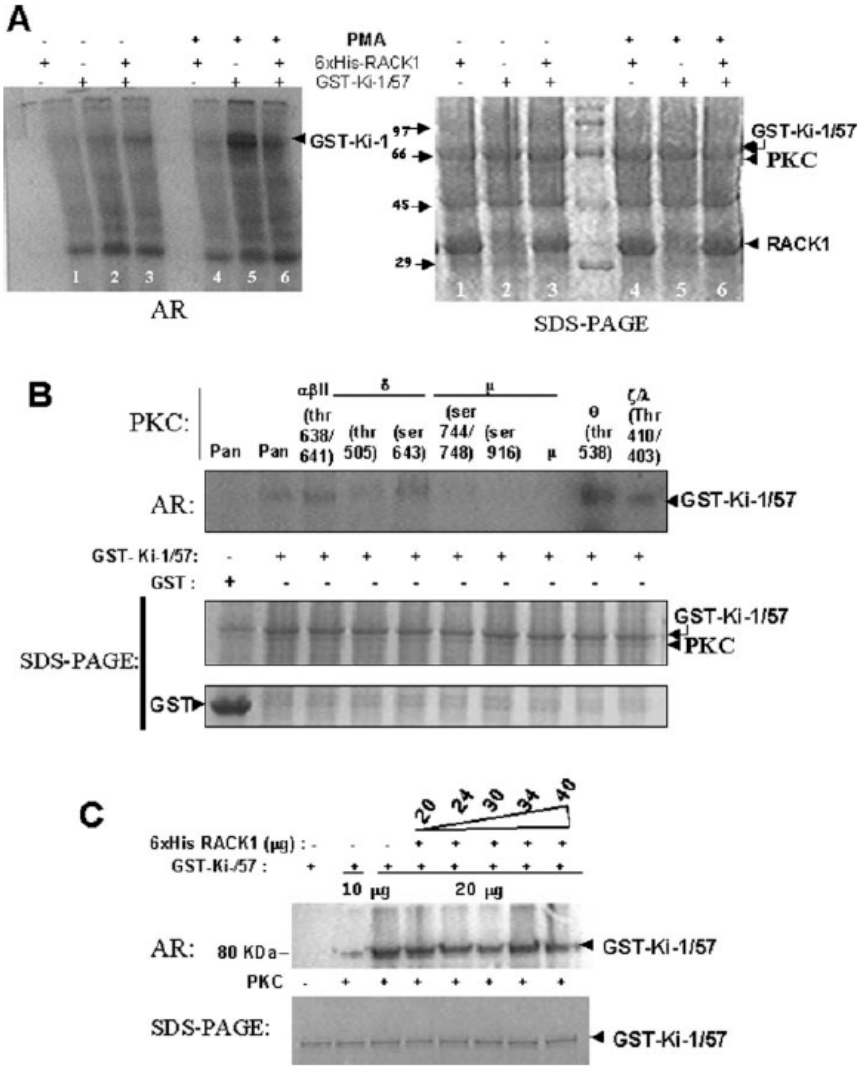

FIG. 3. ***In vitro* phosphorylation of Ki-1/57 by PKC.** *A*, L540 cells were or were not treated for 4 h with PMA. PKC was immunoprecipitated (*IP*) from the lysate of 10^7 L540 cells with anti-phospho-PKC-Pan antibody and then incubated with His_6-RACK1 or GST-Ki-1/57 or both in the presence of [γ-^{32}P]ATP, 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

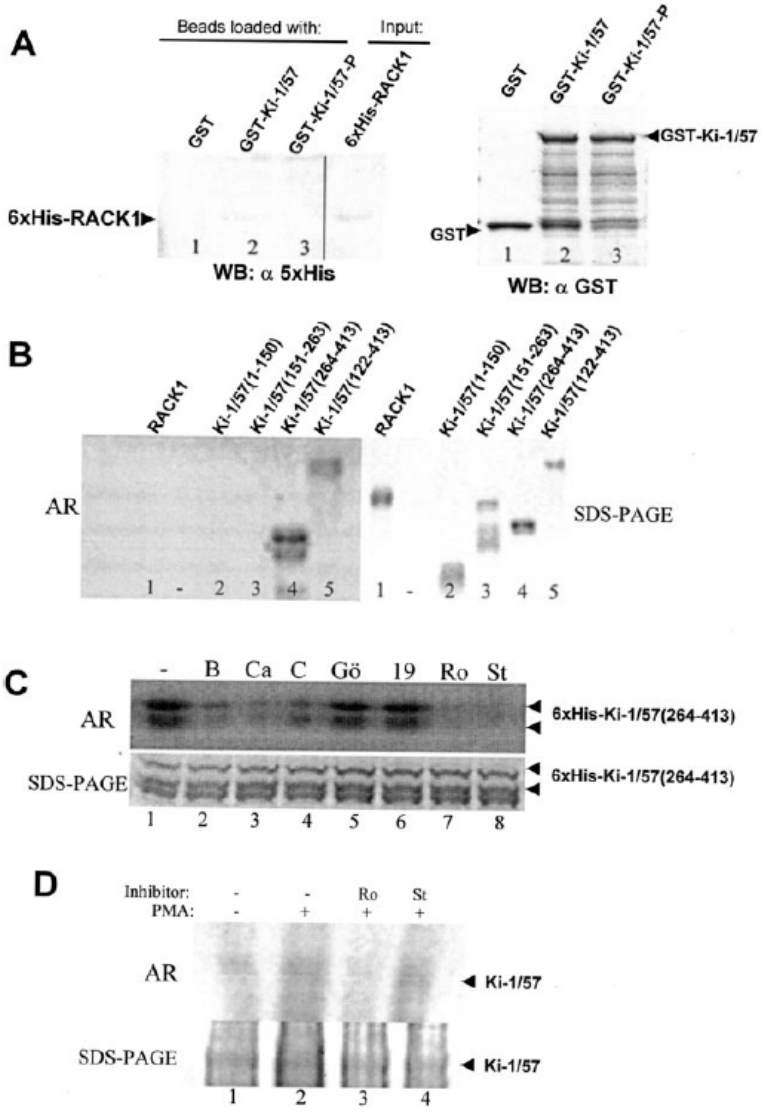

FIG. 4. **The interaction between Ki-1/57 and RACK1 *in vitro* is abolished by the phosphorylation of Ki-1/57.** *A*, *left panel*, GST-Ki-1/57 *in vitro* phosphorylated by purified PKC-Pan no longer co-precipitates RACK1 *in vitro*. *Right panel*, Western blot (*WB*) anti-GST for demonstration of equal 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

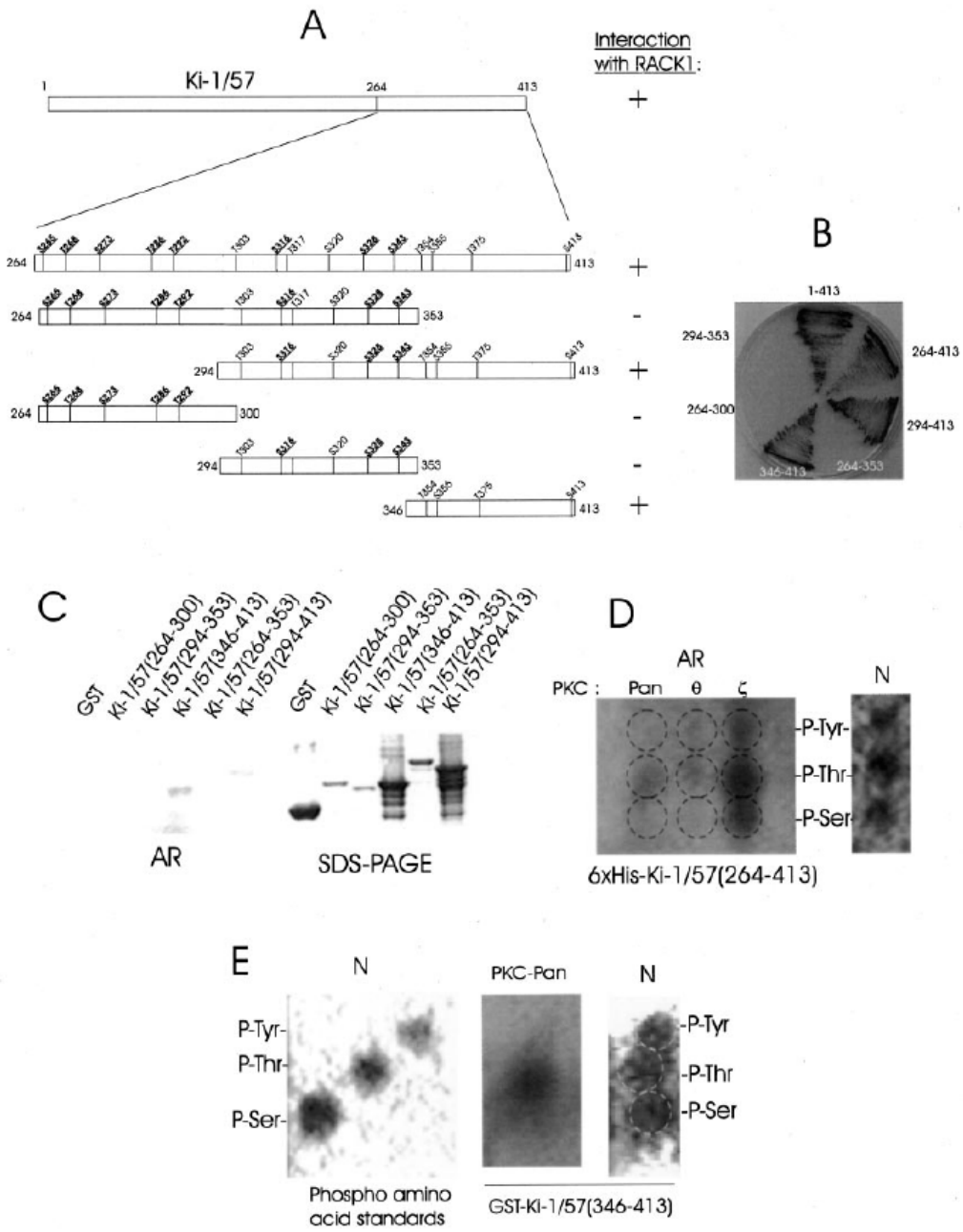

FIG. 5. **Mapping of the phosphorylation and interaction characteristics of Ki-1/57 C-terminal region (264–413) and phosphoaminoacid analysis.** ***A***, schematic representation of the C-terminal region of Ki-1/57 with indication of the present Ser and Thr residues. *Underlined bold type* indicates 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

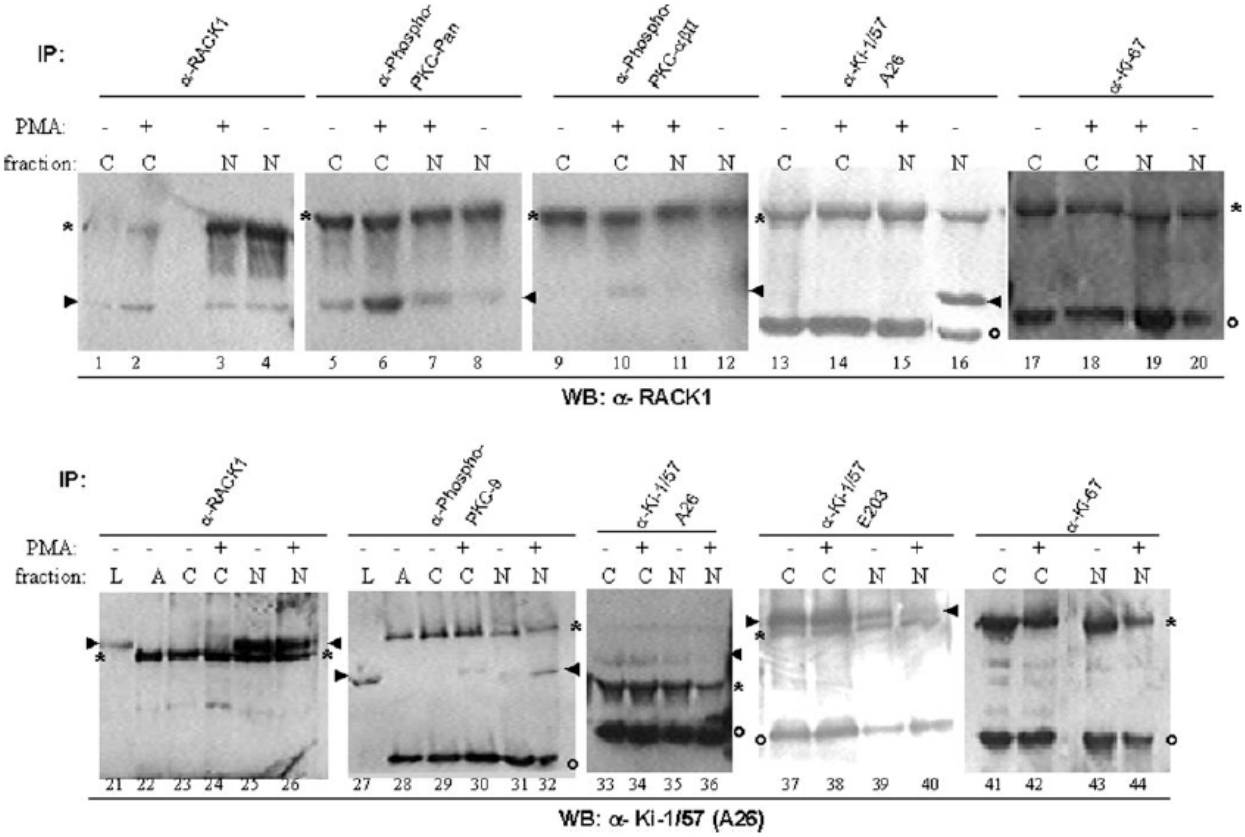

FIG. 6. **The nuclear association between Ki-1/57 and RACK1 is abolished upon PMA activation of the L540 cells.** L540 cells were or were not stimulated for 4 h with PMA. The cytoplasmic (*C*) or nuclear (*N*) fractions of their cell lysates were immunoprecipitated (*IP*) with antibodies anti-RACK1, 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

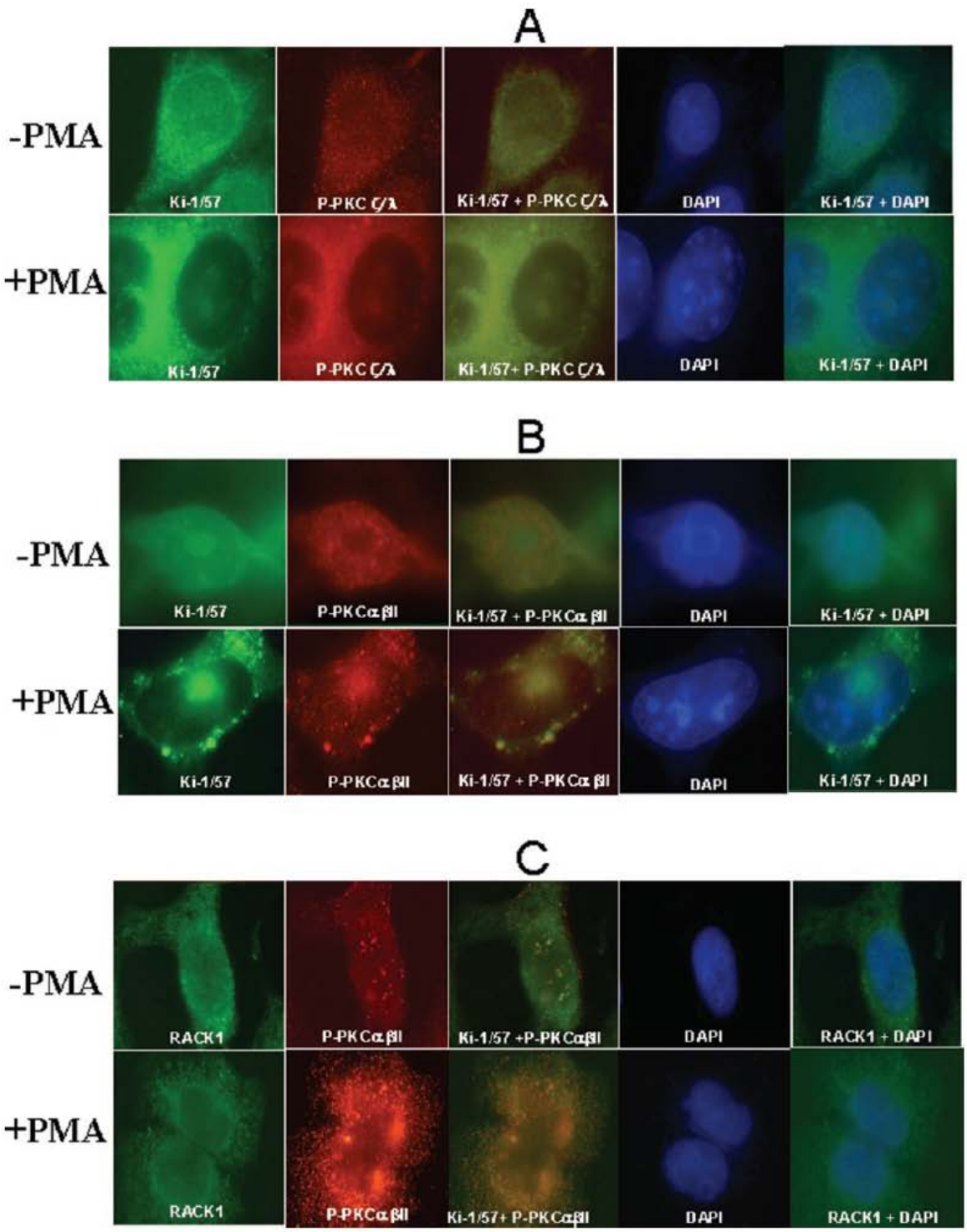

FIG. 7. **Subcellular localization of endogenous RACK1, Ki-1/57, phospho-PKC$\alpha\beta_{II}$, and phospho-PKCζ/λbefore and after treatment of cells with PMA.** HeLa cells grown on glass coverslips were stimulated or not with PMA for 4 h at 37 °C. The cells were fixed with 100% methanol, and the proteins 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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3.3 Artigo III:

Ki-1/57 interacts with p53 and interferes negatively in its transcriptional activation function

Flávia C. Nery, Edmilson Rui, and Jörg Kobarg

(Artigo submetido)

Ki-1/57 interacts with p53 and interferes negatively in its transcriptional activation function

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Abstract:
Ki-1/57 is a cytoplasmic and nuclear phospho-protein of 57 kDa and interacts with the adaptor protein RACK1. Upon cellular activation with PMA the protein kinase C phosphorylates Ki-1/57 on several C-terminal Thr and Ser residues and binds now itself to this newly generated docking sites. The interaction of Ki-1/57 with RACK1 after phosphorylation on the other hand is completely abolished. Aside this Ki-1/57 also interacts with a series of nuclear proteins including CHD3, Daxx and Topors, suggesting that it might be involved in the regulation of transcription. Here we describe yeast two-hybrid studies that identified a total of 11 proteins interacting with Ki-1/57, all of which interact or are functionally associated with p53 itself or other members of the p53 family of proteins. We found that Ki-1/57 is able to interact with p53 in the yeast two-hybrid system when the interaction was tested directly. This interaction could be confirmed by pull down assays with purified proteins in vitro and by a co-immunoprecipitation assay of the proteins from the human Hodgkin analogous lymphoma cell line L540. Furthermore, we found that the in vitro phosphorylation of p53 by PKC abolishes its interaction with Ki-1/57. Finally, we found in a transactivation assay in yeast cells that the co-expression of Ki-1/57 inhibits the p53 dependent expression of a beta-galactosidase reporter gene. Altogether, we identified Ki-1/57 as a new p53 interacting protein, which can negatively influence its transactivation function and found further support for the hypothesis that Ki-1/57 may be involved in the regulation of transcription.

Keywords: protein-protein interaction, yeast two-hybrid system, transactivation, cellular localization.

Abbreviations: MM, minimal medium; CHD-3, Chromobox Helicase DNA-binding Domain protein 3; RACK1, Receptor of Activated Kinase 1; PKC, protein kinase C; PMA, phorbol 12-acetate; PAI, plasminogen activator inhibitor; IHABP4, intracellular hyaluronan-binding protein 4.

1. Introduction

The protein antigen Ki-1/57 was originally identified by the cross-reaction of the first CD30 monoclonal antibody Ki-1 [1-4]. Ki-1 detected not only the 120 kDa transmembrane protein CD30 but also the cytoplasmic and nuclear Ki-1/57 in the malignant Hodgkin and Sternberg-Reed cells in Hodgkin lymphoma [1] and in the Hodgkin analogous tumor cell line L540 [4]. Ki-1/57 is a phospho-protein [2, 3] and when isolated from the L540 cells is associated with a serine/threonine protein kinase activity [4]. Electron microscopic analyses 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 and other nuclear bodies [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. Another group cloned the full length cDNA clone [7], found that Ki-1/57, due to its many positively charged Arg and Lys residues is able to interact with several negatively charged macromolecules, including glycosaminoglycans and RNA. Huang and co-workers called Ki-1/57 Intracellular hyaluronan-binding protein 4 (IHABP4) because of its binding to hyaluronat [7]. 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 with 40,7 % identity and 67,4 % similarity with Ki-1/57 [8]. Recent studies from our group suggest that both Ki-1/57 and CGI-55 are proteins that could be involved in nuclear functions such as the regulation of transcription [8]. We found that both proteins interact with the chromatin remodeling protein CHD-3 [8] and with several other transcriptional regulators including Topors and Daxx [9]. Furthermore, we found that Ki-1/57 interacts and forms a stable complex in human cells with the adaptor protein RACK1, which itself is physically associated with activated PKC [9]. This interaction provided a possible explanation for the initial observation of the association of the Ki-1/57 antigen with a Ser/Thr kinase activity. Ki-1/57 is also able to associate with PKC and serves as its substrate for phosphorylation in vitro and in vivo. The stable interaction of Ki-1/57 with RACK1 is abolished by the PMA mediated PKC dependent phosphorylation of Ki-1/57 [9]. These data suggest that the nuclear functions of Ki-1/57 could be regulated by a cytoplasmic signaling cascade involving PKC and RACK1. Most interestingly, it has been described recently

that RACK1 has a negative regulatory function on the transcription activation function of p73, a member of the p53 family of proteins [10].

P53 is functionally a tumor suppressor protein and belongs to the large protein group of transcription factors. It displays its growth suppression properties by the transcriptional regulation of gene expression. Specifically, p53 is involved in safeguarding the genomic integrity [11], sensing of DNA damage [12], monitoring of the G1 checkpoint of the cell cycle [13] and activation of the cellular apoptosis program to kill cells with damaged genomes [14]. Genes controlled by p53 include p21 [15], GADD45 [13], cyclin G [16] and bax [17] among many others. The inactivation of p53 function through mutations or by interaction with viral and altered cellular proteins is a common event in human carcinogenesis and the majority of human cancers have mutations in the p53 gene [18]. Among several other cellular proteins Daxx [19], Topors [20] and MDM2 [21] have been described to directly interact with p53 or have an influence on its function. The direct interaction of regulatory proteins with p53 can interfere with p53 functions, like its multimerization, its transactivation function or its sequence specific DNA binding [22]. Furthermore the function pf p53 also seems to be tightly controlled by post-translational mechanisms, including phosphorylation and acteylation among others [23, 24, 25]. Here, we report that the human protein Ki-1/57 interacts with several p53 interacting proteins as well as with p53 itself in the yeast two-hybrid system. The interaction of p53 and Ki-1/57 could be confirmed by independent assays in vitro and in vivo. We also show that Ki-1/57 negatively influences the p53 dependent transcription and that phosphorylation of p53 blocks the interaction with Ki-1/57 in vitro. Our results suggest that Ki-1/57 is a protein that might be involved in transcriptional regulation processes and identify it as a new p53 interacting protein with inhibitory functions.

2- Materials and Methods

2.1. Plasmid construction, antibodies and cell culture

A pGEX plasmid containing the full length human p53 in frame with GST was kindly provided by Dr. Gianni Del Sal (Laboratorio Nazionale CIB, Trieste, Italy). The gene coding human p53(1-393) protein was amplified from this vector with specific primers and inserted in frame with the Gal4 activation domain, via EcoRI and BamHI sites, into the cloning site of the yeast expression vector pGAD424 (Clontech). The correctness of all vector constructs was confirmed by DNA sequencing. The Ki-1/57 constructions in the yeast vectors pBTM116, pGAD424 and the bacterial vectors pET-28a have been described previously [9]. The large T-Gal4-AD vector had been kindly provided by Dr. M. Bucciarelli Rodrigues (UFMG, Belo Horizonte, Brasil). Antibodies A26 [6] and Ki-1 [1] and cell culture procedures for the L540 cells have been described previously elsewhere [26, 9].

2.2. Yeast Two-hybrid Screening and Interaction Analysis

The pBTM116-Ki-1/57(1-150) and pBTM116-Ki-1/57(122-413) vectors were used to express the protein Ki-1/57 (C-terminal) linked to the C-terminus of LexA DNA-binding domain peptide in Saccharomyces cerevisiae strain L40 [27, 28, 29]. 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 growth of co-transformands (on –W, -L, -H culture medium plates), β-galactosidase activity test, plasmid DNA extraction and sequencing were performed as described [30].

2.3. Bacterial expression, protein purification and in vitro protein phosphorylation

GST-p53 protein and 6xHis-Ki-1/57 proteins were expressed in E. coli BL21-CodonPlus-RIL (Stratagene), and purified using Glutathione-sepharose 4B (Amersham) or Ni-NTA sepharose beads (Qiagen) according to procedures provided by the manufacturers [30, 9]. In vitro protein phosphorylation has been described previously [9].

2.4. In vitro binding assay and western blot analysis

1 μg of GST-p53 fusion protein was coupled to glutathione sepharose beads. Next, 1 μg of phosphorylated or non-phosphorylated 6xHis-Ki-1/57 proteins were incubated on and end-over-end mixer for 2 h with the GST-p53 beads and then washed with buffer (20 mM Tris-HCl, pH 7.5, 100 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 or mouse anti-5xHis antibody and secondary anti-mouse IgG-HRP conjugate for detection.

2.5. In vivo binding assay and western blot analysis

1.0 x 10^7 L540 were collected and lysed in 1 ml buffer W (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 0.5 mM DTT, 0.1 mM EDTA, 1 mM PMSF, 0.1% Triton X-100, mix of 5 protease inhibitors). Lysate was treated with DNAse (Promega) and cleared at 14.000 x g for 15 min. Next 20 µl protein A sepharose beads (Pharmacia) were loaded with the indicated antibodies, washed with buffer W and incubated or not with the indicated lysates for 2 h at 4 °C. After further two washes with buffer W the beads were re-suspended in SDS-PAGE loading buffer, boiled and analyzed by Western blot using different monoclonal antibodies for detection. Western blots were developed by chemiluminescence as described [30].

2.6. Analysis of protein by fluorescence microscopy

L540 and Cos7 cells were maintained at 37 ºC in 5% CO2 in Dulbecco´s modified Eagle´s medium containing 20% or 10% heat inactivated fetal calf serum respectively. Cells were cultured on glass cover slips for 24 h, fixed with 100% methanol and incubated with the goat anti-p53 (C-19, Santa Cruz Biotech.) polyclonal and mouse anti-Ki-1 monoclonal antibodies. Fluorescein-coupled anti-mouse (green) or rhodamine-conjugated anti-goat antibodies (red) were used as secondary antibodies. DAPI staining (blue) was used to show the position of the nuclei. Cover slips were then mounted in 80% glycerol / 10 mM Tris-HCl (pH 7.5) on cover glasses and analyzed with a fluorescence microscope (Eclipse E600, Nikon).

2.7. Yeast one-hybrid system based transcriptional activation assay

The vector p53blue (Clontech) was generated by inserting three tandem copies of the consensus p53 binding site via EcoRV and SalI sites into the vector pLacZi (Clontech). This vector was linearized with ApaI and transformed into yeast strain W303 (genotype: ade2-1/ade2-1; ura3-1/ura3-1; his3-11,15/his311,15; trp1-1/trp1-1; leu2-3,112/leu2-3,112) for integration into its genome and generation of the yeast reporter strain W303-p53blue, which was used for the yeast one-hybrid transcriptional activation assay described below. Next, W303-p53blue cells were co-transformed with the plasmid constructions indicated in Fig. 5. The degree of activation of the p53 promotor lacZ reporter construct was then assessed by an ONPG (o-nitro phenyl B-D-galactopyranoside) liquid assay (Clontech). Briefly, equal quantities of yeast cells were centrifuged and disrupted in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) by freeze-thawing. 25 mM 2-mercaptoethanol and 10 mM ONPG (each dissolved in Z-buffer) were added to each reaction. After 1 h at 30 °C the reaction was stopped by adding 0.3 volumes of 1 M Na_2CO_3. After mixing and centrifugation the OD of the supernatants was measured at 420 nm using a spectrophotometer. All experiments were done in triplicate and the average value was plotted as arbitrary units as described previously [31].

2.8. Phosphorylation of p53

For phosphorylation of p53 before In vitro binding assay, the protocols were adjusted in order to avoid the thermal denaturation of p53 during prolonged incubation at 30 ^{o}C. p53 was phosphorylated for 10 min at 30 ^{o}C with 20 mU of PKC (Promega). Only unlabelled ATP was used for this assay. The PKC reaction (20 μl) was performed in a buffer containing 20 mM Tris/HCl, pH 7.5, 5 mM MgCl2, 0.2 mM CaCl2, 0.1 mg/ml PMA, and 10 mM ATP.

3. Results and Discussion

3.1. Yeast two-hybrid screens employing the N- and C-terminal regions of Ki-1/57

To gain functional insights via the identification of interacting proteins of Ki-1/57, the yeast two-hybrid system [27-29] was employed, utilizing a human fetal brain cDNA library. We performed two screens for Ki-1/57 using the construct pBTM116-Ki-1/57(122-413) encompassing the C-terminal of Ki-1/57 and pBTM116-Ki-1/57(1-150), spanning the N-terminal region of Ki-1/57. We sequenced a total of aprox. 100 bait plasmids for Ki-1/57(122-413) and another 100 for Ki-1/57(1-150).

Interestingly, we identified a series of proteins interacting with both types of constructs that are functionally associated with p53 (Table 1). In fact all identified proteins shown in Table 1 either interact directly with p53 or with another member of the p53 protein family (e.g. p73). Further interacting proteins that interact with several of the Ki-1/57 interacting proteins include SUMO, Topoisomerase 1 and PML. All of these proteins are functionally interconnected and either directly or indirectly associated to the regulation of transcription and this emphasizes the potential functional relevance of the newly discovered interactions.

We did however not identify p53 itself in the yeast two-hybrid screen. This is most likely due to the fact that p53 only interacts with full length Ki-1/57 (see next section) and we used two fragments of Ki-1/57 in the two screens. However, we found the appearance of so many p53 interacting proteins intriguing and speculated that Ki-1/57 might also be able to interact with p53 and could be functionally associated to it. To test this hypothesis we inserted murine (72-390) p53 (mp53) and full-length human p53 (hp53) into the two-hybrid vectors pBTM116 or pGAD424 (see below) and tested the interaction of Ki-1/57 and p53 in a direct assay one on one.

Table 1: Human Ki-1/57 N- and C-terminal interacting proteins identified by two yeast two-hybrid system screens that are functionally or physically associated with members of the p53 family of proteins.

protein that interacted with Ki-1/57 (synonyms)*	bait	also interacts or is functionally associated with**	main domain composition/ function***	Reference
RACK1	Ki-1/57(122-413) (C-terminal)	P73 (p53 family)	7 WD40 repeats/ adaptor protein	9, 10
CHD-3		P73 (p53 family), SUMO	2 PHD, 2 CHROMO, 1 helicase, 1 DNA-binding / remodelling of chromatin	8, 34
Topors (p53 binding protein, LUN)		P53, SUMO, PML, Topoisomerase 1	RING/trascnritional regulation	20, 35
DAXX		P53, p73, PML	SAP/ transcriptional regulation	19, 36
PIAS-3		P53, SUMO	SAP, zf-MIZ1/SUMO ligase, (transcription ?)	37
HMG-protein 2 – like1		P53	HMG-Box/ transcription	38, 39
TIP-60		P53	Histone Acetyl Transferase	40
UBC9	Ki-1/57(1-150) (N-terminal)	P53, SUMO, PML	E2I catalytic domain/ubiquitination, E2 SUMO ligase	32
GADD34		P53	Growth arrest and DNA damage sensing	41
YB-1, Y-box binding protein (NSEP-1)		P53	C-domain, 4 basic, aromatic islands/ multifunctional, p53 activator	22
SF2p32		P53, topoisomerase 1	RRM/splicing	42

*other synonyms may be known, ** other interacting proteins may be known, *** other domains and functions may be known

3.2. Test of direct interaction between Ki-1/57 and p53 in the yeast two-hybrid system

To directly test the interaction between Ki-1/57 and mp53(72-390) a series of bait and prey construct were generated in the yeast two-hybrid system vectors pBTM116 (lexA-DNA-binding domain) and pGAD424 (Gal4-activation domain) and co-transformed as indicated in Fig.1. We found that only full length Ki-1/57 is capable to interact with mp53, whereas three different deletion constructions all failed to interact with mp53. Since two of these constructs (Ki-1/57 1-150 and Ki-1/57 122-13) were used in our library screenings, this might explain why we did not find p53 as an interacting protein. Interestingly, the interaction could be confirmed when the inserts and vectors were interchanged. Ki-1/57 in fusion to either lexA or Gal4AD interacts equally well with mp53 fused to Gal4AD or lexA, respectively (Fig.1, compare sections 3 and 4). Since the murine p53 constructs lacks the amino acids 1-72 the interaction does not seem to involve the N-terminal domain of mp53. As controls we used empty

vector (Fig.1, section 2) and a construct of large T, which interacted as expected with p53 (Fig.1, section 1).

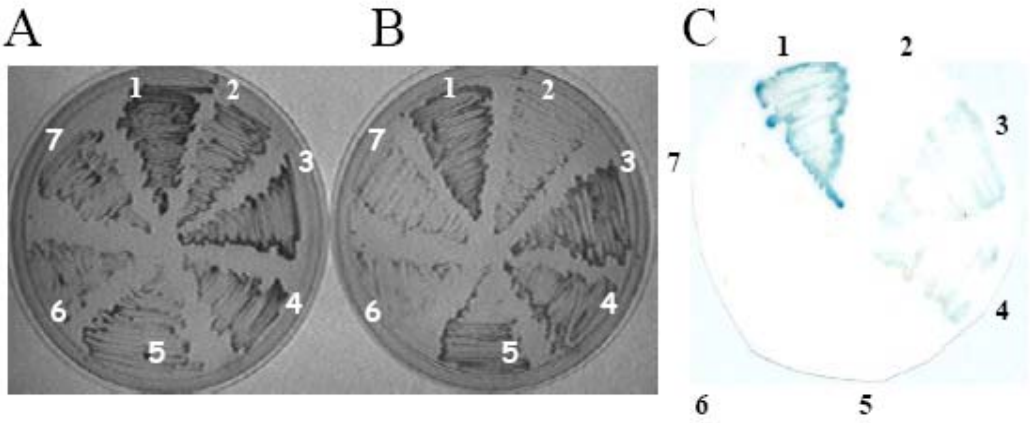
We also tested all deletions and full length Ki-1/57 in pBTM116 against full-length human p53 cloned in pGAD424 and obtained the same results as for mp53 above (data not shown). These data suggested that Ki-1/57 interacts with p53 in a specific and reproducible manner.

3.3. In vitro confirmation of the interaction between Ki-1/57 and p53 with purified recombinant proteins

In order to test the newly identified interaction between Ki-1/57 and p53 we performed a series of independent control experiments. First, we carried out a pull down assay with purified recombinant proteins that had been expressed in E. coli (GST, GST-p53, 6xHis-Ki-1/57) to confirm the interaction between Ki-1/57 and p53 in vitro. As shown in Fig. 2A GST-p53 or GST control protein bound to the glutathione beads. After wash and incubation with 6xHis-Ki-1/57 protein in the supernatant the bound protein was detected by anti-5xHis monoclonal antibody (Fig.2B).

In a parallel set of experiments we also analyzed if the in vitro phosphorylation of either 6xHis-Ki-1/57 or p53 by PKC had any influence on the interaction of the two proteins (Fig.2, GST-p53-P, 6xHis-Ki-1/57-P). We previously reported that the interaction of Ki-1/57 with the adaptor protein RACK1 is abolished in vitro and in vivo by the phosphorylation of Ki-1/57 on some C-terminal Thr and Ser residues [9]. Here we found that the phosphorylation of Ki-1/57 does not influence its capacity to interact with p53.

The in vitro phosphorylation of p53 on the other hand totally blocked the interaction between p53 and Ki-1/57 as well as Ki-1/57-P. The phosphorylation of p53 can have both an inhibitory [23] as well as a stimulatory effect [21] on the DNA binding affinity and transactivation function of p53. This depends on the kinase that acts on p53, its target Ser, Thr or Tyr residue and its localization in one of the four principal domains (trans-activation, DNA-binding, tetramerization and C-terminal negative regulatory domains) of p53. The phosphorylation may positively or negatively influence p53 interaction with the target promotor DNAs or with regulatory proteins including UBC9 [32].



D

#	Bait vector (pBTM116)	Prey vector (pGAD424)
1	lexA-mp53(72-390)	Gal4-AD-large T (positive control)
2	lexA-mp53(72-390)	Gal4-AD without insert (negative control)
3	lexA-mp53(72-390)	Gal4-AD-Ki-1/57(1-413)
4	lexA-Ki-1/57 (1-413)	Gal4-AD-mp53(72-390)
5	lexA-Ki-1/57 (151-263)	Gal4-AD-mp53(72-390)
6	lexA-Ki-1/57 (1-150)	Gal4-AD-mp53(72-390)
7	lexA-Ki-1/57 (122-413)	Gal4-AD-mp53(72-390)

Figura 1. Only full length Ki-1/57 interacts with the mp53 in the yeast two-hybrid assay.

(A)-(C) Direct test for interaction between mp53(72-390) and Ki-1/57 and mapping of the regions of Ki-1/57 that interact with p53. N- and C-terminal truncations of human Ki-1/57 were fused in frame to indicated domains in plasmids pBTM116 or pGAD424 and co-transfected into yeast L40 cells with vectors containing the fusion of mp53(72-390) in these vectors (see table D for details). Interaction was determined by the ability of the co-transformand cells to grow on MM,-W,-L,-H (B). Presence of "bait" and "prey" plasmids in the co-transformed cells was controlled by growth on MM-W,-L (A). (C) Blue-color production from the transcriptional activation of the lacZ reporter gene in case of productive interaction between fusion proteins. D: Table indicating the used vector constructions of Ki-1/57, mp53 and control fusions for the co-transfections 1-7.

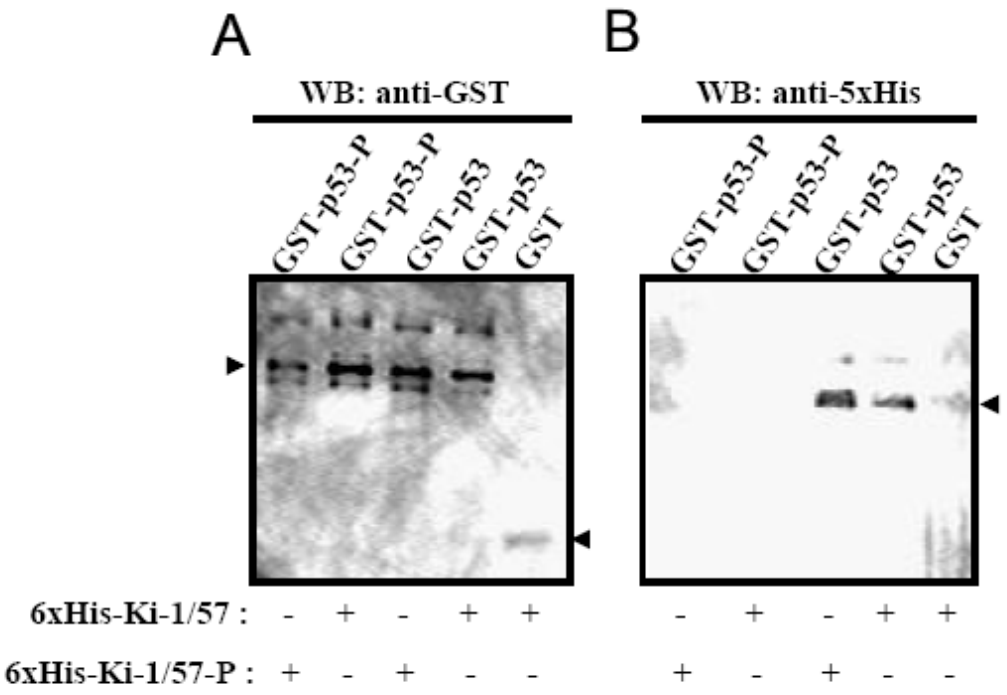


Figura 2. In vitro pull down assay with recombinant immobilized GST-p53 and 6xHis-Ki-1/57 protein, but only in its non-phosphorylated form.

Briefly, glutathione sepharose beads were coupled with GST-p53 fusion protein. Next the loaded beads were washed then incubated with the proteins: wild type 6xHis-K-1/57 or 6xHis-Ki-1/57P, which had been previously phosphorylated in vitro by PKC. After washing the bound proteins were analyzed by anti-GST (A) or anti-5xHis (B) Western blots. Detected fusion proteins (GST or 6xHis-tags) are pointed out by arrows on the left side and right side of the panels.

3.4. Co-immunoprecipitation of Ki-1/57 and p53 from human L540 cells

In order to test if Ki-1/57 and p53 can form a stable complex in human cells we performed co-immunoprecipitation experiments with anti-Ki-1/57, anti-53 and control antibodies. We used the human Hodgkin's disease analogous cell line L540 [26] for these experiments since both Ki-1/57 [9] and p53 [33] are expressed in these cells. We found that when we immuno-precipitated p53 from the L540 cells that Ki-1/57 co-precipitates (Fig. 3, lane 7). This co-precipitation is specific, since it does not occur with an anti-mouse IgG control antibody (lane 3). The Ki-1/57 protein band was identified by both analysis of the L540 lysate directly (lane 1) as well as by immunoprecipitation with the Ki-1/57 specific antibody A26 (lane 4). Bands derived from the antibodies heavy or light chains were controlled by running out antibodies loaded to protein sepharose beads prior to incubation with L540 lysate (lanes 2, 4, 6).

3.5. Sub-cellular co-localization of Ki-1/57 and p53

Next we tested if p53 and Ki-1/57 co-localize in mammalian cells. We used Cos7 cells, since they express p53 and have a similar morphology as adherent Hela cells, which do not express p53. Cos7 cells were grown on glass cover slips, fixed and stained with DAPI. Ki-1/57 was immunodetected with mouse anti-human A26 antibody and sheep anti human p53 antibody. Anti mouse FITC coupled and anti sheep rhodamine antisera were used as secondary reagents for detection. As previously described Ki-1/57 is localized both in the cytoplasm and in the nucleus [9] and p53 is predominantly localized in the nucleus (Fig. 4). The nuclear localization of both proteins is confirmed by the nuclear DAPI staining. When both proteins stainings are merged there is a clear orange colored overlap of the two labelings, especially pronounced in the region directly underneath the nuclear envelope.

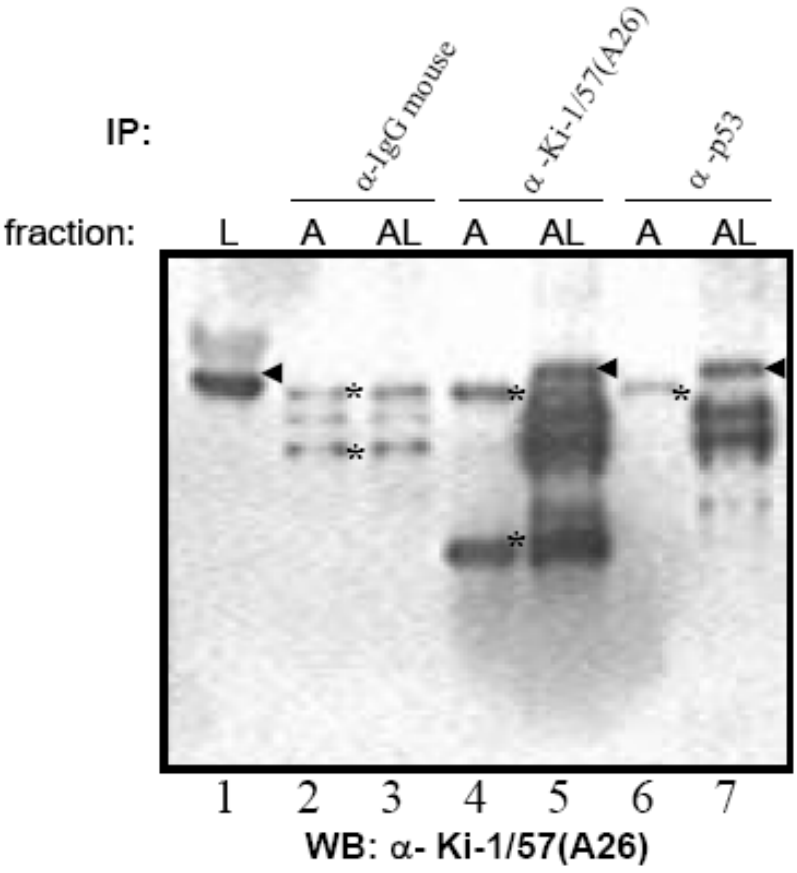


Figura 3. Co-immunopreciptiation of Ki-1/57 and p53 from the lysate of L540 cells.

10^7 L540 Hodgkin analogous cells were lysed. The lysate was (AL) immunoprecipitated (IP) with the indicated antibodies against Ki-1/57, p53 or IgG (negative control). Also run out on the gel were additional controls of the same amounts of antibodies (A) which had not been incubated with lysate as well as lysate itself (L). The Western blot (WB) was then developed by A26 antibody, which detects Ki-1/57. The arrow heads indicate the Ki-1/57 protein and the asterisk the heavy and light chains of the used antibodies.

3.6. Ki-1/57 inhibits p53 dependent transactivation of beta-galactosidase expression in the yeast

It has been previously described that p53 is a transcriptional activator of many different promoters [13, 15-17]. Several other proteins have been described that either co-activate or inhibit the transactivation function of p53 most likely by physically interacting with the p53 protein (Table 1). We wanted to test if Ki-1/57 is capable of influencing the transcription activation function of p53 on a target promotor. Therefore, we established a yeast one-hybrid system based transcription activation system. The yeast strain W303 was transfected with a plasmid containing the LacZ gene (encoding beta-galactosidase) under control of a p53 promotor region. Next, additional co-transfections of the yeast expression plasmids (pGAD424) encoding human p53 in fusion with the Gal4 activation domain or empty vector on the one hand and of a second yeast expression plasmid (pBTM116) encoding full length Ki-1/57 protein fused to the lexA DNA binding domain or empty plasmid were performed (Fig. 5). The co-transfected yeast clones were than tested for the activation of the p53 reporter gene encoding beta galactosidase (ONPG enzymatic assay of the beta-galactosidase activity).

As expected, the presence of only the p53 encoding plasmid caused a relatively strong activation of the reporter gene (Fig 5., 2 column) but remained on approximately the same level when an empty pBTM116 had been co-transfected into the cells (column 3). The co-transfection of a pBTM116 plamid containing the cDNA coding for full length Ki-1/57 caused a decrease in the level of beta-galactosidase expression by 56% in these cells (column 4). Expression of Ki-1/57 alone on the other hand had no significant capacity to increase the p53 promotor activity (column 5) leaving the beta-galactosidase activity on the background level observed for cells transfected with empty pGAD424 vector (Gal4-AD, column 1) or with empty pBTM116 vector (lexA-BD, data not shown).

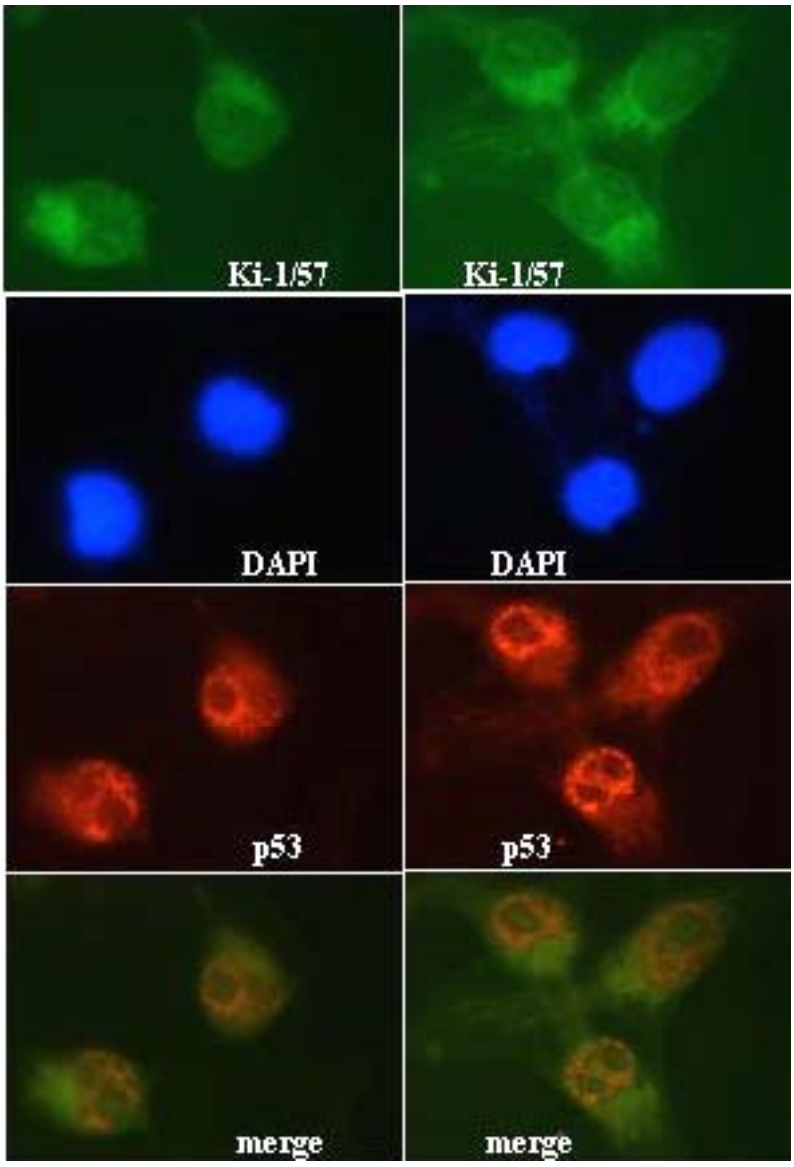


Figura 4. Sub-cellular localization of endogenous Ki-1/57 and p53 in Cos7 cells. Cos7 cells were grown on glass cover slips and after 24 h were fixed with 100% methanol and proteins were detected and analyzed on a Nikon fluorescence microscope using the following primary antibodies: Ki-1 monoclonal mouse antibody and goat anti-p53 monoclonal antibody. Fluorescein-coupled anti-mouse (green) or rhodamine-conjugated anti-goat antibodies (red) were used as secondary antibodies. DAPI staining (blue) was used to show the position of the nuclei. Superimposing the two colors (merge) results in a yellow/orange color.

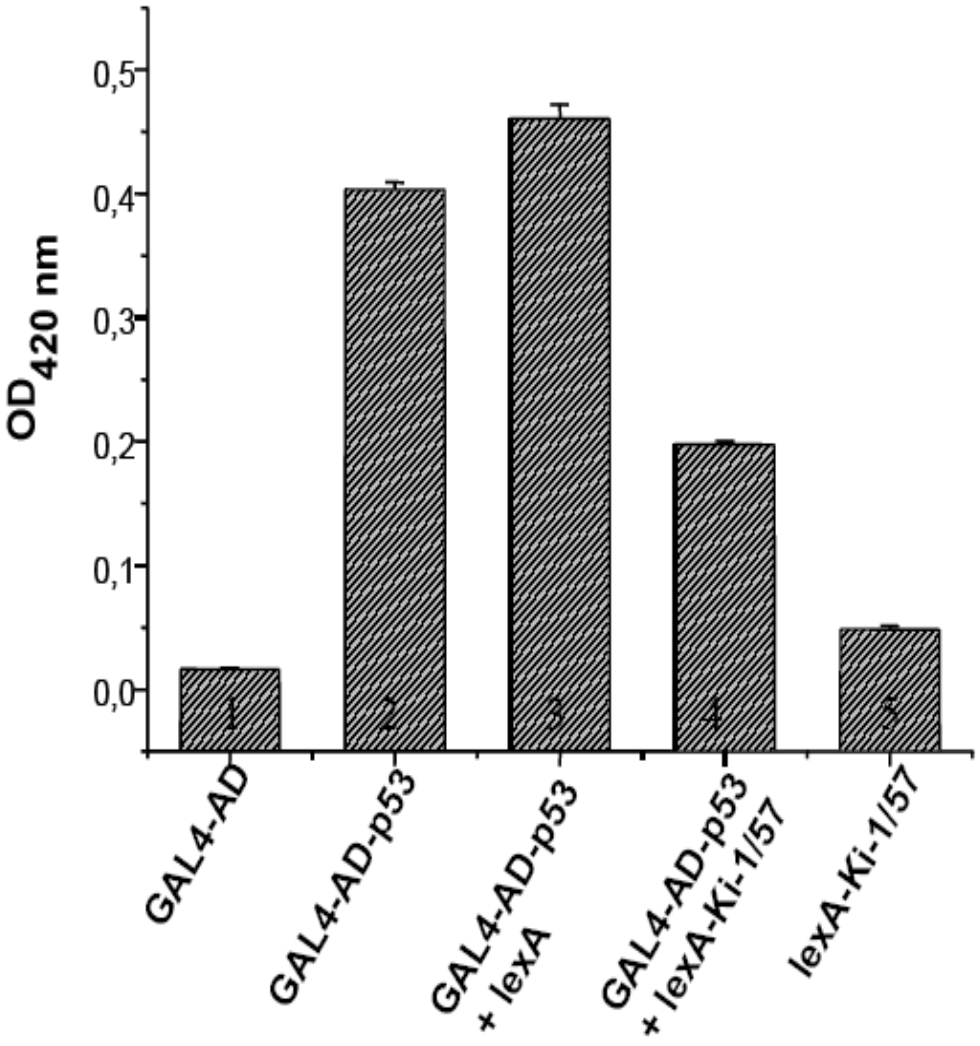


Figura 5. Ki-1/57 inhibits p53 dependent transactivation of beta-galactosidase expression in the yeast

W303-p53blue yeast cells were transfected or co-transfected with the indicated plasmid constructs: Gal4 (empty pGAD424 vector), LexA (empty vector pBTM116), Gal4-p53 (pGAD424 vector containing full length human p53 cDNA), LexA-Ki-1/57 (pBTM116 vector containing full length human Ki-1/57 cDNA). Freshly grown transfected colonies were then grown in liquid culture. After adjusting equal cell numbers the cells were pelleted, lysed and their cell lysates were submitted to a quantitative beta-galactosidase assay based on the OPNG reaction (see Mat. and Meth. section for details). Each point represents the average value of three independent experiments in arbitrary units expressing the amount of the generated yellow reaction product. The error bar at the top of each column indicates the standard deviation.

In conclusion, the newly identified p53 interaction partner Ki-1/57 could have a negative regulatory role for p53. It is particularly interesting that the interaction between Ki-1/57 and p53 is interrupted by the in vitro phosphorylation of p53 by PKC. Previous studies had identified Ki-1/57 as protein, which interacts with RACK1 and is a target for PMA activated PKC in vivo [9]. Since RACK1 is also functionally associated to the p53 protein family as a negative regulator of p73 dependent transcription [10] it is tempting to speculate that a regulatory cytoplasmic pathway involving the proteins PKC, RACK1 and Ki-1/57 could be important for the fine regulation of p53 function in dependence of the cells activation status. Future investigations are required to determine Ki-1/57 exact role in relation to p53 and whether the in vivo phosphorylation of p53 by different protein kinases has an influence on its activity and if its functional inactivation or inhibition upon mitogenic activation (PMA, PHA) in leukocytes might be an important step to prevent p53 dependent blockage of the cell in the interphase and allow cells to enter in mitosis.

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3.4 Artigo IV:

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

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

(Artigo submetido)

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

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Abstract:
Ki-1/57, the 57-kDa cytoplasmic and nuclear antigen recognized by the CD30 antibody Ki-1, is associated with protein kinase activity and regulated by activation dependent phosphorylation. In recent studies we employed the yeast two-hybrid system in order to identify proteins interacting with Ki-1/57 and thereby get clues on its cellular functional context. Ki-1/57 interacts with the Receptor of Activated Kinase-1 (RACK1), a signalizing adaptor protein that binds activated PKC. Furthermore, Ki-1/57 is a substrate for PKC phosphorylation *in vitro* and *in vivo* and co-precipitates with PKC when isolated from PMA L540 cells. In this work, we analyze the biophysical characteristics of the individual proteins and of the interaction between RACK-1 and two protein fragments of Ki-1/57 (122-413, 264-413). The far-UV circular dichroism spectrum of RACK1, a WD repeat protein, showed an unusual positive ellipticity at 229 nm. In other proteins of the WD family, an unusual positive ellipticity at 229 nm has been attributed to surface tryptophans since the maximum could be abolished by modification of the tryptophans with N-bromosuccinimide (NBS). Our results showed that NBS, Ki-1/57(122-413) and Ki-1/57(264-413) can abolish this unusual positive ellipticity of RACK1. The additive far UV-CD spectrum profiles of the Ki-1/57(122-413) or (264-413) with RACK1 confirm that the proteins interact. In summary, the circular dichroism and fluorescence studies implicate that the tryptophans residues of RACK1 are involved in the interaction with Ki-1/57. Furthermore, we observed that the phosphorylation of Ki-1/57 changes its secondary structure, which might contribute to the complete dissociation of RACK1 and Ki-1/57 upon Ki-1/57 phosphorylation by PKC.

Keywords: RACK1, WD family, Ki-1/57, Circular Dichroism, Fluorescence, protein phosphorylation.

Abbreviations: Luria-Bertani medium (LB), Isopropyl-1-thio-ß-D-galactopyranoside (IPTG), *N*-bromosuccinimide (NBS), 2-mercaptoethanol, dithiothreitol (DTT), sodium dodecyl sulfate (SDS), Protein Kinase C (PKC).

1. Introduction

Ki-1/57, the 57-kDa human protein antigen recognized by the CD30 antibody Ki-1 in Hodgkin and Sternberg-Reed cells in Hodgkin lymphoma [1], is a cytoplasmic and nuclear protein, which is phosphorylated on serine and threonine residues [2, 3]. When isolated from the Hodgkin's lymphoma analogous cell line L540, Ki-1/57 co-immunoprecipitates with a Thr/Ser protein kinase activity [4]. 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 [5]. In recent studies, where the yeast two-hybrid system was used to identify proteins interacting with Ki-1/57, we found that Ki-1/57 engages in specific interactions with the Chromatin-Helicase-DNA-binding domain protein 3 (CHD3) [6], a nuclear protein involved in chromatin remodeling and transcription regulation, and with the Receptor of Activated Kinase C-1 (RACK1) [7], an adaptor protein that interacts itself with activated Protein Kinase C (PKC) [8].

Ki-1/57 has also been termed IHABP4 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* [9]. It has not been shown yet whether such binding actually occurs in vivo. PAI-1 mRNA-binding protein is an alternative splice variant of CGI-55, the putative paralogue of Ki-1/57, and has been shown to specifically bind to the mRNA of type-1 plasminogen activator inhibitor (PAI-1) [10]. This protein called PAI-RBP1 is thought to be involved in regulation of mRNA stability.

RACK1, that interacts with Ki-1/57 but not with CGI-55, is an adaptor protein from the WD repeat proteins family. RACK1 is involved with very diverse cellular functions including regulation of signal transduction [11-12], pre-mRNA processing [13], gene transcription [14], cell cycle progression [15], translation [16] and actin cytoskeleton rearrangement [17]. RACK1 is a member of WD repeat family of proteins that are made up od highly conserved repeating units called WD repeats [18]. Each repeating unit consists of a core region of approximately 40 amino acids typically bracketed by GH (glycine-histidine) and WD (tryptophan-aspartate), and a variable region of 6-94 amino acids [18-19]. This repeating unit was first recognized in the G

protein β subunit that transduces signals across the plasma membrane [20]. Like Gβ subunit the amino acid sequence deduced from the cDNA shows that RACK1 is made up of seven WD repeats, but with minimal N- and C-terminal extensions, that have been predicted to all have a similar architecture [19, 20-22]. Different from other researchers [18], we have expressed RACK1 in *Escherichia coli* and were able to purify it in large quantities in soluble form, making it suitable for structural studies and the assessment of its biophysical properties through spectroscopic analyses.

Here we investigated the abrogation of the interaction between RACK1 e Ki-1/57, after the phosphorylation of Ki-1/57 by PKC *in vitro*, using circular dichroism (CD) and emission fluorescence spectroscopy. We observed that the RACK1 CD spectrum shows an unusual positive ellipticity at 229 nm that it is attributed to tryptophans, since the 229 nm maximum could be abolished by modification of these tryptophans by N-bromosuccinimide (NBS) [23]. Other important observation is the decrease of fluorescence emission spectrum by NBS modification [24-26]. It demonstrates that the tryptophans are exposed in the molecule.

Furthermore, our results showed that the C- terminal amino acids 264-413 of Ki-1/57 can abolish RACK1 mentioned unusual positive ellipticity at 229 nm, and thereby confirming this interaction. Besides, the emission fluorescence intensity of RACK1 decreased with NBS. We observed that 0,4 mM NBS abolished the tryptophan emission fluorescence. The same two fragments of Ki-1/57 that interacted with RACK1 also decreased the RACK1 emission fluorescence intensity. Our result seems to indicate that Ki-1/57 could interact with surface tryptophans of RACK1.

2. Materials and Methods

2.1 Plasmid construction

Several sets of oligonucleotides were designed to allow sub-cloning of the full cDNA and the constructs of Ki-1/57 coding the indicated regions in bacterial expression vectors. We inserted the full cDNA of Ki-1/57 (1-413) and the constructs: the N-Terminal Ki-1/57(1-150), C-terminal Ki-1/57(122-413), C-terminal Ki-1/57(151-263), C-terminal Ki-1/57(264-413), into the *EcoR*I and *BamH*I sites of vector pPROEX (Invitrogen) to obtain 6xHis-tagged proteins. The full cDNA encoding the protein RACK1 was amplified and inserted into the *Nde*I and *Hind*III of vector pET-28a (Novagen) to obtain the 6xHis-tagged protein.

2.2 Bacterial expression and protein purification

Ki-1/57 constructs were expressed in *E. coli* BL21-CodonPlus-RIL (Stratagene) and 6xHis-RACK-1 protein was expressed in *E. coli* BL21 (DE3) (Stratagene). Overnight cultures of *E. coli* transformed with 6xHis-Ki-1/57 recombinant constructs in pPROEX vector were diluted 1:50 in 1000 mL of fresh LB media (100 μg/mL ampicillin, 34 μg/mL cloranfenicol) and grown at 37 ^{o}C until the OD_{600} reached 0.7-0.8. Then we added isoprenyl β-D-thiogalactopyranoside (IPTG) to 1.8 mM. After a further 4 h of growth, cells were pelleted and resuspended in 10% of the culture volume of buffer A (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol, 1mM PMSF). Cells were lysed by sonication on ice. The lysis supernatant was cleared by centrifugation and then submitted to purification on a HiTrap™ Chelating HP Column (Amersham Biosciences). Overnight cultures of *E. coli* transformed with 6xHis-RACK1 recombinant pET28a vector were diluted 1:50 in 1000 mL of fresh LB media (50 μg/mL kanamicin) and grown at 37 °C until the OD_{600} reached 0.7-0.8 and before adding IPTG to 1.8 mM, while the temperature was lowered to 30 ^{o}C. After a further 4 h of growth, cells were pelleted and resuspended in 10% of the culture volume of buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, 0.5 mM DTT and 0.5 mM EDTA). Cells were lysed by sonication on ice, followed by centrifugation at 6000xg for 30 min. The supernatant was purified with 2 ml of Ni-NTA (nitrilotriacetic acid) resin (QIAGEN) preequilibrated

with buffer A at about 10 mL/h. The column was washed with 50 ml of buffer A or until the OD_{280} of the flow-through was less than 0.01. The recombinant proteins were eluted with a 20-mL linear gradient of 0-0.5 M imidazole in buffer B (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 % Glycerol, 0.5 mm EDTA, 0.5 mm DTT e 0.5 M Imidazole). Fractions of 1 mL each were collected, and aliquots were analyzed by SDS-PAGE on 10%. The peak fractions containing of 6xHis-Ki-1/57 constructs and 6xHis-RACK1 recombinant proteins were dialyzed against 20 mM Tris–HCl pH 7.5, 5 % Glycerol, 1 mm DTT, 1 mM EDTA e 1 mM phenylmethylsulfonyl fluoride (PMSF) and concentrated using UltraFree4 (Millipore) by centrifugation of 3000 g and 4 ^{o}C. After concentration these protein were dialyzed against 20 mM Tris–HCl pH 7.5, 20 % Glycerol, 1 mM DTT, 1 mM EDTA and 1 mM PMSF and analyzed by SDS–PAGE.

2.3 Far-UV circular dichroism (CD) spectroscopy

CD spectra of RACK1 or Ki-1/57 and constructs were recorded on a Jasco J-810 spectropolarimeter with temperature controlled by a Peltier Type Control System PFD 425S. Spectra were measured at a temperature of 25 ^{o}C. Samples were examined in 0.1 cm cubettes at a concentration of protein, 10 μM, chosen to maintain the dynode voltage below 650 V in the wavelength region 260-200 nm or 260-205nm. Protein concentration was determined using absorption at 280 nm and an extinction coefficient [27], which was calculated from the composition of the protein using '*Protparam*'. The spectrum reported for each sample represents the average of 20 individual spectra for each preparation and has been corrected for baseline contribution due to buffer (0.1 M Phosphate buffer, pH 7.5). Scanning rate was 50 nm/min with spectral band width of 1 nm and response time was two seconds. CDNN Deconvolution software (Version 2- http://bioinformatik.biochemtech. uni.halle.dee/cdnn) was employed for secondary structure prediction. For thermal denaturation studies, measurements were made at fixed wavelength (229 nm), the scan rate was varied from 1 ^{o}C / min and samples were heated from 5 to 90 ^{o}C (by using scan rates of 50 ^{o}C/h). The reversibility of unfolding was tested by performing several consecutive up and down scans and by scan rate variation. Molar ellipticity values were determined as follows: $[\theta](deg.cm^2/dmol)=[\theta\text{-}(MRW)]/[(10)(lc)]$, where θ represents the displacement from the baseline value X full range in degrees, MRW

equals the mean residue weight of an amino acid, l is the path length of the cell in cm, and c equals the concentration of protein in g/mL. All reported spectra were normalized to molar ellipticity values in deg.cm^2/dmol. For the analyses of tryptophan modification of 6xHis-RACK1 by N-bromosuccinimide (NBS), samples were treated with 0.5 mM NBS in buffer 0.1 M sodium phosphate buffer, pH 7.5 for 5 minutes and CD spectra were recorded immediately afterwards [19]. Base lines were run tree times in all the conditions mentioned above.

2.4. Fluorescence spectroscopic studies

The experiments were performed with an Aminco Bowman® Series 2 (SLM-AMINCO) spectrofluorimeter equipped with a 450 W lamp. Experiments using the fluorescence of RACK1 or Ki-1/57 constructs were carried out at 25 °C in a buffer containing 0.1 M Phosphate (pH 7.5). The recombinant proteins were analyzed at concentrations of 1.0 μM. The tryptophan intrinsic fluorescence was investigated with the excitation at 295 nm and the emission was measured from 300 nm to 430 nm with a spectral bandpass of 4 nm for excitation and 4 nm for emission. For the analyses of tryptophan modification of 6xHis-RACK1 by N-bromosuccinimide (NBS), samples were treated with 0.5 mM NBS in buffer 0.1 M sodium phosphate buffer, pH 7.5 for 5 minutes and fluorescence spectra were recorded immediately afterwards.

3. Results

3.1-Expression and Purification of RACK1 and the Ki-1/57 constructs

To facilitate the purification of RACK1 and Ki-1/57 constructs from *E. coli*, we expressed them as fusion proteins containing an N-terminal 6xHis tag. The expression of RACK1 was induced by 1.8 mM IPTG at 30^{o} by four hours. Purification of 6xHis-RACK1 from the soluble fraction on Ni-NTA resin resulted in large protein quantities, which were more than 95% pure as confirmed by SDS-PAGE (Figure 1A). This protein was however unstable at low ionic strength or in the absence of glycerol. It remained however soluble in the presence of 150 mM NaCl and 20% glycerol. The presence of 1 mM EDTA and 1 mM DTT further helped to prevent the formation of aggregates of this protein. RACK1 is a monomer in solution as confirmed by gel filtration chromatography and mass spectroscopy analyses (data not shown). The purification of 6xHis-RACK1 from 1 L of culture yielded about 3 mg of protein.

The cDNA regions encoding for four Ki-1/57 constructions: one encoding amino acids 122-413, 264-413, 151-263 or 1-150 of Ki-1/57 were amplified by PCR and subcloned into the pProex expression vector, downstream from the sequence coding for a histidine tag. Recombinant vectors were than transformed into E. coli BL21 (DE3) codonplus RIL, because some regions of Ki-1/57 sequence contain codons that are rare in bacteria and could cause premature termination of translation or low expression rates. Western blot analysis with an anti-4xHis antibody confirmed that all Ki-1/57 protein constructs contain the N-terminal 6xHis-tag (data not shown). The expression of the Ki-1/57 constructs was induced by addition of 1.8 mM IPTG at 37 ^{o}C by four h. Proteins in the column eluate were separated by SDS-PAGE (Fig. 1B). The purification from 1 L of culture yielded approximately 0.5 mg of protein of constructs 6xHis-Ki-1/57(1-150), 3 mg of protein for 6xHis-Ki-1/57(151-263), 1 mg of protein for 6xHis-Ki-1/57(254-413) and 2 mg of protein construct 6xHis-Ki-1/57(122-413).

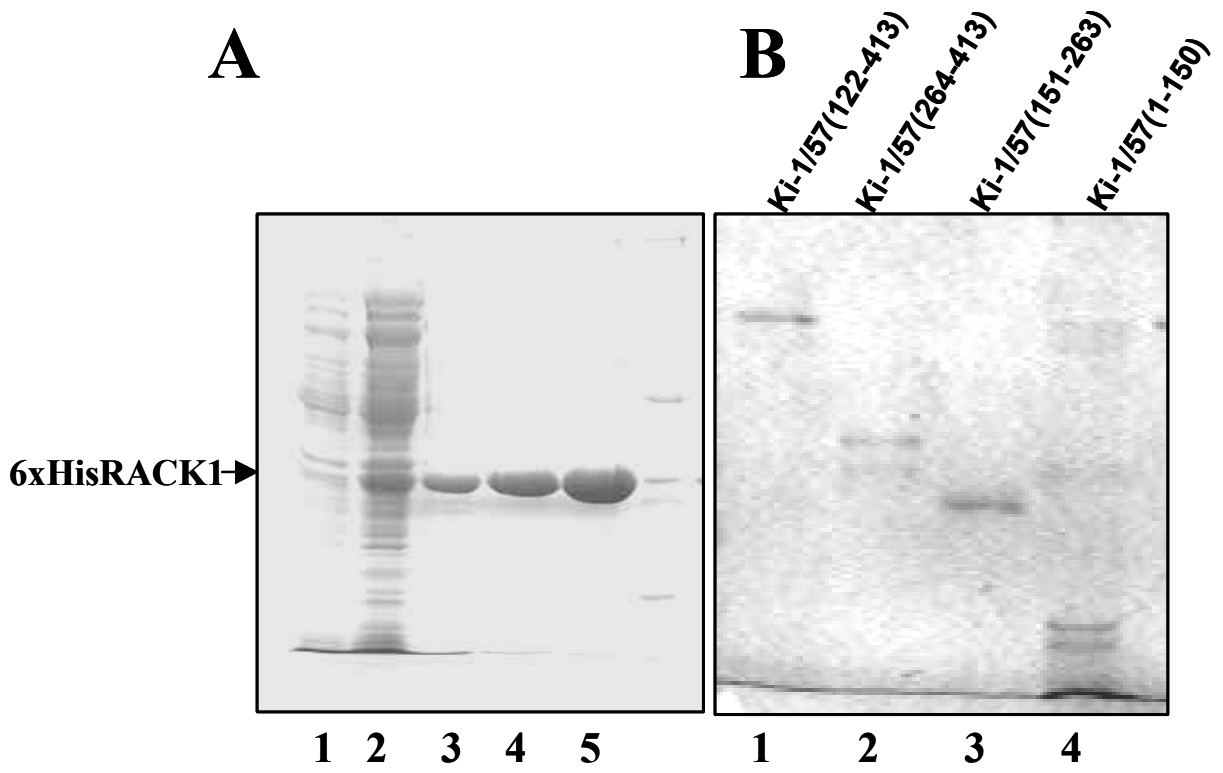

Figura 1. SDS-polyacrylamide gel electrophoresis analysis of purified 6xHis-RACK1 protein and of Ki-1/57 protein fragments. Expression and purification of 6xHis-RACK1 from BL21 (DE3) cells was shown in A: the fraction not induced with IPTG (lane 1), the fraction induced (lane 2) and the fractions after purification (lanes 3-5), lane 6 marker proteins. B. Expression of Ki-1/57 constructs from BL21 (DE3) codon plus RIL cells: lane 1, C-terminal Ki-1/57 (122-413); lane 2, C-terminal Ki-1/57 (264-413); lane 3, C-terminal Ki-1/57 (151-263) and lane 4, N-Terminal Ki-57 (1-150).

3.2- Circular Dichroism

The overall percentage of α-helices calculated from the RACK1 CD spectrum was estimated to be less than 5% calculated from the molar ellipticity at 200-260 nm (CDNN program). The far-UV-CD spectrum of 6xHis-RACK1 at room temperature shows a pronounced maximum at 229 nm (Fig. 2A). In other proteins, the positive molar ellipticity at around 230 nm has been ascribed to the presence of disulfide bonds or interactions between aromatic residues, such as tryptophans, phenylalanines, and tyrosines [28,29]. RACK1 has 13 tryptophans, 8 phenylalanines and 6 tyrosines. In order to determine the source of the positive ellipticity of 6xHis-RACK1 at 229 nm, the tryptophan residues were modified with 0.5 mM of NBS. N-Bromosuccinimide (NBS) is a known protein reagent able to modify proteins through the oxidation of mainly tryptophan residues. These properties make NBS a suitable reagent to selectively block tryptophan residues. Fig. 2A shows the CD spectra of 6xHis-RACK1 before and after treatment with NBS. Treatment with NBS completely abolishes the maximum at 229 nm, indicating that interactions between tryptophan residues may be responsible for the maximum at 229 nm.

We observed by experiments of thermal stability of RACK1 that it was unstable at temperatures above 45 ^{o}C because the maximum at 229 nm disappears at this temperature (data not shown). This supposed protein denaturation was irreversible.

The experiments of circular dichroism of pre-incubated RACK1 and Ki-1/57 constructs (Fig 2B) showed that the positive ellipticity at 229 nm of RACK1 disappears in the presence of the Ki-1/57 fragments 122-413 or 264-413 (Fig. 2B and data not shown). The experimental additive spectrum profiles of either Ki-1/57(122-413) or Ki-1/57(264-413) together with RACK1 clearly suggest that both proteins interact. This confirms the previously obtained interaction data from independent two-hybrid and pull-down experiments [7], and further suggest that Ki-1/57 interacts with the surface tryptophans of RACK1.

We observed previously that the phosphorylation of Ki-1/57 on its extreme C-terminal, abolished the interaction with RACK1. Here we found that the phosphorylation of Ki-1/57 does not abolish RACK1 unusual positive ellipticity at 229 nm (Fig. 2B). This shows that the phosphorylation of Ki-1/57 really prevents any interaction with RACK1.

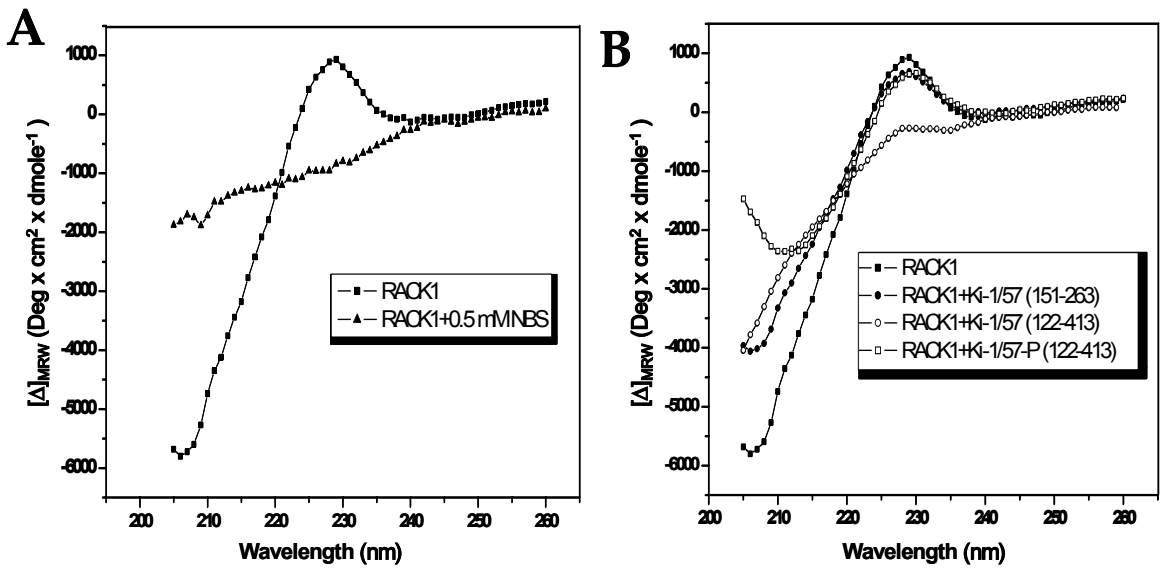

Figura 2. CD spectra from 200 to 260 nm of purified 6xHis-RACK1 and Ki-1/57 constructs.

(A) 6xHis-RACK1 in 0.1 M sodium phosphate buffer, pH 7.0 at 25 ºC, in the absence and presence of 0.5 mM of NBS. (B) Ki-1/57 interference in the positive ellipticity at 229 nm of RACK1 (see text for detailed explanation).

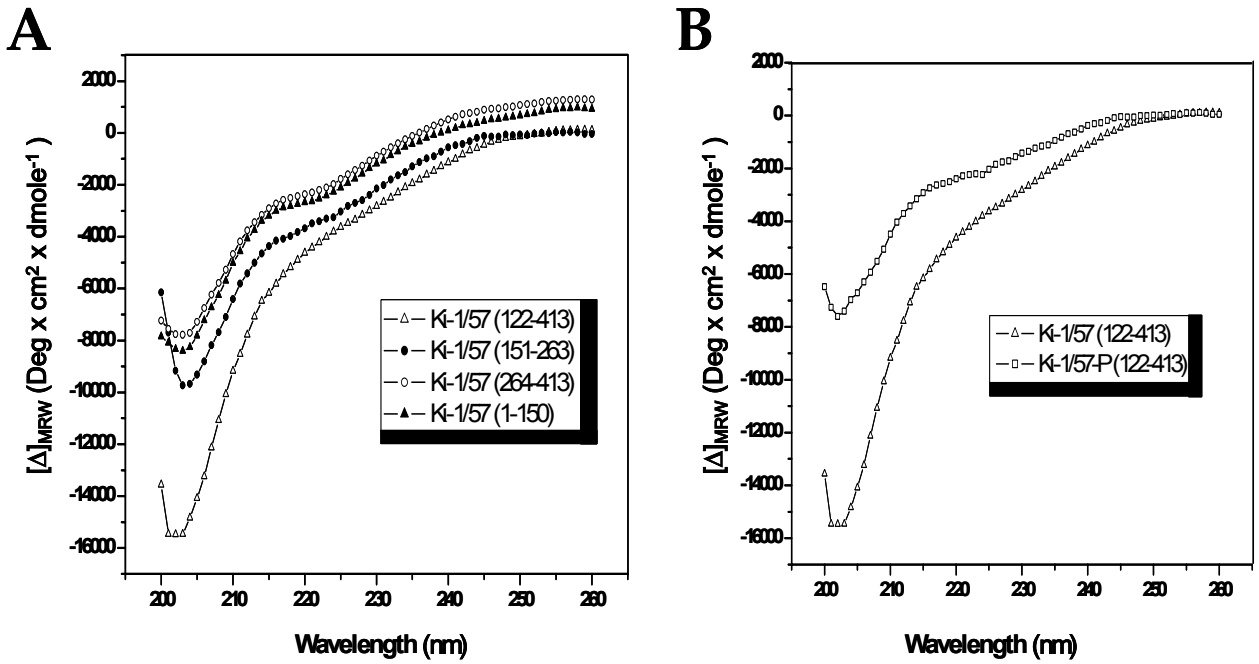

Figura 3. CD spectra from 200 to 260 nm of purified Ki-1/57 fragments at room temperature. (A); (B): Difference between the phosphorylated C-terminal Ki-1/57 fragment (122-413) (-P) and its unphosphorylated form.

Furthermore, the addition of Ki-1/57(151-263), a fragment of the protein that does not interact with RACK1 [7], did not diminish the positive molar ellipticity of RACK1 (data not shown). This shows that only the fragment Ki-1/57(122-413) can interact with the surface tryptophans of RACK1. An argument might be made that the application of NBS could affect the general folding of RACK1. However, the selective modification of RACK1 tryptophans was confirmed by other spectroscopic method. Furthermore, the interference of both C-terminal Ki-1/57 constructs with RACK1 tryptophans was also confirmed by fluorescence studies (see below).

The overall percentage of α-helices calculated from the CD spectrum of Ki-1/57 fragments was estimated to be less than 8% calculated from the molar ellipticity at 200-260 nm (CDNN program) (Fig. 3A). In order to determine if the phosphorylation interferes with the Ki-1/57 secondary structure we phosphorylated Ki-1/57(122-413) in vitro with PKC as described previously [7], and used it in circular dichroism spectroscopy experiments. We observed that phosphorylated Ki-1/57(122-413), has about 2.3% less alpha-helical content (decreased from 9% to 6.7%) and that the percentage of anti-parallel beta-sheet increased about 5% (increase from 31.6% to 36.5%). The minimum at 208 nm of un-phosphorylated Ki-1/57 in relation to phosphorylated Ki-1/57 increases from -7500 to –14000 molar ellipticity (Fig. 3B). This corroborates our hypothesis that phosphorylation may modify Ki-1/57 secondary structure and such a change in secondary may at least contribute to the complete abrogation of the interaction with RACK1 upon phosphorylation of Ki-1/57.

3.3- Intrinsic Fluorescence Studies

The fluorescence emission spectrum of RACK1 is typical of a tryptophan-rich protein. When excited at 295 nm, there is a strong emission maximum at 330 nm (Fig. 4A). The effect of NBS modification on the fluorescence emission spectrum of the RACK1 is shown in Fig. 4A. There is an increasing loss of fluorescence at gradually increasing concentrations of NBS that corresponds to the progressive oxidation of the tryptophans. At 0.4 mM NBS completely abolishes the tryptophan fluorescence.

Another way to characterize the influence of Ki-1/57 binding to RACK1 is to measure the fluorescence emission upon titration of RACK1 with Ki-1/57(151-413) or (122-413). In both cases, the binding of the Ki-1/57 constructs to RACK1 significantly

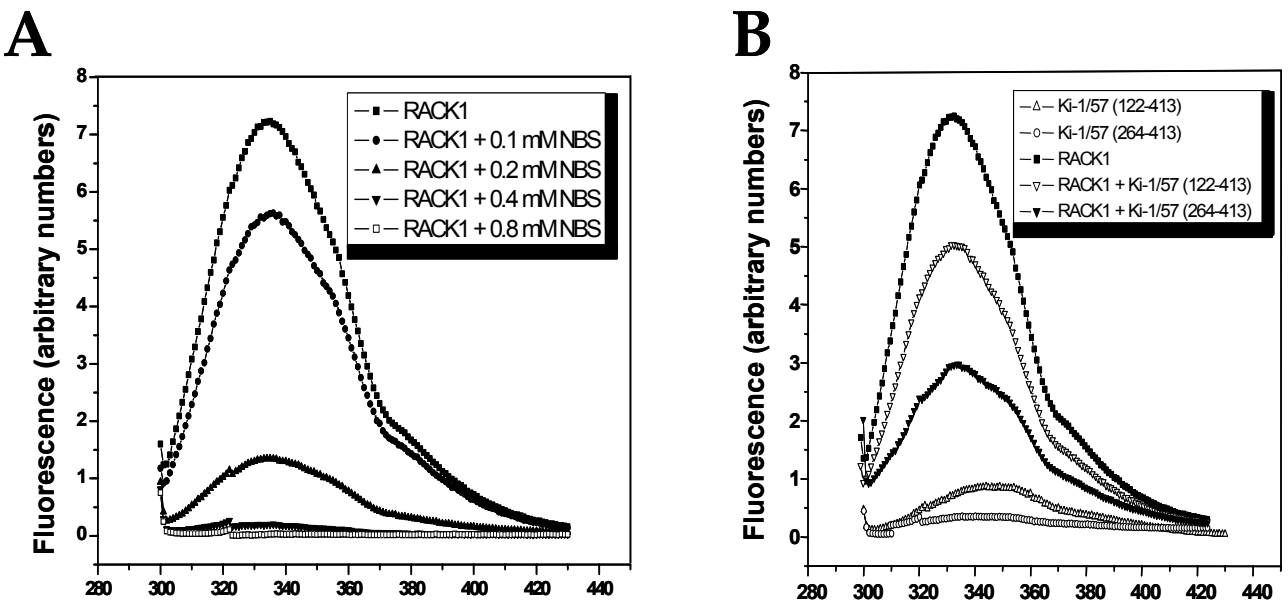

Figura 4. Fluorescence emission spectra of RACK1 and Ki-1/57.

(A) 6xHis-RACK1 was treated with different concentrations of NBS. (B) 6xHis-RACK1 was incubated with Ki-1/57(122-413) and Ki-1/57(264-413). 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.

decrease the relative fluorescence emission of RACK1. Depending on the Ki-1/57 fragment used the decrease in fluorescence varied from 30 to 60%. Ki-1/57(264-413) was more efficient in decreasing the RACK1 fluorescence than the larger fragment Ki-1/57(122-413). This indicates again that both Ki-1/57 constructs interact with the surface tryptophans of RACK1.

4. Discussion

In order to address the mechanism by which phosphorylation of Ki-1/57 abolishes the interaction with RACK1 and to test if the positive ellipticity at 229 nm of RACK1 can be attributed to surface tryptophans we performed a spectroscopic studies of the interaction between RACK-1 and two C-terminal Ki-1/57 constructions (264-413 and 122-413).

First, we wanted to address if phosphorylation changes the secondary structure of Ki-1/57. We found significant and reproducible changes in the secondary structure of Ki-1/57. The α-helices content decreases from 9% to 6.7% and the percentage of anti-parallel beta-sheet increase 31.6% to 36.5% upon phosphorylation. Besides, the minimum at 208 nm of un-phosphorylated Ki-1/57 in relation to phosphorylated Ki-1/57 increases from -7500 to –14000 molar ellipticity.

In a recent study, Bjorndal and collaborators showed that CD spectra of RACK1 did not show the positive ellipticity at 229 nm [30]. We believe that their protein may not have been completely folded because different of them our CD data from RACK1 correspond well with that of spectra of other member of the WD protein family [23]. The positive peak around 225 to 300 nm in RACK1 reflects contributions made by tryptophan side chains located in β–strands [31-35] and it is observed in members of the WD domain [23] WW domain families [36-38] and in proteins that do not belong these families but show a β-propeller structure [39, 40]. Members of the the WW domain family are small proteins with three-stranded antiparallel β-sheet topology present a positive peak the 230 nm which disappears at 90 °C [36]. This indicates a disruption of the tertiary structure as observed in the WD domain protein family, too. The parameters for thermal denaturation of the WW and WD domain families are similar. In both families the proteins start to unfold around 45 °C, probably due to

increased side chain motion of the surface set of tryptophans [23]. It also was observed that other proteins predicted to form a beta-propeller structure, as hemopexin, also exhibit this same positive ellipticity around the 230 nm region [38]. Since positive ellipticity signals have been noted for proteins with predominantly β-sheet and β-turn secondary structures that contain aromatic amino acids, our data are compatible with the presence of these elements in the RACK1 protein.

Tryptophan is an amino acid that generally exists in the hydrophobic core region of protein. The nitrogen atom on the indole group usually forms a hydrogen bond with other groups on the main or side chains [41]. The chemical modification of tryptophan might affect the nearby hydrogen bond and hydrophobic microenvironment. Chemical modification of RACK1 by tryptophan-specific reagents, such as NBS, corroborated with our earlier results that tryptophans participate of in the binding between RACK-1 and Ki-1/57 proteins.

Another observation is that different of other members of WD domain family RACK1 and SEC13 contain only WD-repeats with little or no amino or carboxyl terminal extensions. This indicates that the secondary structure of these proteins can be similar. Our date showed very similar CD spectra for RACK1 and SEC13.

We obtained the 6xHis-RACK1 in soluble form expressed in *E. coli*. Other researchers have described RACK1 as insoluble protein [17] and explained their results by a possible association of RACK1 with the membrane or insoluble fraction of the mammalian cell. We speculate that this in vivo association with the membrane and insoluble fraction is caused by interactions with other proteins [23].

In summary, our data demonstrate that Ki-1/57, which we previously demonstrated to interact with RACK1, can interfere in the signals of CD and fluorescence of its interacting protein 6xHis-RACK1. Besides, the changes in the secondary structure of Ki-1/57 upon phosphorylation may contribute to the observed interruption of the interaction between these proteins.

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4 RESULTADOS COMPLEMENTARES

4.1 Caracterização da proteína Ki-1/57

A proteína Ki-1/57 não possui domínios característicos descritos na literatura, no entanto, esta proteína contém regiões ricas em arginina (aa 43-218), em ácido glutâmico (aa 257-295) e rica em glicina (aa 184-244), segundo a análise com programa *Motif Scan* (http://myhits.isb-sib.ch/cgi-bin/motif_scan). Além disso, apresenta um motivo denominado HABP4_PAI-RBP1 (Huang et al., 2000, Heaton et al., 2001), descrito em banco de dados, que no caso de Ki-1/57 se encontra entre os aminoácidos 183-360, sendo indicado por ligar a hialuronato ou RNA. Apresenta também um motivo de importação nuclear (RKRR- aa 55-58) e um potencial motivo de ligação a ATP (G-X-G-XX-G-X(X)$_{13-22}$–K) entre os aminoácidos 194-230 (Lemos et al., 2003).

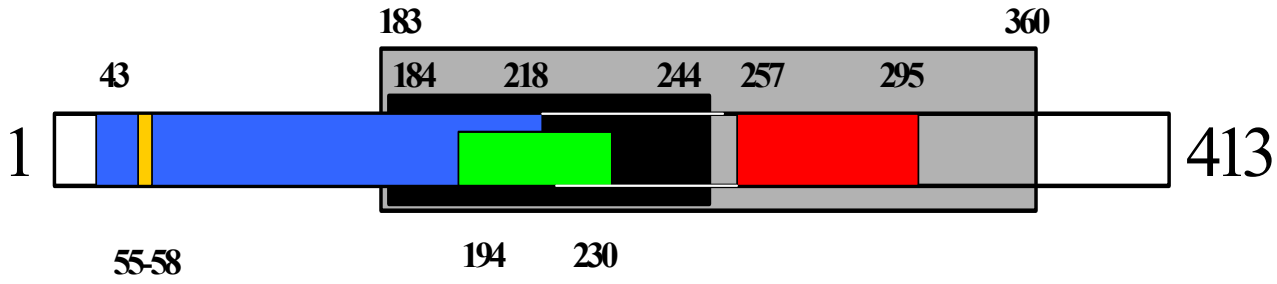

Figura 6: Caracterização da proteína Ki-1/57 em motivos protéicos.
Legenda:

- Motivo rico em arginina
- Motivo de exportação nuclear
- Motivo HABP4_PAI_RBP1
- Motivo rico em glicina
- Motivo de ligação a ATP
- Motivo rico em ácido glutâmico

4.2 O sistema de duplo híbrido em levedura

O sistema de duplo híbrido em levedura é uma poderosa metodologia que foi desenvolvida para identificar genes que codificam proteínas (presas) que interagem com uma dada proteína alvo (isca) *in vivo* (Chien *et al*., 1991; Fields e Song, 1989; Fields e Sternglanz, 1994). A base para o sistema de duplo híbrido em levedura está na estrutura de um fator de transcrição que tem dois domínios fisicamente separados: o domínio de ligação ao DNA (BD) e o domínio de ativação da transcrição (AD). O domínio da ligação ao DNA se liga a uma seqüência promotora específica, que se situa no início de um gene repórter, este por sua vez interage com o domínio de ativação da transcrição, o qual atrai os componentes críticos do complexo de iniciação de transcrição. No sistema de duplo híbrido o gene que codifica proteína de interesse é fusionado ao gene que codifica o domínio de ligação ao DNA, enquanto uma biblioteca de cDNA, a qual codifica várias proteínas potencialmente interativas a serem testadas, é fundida ao gene que codifica o domínio de ativação de transcrição. Quando ocorre uma interação entre a proteína de interesse e uma proteína da biblioteca, o fator de transcrição é reconstituído e os genes repórteres que estão sob seu controle são ativados.

Antes de iniciar com a triagem de possíveis proteínas que interagem com as proteínas Ki-1/57 e CGI-55, um possível parólogo de Ki-1/57, como será discutido no tópico 4.3, nós comparamos a expressão destas proteínas em diferentes tecidos por *Northern blot* (Resultados: Artigo I, fig.2). Nós observamos que a proteína CGI-55 foi predominantemente expressa em: coração, músculo esquelético, rins, placenta, fígado e cérebro (em ordem decrescente de intensidade); a proteína Ki-1/57 é predominante expressa em: cérebro, rins, coração e músculo esquelético (em ordem decrescente de intensidade). A alta expressão de Ki-1/57 no cérebro sugeriu a utilização de uma biblioteca de cérebro fetal humano para as triagens de interação proteína-proteína pelo sistema de duplo híbrido em levedura.

4.2.1 Ensaio de duplo híbrido em levedura com a porção C-terminal de Ki-1/57 (122-413)

Depois de realizados todos os testes de auto-ativação da proteína Ki-1/57 C-terminal (122-413), a qual o gene foi inserido no plasmídeo pBTM116 fusionado ao

domínio de ligação a DNA (BD) da proteína LexA, esta foi utilizada como "isca" para a triagem de possíveis ligantes codificados por uma biblioteca comercial de cDNA de cérebro fetal humano (Clontech) fusionada ao domínio de ativação (AD) de GAL4. A interação entre Ki-1-57 C-terminal e as proteínas ligantes foi detectada pelo crescimento em meio sem histidina e pela produção da β-Galactosidase. A transformação da cepa L40 de *Saccharomyces cerevisiae* expressando o plasmídeo "isca" com a biblioteca de cDNA, teve uma eficiência de $1,3x10^6$, resultando em 565 transformantes que foram capazes de crescer em meio sem histidina e entre estes clones, 184 foram positivos para o ensaio de expressão de β-Galactosidase em papel de filtro. Destes 184 clones, apenas 54 foram reconfirmados por co-transformação da cepa L540 e, finalmente, 51 sequências foram obtidas e analisadas pelo programa BLAST, levando a identificação de 11 proteínas, como apresentado na tabela 1.

Tabela 1: Proteínas que interagem com a região C - terminal da proteína Ki-1/57 (122-413) pelo sistema duplo híbrido em levedura.

KDa	Proteínas	Número de clones	Domínio/ função	Ref. bibliográfica
36	(RACK1) Receptor para quinase C ativada *(Receptor for Activated Kinase C-1)*	27	Repetições WD40 *(WD40 repeats*) / Ligam-se à PKC ativada, ao ribossoma (levedura) e ao RNA.	Guillemot *et al*., 1989. Ron, D. *et al*., 1994.
~93	(TOPORS) Proteína que se liga a topoisomerase I rica em argina e serina (topoisomerase I binding, arginine/serine-rich protein)	4	Domínio dedo de anel *(RING finger) e* região rica em arginina e serina/ ativação transcricional	Haluska *et al.,*1999. Zhou *et al*., 1999.
~240	(CHD-3) Proteína com domínios cromo-helicase-de-ligação ao DNA. *(Chromodomain Helicase DNA binding protein-3)*	3	Domínio dedos de Zinco (*zinc finger*), Domínio CHROMO: organizador e modificador de cromatina (Chromatin Organization Modifier), /Família das Helicases , regulação da transcrição e remodelagem de cromatina	Woodage *et al*., 1997.
~149	(ZFP106) Proteina com dedos de zinco 106 *(Zinc finger protein 106)*	3	Zinc finger, repetições WD40 (*WD40 repeats*) / transcrição.	Grasberger *et al*., 2005.
~41	(HRMT1L2) Arginina Metil transferase 1 *(Argine N-Methytransferase 1L2)*	2	Motivo de ligação ao RNA / Modificação pós transcricional através da metilação de arginina de proteínas alvos.	Scott *et al*., 1998.

Tabela 1: (continuação)

KDa	Proteínas	Número de clones	Domínio/ função	Ref. bibliográfica
~67	(PIAS-3) Proteína inibidora de STAT3 ativada *(Protein inhibitor of activated STAT3)*	2	Domínio SAP – possível motivo de ligação ao DNA - (bihelical)	Chung *et al.,* 1997.
120	(ALEX2) Proteína com repetição Armadillo *(Armadillo repeat protein X chromosome,2)*	2	Segmento transmembrana e uma repetição Armadillo (arm) /supressor de tumor	Kurochkin *et al.,* 2001.
~53	(HMGBCG) Similar a proteína *High-MobilityGroup* 2-Like 1	2	Domínios HMG/ fator de transcrição	Seroussi *et al.*, 1999.
120	(DAXX) Proteína associada à morte *(Death-associated protein)*	2	Região de ligação à proteína Fas/ Apoptose e transcrição	Li *et al.,* 2000. Koer al., 2001.
~42	(ACTB) Actina beta	1	Domínio de Actina e Domínio ATPase similar aos domínios de Hexoquinase e proteínas Hsp 70.	Ng *et al.*, 1985.
100	(p100) um co-ativador do fator de transcrição do vírus Epstein-Barr 2 ou EBNA-2	1	Domínio Tudor homólogo a nuclease de *Staphylococcus* e Motivo de ligação ao RNA /coativador de STAT6 e transcrição	Broadhurst e Wheeler, 2001.

4.2.2 Ensaio de duplo híbrido em levedura com a porção N-terminal de Ki-1/57 (1-150)

Da mesma forma como mencionado anteriormente, nós testamos quanto uma possível auto-ativação da proteína Ki-1/57 N-terminal (1-150). Nós observamos que esta "isca" era capaz de crescer em meio sem histidina, no entanto, as colônias transformadas com esta "isca" não eram capazes de crescer na presença de 3-aminotriazol, mesmo que em concentrações mais baixas como 2 mM. A interação entre Ki-1-57 N-terminal e as proteínas ligantes foi detectada pelo crescimento dos clones em meio sem histidina e com 10 mM de 3-aminotriazol e pela produção da β-Galactosidase. A transformação da cepa L40 de *Saccharomyces cerevisiae* expressando este plasmídeo "isca" com a biblioteca de cDNA, teve uma eficiência de 1,2 $x10^5$, resultando em 79 transformantes que foram capazes de crescer em meio

sem histidina e com 10 mM de 3-aminotriazol e entre estes clones, 60 clones foram positivos para o ensaio de expressão de β-Galactosidase. Foram obtidas 39 seqüências de DNA e estas foram analisadas pelo programa BLAST. Esta análise resultou na identificação de 20 proteínas que interagem com N-terminal de Ki-1/57 e estão descritas na tabela 2.

Tabela 2: Proteínas que interagem com a região N- terminal da proteína Ki-1/57 (1-122) pelo sistema duplo híbrido em levedura.

KDa	Proteínas	Número de clones	Domínio/ função	Ref. bibliográfica
40	(HRMT1L2) Proteína arginina metil transferase1 *(arginina metil transferaseT1L2)*	6	Motivo catalítico/ Atividade de metil-transferase para resíduos de argina	Scott *et al*., 1998
~18	(UBC9) Enzima conjugadora de ubiquitina E2I (*UBE2I: ubiquitin-conjugating enzyme E2I*)	5	Domínio catalítico/ degradação protéica no proteasomo por ubiquitinação.	Watanabe *et al*.,1996.
19	*(*CIRBP) Proteína que se liga ao RNA induzida por frio (*cold inducible RNA binding protein)*	3	*Motivo* de reconhecimento de RNA (RRM) / regulação de splicing alternativo.	*Nishiya et al.,* 1997.
~70	(FXR1P) Proteína relacionada com retardamento mental X-frágil (*Fragile X mental retardation-related protein 1*)	3	Domínio KH e caixas RG/ associam com os polissomos (60 S) e poli A de RNAm.	Siomi *et al*., 1995.
~36	(YB-1 ou NSEP1) Proteína de ligação ao elemento sensível a nuclease 1 *(nuclease sensitive element binding protein 1*)	3	Domínio choque-frio (*Cold-shock)* de ligação ao DNA-RNA / interação com elementos regulatórios no DNA.	Didier *et al*., 1988.
52.7	(BTBD2) Proteína contendo 2 domínios BTB(POZ) (*BTB (POZ) domain containing 2)*	2	Domínio BTB (POZ) / Interage com histona-desacetilase.	Carim-Todd *et al*., 2001.
~72	(APLP1) Proteína similar a proteína amilóide precursora 1 (*Amyloid-precursor-like protein 1)*	2	Domínio homólogo aos inibidores de serina proteases/ glicoproteína associada a membrana plasmática e envolvida na doença de Alzheimer. APLP1 é processada pela gama-secretase.	Lenkkeri *et al*., 1998.
8	(NDUFAB1) Proteína NADH desidrogenase, subcomplexo alfa/beta 1 *(NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1)*	2	Transporte de elétrons do NADH para ubiquinona.	Loeffen *et al*., 1998.

Tabela 2: (continuação)

KDa	Proteínas	Número de clones	Domínio/ função	Ref. bibliográfica
73	GADD34 Proteína de parada de crescimento celular induzida por danos ao DNA 34 *(growth arrest and DNA-damage-inducible 34)* ou PPP1R15A / 674 aa	2	GADD 34 atua na proliferação celular em melanoma, stress celular e apoptose.	Holander *et al.*,1997.
106	(EB1) Proteína associada a E2a-Pbx1 *(E2a-Pbx1 associated proteína)*	1	Domínios: PTB/PID e SAM/ domínio de ligação à fosfotirosina/ interage com receptores após fosforilação da tirosina.	Fu *et al.*,1999.
22	(RPL38) Proteína ribossomal L38 *(ribosomal protein L38)*/ 189 aa	1	Ligação à fosfatidil-etanolamina / liga-se a lipídeos, interage com a maquinaria de trandução protéica.	Kenmochi *et al.*, 2001.
35	(RIL) Proteína *reversion-induced LIM protein* / 328 aa	1	Domínios: PDZ, LIM e de ligação a zinco/ interação com tirosina quinase.	Bashirova *et al.*,1998.
154	KIAA1883 protein	1	Domínio catalítico, fosfotransferase, quinase Ser/Ther	NCBI Annotation Project.
92	(EPS8): Substrato 8 do receptor de fator de crescimento epidermal (*epidermal growth factor receptor pathway substrate 8)*	1	Domínio de ligação à fosfotirosina, motivo SH3/ proliferação celular	Wong *et al.*, 1994
26	(SFRS9) Fator de splicing rico em arginina/serina 9 (*splicing factor argine/serine rich 9*)	1	Motivo RRM / Interage com NSEP 1, controle de hnRNP A1 por splicing alternativo, funciona como repressor do sitio 3'de splicing.	Simarde e Chabot, 2002.
293	Miosina IXA	1	Dominíos: associação com Ras (RalGDS/AF-6), Miosina, ligação a ATPases, DAG, Região ester de forbol, região de PKC 1, rico em cisteínas, ligação a GTPase e domínio RhoGAP	NCBI Annotation Project.
2	(SF2/p32) Fator de splicing 2/p32	1	Motivo de ligação ao RNA/ fator protéico essencial para pré-mRNA splicing em HeLA. SF2 é essencial para a acurácia do splicing e na regulação do splicing alternativo	Krainer, et al., 1991.
73	Receptor de crescimento opióide *(Opioid Growth receptor)*	1	Domínio efetor *Rabphilin-3*^{A}/ envolvida no transporte de vesículas sinápticas.	NCBI Annotation Project.

4.3 A proteína Ki-1/57 possui um parálogo humano: CGI-55 e possíveis ortólogos em outros organismos vertebrados

Podemos observar que as seqüências de aminoácidos de possíveis ortólogos de Ki-1/57 em diferentes mamíferos apresentam alta identidade seqüencial (*Mus musculus*, *Rattus norvegicus e Homo sapiens).* No entanto, podemos observar que um possível ortólogo de Ki-1/57 em aves: *Gallus gallus,* apresenta uma identidade seqüencial menor, comparando-se com os mamíferos (figura 7). Dentro da espécie humana, a proteína humana Ki-1/57 possui alta identidade com outra proteína, chamada CGI-55, também com função desconhecida. As seqüências das proteínas CGI-55 e de Ki-1/57 apresentam uma similaridade de aproximadamente 67 % na seqüência de aminoácidos (40 % dos resíduos são idênticos, 27 % são similares), segundo o programa Clustal W (Resultados: Artigo I, fig. 1; figura 8). Esta alta similaridade sugere que estas duas proteínas humanas podem ser parálogas e podem ter funções similares ou redundantes na célula.

Uma outra isoforma de CGI-55 é conhecida por PAI-RBP1: "*PAI-1 mRNA-binding protein*". A proteína PAI-RBP1 foi identificada originalmente como uma proteína com afinidade para um elemento rico em AU na região 3'-não traduzida do RNA de "*plasminogen activator inhibitor"* (PAI), e, possivelmente, envolvida na relação da estabilidade desse RNAm. (Heaton *et al.* 2001).

As proteínas Ki-1/57, CGI-55 e PAI-RBP1 apresentam um motivo em comum (HABP4_PAI-RBP1) no seus C-terminais. Esta seqüência é muito conservada, como se pode observar ao alinhar as seqüências de aminoácidos destas proteínas (figura 8). No caso de Ki-1/57, esse motivo se encontra entre os aminoácidos 183-360 (figura 8).

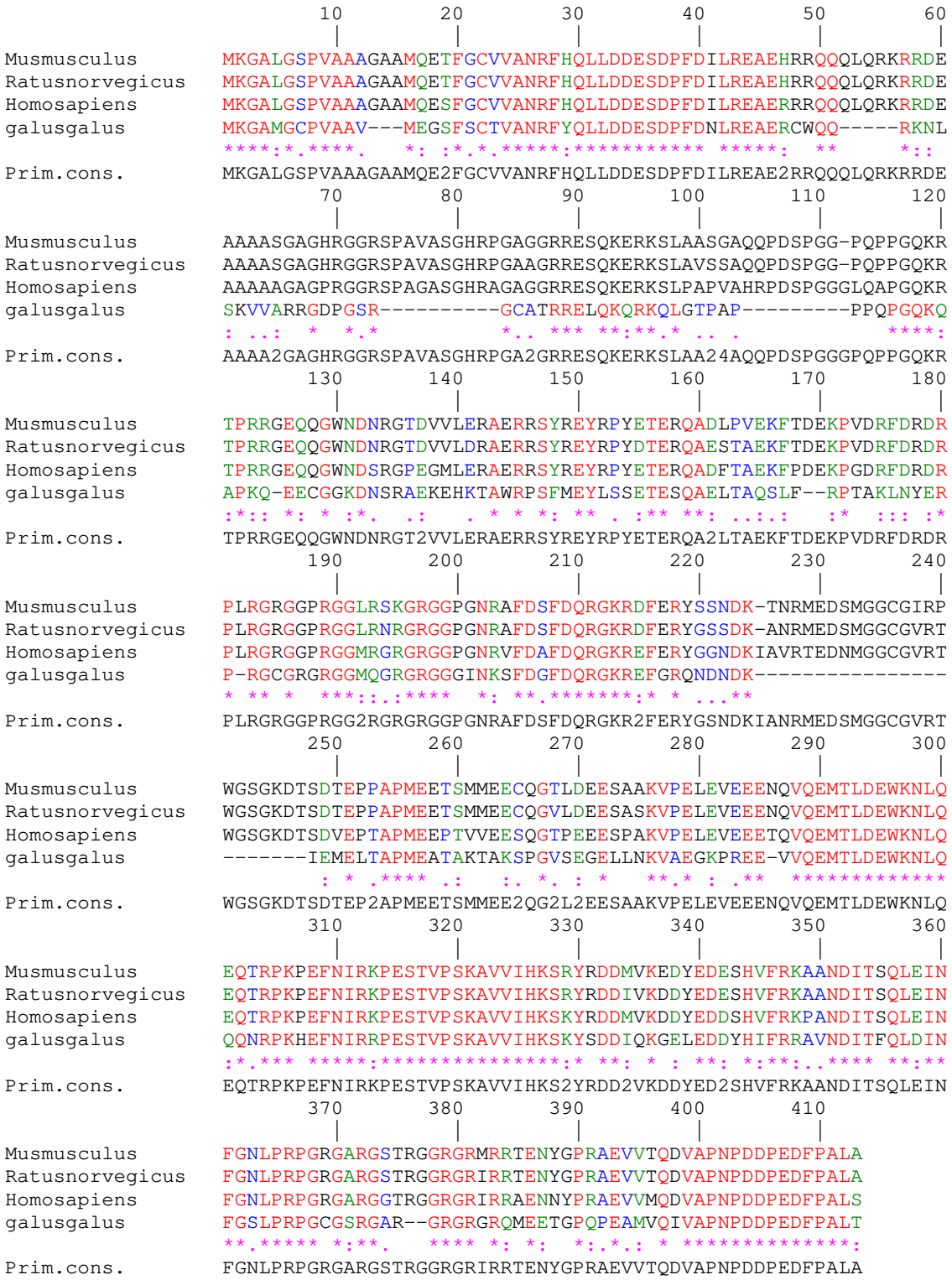

Figura 7: Alinhamento das seqüências de aminoácidos de possíveis proteínas ortólogas de Ki-1/57. Seqüências alinhadas das espécies: *Mus musculus, Rattus norvegicus, Homo sapiens e Gallus gallus* pelo program Clustal W (www.clustaw.genome.ad.jp).

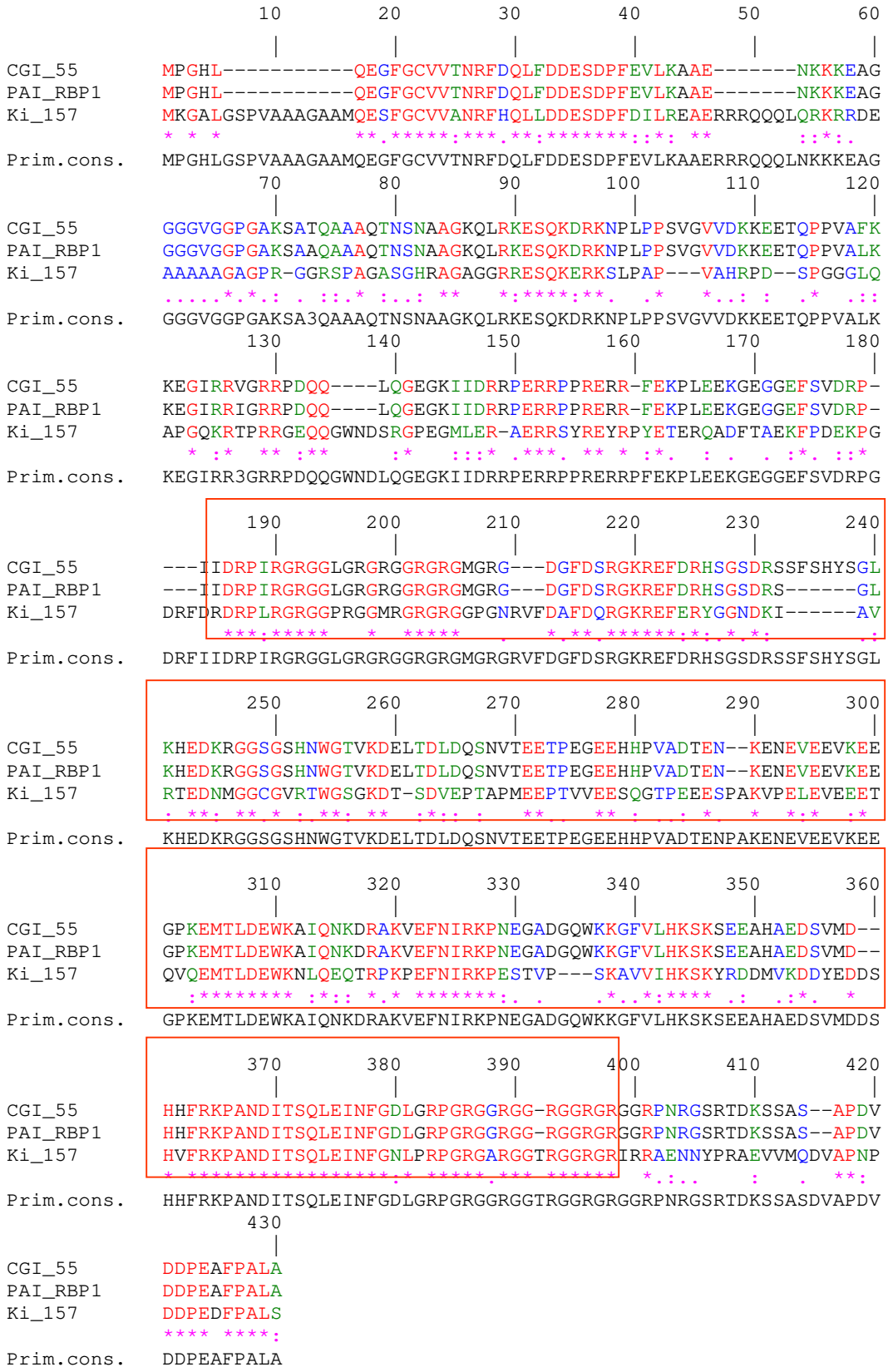

Figura 8: Alinhamento da seqüência de aminoácidos das proteínas CGI-55, PAIRBP1 e Ki-1/57. A caixa corresponde ao motivo “HABP4_PAI-RBP1” comum entre elas.

4.4 Ensaios de cristalização

Os ensaios de cristalização destas proteínas foram realizados por difusão de vapor em gota sentada com reagentes das empresas Hampton Research (HR), Jena Bioscience (JBS) e Cristal Screen (CS). Estes sistemas são compostos por diferentes tampões de cristalização. As placas de cristalização foram mantidas a 18°C e analisadas semanalmente. Os ensaios de cristalografia foram realizados em colaboração com a Dra. Beatriz Gomes Guimaraes (LNLS).

4.4.1 Utilizando a proteína recombinante 6his-RACK-1

A proteína RACK1 mostrou formação cristalina do tipo agulha em várias condições, após 24 horas da realização do ensaio (figura 9 A), na concentração final de 10 mg/ml.

A partir de uma das condições encontradas (20% PEG 10000/20% Glicerol/ Tris.HCl, pH 8,0/ 0,1 M NaCl), onde observamos a formação de agulhas finas (figura 9A), nos realizamos um ensaio com diferentes concentrações de DTT e encontramos que a proteína RACK-1 na presença de 5 mM DTT formou cristais maiores do que os encontrados anteriormente e também mais espessos (figura 9 B).

4.4.2 Utilizando os fragmentos C-terminais de Ki-1/57: KiC (aa 122-413) e KiC2 (aa151-263)

A indução da expressão da proteína 6xHis-Ki-1/57 completa foi mais baixa que a indução dos fragmentos de Ki-1/57. Desta forma, o baixo rendimento protéico impossibilitou o uso da proteína 6xHis-Ki-1/57 completa nos experimentos de cristalização.

Os fragmentos C-terminais de Ki-1/57: KiC (aa 122-413) e KiC2 (aa151-263) mostraram ser mais puros e estáveis e por isso decidiu-se dar início aos ensaios de cristalização. Em um período de 24 horas, observamos que as proteínas KiC e KiC2 mostraram formações cristalinas (rosetas). A proteína truncada KiC2 apresentou formação cristalina nas condições; 0,1 M Tris.HCl pH 8, 5 e 2 M sulfato de amônio (CS1/4) e 0,1 M Citrato de sódio pH 5,6; 30% PEG 4000 e 2 M acetado de amônio (CS1/9) e a proteína truncada KiC apresentou formação cristalina na condição 0,1 M

Na Hepes pH 7,5; 5% PEG 4000 e 30% MPD e (JBS 7/C4) (figura 9C e 9D). A co-cristalização pode ser uma necessidade neste caso, no entanto, continuaremos com a tentativa de cristalizar as proteínas RACK1, KiC2 e KiC independentes.

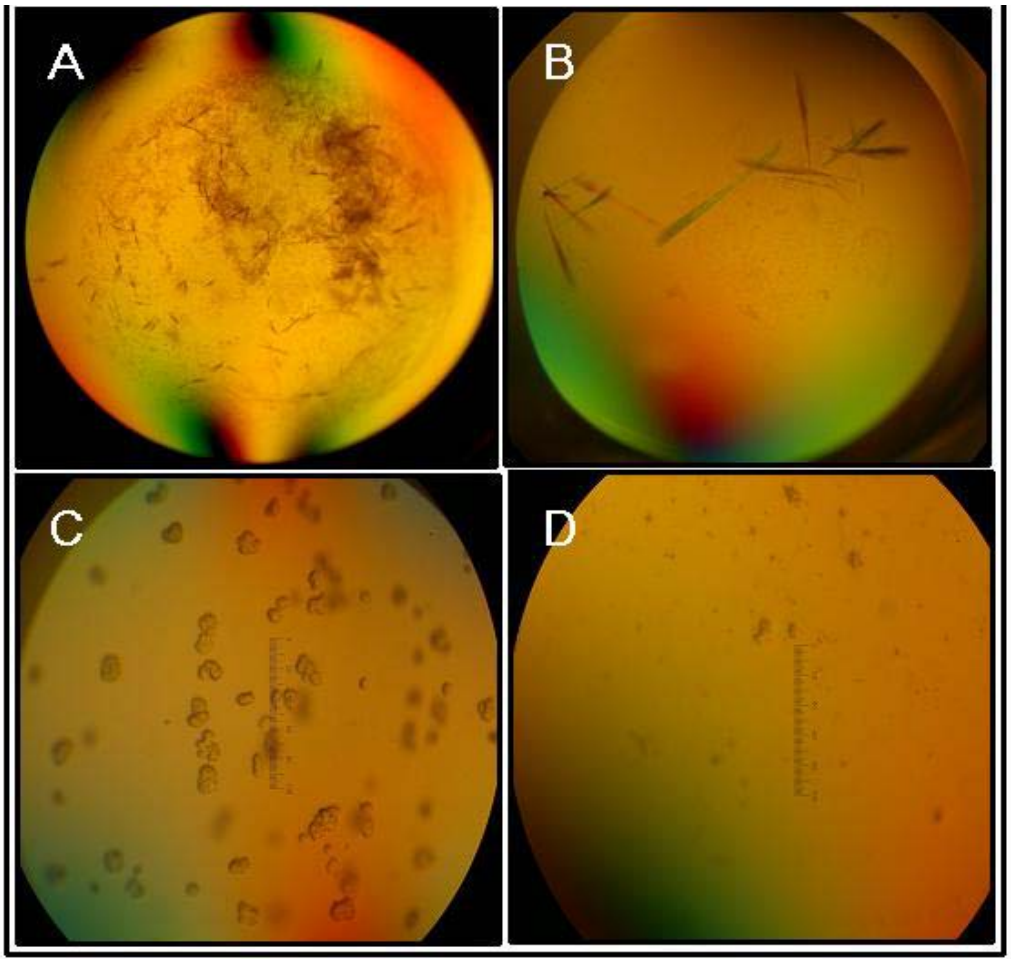

Figura 9: Ensaios de cristalização das proteínas recombinantes RACK1 (A-B) e Ki-1/57 (151-263) (C)/ e Ki-1/57 (122-413) (D). Em A, observou-se que a proteína RACK1 formou pequenas agulhas em tampão: 0,1 M Tris.HCl, pH 8, 0.1 M NaCl, 20% PEG 10.000, 20% Glicerol. Durante o refinamento observamos um aumento significativo no tamanho das agulhas e na sua espessura, mostrado em B, cujo tampão utilizado o mesmo utilizado em A e com 5 mM DTT. Em C, formações cristalinas foram encontradas durante os ensaios de cristalização com a proteína KiC2 no tampão: 0,1 M Tris.HCl pH 8, 5 e 2 M Sulfato de Amônio e em D, durante o ensaio com a proteína KiC no tampão: 0,1 M Na Hepes pH 7,5; 30% MPD e 5% PEG 4000.

5 DISCUSSÃO

5.1 O ensaio de duplo híbrido em levedura pode sugerir possíveis contextos funcionais para a proteína Ki-1/57

Para obter informações funcionais sobre uma proteína com função desconhecida, como a proteína Ki-1/57, existem várias diferentes abordagens e métodos de biologia molecular. Uma delas é o sistema de duplo híbrido em levedura (*yeast two- hybrid system*).

Inicialmente nós não tínhamos o gene completo que codifica a proteína Ki-1/57 e por isso, iniciamos a triagem com o fragmento Ki-1/57(122-413). Posteriormente, nós conseguimos amplificar a região que codifica o N-terminal de Ki-1/57 e realizamos um novo ensaio com esta região da proteína.

Com este objetivo de identificar proteínas que interagem com Ki-1/57 pelo sistema de duplo híbrido em levedura, nós transformarmos a cepa L40 de *Saccharomyces cerevisiae* com duas iscas diferentes: a)pBTM116-Ki-1/57(122-413) e b)pBTM116-Ki-1/57(1-150) e usamos como presa uma biblioteca de cDNA de cérebro fetal humano clonada no vetor pACT2 (Clontech) como presa. Nos ensaios de duplo híbrido em levedura, nós identificamos 11 proteínas diferentes (tabela 1, página 79) que interagem com Ki-1/57(122-413) e 20 proteínas diferentes que interagem com Ki-1/57(1-150) (tabela 2, página 81). Para uma melhor compreensão das diversas proteínas encontradas no ensaio de duplo híbrido, consulte a tabela 1 e 2.

Na triagem de proteínas que interagem com Ki-1/57 C-terminal pelo sistema de duplo híbrido em levedura, destacaram-se as proteínas RACK1 *(Receptor for Activated Kinase –1)* e CHD3 (*Chromo-Helicase DNA-binding domain protein 3*). Isto porque a proteína RACK1 foi encontrada em 54% das seqüências analisadas e CHD3 porque ela além de interagir com Ki-1/57 esta também interage fortemente com a proteína CGI-55, aparecendo em 42% das seqüências analisadas (Resultados: Artigo I).

Interessantemente, nós encontramos a proteína *HRMT1L2* (*Arginina Metil-transferase)* em ambos os ensaios: com o N- e C- terminal de Ki-1/57*. S*abe-se que esta proteína é responsável pela metilação de resíduos de arginina presente em

motivos denominados por RG Box em proteínas alvo. Estes motivos estão presentes na seqüência que codifica a proteína Ki-1/57.

Analisando ambos os ensaios nós podemos agrupar as proteínas identificadas nas seguintes classes funcionais, levando em consideração que uma única proteína pode estar agrupada em mais de uma classe funcional (ver também figura 10):

1) 62% dos clones encontrados codificam proteínas envolvidas na regulação da transcrição ou na ligação ao DNA: RACK1, CHD3, TOPORS, ZFP106, ZFP189, TIP60, BTBD2, YB-1 (NSEP1), GADD34, DAXX, PIAS, p100 e HMG;
2) 51% dos clones encontrados codificam proteínas envolvidas em cascatas de sinalização celular: RACK1, HRMT1L2, EB-1, RIL, ALEX 2, CTGF e EPS8, APLP1, Miosina IXA e receptor de crescimento opióide (*opioid growth receptor*);
3) 45% dos clones encontrados codificam proteínas envolvidas em processamento de RNA e na ligação ao RNA mensageiro ou RNA ribossômico ou polissomos: RACK1, CIRBP, YB-1 (NSEP1), SFRS9 e SF2/p32, RPL38, p100 e FXRP;
4) 15% dos clones encontrados codificam proteínas envolvidas na degradação de outras proteínas, no metabolismo, no citoesqueleto e na apoptose: UBC9, GADD34, DAXX, NAPH Desidrogenase 1, Miosina IXA e ACTB.

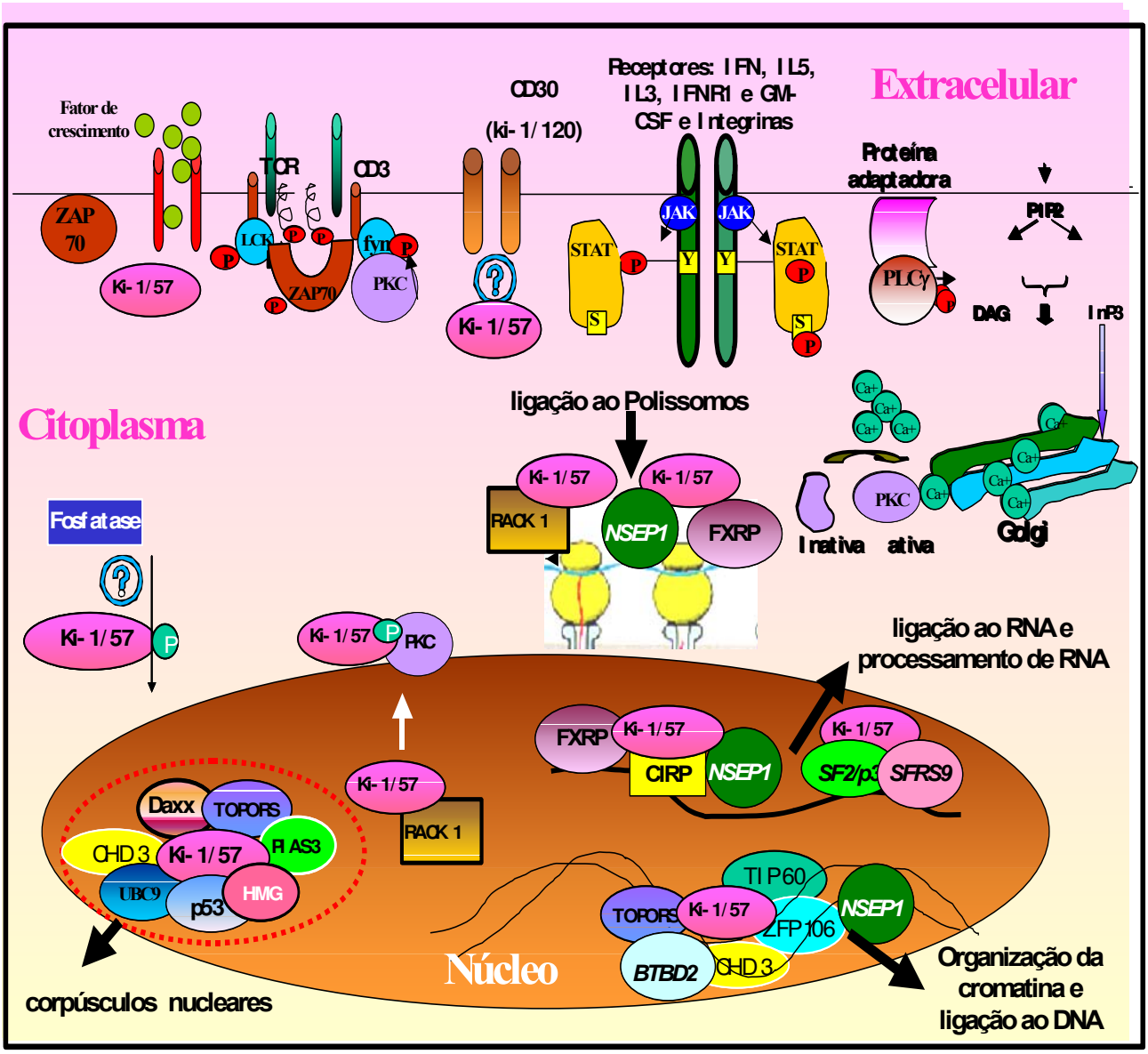

Figura 10: Associação a possíveis contextos funcionais da proteína Ki-1/57 através da identificação de proteínas que interagem com ela, usando o sistema duplo híbrido em levedura. Associamos as proteínas à três papeis funcionais: 1) regulação da transcrição e organização da cromatina ou ligação ao DNA; 2) cascatas de sinalização celular e 3) processamento de RNA, ligação ao RNA e aos polissomos (vide o texto para mais detalhes).

5.2 As proteínas Ki-1/57 e CGI-55 podem apresentar funções similares

A proteína CGI-55 assim como a proteína Ki-1/57 interagem *in vivo* e *in vitro* com a proteína CHD3 (*Chromo-Helicase DNA-binding domain protein 3*), como identificado na técnica do duplo híbrido em levedura (Resultados: Artigo I). Usando-se a seqüência que codifica a proteína CGI-55 como isca, observou-se que 42% dos clones encontrados mostraram interagir com a região C-terminal da proteína CHD3 (Resultados: Artigo I, fig. 3). Ao realizamos o ensaio de duplo híbrido com a região C-terminal da proteína Ki-1/57 (122-413), identificamos também a proteína CHD3, mas desta vez em apenas 4% dos clones encontrados (Resultados: Artigo I, fig. 3). As interações das duas proteínas CGI-55 e Ki-1/57 com CHD3 foram confirmados em ensaios *in vitro* com proteínas recombinantes purificadas (CGI-55) e *in vivo* (CGI-55, Ki-1/57) através de estudos de co-imunoprecipitação das proteínas recombinantes expressas em células de insetos (Reusultados: Artigo I, fig. 5).

As proteínas CHD são membros de uma família de proteínas envolvidas na regulação da transcrição e na remodelagem da cromatina (Woodage *et al.*, 1997; Tong *et al.*, 1998). A proteína CHD3 possui na porção central um domínio Helicase/ATPase o qual é normalmente encontrado em uma família de proteínas, as quais estão associadas a uma vasta gama de funções, tais como: replicação, recombinação, reparo de DNA, transcrição, processamento e reparo de RNA. Em alguns casos estas proteínas fazem parte de um complexo que contém uma subunidade de desacetilação de histonas que está correlacionado com a inativação da transcrição (Ogas *et al*, 1999).

Nós encontramos que além de CHD3, tanto a Ki-1/57 como a CGI-55 interagem com outras proteínas nucleares, relacionadas com a remodelagem de cromatina e a regulação da transcrição. Estas proteínas são: TOPORS (proteína que se liga a topoisomerase I), DAXX (proteína que se liga a Fas) e PIAS (inibidor de STAT ativada). No entanto, como já foi mencionado, Ki-1/57 interage com várias outras proteínas envolvidas com a regulação da transcrição: RACK1, ZFP106, ZFP189, TIP60, BTBD2, YB-1 (NSEP1), FXR1P, UBC9, GADD34, p100 e HMG. Curiosamente, a proteína Tip60 também possui o domínio CHROMO, encontrado em CHD3 e envolvido na remodelagem da cromatina. Estes dados sugerem que Ki-1/57 e CGI-55

podem pertencer a uma família de proteínas com funções em comum: remodelagem da cromatina e regulação da transcrição.

Pela análise da localização celular das proteínas CGI-55-GFP (Resultado: Artigo I, figura 6) e Ki-1/57-GFP (dados não publicados) nós constamos que as proteínas CGI-55 e Ki-1/57 apresentam características em comum. Ambas apresentam um padrão granular na região nuclear e perinuclear. Isto pode sugerir que ambas estão envolvidas na formação de corpúsculos nucleares.

Sabe-se que o núcleo de mamíferos pode conter de 10 a 30 corpúsculos entre outras estruturas (Matera, 1999, Muratani *et al.,* 2002) (figura 11A). Entre estes corpúsculos estão os corpúsculos PML (ND10 ou pODs). Estes corpúsculos correspondem a um sub-compartimento nuclear, contendo várias proteínas não-histonas e sem a presença de cromatina ou RNA, sendo encontrados próximos a outras estruturas nucleares tais como cromatina e os corpúsculos de Cajal (Maul *et al*., 2000, Boisvert *et al.,* 2000) (figura 11B). Os corpúsculos de PML estão envolvidos na supressão de tumores, regulação da expressão gênica, regulação do ciclo celular e transcrição, estrutura da cromatina, apoptose e na infecção viral (Seeler e Dejean, 1999; Reyes, 2001). Embora a proteína de PML seja essencial para a composição dos corpúsculos de PML (Zhong *et al.,* 1999), muitas outras proteínas estão presentes nestes corpúculos, tais como: CHD3, Topors, Daxx e proteínas da família PIAS, Ubc9, p53, PZLF, HMG, GADD, Sp100, SUMO1 e CBP (Seeler e Dejean, 1999; Maul *et al.,* 2000). A proteína CGI-55 mostrou interagir com as cinco primeiras proteínas e Ki-1/57 mostrou interagir com as dez primeiras proteínas (figura 11C). Isto pode ser uma forte evidência de que as proteínas Ki-1/57 e CGI-55 podem estar envolvidas com a composição ou com a função destes corpúsculos nucleares.

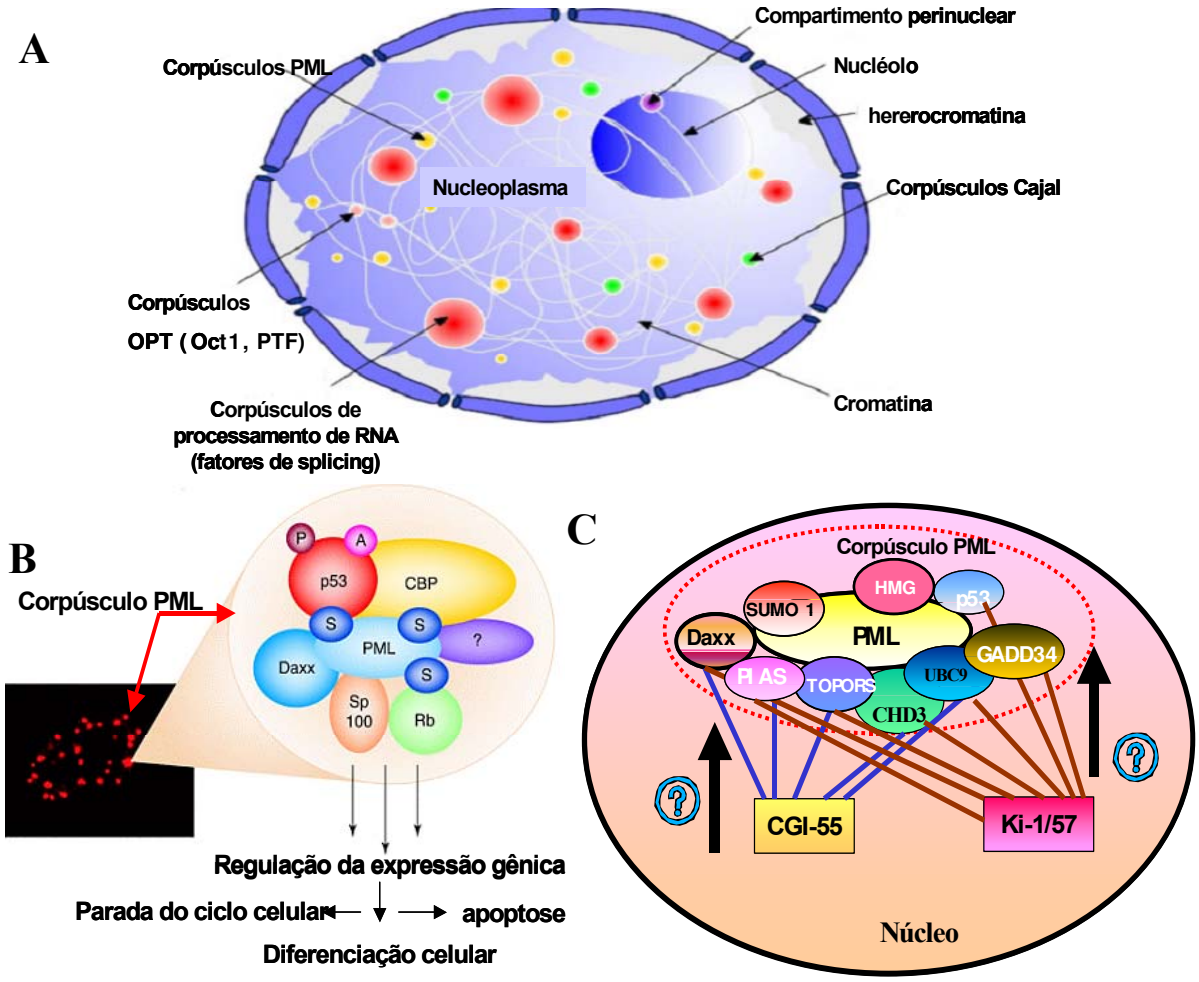

Figura 11: Arquitetura do núcleo e dos corpúsculos nucleares. A: figura esquemática do núcleo e de seus sub-compartimentos: cromatina, nucléolo, heterocromatina, e os corpúsculos: PML, OPT, Cajal e de processamento de RNA (figura adaptada do site: http://www.erin.utoronto.ca/~w3bio315/complexity%20nucleus.htm); B: a figura a esquerda mostra um núcleo marcado com anticorpo anti-PML, mostrando os corpúsculos PML e a esquerda uma representação esquemática deste corpúsculo com algumas proteínas que o compõem e as funções desempenhadas (figura retirada do artigo de Gottifredi e Prives, 2001); C: representação esquemática de várias proteínas já foram descritas participar da formação de corpúsculos PML e sua possível relação com as proteínas CGI-55 e Ki-1/57. As setas em azul indicam que CGI-55 interage com as proteínas interligadas e as setas em vermelho indicam que Ki-1/57 interage com as proteínas interligadas. No entanto, não sabemos ainda (interrogação) se as proteínas Ki-1/57 e CGI-55 estão presentes em corpúsculos PML.

5.3 A proteína Ki-1/57 interage com o receptor para quinase C ativada-1

Através do sistema duplo híbrido em levedura, identificamos que a proteína Ki-1/57 interage especificamente com a proteína adaptadora RACK-1 (“Receptor of activated C kinase 1”) que está envolvida em mecanismos de transdução de sinal envolvendo a proteína quinase C (Ron *et al*., 1994; Chang *et al*., 1998; Usacheva *et al*., 2001). No entanto, a proteína CGI-55 não interagiu com RACK1 (Resultados: Artigo II).

A proteína RACK1 foi inicialmente identificada e clonada de cDNA de fígado de galinha e de cDNA de linfócito B humano (Guillemot *et al*., 1989), descrita com os nomes C12.13 ou H12.3, respectivamente. A proteína RACK1 foi também descrita como "Guanine nucleotide-binding protein β-subunit-like" devido a sua similaridade seqüencial com a subunidade beta da proteína G (Ron *et al.*, 1994). A proteína RACK1 tem 36 kDa, consiste de 317 aminoácidos e pertence à família de proteínas que possuem repetições internas do tipo WD (triptofano e ácido aspártico). As repetições WD são compostas por aproximadamente 40-60 aminoácidos. Nestas repetições, encontram-se dipeptídeos compostos por glicina e histidina (GH) entre os resíduos 11-24 na região N-terminal. E estas terminam com triptofano e ácido aspártico (WD) (Smith *et al.,* 1999). Sete repetições WD são encontradas em RACK1. A proteína RACK1 pode se ligar a várias enzimas envolvidas na sinalização celular (Garcia-Higuera *et al*., 1996 ; Chang *et al*., 1998; Yu *et al.,* 2000; Smith *et al.,*2000; Schechtman e Rosen, 2001; Usacheva *et al*., 2001). Esta proteína pode ser uma proteína de ancoragem não somente para PKC, mas também para tirosina quinases, por exemplo a Src e para tirosina fosfatases, por exemplo a PTPμ (Schechtman e Rosen, 2001; McCahill *et al.,* 2002).

Várias outras funções são atribuídas às proteínas da família *WD-repeat*, além da participação em transdução de sinal. Entre estas funções estão: participação em processamento de RNA **(**Takagaki e Manley, 1992), como por exemplo na formação de partículas snRNP (Angenstein et al. 2002); regulação da formação de vesículas (Golgi) (Gerich *et al*., 1995); remodelagem de cromatina (Ahmad *et al*., 1999); remodelagem do citoesqueleto (Hamill *et al.*, 1998); divisão celular (Weinstein *et al.,* 1997, Kim *et al.,* 1998) e morte celular (Rodriguez *et al.* 1999).

Uma análise estrutural dos membros da família de repetições *WD* mostrou que estes apresentaram uma estrutura do tipo hélice-β *(β-propeller*). Entre essas proteínas da família WD que tiveram sua estrutura terciária resolvida com o tipo β-hélices estão: a subunidade beta da proteína G (Wall *et al.,* 1995; Neer e Smith, 1996; Lambright *et al*., 1996); envolvida em trandução de sinal; Bub3p (Wilson *et al.,* 2005), uma proteína envolvida no ciclo celular; Clathrin (ter Haar *et al*., 1998), uma proteína envolvida na formação de vesículas; Tup1 (Sprague *et al.* 2000), um co-repressor transcricional; Aip1p (Voegtli *et al*., 2003), uma proteína envolvida com a despolarização de filamentos de actina e Ski8p (Madrona e Wilson, 2004), uma proteína envolvida na degradação de RNA.

A proteína RACK1 interagiu especificamente com a porção C-terminal de Ki-1/57 (122-413) pela técnica do duplo híbrido em levedura (Resultados: Artigo II, fig.1). Nós testamos a interação entre RACK1 e a porção N-terminal de Ki-1/57 (aa 1-150) e estas não interagem (Resultados: Artigo II, fig.1A). Isto indica que a mínima região de interação de Ki-1/57 responsável por sua interação com RACK1 deve estar presente no seu C-terminal. Por outro lado, nós não conseguimos mapear o sítio de interação de RACK1 responsável pela ligação com Ki-1/57, pois a interação somente foi confirmada com a proteína RACK1 completa (Resultados: Artigo II, fig.1B). Desta forma, uma vez que as repetições WD são conhecidas como responsável pela interação proteína-proteína, nós acreditamos que o sítio de ligação à Ki-1/57 presente na proteína RACK1 não é baseado em uma seqüência de aminoácido específica, mas em triptofanos estruturalmente próximos.

5.4 Ki-1/57 é substrato para a proteína quinase C ativada por PMA

Experimentos anteriores com a proteína Ki-1/57 mostraram que esta proteína quando imunoprecipitada de células humanas co-precipita com atividade quinase em resíduos de serina e treonina (Ser/Thr) (Rhode *et al*., 1992). Não se conhecia, no entanto, se Ki-1/57 seria mesmo uma quinase ou se ela está associada a alguma quinase.

A descoberta da interação entre Ki-1/57 e RACK1, uma proteína que por sua vez está diretamente associada com PKC ativada, sugeriu uma hipótese que resolvemos testar: Ki-1/57 pode estar associado com PKC na sua forma ativada e Ki-1/57 pode ser

alvo da fosforilação por PKC? Desta forma, nossos experimentos tentaram elucidar o significado da interação entre Ki-1/57-RACK1 focalizando em compreender se RACK1 atua como proteína de ancoragem para PKC e se a fosforilação interfere com esta interação.

Ki-1/57 co-precipita RACK1 no compartimento nuclear sem indução com PMA. Isto pode indicar que *in vivo* Ki-1/57 interage com RACK1 antes de ser fosforilado. Analisando a seqüência primária da proteína Ki-1/57, nós observamos que esta apresenta sítios potenciais de fosforilação por PKC. Ao isolamos a PKC de células L540 ativadas com o estimulador PMA conseguimos demonstrar que Ki-1/57 é um excelente substrato *in vitro* da PKC ativada (por PMA) e que Ki-1/57 é pouco fosforilado pela PKC isolada de células não ativadas (Resultados: Artigo II, fig. 3A, canaletas 5 e 6).

Enzimas da família proteína quinase C desempenham importantes papéis na diferenciação celular, proliferação e apoptose (Altman and Villalba, 2002). Naturalmente dentro das células, as proteínas PKC são alvos de ativação por DAG (1,2- diacilglicerol) de forma reversível. No entanto, esteres de forbol sintéticos como PMA (4α –forbol, 12-miristato, 13-acetato) podem ativar PKC de forma irreversível e por isso são conhecidos por propagar tumores.

A família PKC consiste de várias subfamílias: PKCs convencionais, dependentes de Ca^{2+} e DAG (cPKC: α, βI, βII e γ); PKCs novas, independentes de Ca^{2+} e dependentes de DAG (nPKCs: δ,ε,η, e θ); PKCs atípicas, independentes de Ca^{2+} e DAG, geralmente ativadas por PIP3 e ceramida (aPKCs: ζ e λ). Além disso, existe uma outra subfamília de PKC conhecidas como PKCD (PKCμ, PKCv, PKCD2) (Lint *et al*., 2002). Nesta subfamília, o DAG não é o único mensageiro secundário. As PKCD podem ser também ativadas por outros estímulos, tais como: neuropeptídeos, fator de crescimento, receptores TNF e quando ocorre a sinalização entre receptores de células B e T.

Nós acreditamos então, que a proteína Ki-1/57 não se autofosforila, uma vez que não apresenta nenhum domínio conservado de quinase, no entanto, é fosforilada por PKC e, além disso, esta fosforilação é dependente da ativação por PMA. A fosforilação é uma modificação pós traducional muito importante e pode resultar numa mudança conformacional na proteína, influenciando, por exemplo, a interação desta

com outras proteínas. Baseando-se neste conhecimento, nós investigamos se a fosforilação interferia positivamente ou negativamente na interação com RACK1.

Nós observamos que após a fosforilação da proteína Ki-1/57 *in vitro* e *in vivo*, esta perde sua capacidade de interagir com RACK1 (Resultados: Artigo II, fig. 4A, canaletas 2 e 3 e Artigo II, fig. 6, canaletas 16 e 15) e migra do núcleo para o citoplasma (Artigo II, fig. 7A e B). Nós também investigamos se a proteína RACK1 poderia interferir na fosforilação de Ki-1/57 por PKC e observamos que não interfere (Resultados: Artigo II, fig. 3C).

Após a dissociação da proteína RACK1, a atividade da proteína Ki-1/57 na célula provavelmente será alterada, já que ela provavelmente interage no núcleo com uma série de proteínas envolvidas na remodelagem da cromatina, como por exemplo, CHD-3, entre outras.

O trânsito de Ki-1/57 entre núcleo e citoplasma foi observado tanto nas análises de *Western blot* das frações citoplasmáticas e nucleares das células L540 (Resultados: Artigo II, fig. 6, canaletas 35, 36 e 39 e 40) como em estudos de microscopia de fluorescência, onde a saída de Ki-1/57 do núcleo foi observada após tratamento com PMA (Resultados: Artigo II, fig. 7AeB). Outro efeito da fosforilação de Ki-1/57, após a ativação com PMA, é que esta ganha a capacidade de interagir com a PKC-theta no núcleo e no citoplasma de maneira estável (Resultados: Artigo II, fig. 6, canaletas 32 e 30).

Especulamos se a fosforilação de Ki-1/57 ocorreria na região responsável pela ligação em RACK1. Para testar esta hipótese nos expressamos diferentes fragmentos da proteína Ki-1/57: 6xHis-Ki-1/57(1-150), (151-263), (264-413), (122-413) e submetemos à fosforilação *in vitro*. Observamos que os fragmentos C-terminais 6xHis-Ki-1/57(122-413) e (264-413) foram fosforilados (Resultados: Artigo II, fig. 4B, canaletas 4 e 5).

Para conhecer especificamente quais resíduos na proteína Ki-1/57 são alvos da fosforilação pela PKC, nós realizamos uma predição inicial através do programa *NetPhos 2.0 Server* (http://www.cbs.dtu.dk/services/NetPhos/). Com o programa, nós encontramos no fragmento C-terminal extremo (264-413) um total de 15 resíduos de Ser e Thr com probabilidade de serem fosforilados por proteína quinase C (Resultados: Artigo II, fig. 5A). Esses fragmentos foram usados para avaliar seu potencial em servir como substrato de fosforilação pela PKC *in vitro*. Neste caso,

vimos que somente 2 dos 5 fragmentos, Ki-1/57(294-413) e Ki-1/57(346-413) foram fosforilados (Resultados: Artigo II, fig. 5C). Interessantemente, estes mesmos fragmentos foram também capazes de interagir ainda com RACK1, no sistema de duplo híbrido em levedura (Resultados: Artigo II, fig. 5B).

A proteína 6xHis-Ki-1/57 (264-413) foi submetida a uma análise de fosfo-aminoácido que revelou que principalmente Thr, mas também resíduos Ser sofrem fosforilação (Resultados: Artigo II, fig. 5D). Baseado neste achado, nós também fizemos uma análise dos fosfo-aminoácidos da proteína Ki-1/57 (346-413). Achamos que esta proteína sofre fosforilação apenas em resíduos de Thr (Resultados: Artigo II, fig.5 E). Isso sugere que dos 34 resíduos de Thr e Ser na proteína Ki-1/57 e possíveis alvos de fosforilação por PKC, analisados pelo programa *NetPhos 2.0 Server* (http://www.cbs.dtu.dk/services/NetPhos/), apenas T354 e T375 são fosforilados pela PKC *in vitro*.

A figura 12 é uma representação esquemática do modelo proposto por nós da via de sinalização entre Ki-1/57/RACK1/PKC a qual regula a localização subcelular de Ki-1/57.

Estes resultados confirmam o observado por Dr. H. Lemke, onde este observou que Ki-1/57 encontra-se no citoplasma na forma fosforilada e no núcleo desfosforilada (dados não publicados).

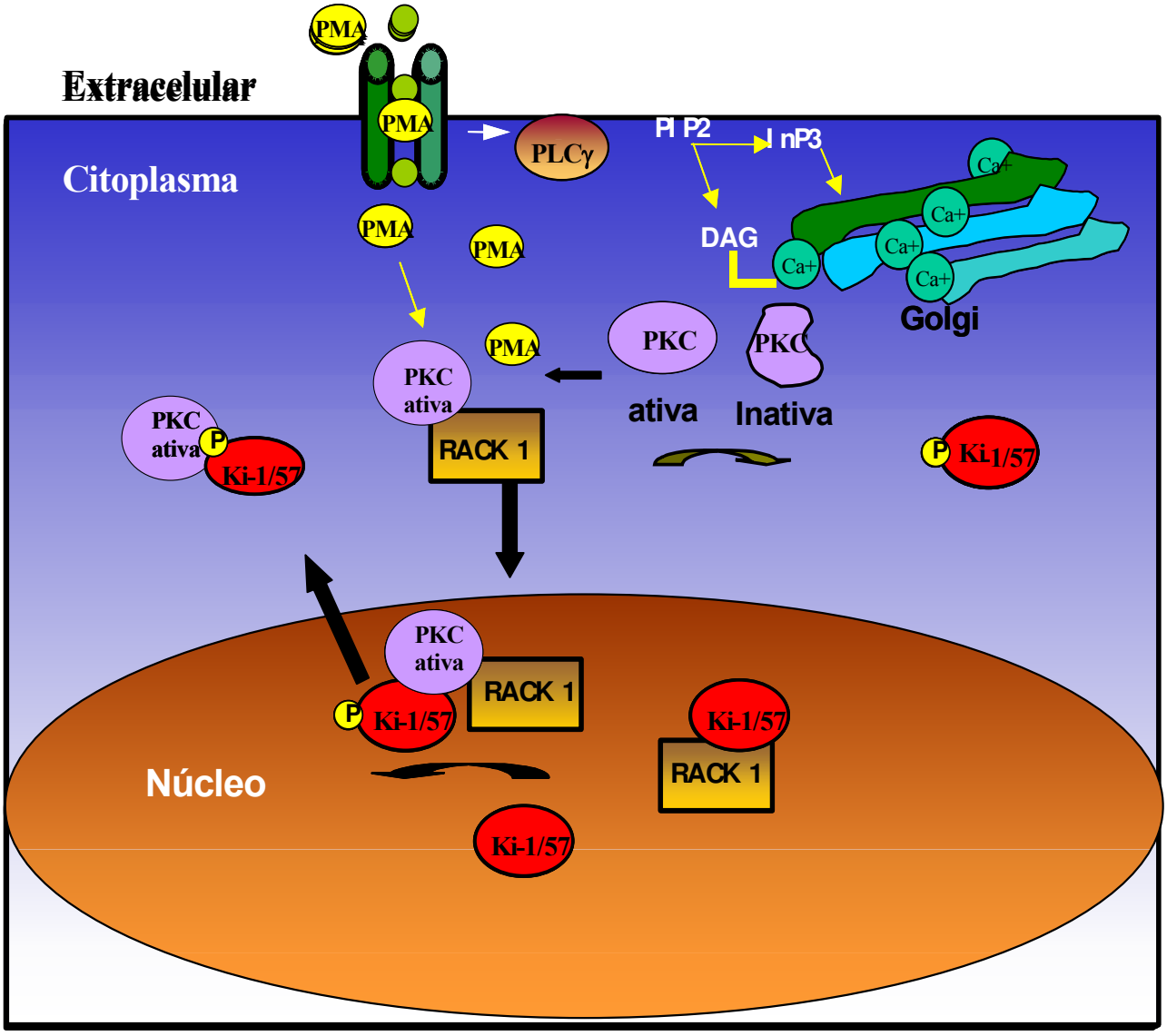

Figura 12: Modelo preliminar da regulação da localização subcelular da proteína Ki-1/57 por fosforilação. A ativação de um receptor pode ativar a fosfolipase C γ (PLCγ) que faz a hidrólise de PIP2 em DAG e InP3. Estes mensageiros secundários, junto com o cálcio levam à ativação de PKC. PKC também pode ser ativada diretamente por PMA. Quando PKC está ativa, ela se liga a RACK1. Nós creditamos que Ki-1/57 interage com RACK1 no núcleo, é fosforilada por PKC neste compartimento celular e, finalmente, a interação com RACK1 é interrompida. Ki-1/57 fosforilada migra do núcleo para o citoplasma e agora pode interagir diretamente com PKC.

5.5 A proteína Ki-1/57 interage com a proteína p53 e pode atuar como seu repressor transcricional

Nos nossos dois ensaios de duplo híbrido, onde utilizamos a porção maior C-terminal de Ki-1/57 (122-413) e a porção N-terminal de Ki-1/57 (1-150), encontramos 11 proteínas capazes de interagir também com p53 e ou com outros membros da família p53 (ex. p73) (Resultados: Artigo III, tabela 1), no entanto não encontramos nenhum membro da família p53. Isto pode ser explicado pelo fato que somente a proteína Ki-1/57 completa se mostrou capaz de interagir com p53 (Resultados: Artigo III, fig. 1B, números 3 e 4).

A proteína p53 foi descoberta por acaso em 1979, quando esta foi imunoprecipitada com o antígeno T, ou "Large-T", do vírus SV40, em células transformadas por este vírus (Oren e Rotter, 1999). Em 1980, alguns pesquisadores descobriram que muitos cânceres possuem mutações nas quais levam a inativação de p53 (Hofseth *et al.* 2004).

A proteína p53 é codificada por um gene situado no cromossomo de número 17, e seu nome é conseqüência de seu peso molecular de 53 kDa. À proteína p53 foi atribuído um papel importante na carcinogênese (Koumenis *et al*., 2001). Desde então, uma vasta literatura tem sido publicada visando definir melhor o seu papel fisiológico. Sua principal função está relacionada à preservação da integridade do genoma na célula. Durante o ciclo de divisão celular, a proteína p53 desempenha um papel muito importante. A função da proteína p53 é, através de uma cascata de reações, impedir que a célula com o DNA mutado entre em processo de mitose e complete a divisão. Para isto, dois caminhos poderão ser seguidos: a correção da mutação através da ativação de proteínas de reparo ou a indução da morte celular através da apoptose (Vogelstein *et al*. 2000, 2004). Desta forma, a proteína p53 é uma proteína supressora de tumor.

A proteína p53 consiste de quatro domínios: um domínio N-terminal de transativação; um domínio central de ligação ao DNA; um domínio de oligomerização e um domínio C-terminal básico de regulação. A estrutura do domínio de oligomerização consiste de 2 folhas do tipo α-helices anti-paralelas e uma folha β-sheet anti-paralela. (Levine *et al*., 1990; Soussi *et al*., 1990; Lane *et al*. 1990; Levine *et al.*, 1991; Kaelin *et al.*, 1999).

Nós observamos que a proteína Ki-1/57 fosforilada ou não por PKC interage igualmente com p53 *in vitro* pelo sistema *GST-pull down*. No entanto, quando a proteína p53 foi fosforilada por PKC, a sua interação com Ki-1/57 foi interrompida (Resultados: Artigo III, fig. 2B). Curiosamente, a proteína UBC9, a qual interage com ambas p53 e Ki-1/57, também perde a afinidade por p53 quando esta se encontra fosforilada (Lin *et al.,* 2004).

Na literatura nós procuramos se no linfoma de Hodgkin a proteína p53 encontra-se mutada ou ausente. Nós observamos que muitos trabalhos relatam que neste tipo de tumor a p53 se encontra na sua forma selvagem (Sturzenhofecker *et al.,* 2003; Garcia *et al.,* 2003; Maggio *et al.,* 2001). Desta forma, tornou-se possível utilizar as células de linfoma de Hodgkin L540 para estudar a interação *in vivo* entre Ki-1/57 e p53 (Resultados: Artigo III, fig. 3). Uma vez que confirmamos a interação entre Ki-1/57 e p53, nós ainda tínhamos que compreender qual seria a possível função desta interação. Para testarmos a hipótese de que Ki-1/57 poderia influenciar na atividade transcricional de p53 nós utilizamos a técnica de mono-híbrido em levedura.

Nós observamos que Ki-1/57 inibiu a atividade transcricional de p53 em mais de 50 % (Resultados: Artigo III, fig. 5). Por outro lado, nós investigamos se a proteína Ki-1/57 interage com a região promotora alvo de p53, o que poderia explicar a redução na ativação. No entanto, Ki-1/57 foi incapaz de reconhecer esta região, inferindo que a regulação ocorre diretamente com a proteína p53.

Recentemente foi associado à RACK1 à função de ser um regulador negativo de p73, um membro da família p53 (Ozaki *et al*., 2003). RACK1 mostrou inibir a atividade transcricional de p73, no entanto este mecanismo é revertido pela proteína de retinoblastoma (pRb). Curiosamente uma outra proteína que mostrou interagir com Ki-1/57 e também interage com p53 é a proteína YB1. Esta proteína mostrou ser também um regulador negativo de p53. Quando a proteína YB1 é super expressa na célula, a atividade transcricional de p53 é reduzida a níveis mínimos (Lasham *et al*., 2003). Desta forma, a proteína Ki-1/57 parece estar funcionalmente associada as proteínas: RACK1 e YB1 (NSEP1) com a mesma função de repressor transcricional de p53.

5.6 Análises de dicroísmo circular da proteína Ki-1/57

Moléculas opticamente ativas, que possuem um centro de assimetria, interagem com a luz circularmente polarizada e provocam alteração na polarização da luz incidente. A técnica espectroscópica de dicroísmo circular (CD) detecta essa alteração através da medida da diferença da absorção da luz circularmente polarizada à direita e à esquerda após esta passar através de uma amostra (Greenfield e Fasman,1973; Woody, 1995).

As proteínas possuem em sua estrutura dois cromóforos principais: as ligações amídicas e as estruturas aromáticas. As primeiras são responsáveis pelo sinal característico no comprimento de onda (λ) do ultravioleta distante (abaixo de 250 nm) sendo amplamente utilizadas como sondas de estruturas secundárias de proteínas. As estruturas aromáticas das proteínas são responsáveis pelo sinal no ultravioleta próximo (entre 250-300 nm) e são utilizadas para avaliar a estrutura terciária da proteína em questão (Woody, 1995). Considerando estas características, a técnica de CD é utilizada para estimar a quantidade de estrutura secundária de uma determinada proteína e avaliar mudanças conformacionais ocasionadas por ligantes, agentes desnaturantes (acidez, uréia, cloridrato de guanidina, temperatura, etc) (Greenfield, 2004). A forma do espectro de CD de uma proteína específica depende do seu conteúdo de estrutura secundária. Isto permite que as proporções de hélices, estruturas beta, alças (turns) e estruturas randômicas sejam determinadas. Estudos de sinais característicos de diferentes estruturas secundárias nos espectros de CD de proteínas possibilitaram classificá-las em 5 classes (Levitt e Chothia, 1976): 1) principalmente α-hélices, 2) principalmente folhas β-pregueadas, 3) α + β (regiões α e β separadas), 4) α/β (regiões com α e β misturadas), e 5) randômica (predominantemente desordenada).

Pelas análises dos espectros de CD, nós observamos que os fragmentos de Ki-1/57 apresentam uma predominância de folha beta na composição de suas estruturas secundárias (Resultados: Artigo IV, fig. 3A). Estes resultados indicam uma predominância de estruturas do tipo folha β-pregueada anti-paralela em todos os fragmentos analisados de acordo com o programa *CDNN Deconvolution* (versão 2-Bioinformatic.biochemtech.unihalle.dee/cdnn).

No entanto, nós observamos que quando o fragmento de Ki-1/57 (122-413) foi fosforilado *in vitro* e submetido às análises de CD, houve um ganho de estrutura do tipo α-hélice e perda de estrutura do tipo folha β-pregueada anti-paralela (Resultados: Artigo IV, fig. 3B). Desta forma, concluímos que a fosforilação de Ki-1/57 interfere na estrutura secundária desta proteína. Isto poderia explicar nossos dados anteriores (Nery *et al*., 2004) onde a proteína Ki-1/57 quando fosforilada não mais interage com RACK1. Com estes dados podemos entender que algum tipo de mudança estrutural pode ser a causa da interrupção desta interação.

5.7 Análises por dicroísmo circular da proteína RACK1 e de sua interação com Ki-1/57

A proteína RACK1 possui 36 kDa e consiste de 317 aminoácidos. Esta proteína pertence a uma família de proteínas que possuem repetições internas de aminoácidos Trp-Asp (WD-repeats). Sete repetições WD foram encontradas em RACK1. O espectro de CD da proteína RACK1 é indicativo de presença de folhas do tipo β-pregueadas. A proteína RACK1 apresenta um pico de absorção com o máximo de 229-300 nm, típico de proteínas WD-*repeats* (Resultados: Artigo IV). Este pico é geralmente atribuído à presença de pontes de sulfeto ou interações entre resíduos aromáticos como triptofanos, fenilalanina e tirosinas (Saxena, K 1996; Wall *et al*., 1995; Hider *et al*., 1988). No total, a proteína de fusão 6xHis-RACK1 contém 13 triptofanos, 8 fenilalaninas e 6 tirosinas. A proteína RACK1, assim como todas as outras proteínas da família WD, tem muitos triptofanos e, possivelmente, o pico entre 229-230 nm é devido aos triptofanos encontrados em todas as proteínas desta família com espectro de CD descrito na literatura.

Um trabalho descrito na literatura correlaciona o pico entre 229-230 nm aos triptofanos expostos nesta família de proteínas, que são responsáveis por interação proteína-proteína (Saxena *et al.* 1996). Neste trabalho, o pico de absorção desapareceu quando foi adicionado N-bromosuccinimida (NBS), um reagente que reage especificamente com triptofanos.

Para investigar se a proteína Ki-1/57 interage com os possíveis triptofanos expostos da proteína RACK1, nós realizamos experimentos de dicroísmos circular das

proteínas RACK1 na presença de NBS (Resultados: Artigo IV, fig. 2A) e em complexo com os fragmentos Ki-1/57(264-413) e (122-413) (Resultados: Artigo IV, fig. 2B). Os nossos estudos mostraram que o NBS aboliu o pico positivo de RACK1. Além disso, a deleção Ki-1/57(122-413) interferiu com o pico positivo de RACK1, eliminando-o. No entanto, este mesmo fragmento fosforilado por PKC e o fragmento Ki-1/57(151-263), que mostrou não interagir com RACK1, não foram capazes de eliminar o pico positivo de RACK1, o que pode indicar que eles sejam ineficientes na ligação aos triptofanos expostos de RACK1 (Resultadaos: Artigo IV, fig 2B).

5.8 Análises por emissão de fluorescência de RACK1 e Ki-1/57

O estudo espectrofotométrico por fluorescência é baseado em um fenômeno quântico no qual um elétron de um determinado cromóforo, após absorver uma determinada quantidade de energia, atinge um estado excitado e ao retornar ao seu estado basal este emite parte da energia absorvida na forma de luz (Lakowicz *et al.*, 1983). Desta forma, a quantidade de energia emitida na forma de ondas eletromagnéticas é relativamente inferior à energia absorvida (energia de excitação) e o λ da luz emitida é maior do que aquele da luz absorvida. Existem três aminoácidos em proteínas que apresentam o fenômeno de fluorescência. São eles: principalmente triptofano, tirosina e em menor escala fenilalanina (Lakowicz *et al.*, 1983).

Os resultados obtidos pela técnica de fluorescência confirmaram os dados anteriores obtidos por CD, uma vez que a proteína Ki-1/57 parece interagir com os triptofanos de RACK1. Os espectros de emissão de fluorescência de triptofano (excitação em 295 nm) da proteína RACK1 foram comparados com os espectros obtidos quando esta proteína foi incubada com os fragmentos Ki-1/57 (264-413) ou Ki-1/57 (122-413) (Resultados: Artigo IV, fig. 4B). A proteína RACK1 apresenta 13 triptofanos (W17, W43, W83, W90, W132, W150, W170, W177, W219, W247, W259, W291, W310) e seu espectro de fluorescência apresenta um máximo em 335 nm. A proteína Ki-1/57 apresenta somente 3 triptofanos (W130, W241 e W296). Desta forma, uma vez que os espectros foram obtidos na mesma concentração de proteína, o fragmento Ki-1/57 (122-413), com 3 triptofanos, e o fragmento Ki-1/57 (264-413), com apenas um triptofano, apresentam uma intensidade de fluorescência inferior ao observado para RACK1 (Resultados: Artigo IV, fig. 4B).

6 CONCLUSÕES

6.1 - Os resultados do "*Northern blot*" indicam que a proteína Ki-1/57 apresenta uma maior expressão em coração, músculo esquelético, rins e cérebro humano;

6.2- A proteína Ki-1/57 possui um parólogo humano, CGI-55, com o qual apresenta uma similaridade de 67,6 % ao nível da seqüência de aminoácidos (40,7 % dos resíduos são idênticos);

6.3- As proteínas CGI-55 e Ki-1/57 interagem com a proteína CHD3 (*Chromo-Helicase DNA-binding domain protein 3*);

6.4- A proteína Ki-1/57 interage com o receptor para quinase C ativada-1 (RACK-1), mas a proteína CGI-55 não. A interação entre Ki-1/57 e RACK1 foi confirmada por experimento de co-imunoprecipitação e co-precipitação;

6.5- Ki-1/57 é substrato para a proteína quinase C (PKC) ativada por PMA *in vitro* e *in vivo*. A proteína RACK1 não interfere na fosforilação de Ki-1/57 por PKC;

6.6- Após a sua fosforilação a proteína Ki-1/57 perde sua capacidade de interagir com a proteína RACK1 *in vitro* e *in vivo*;

6.7- Quando as células L640 ou HeLa são estimuladas com PMA, a proteína Ki-1/57 migra do núcleo para o citoplasma;

6.8- O extremo C-terminal de Ki-167(366-613) é responsável pela interação com RACK1, como observado pelos experimentos com sistema duplo híbrido em levedura sendo fosforilado pela PKC;

6.9- Análises de fosfo-aminoácidos de Ki-1/57(366-613) mostraram que este fragmento sofre fosforilação apenas em resíduos de treonina. Isso tudo sugere que dos 34 resíduos de Thr e Ser possíveis alvos de fosforilação na proteína Ki-1/57, apenas T366 e T376 são alvos diretos da fosforilação pela PKC *in vitro*.

6.10- A proteína Ki-1/57 interage com a proteína p53 pelo sistema de duplo híbrido em levedura e em experimentos de imunoprecipitação e co-precipitação *in vitro*;

6.11- Nós observamos que a proteína Ki-1/57 fosforilada ou não por PKC interage igualmente com p53 *in vitro*. No entanto, quando a proteína p53 foi fosforilada por PKC a sua interação com Ki-1/57 foi interrompida;

6.12- A proteína Ki-1/57 inibiu a atividade transcricional de p53 em mais de 60 % no ensaio de mono híbrido em levedura;

6.13- Estudos de espectroscopia de dicroísmo circular de Ki-1/57 e seus fragmentos mostraram uma predominância de estrutura secundária do tipo folha-β pregueada e além disso, todas as proteínas apresentaram aproximadamente 36% de regiões de baixa complexidade estrutural ("*random coil*");

6.14- O fragmento de Ki-1/57(122-613) fosforilado *in vitro* e submetido às análises de CD mostrou um ganho de estrutura do tipo α-hélice e perda de estrutura do tipo folha β-pregueada anti-paralela. Desta forma, concluímos que a fosforilação de Ki-1/57 interfere na estrutura secundária desta proteína, assim possivelmente contribuindo com a interrupção da interação com RACK1;

6.15- O espectro de CD da proteína RACK1 é indicativo de presença de folha do tipo β-pregueada. Além disso, esta proteína apresenta um pico positivo com máximo entre 229-300 nm, típico de proteínas da família "*WD-repeat*" ricas em triptofanos. Este pico positivo de RACK1 desapareceu quando esta foi tratada com NBS e quando esta foi complexada aos fragmentos Ki-1/57 (266-613) e Ki-1/57 (122-613). Isto sugere que Ki-1/57 interage com os triptofanos da superfície da proteína RACK1.

7 PERSPECTIVAS

Neste trabalho de doutoramento foram realizados estudos funcionais da proteína Ki-1/57, caracterizando sua interação com as proteínas RACK1 e p53. No entanto, vários experimentos devem ser feitos ainda para melhor caracterizar o papel de Ki-1/57 dentro da via de sinalização destas proteínas.

Uma outra abordagem foi o estudo estrutural e espectroscópico das proteínas RACK1 e Ki-1/57. Inicialmente nós tentamos cristalizar as proteínas recombinantes: 6xHis-RACK1, 6xHis-Ki-1/57(122-613) e 6xHis-Ki-1/57(161-263). As proteínas 6xHis-Ki-1/57(122-613) e (161-263) apresentaram uma cristalização na forma de pequenas rosetas e a proteína 6xHis-RACK1 apresentou uma cristalização na forma de finas agulhas. No entanto, estes resultados obtidos não possibilitaram dar início aos experimentos de difração por raios-X e resolução de suas estruturas terciárias. Mas o fato destas proteínas já terem apresentado uma formação cristalina pode ser uma forte indicação que elas estão corretamente enoveladas e estamos perto de conseguir cristais que possam ser usados em experimentos de difração de raios-X e tentar resolver a estrutura destas proteínas. Além disso, a RACK1 possui alta identidade seqüencial com a subunidade beta da proteína G e essa somente foi cristalizada em complexo com as outras subunidades. Isto sugere que a co-cristalização pode ser uma necessidade para uma formação cristalina favorável das proteínas Ki-1/57 e RACK1 para poder dar início aos experimentos de difração de raios-X. Já os estudos espectroscópicos foram bem sucedidos e a sua padronização possibilita novos estudos de interação entre Ki-1/57 e outras proteínas, como por exemplo p53.

A proteína Ki-1/57 pode ser uma proteína filogeneticamente antiga, pois além de estar presente nos vertebrados, nós podemos encontrar possíveis ortólogos em organismos invertebrados, tais como: *Drosophila melanogaster* (gi:26686396), *Caenorhabditis elegans* (gi:26168369), e *Aedes aegypti* (gi:16210666). Pouco se sabe a respeito da função de Ki-1/57 e desta família de proteínas, no entanto, todos os possíveis ortólogos parecem estar relacionados com o metabolismo, transporte ou processamento de RNA. Alinhando-se a seqüência de Ki-1/57 com as proteínas de invertebrados pelo programa *ClustalW:* (www.clustaw.genome.ad.jp/) nós observamos que Ki-1/57 apresenta uma similaridade de 61,6% ao nível da seqüência de aminoácidos com a proteína VIG de Drosophila (22,18% dos resíduos são idênticos);

uma similaridade de 66,9% ao nível da seqüência de aminoácidos com a proteína HABP4 de *C. elegans* (26,26% dos resíduos são idênticos); uma similaridade de 67,11 % ao nível da seqüência de aminoácidos com a proteína HABP4 de *A aegypti* (22,68 % dos resíduos são idênticos). Neste alinhamento retiramos os 16 aminoácidos iniciais da proteína Ki-1/57, porque estes se diferem de todas as proteínas estudadas.

Entre os possíveis ortólogos encontrados em invertebrados, podemos dizer que a proteína VIG de *Drosophila* é a melhor caracterizada (Caudy *et al*., 2002; Caudy *et al*., 2003; Caudy e Hannon, 2004). A proteína VIG não tem nenhum domínio reconhecido, com exceção de uma região rica em arginina e glicina denominada por "RG Box". Esta região é responsável por ligar a RNA e também está presente em Ki-1/57. Interessantemente, a proteína VIG interage com as proteínas as proteínas da família X frágil de *Drosophila*: dFXRP1, dFXRP2 e dFMR1 (Caudy *et al.*, 2002) e nós encontramos no ensaio de duplo híbrido em levedura que Ki-1/57 interage com a proteína humana: hFXRP2. As proteínas da família X frágil são bem conservadas em humanos e em *Drosophila* e estas contém dois domínios de KH e um motivo RG. O domínio KH é essencial para ligação ao RNA em ambas as espécies: *Drosophila* e humanos (Wan *et al.,* 2000). As proteínas da família X frágil se ligam aos poliribossomos, predominantemente na subunidade 60S do ribossomo e desempenham um importante papel no desenvolvimento da síndrome de retardamento mental X frágil. A proteína VIG além de se associar com várias proteínas da família X frágil associa-se também com as proteínas RISC e Ago-2 envolvidas com o mecanismo de silenciamento gênico através do RNA de interferência (RNAi). RNAi é realizado por uma via endógena muito importante para o desenvolvimento normal de muitos organismos, como em *Drosophila* e *C. elegans*. Estes organismos expressam centenas de micro RNAs diferentes (miRNAs) com a função de suprimir a expressão de genes endógenos. Estes miRNAS de interferência são úteis para estudar os aspectos da maquinaria de RNAi e utilizá-la para benefício humano.

Além da proteína FXRP2, a proteína Ki-1/57 mostrou interagir com outras proteínas que se ligam a RNA como: YB-1 (NSEP1) e CIRP, além de proteínas envolvidas no processamento de mRNA (SFRS9 e SF2/p32) e interessantemente com proteínas que se ligam a polissomos (RPL38, NSEP1, FXRP e RACK1). Estes dados abrem novas hipóteses funcionais da proteína Ki-1/57: será que Ki-1/57 interage com RNA? Será que ela está envolvida com a regulação e maturação do RNA?

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Lista de endereços eletrônicos na rede mundial de computadores (Internet):

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ABRALE: www.abrale.org.br

***BLAST*:** www.ncbi.nlm.nih.gov/BLAST /

CDNN Deconvolution (versão 2): Bioinformatik.biochemtech.uni-halle.dee/cdnn

ClustalW: www.clustaw.genome.ad.jp/

Motif Scan: http://myhits.isb-sib.ch/cgi-bin/motif_scan

NCBI: www.ncbi.nlm.nih.gov

PDB: www.pdbbeta.rcsb.org/pdb/Welcome.do

NetPhos 2.0 Server*:* *http://www.cbs.dtu.dk/services/NetPhos/*

PredictProtein: www.cubic.bioc.Columbia.edu/predictprotein/

ProtParam Tool: www.ca.expasy.org/toos/protparam.html

PSORT II prediction*:* (http://psort.nibb.ac.jp/form2.html)

Swiss-Model: www.expasy.ch/swiss-mod/SWISS-MODEL.html

http://www.escolavesper.com.br/defesasdocorpo.html

http://www.healthcentral.com/mhc/img/img1225.cfm

http://researchpath.hitchcock.org/levylecture/selfasspln.html

http://hits.isb-sib.ch/cgi-bin/PFSCAN_pars