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IDENTIFICAÇÃO DE PROTEÍNAS QUE INTERAGEM COM A PROTEÍNA CGI-55 PARA A CARACTERIZAÇÃO DO SEU CONTEXTO FUNCIONAL

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Taíla Andrade Lemos
e aprovada pela Comissão Julgadora.

Tese apresentada no Instituto de Biologia da Universidade Estadual de Campinas, para obtenção do título de Doutor em Genética e Biologia Molecular-Área de Genética Humana e Médica

Orientador: Prof. Dr. Jörg Kobarg

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Amar o perdido
deixa confundido
este coração.

Nada pode o olvido
contra o sem sentido
apelo do Não.

As coisas tangíveis
tornam-se insensíveis
à palma da mão.

Mas as coisas findas,
muito mais que lindas,
essas ficarão.

Carlos Drummond de Andrade

Ao meu marido Fernando, que com muita paciência e amor acompanhou a realização deste trabalho, Dedico

Aos meus pais Zenon e Cidinha, aos meus irmãos Juliana, Zenon e Fernando, e à minha nova família, Valdemar, Lia, Raquel, Alexandre, Camila, e a mais nova integrante Laurinha, agradeço por toda confiança, amor e dedicação.

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ABREVIAÇÕES

APL	(*Acute Promyelocytic Leukemia*)- leucemia promielocítica aguda
AD	(*Activation domain*) – Domínio de ativação da transcrição
Aos1	(*SUMO-1 activating enzyme subunit 1*) - Subunidade 1 da enzima ativadora de SUMO-1
AR	(*Androgen Receptor*) – Receptor de andrógenos
ASK-1	(*Apoptosis signal-regulating kinase 1*) – Quinase 1 reguladora de sinal de apoptose
ATP	(Adenosine triphosphate) – Adenosina trifosfato.
AVV2	(*Adeno-Associated Virus 2*) – Vírus associadao ao adeno vírus 2
Bcl2	(Oncoprotein *B-Cell Lymphoma 2*) – Oncoproteína Bcl2
BD	(*Binding Domain*) – Domínio de ligação ao DNA
BML	(*Bloom syndrome protein*) –Proteína da sindrome de Bloom
CGI-55	(*Comparative genome identified 55*) – Identificada por comparação genômica, 55kDa
CHD3	(*Chromodomain helicase DNA binding protein 3*) – Proteína domínios cromo-helicase-ligação de DNA 3.
CPB	(*CAMP response element-binding protein*) – Proteína que se liga ao elemento de resposta ao CAMP
c-Jun	(Cellular *Jun oncoprotein*) - Oncoproteína c-Jun
Daxx	(*Fas binding protein*) – Proteína que se liga a Fas.
Dek	(*Dek oncoprotein*) – Oncoproteína Dek
DNA	(*Deoxyribonucleic acid*) - Ácido desoxiribonucléico.
ESTs	(*Expressed Sequence Tags*) - Seqüências de cDNA expressas
EST-1	(*Estrogen sulfotransferase 1*) – Estrógeno Sulfotransferase 1
Fas	(*Factor of apoptosis*)- Fator de apoptose.
FISH	(*Fluorescence in situ hybridization*) – Fluorescência de hibridação in situ
GR	(*Glucocorticoid receptor*) – Receptor de hormônio glucocorticóide
H2A	(*Histone 2 A*) – Histona 2A
H2B	(*Histone 2 B*) – Histona 2B
H3	(*Histone 3*) – Histona 3
H4	(*Histone 4*) – Histona 4
HDAC II	(*Histone deacetylase* II) – Histona desacetilase II
HPC2	(*Human polycomb protein*) – Proteína polycomb humana 2
HSV-1	(*Herpes Simplex Virus type 1*) – Vírus da *Herpes simplex* tipo 1
ICPO	(Herpes simplex virus type 1 protein) – Proteína do vírus *Herpes simplex* tipo 1
IHABP4	(*Hyaluronan-binding protein 4*)-Proteína 4 que se liga a hialurona
JNK	(*c-jun kinase*) – Quinase de c-jun
Ki-1/57	(*Ki-1 antigen of 57 kDa*) – antígeno Ki-1 de 57 kDa
MRNA	(*messenger RNA*) – RNA mensageiro
ND10	(*Nuclear Bodies* 10) – Corpúculos nucleares 10.

p53	(*Tumor protein p53*) – Proteína de tumor p53, 53 kDa
P73α	(*Tumor protein p73*) - Proteína de tumor p73, 73 kDa
PAI-RBP1	(*PAI-1 mRNA- binding protein*) – Proteína que se liga ao mRNA de PAI-1.
Pax-3	(*Paired box protein Pax-3*) – Proteína Pax 3
Pax-5	(*Paired box protein Pax-5*) - Proteína Pax 5
PIAS	(*protein inhibitor of activated STAT*) – Proteína inibidora de STAT ativado
PIC1	(*PML-interacting clone 1*) – Clone 1 que interage com PML
PML	(*Protein of Promielocytic leukemia*) – Proteína da leucemia promielocítica.
PML NBs	(*PML Nuclear Bodies*) – Corpúculos nucleares associados à proteína PML.
PODs	(*Promyelocytic Oncogenic Domains*) – Domínios da oncogênese promielocítica.
RanBP2	(*RAN binding protein 2*) –Proteína 2 que se liga a RAN (*Ras-related nuclear protein*)
RARα	(Retinoic acid receptor, alpha) – Receptor alfa de ácido retinóico
Rb	(Protein *Retinoblastoma*) – Proteína de Retinoblastoma
RBCC	(*Ring finger, B-box- Coiled coil*) – Domínio RBCC
rRNA	(*Ribossomal RNA*) – RNA ribossomal
RNA	(*Ribonucleic acid*) – Ácido ribonucléico.
Sp100	(*Nuclear antigen Sp100*) – Antígeno nuclear Sp100
Sp140	(*Nuclear antigen Sp140*) - Antígeno nuclear Sp140
Sp3	(*Sp3 transcription factor*) – Fator de transcrição Sp3
STAT	(*Signal transducer and activator of transcription*) – Sinalizador e ativador da transcrição
SUMO-1	(*Small Ubiquitin-related MOdifier*) – modificador pequeno relacionado a ubiquitina
TDG	(*Thymine DNA Glycosylase*) – Timina DNA-glicosilase
TNF	(*Tumor Necrosis Factor*) – Fator de necrose de tumor
Topors	(*Topoisomerase I binding protein*) – Proteína que se liga a topoisomerase I.
UBA2	(*SUMO-1 activating enzyme subunit 2*)- Subunidade 2 da enzima ativadora de SUMO-1
UBL1	(*Ubiquitin-Like1*) – Proteína 1 semelhante à ubiquitina
UBACT	(*Repeat in ubiquitin-activating (UBA) protein*) – Repetições nas proteínas ativadoras de ubiquitina
Zf-MIZ	(*MIZ zinc finger*) – Dedo de zinco MIZ (*Msx-interacting-zinc finger*)

RESUMO

A fosfoproteína de 57kDa, antígeno Ki1/57, foi primeiramente identificada através de reação cruzada com o anticorpo monoclonal anti-CD30, chamado Ki-1. Sua função permanece desconhecida, mas sabe-se que está localizada no citoplasma e núcleo, está associada à atividade de proteinoquinase e é fosforilada após ativação por mitógenos, sugerindo que esta proteína possa ser uma proteína reguladora.

Utilizando a seqüência de aminoácidos do antígeno Ki-1/57, identificamos uma proteína humana homóloga denominada CGI-55, através de busca por comparação de similaridade no banco de dados do NCBI. A alta similaridade dessas duas seqüências (67%) sugere que CGI-55 e Ki-1/57 possam ser parálagos e possuam funções relacionadas. O objetivo principal deste projeto foi identificar o possível contexto celular funcional da proteína CGI-55, através da identificação das proteínas associadas a ela, utilizando a técnica do duplo-híbrido de levedura.

A análise da proteína CGI-55 através do método do duplo-híbrido de levedura resultou na identificação de nove proteínas interativas: CHD-3 (*Chromo-Helicase-DNA-binding domain protein-3*), Daxx (*Fas binding protein*), Topors (*Topoisomerase I binding protein*), HPC2 (*human polycomb protein*), TDG (*Thymine DNA Glycosylase*), UBA2 (*SUMO-1 activating enzyme subunit 2*) e PIAS-1, -3 e –y (*Protein inhibitor of activated STAT*). A maioria destas interações pôde ser confirmada através de ensaios de co-precipitação *in vivo* e *in vitro* (CHD-3) ou através de estudos de colocalização das proteínas fusionadas a fluoróforos, *in vivo*, em células HeLa (Daxx, Topors, PIASy, UBA2). O mapeamento do sítio de interação de CGI-55 com estas proteínas, novamente utilizando a técnica do duplo-híbrido de levedura, resultou na identificação de três padrões de interação, proteínas que interagem apenas com CGI-55 completo, apenas com a porção C-terminal e com as porções C- e N-terminais.

A CHD-3, Topors e HPC2 são proteínas nucleares envolvidas no remodelamento da cromatina e na regulação da transcrição e a TDG é uma enzima de reparo de DNA. A maioria das proteínas que interagiram com CGI-55 estão estruturalmente (Daxx, Topors) ou funcionalmente (PIAS-1, PIAS-3, PIASy, UBA2) associadas com os corpúsculos nucleares PML (PML-NBs) e com o processo de sumolização de proteínas (PIAS, UBA2). Os PML-NBs são subdomínios nucleares importantes envolvidos na regulação

da expressão gênica, apoptose e oncogênese. O fato de tanto a CGI-55 fusionada à GFP quanto a CGI-55 endógena, detectada pelo anticorpo monoclonal específico 10.5.6, terem apresentado localização granular no citoplasma e no núcleo, assim como sua associação com proteínas que se localizam nos PML-NBs, sugere que CGI-55 possa estar associada aos PML-NBs. Por isso, nós testamos, se CGI-55, assim como as proteínas com as quais interage, colocalizam com a proteína PML, o principal componente dos PML-NBs. Nós encontramos que Topors e Daxx colocalizam-se com os PML-NBs e que PIASy, UBA2 e CGI-55 colocalizam-se no citoplasma com a proteína PML, mas só parcialmente com os PML-NBs no núcleo.

Nossos dados sugerem que CGI-55 associada às proteínas citadas anteriormente esteja envolvido com os processos nucleares de remodelamento de cromatina e regulação da expressão gênica.

ABSTRACT

The 57 kDa human phospho-protein antigen Ki-1/57 was first identified by a cross reaction of the anti-CD30 monoclonal antibody Ki-1. Its function is still unknown, but the findings that it is localized in the cytoplasm and nucleus, is associated with protein kinase activity and phosphorylated after activation with mitogens, suggested that it might be a regulatory protein.

Using the amino acid sequence of the Ki-1/57 antigen we identified a homologous human protein tentatively called CGI-55 by blast-searches in the NCBI data bank. The high sequence similarity of 67% suggested that the CGI-55 and Ki-1/57 might be paralogs and have related functions. The main objective of this project was to identify the possible functional cellular context of the protein CGI-55 by identifying its interacting protein partners using the yeast two-hybrid technique.

The analysis of the protein CGI-55 by the yeast two-hybrid method resulted in the identification of nine interacting proteins: CHD-3 (Chromo-Helicase-DNA-binding domain protein-3), Daxx (Fas binding protein), Topors (Topoisomerase I binding protein), HPC2 (human polycomb protein), TDG (Thymine DNA Glycosylase), UBA2 (SUMO-1 activating enzyme subunit 2) and PIAS-1, -3 and –y (Protein inhibitor of activated STAT). The majority of these interactions could be confirmed by *in vitro* and *in vivo* pull down assays (CHD-3) or *in vivo* co-localization studies with fluorescent fusion proteins in Hela cells (Daxx, Topors, PIASy, UBA2). Mapping of the CGI-55 interaction site with these proteins, again using the yeast two-hybrid technique, resulted in the identification of three different patterns of interaction, proteins that interact only with complete CGI-55 or only its C-terminal region and those that interact with both its C- and N-terminal region.

The CHD-3, Topors and HPC2 are nuclear proteins involved in the chromatin remodeling and transcriptional regulation and TDG is a DNA repair enzyme. Most of the CGI-55 interacting proteins are either structurally (Daxx, Topors) or functionally (PIAS-1, PIAS-3, PIASy, UBA2) associated with PML-nuclear bodies (NBs) and with the process of protein sumoylation (PIAS, UBA2). PML-NBs are important nuclear sub-domains that are involved in the regulation of gene expression, apoptosis and oncogenesis. The fact that CGI-55-GFP as well as endogenous CGI-55, detected by the

specific monoclonal antibody 10.5.6., showed a speckled localization both in the cytoplasm and in the nucleus together with its interaction with a series of proteins found in PML-NBs, suggested that CGI-55 might be associated with PML-NBs. Therefore, we tested if CGI-55 as well as its interacting proteins co-localize with the protein PML, the major component of PML-NB. We found that Topors and Daxx co-localize with PML-NBs and that PIASy, UBA2 and CGI-55 co-localize with the PML protein in the cytoplasm but co-localized only partially with the PML-NB in the nucleus.

Our data suggest that CGI-55 associated with the above mentioned proteins is involved in nuclear processes such as chromatin remodeling and the regulation of gene expression.

INTRODUÇÃO

1. Proteína Ki-1/57

Ki-1 foi o primeiro anticorpo monoclonal que detectou especificamente células malignas de Hodgkin e células Sternberg-Reed no linfoma de Hodgkin (*Morbus Hodgkin*) (Schwab et al., 1982). Este anticorpo detecta dois diferentes antígenos, um na superfície e outro no citoplasma das células de Hodgkin (Froese et al., 1987; Hansen et al., 1989). O primeiro é a glicoproteína de superfície de membrana de 120 kDa chamada CD30, assim como o seu precursor intracelular de 90 kDa. Este receptor de superfície foi subseqüentemente clonado e seqüenciado e pode ser caracterizado como um membro da grande família dos receptores de fatores de necrose de tumor (*TNF-receptor family*) (Dürkop et al., 1992). O segundo antígeno do Ki-1, chamado Ki-1/57 é uma fosfoproteína intracelular de 57 kDa (Hansen et al., 1989).

O antígeno Ki-1/57 foi primeiramente isolado de células tumorais e apresenta atividade de serina/treonina quinase. Os substratos fosforilados por Ki-1/57 foram CD30, histonas e anticorpos, assim como a própria proteína Ki-1/57 (Hansen et al., 1990).

Através de análises de microscopia eletrônica, o antígeno Ki-1/57 foi localizado no citoplasma, nos poros nucleares, no núcleo e também associado ao nucléolo (Rohde 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 e treonina. A forma nuclear não é fosforilada (Hansen et al., 1990).

Ki-1/57 é expresso em células de uma grande variedade de tipos de câncer, tais como linfoma de células T, adenocarcinoma, carcinoma de próstata, carcinoma de bexiga

e é fosforilado em leucócitos do sangue humano ativados com mitógenos (Kobarg et al.,1997).

A análise da localização cromossômica do gene Ki-1/57 por FISH (*Fluorescence In Situ Hybridization*) mapeou o gene na banda 9q22.3-q31 no braço longo do cromossomo 9 humano (Kobarg et al.,1997). Esta região é freqüentemente afetada por deleções secundárias na leucemia mielóide aguda do tipo M2 [translocação t(8;21)] e tipo M3 [translocação t(15;17)] (Heim e Mitelman, 1995).

Estes dados sugerem que o antígeno Ki-1/57 possa ser uma molécula sinalizadora de crescimento celular e um provável candidato a oncoproteína.

Através de busca em bancos de dados por proteínas relacionadas, descobrimos um possível parálogo humano do antígeno Ki-1/57 cuja função é desconhecida, que foi denominado de CGI-55 (número de acesso AF151813.1) e PAI-RBP1 (número de acesso AL080119.1)(Heaton et al., 2001).

2. A proteína CGI-55: um possível parálogo do antígeno Ki-1/57

Um grande número de seqüências de DNA que codificam proteínas humanas está depositado nos bancos de dados internacionais como seqüências de cDNA ou ESTs (*Expressed Sequence Tags*). A função de muitos destes produtos gênicos permanece desconhecida. A primeira pista sobre a função de uma proteína pode ser obtida através de bioinformática e busca de similaridade. Usando a seqüência parcial de cDNA que codifica o antígeno Ki-1/57 (número de acesso U77327), um novo gene que codifica uma proteína hipotética chamada CGI-55 (número de acesso AF151813.1) foi identificado. A

busca foi realizada no banco de dados NCBI *(Nacional Center of Biotechnological Information*; http://www.ncbi.nlm.nih.gov/blast/).

As seqüências das proteínas CGI-55 e Ki-1/57 apresentam uma similaridade total de 67,4 % ao nível da seqüência dos aminoácidos (40,7 % dos resíduos são idênticos, 22,8 % resíduos conservados). Esta alta similaridade sugere que estas duas proteínas humanas podem ser parálogas e podem ter funções similares ou redundantes na célula.

A proteína ortóloga de CGI-55 do *Caenorhabditis elegans* (Número de acesso: AF016672) apresenta uma similaridade total de 55.3 % com a proteína "CGI-55" humana. O fato das seqüências destas proteínas ortólogas serem tão conservadas entre duas espécies filogeneticamente distantes sugere que a proteína deve ter uma importante função na célula.

Recentemente foi identificado uma forma variante da CGI-55, que interage com a região 3'do mRNA de PAI-1(*Plasminogen Activator Inhibitor, type 1*), chamado PAI-RBP1 (*PAI-1 mRNA- Binding Protein*). A PAI-RBP1 difere de CGI-55 pela ausência de seis aminoácidos na posição 202 (Heaton et al., 2001)

3. Sumolização

Várias proteínas possuem sua função regulada por modificações pós-traducionais. Existem muitos mecanismos de modificações, tais como acetilação, metilação, fosforilação, carboxilação, adenilação, glicosilação, ubiquitinação e sumolização.

SUMO-1 (*Small Ubiquitin-related MOdifier*), também conhecida como PIC1, UBL1, Sentrin, GMP1 e Smt3, é membro da superfamília das proteínas semelhantes à ubiquitina. Para a sumolização é proposta uma seqüência consenso de aminoácidos K-X-

E. Isto contrasta com o processo de ubiquitinação que não exige um motivo consenso. Até o momento, acredita-se que a sumolização ocorra no núcleo (Melchior, 2000).

O processo de sumolização consiste na maturação inicial, onde a extremidade C-terminal é hidrolisada e motivos contendo dois resíduos de glicina adjacentes são expostos. A ativação dependente de ATP é realizada pela enzima heterodimérica E1, composta pelas proteínas SAE1/SAE2 (também denominadas de Aos1 e UBA2, respectivamente). O próximo passo é a conjugação, realizada pela enzima E2 conjugadora de SUMO (Ubc9) (Fig. 1). Ao contrário do processo de ubiquitinação, a sumolização não requer uma ligase E3 de SUMO-1. No entanto, existem outros cofatores que atuam como uma E3 e facilitam o processo de sumolização. As proteínas da família PIAS e RanBP2 agem como uma E3 e causam a multimerização de SUMO, o que se assemelha a poliubiquitinação, porém isto só foi observado *in vitro* (Melchior, 2000).

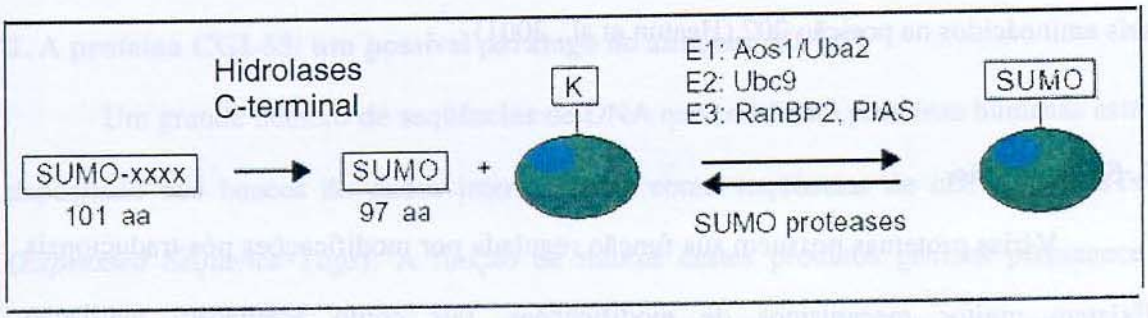

Figura:1 Esquema do processo de sumolização adaptado de Gill Grace (2003). SUMO-1 é sintetisado como um peptídeo de 101aa e é processado para 97aa por hidrolases na porção C-teminal. O SUMO maduro é conjugado à lisina (K) do substrato através de uma série de reações enzimáticas, envolvendo a enzima heterodimérica E1 (Aos1/UBA2), a enzima conjugadora E2 (Ubc9) e várias ligases E3 (RanBP2 e proteínas da família PIAS). SUMO pode ser dissociado do substrato através de proteases (Semp1, Semp2 etc).

Não foi encontrada ainda nenhuma evidencia de que as enzimas E1, E2, E3 ou mesmo SUMO-1 estejam envolvidas no processo de carcinogênese. Porém, a vasta lista de proteínas que já possui seu papel descrito no processo de carcinogênese e que são modificadas por SUMO é cada vez maior (Alarcon-Vargas e Ronai, 2002).

Das várias oncoproteínas identificadas que são modificados por SUMO-1 destacam-se as proteínas PML (*Protein of Promielocytic leukemia*) (Ishov et al., 1999, Muller et al., 1998, Zhong et al., 2000), Sp100 (Sternsdorf et al., 1997, Sternsdorf et al. , 1999, Seeler et al. , 2001), c-Jun (*Jun oncogene*) (Muller et al., 2000, Schimidt e Muller, 2002), p53 (*tumor protein p53*) (Gostissa et al., 1999, Muller et al., 2000, Rodriguez et al., 1999) e p73α (*tumor protein p73 alfa*) (Minty et al., 2000). Das proteínas modificadas por SUMO-1 a mais estudada é a proteína PML.

Recentemente, alguns fatores de transcrição também foram encontrados associados a modificações por SUMO-1, tais como AR (*Androgen Receptor*) (Kotaja et al., 2002, Nishida e Yasuda,2002), GR (Glu*cocorticoid Receptor*) (Tian et al., 2002), Sp3 (Ross et al., 2002). Além dos processos oncogenéticos e regulação da transcrição, a sumolização também pode estar relacionada aos reparos nos danos de DNA. Hardeland et al. (2002) determinaram que a proteína TDG (*Thymine DNA Glycosylase*) perde sua capacidade de reparo de DNA quando não está associada à SUMO-1.

4. Corpúsculos PML

O núcleo, tal como a célula, é compartimentalizado em regiões altamente organizadas estrutural e funcionalmente. Várias desta estruturas subnucleares estão associadas a funções específicas como, por exemplo, o nucléolo está associado à síntese e

processamento do rRNA, os corpúsculos *coiled* aos fatores de *splicing* (Matera, 1999). Os corpúsculos nucleares PML (PML NBs), também denominados de domínios oncogênicos promielocíticos (PODs), domínios nucleares 10 (ND10) ou corpúsculos Kremer, parecem estar envolvidos em vários processos celulares, tais como regulação da transcrição, oncogênese, infecção viral e outros (Hodges et al., 1998; Maul, 1998).

Os corpúsculos nucleares PML são estruturas de aproximadamente 0,5 μm de diâmetro e são melhor observados durante a fase G1do ciclo de divisão celular (Borden, 2002). A proteína PML é o principal componente destes corpúsculos. No entanto, além da proteína PML, outras proteínas foram identificadas nestes corpúsculos, tais como Rb, Sp100, Sp140, SUMO-1, Daxx, Bcl2, BML, p53 etc (Zhong et al., 2000c) (Fig.2).

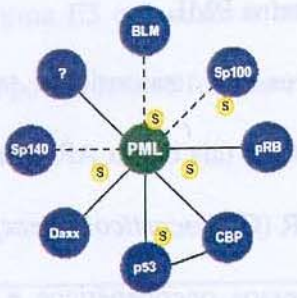

Figura 2: Representação esquemática das proteínas estruturalmente associadas aos corpúsculos PML. As interações diretas estão indicadas pelas linhas cheias e as interações indiretas pelas linhas pontilhadas. PML ocupa o lugar central por ser a principal proteína formadora dos corpúsculos. Os círculos amarelos (s) indicam a sumolização.

Pacientes com leucemia promielocítica aguda não apresentam os corpúsculos PML. Daniel et al. (1993) descobriram que estes pacientes apresentavam uma translocação (t15;17:q22;q21) envolvendo os genes PML e RARα. O tratamento destes pacientes com acido retinóico causa a remissão do câncer e a retorno da proteína PML aos corpúsculos (Sternsdorf et al., 1997; Maul, 1998).

A localização de proteínas associadas aos corpúsculos PML, tais como SUMO-1, Sp100, Sp140, CBP e Daxx é alterada quando estas são expressas em células que não expressam PML, sugerindo que PML seja a proteína formadora destes corpúsculos (Zhong et al., 2000).

A proteína PML possui três resíduos de lisina aos quais SUMO-1 se liga covalentemente. Foi verificado que a proteína PML, quando não está associada à proteína SUMO-1, encontra-se dispersa no citoplasma e, quando sumolizada, acumula-se nos corpúsculos nucleares. A localização de outros componentes dos corpúsculos PML também está associada à modificação por SUMO-1. Zhong et al. (2000) sugeriram um modelo no qual PML é primeiramente modificada por SUMO-1 e, então, recruta os outros componentes, causando a formação dos corpúsculos nucleares (Fig. 3).

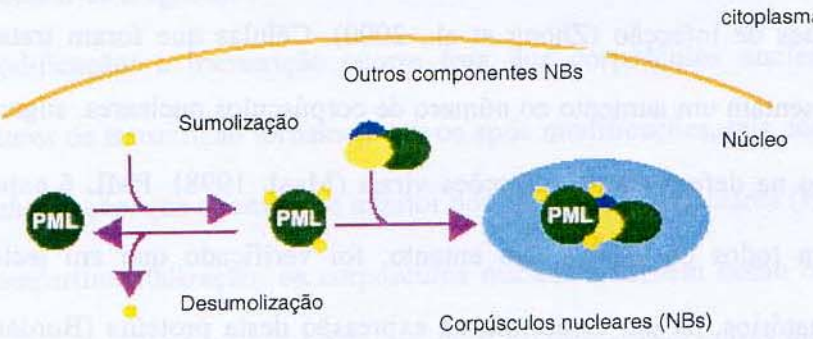

Figura 3: Esquema da formação dos corpúsculos PML adaptado de Zhong et al. (2000). Após a sumolização, a proteína PML recruta os outros componentes para a formação dos cospúsculos nucleares.

PML pertence à família de proteínas caracterizadas pela presença do motivo RBCC (*"RING B-box coiled-coil"*). O motivo RBCC consiste do motivo dedo de zinco (*"zinc-finger"*) C3HC4 e de um ou dois motivos de ligação de zinco ricos em cisteína (*"B-boxes"*) seguidos de região contendo hélices superespiraladas (*"coiled-coil"*) (Fig. 4).

O motivo RBCC na proteína PML está associado à homodimerização de PML, interação proteína-proteína e localização nos corpúsculos nucleares.

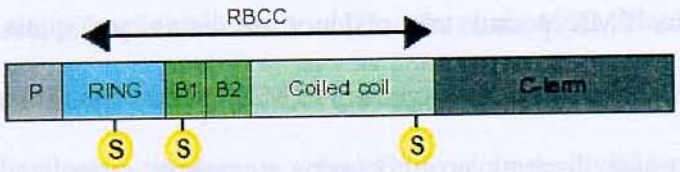

Figura 4: Esquema da proteína PML. Adaptado de Zhong et al. (2000)

4.1 PML e infecções virais

Os corpúsculos nucleares PML são afetados por infecções virais e são desfeitos nas primeiras fases de infecção (Zhong et al., 2000). Células que foram tratadas com interferon-γ apresentam um aumento no número de corpúsculos nucleares, sugerindo que seu envolvimento na defesa contra infecções virais (Maul, 1998). PML é expressa em baixos níveis em todos os tecidos. No entanto, foi verificado que em tecidos com processos inflamatórios, há um acréscimo na expressão desta proteína (Borden, 2002). Além disso, foi verificado que ICPO, uma proteína do HSV-1, quando associada aos corpúsculos PML, causa a destruição destes corpúsculos, visto que, a proteína ICPO também pertence à família das proteínas RING e compete com SUMO-1 pelo sítio de acoplamento á PML. Estas evidências sugerem que ICPO pode causar a ruptura dos corpúsculos nucleares através da inibição da sumolização (Maul, 1998). Estudos com células nocaute PML (PML-/-) demonstraram que estas são mais susceptíveis a infecções

virais, reforçando a hipótese de que PML está envolvida na proteção contra viroses (Salomoni e Pandolfi, 2002).

4.2 PML NBs e regulação da transcrição

Recentemente, foram identificados vários fatores de transcrição e de remodelamento da cromatina associados aos PML-NBs, tais como Rb, CPB e p53 (Borden, 2002). Os corpúsculos PML podem funcionar como repressores ou ativadores da transcrição (Borden, 2002). Zhong et al. (2000) propuseram um modelo de ação dos corpúsculos PML na regulação da transcrição:

a) Iniciação: Os corpúsculos funcionam apenas como locais de estocagem que liberam os fatores de transcrição para a matriz nuclear, à medida que são necessários (Fig. 5a).
b) Modificação: a transcrição ocorre fora dos corpúsculos nucleares, porém os fatores de transcrição tornam-se ativos após modificações, tais como acetilação e sumolização, que ocorrem no interior dos corpúsculos nucleares (Fig. 5b).
c) Compartimentalização: os corpúsculos nucleares servem como compartimentos, onde os fatores de transcrição, através de interação com outras proteínas, mudam de função. Por exemplo, um repressor de transcrição, após sair do corpúsculo nuclear, pode atuar como um ativador de transcrição (Fig. 5c).

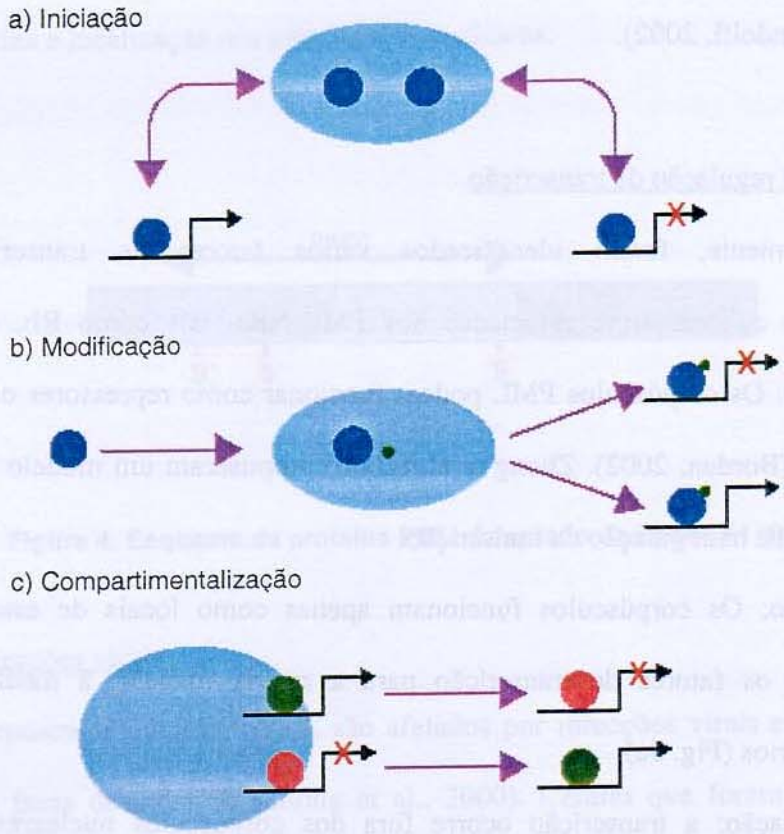

Figura 5: Esquema do modelo de ação dos corpúsculos PML na regulação da transcrição (adaptado de Zhong et al., 2000). a) Iniciação: o corpúsculo regula a concentração dos fatores de transcrição no núcleo; b) Modificação: modificações, tais como sumolização ocorrem dentro dos corpúsculos modulando a atividade transcricional; c) compartimentalização: talvez os corpúsculos nucleares sejam o centro da compartimentalização subnuclear, onde fatores de atividade dupla podem mudar sua função, um fator de ativação de transcrição (verde) após passar pelos corpúsculos nucleares torna-se um fator de repressão da transcrição (vermelho) ou vice-versa

4.3 PML e apoptose

PML participa de diferentes vias que levam à apoptose incluindo a via ativada por p53 e a via das caspases ativada por Daxx/Fas (Salomoni e Pandolfi, 2002). Em resposta a danos no DNA, hipóxia e estresse celular, o supressor de tumor p53 induz a apoptose. Células irradiadas com raios gama apresentam um aumento da expressão de p53 e de PML proporcional ao aumento de apoptose (Gottifredi e Prives, 2001). A proteína Daxx,

originalmente identificada como ligante à Fas, age como uma proteína ativadora das vias de apoptose dependentes de Fas e TGFβ (Zhong et al., 2000b). Daxx está localizada nos corpúsculos PML. Quando da ausência da proteína PML, Daxx encontra-se difusa no núcleo e perde a capacidade de induzir a apoptose via Fas (Salomoni e Pandolfi, 2002). Camundongos e células PML-/- são resistentes à indução de apoptose por Fas, radiação gama, ceramidas e TNF (Salomoni e Pandolfi, 2002). A superexpressão de PML aumenta a taxa de apoptose.

4.4 PML e Câncer:

A leucemia promielocítica aguda (APL) compreende 10% das leucemias mielóides e é caracterizada pelo bloqueio do desenvolvimento celular na fase promielocítica. Mais de 98% dos pacientes APL possuem a translocação cromossômica PML/RARα. Como descrito anteriormente, células que apresentam esta translocação não possuem os corpúsculos nucleares. Nestas células, a proteína PML encontra-se difusa no núcleo. A translocação PML/RARα age como um dominante negativo, anulando a expressão da proteína PML (Rego e Pandolfi, 2002). Camundongos e células PML-/-, quando expostos a agentes carcinogênicos, tornaram-se mais susceptíveis à formação de tumores do que células e camundongos PML+/+, sugerindo que a proteína PML funcione como supressor de tumor (Salomoni e Pandolfi, 2002; Borden,2002).

5 O Sistema Duplo-Híbrido de Levedura

O sistema de duplo-híbrido de levedura ou *"Yeast 2-hybrid system"* é uma metodologia que foi desenvolvida para estudar as interações proteína-proteína *in vivo* (Vidal e Legrain, 1999). As interações proteína-proteína formam a base da ampla variedade de reações bioquímicas e a identificação de proteínas que interagem com a proteína de interesse é um aspecto essencial para a elucidação do funcionamento e da regulação desta proteína (Vidal e Legrain, 1999). O sistema de duplo híbrido de levedura é um método rápido e bastante aplicado para identificação de interações protéicas, mas pode, além disso, ser utilizado para definir domínios ou resíduos de aminoácido que estão envolvidos na interação (Serebriiskii et al., 2001).

O método do duplo-híbrido de levedura se baseia no fato de que um fator de transcrição possui dois domínios fisicamente separados, o domínio de ligação ao DNA (BD) e o domínio de ativação da transcrição (AD) (Fields e Song, 1989). O domínio de ligação ao DNA se liga a uma seqüência promotora específica, que se situa no início de um gene repórter, enquanto o domínio de ativação atrai os componentes críticos do complexo de iniciação de transcrição (Fields e Song, 1989).

No duplo-híbrido de levedura a proteína de interesse é fusionada ao domínio de ligação ao DNA. Uma biblioteca de cDNAs, codificando as proteínas potencialmente interativas a serem identificadas, é fusionada ao domínio de ativação de transcrição. Se ocorrer uma interação entre a proteína de interesse e uma proteína da biblioteca, um fator de transcrição funcional completo é reconstituído e o "gene repórter", que está sob seu controle, será ativado para expressar, por exemplo, a enzima β-galactosidase. Nos clones

onde a β-galactosidase é expressa, na presença do composto X-Gal (*5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside*), é clivada e um subproduto desta reação apresenta coloração azul (Fig. 6) (Fields e Song, 1989).

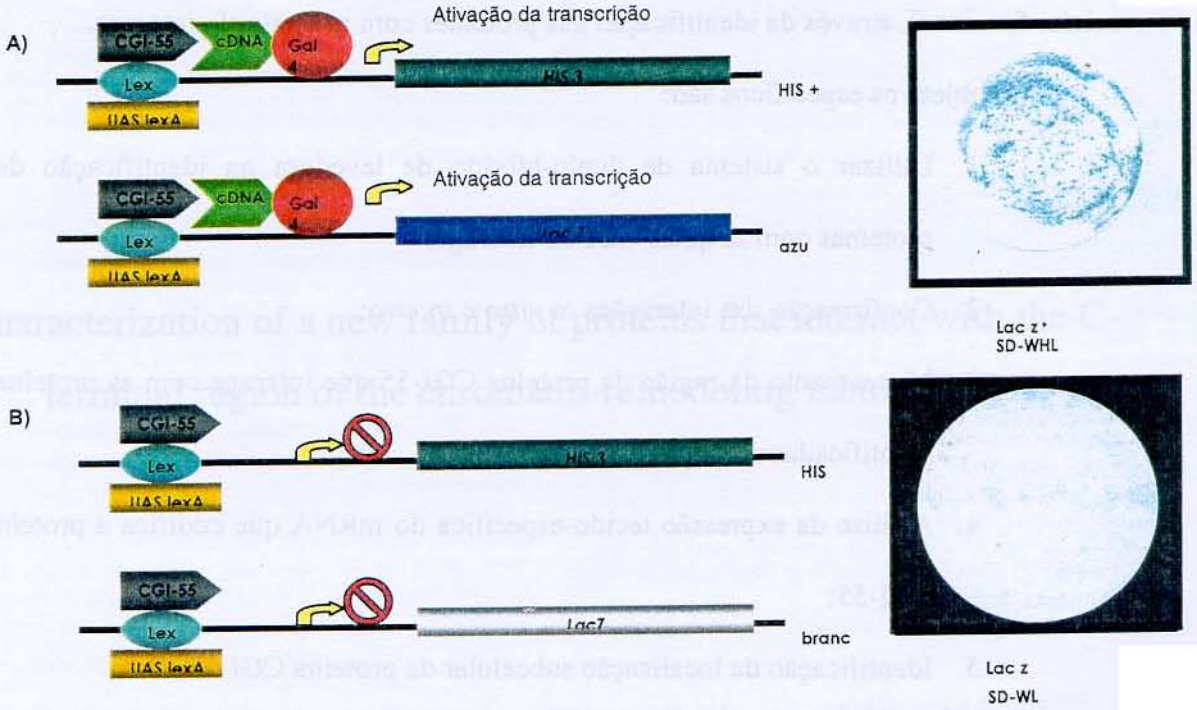

Figura 6: O sistema de duplo-híbrido de levedura. A) A proteína CGI-55 interage com uma proteína da biblioteca o fator de transcrição funcional é reconstituído e os genes repórteres HIS e LacZ transcritos. B) CGI-55 não interage com a proteína da biblioteca e não há a transcrição dos genes repórteres.

A utilização da técnica do duplo híbrido aumentou muito nos anos 90 (Serebriiskii et al., 2001). Utilizamos a técnica do duplo-híbrido para identificar proteínas que interagem com CGI-55 e, assim caracterizar suas possíveis funções na célula.

OBJETIVOS

Este trabalho visa a análise da proteína CGI-55 e a caracterização do seu contexto celular funcional, através da identificação das proteínas com as quais ela interage.

Os objetivos específicos são:

1. Utilizar o sistema de duplo-híbrido de levedura na identificação das proteínas com as quais CGI-55 interage;
2. Confirmação das interações *in vitro* e *in vivo*;
3. Mapeamento da região da proteína CGI-55 que interage com as proteínas identificadas no duplo híbrido;
4. Análise da expressão tecido-específica do mRNA que codifica a proteína CGI-55;
5. Identificação da localização subcelular da proteína CGI-55.

Artigo 1

Characterization of a new 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 *Sf9* 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.
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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

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

2.2. Northern blot analysis

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

2.3. Yeast two-hybrid screening and interaction analysis

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

2.4. Bacterial expression and protein purification

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

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

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

2.6. Production of anti-CGI-55 monoclonal antibody

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

2.7. In vitro binding assay and Western blot analysis

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

2.8. In vivo binding assay and Western blot analysis

1.0×10^7 *Sf*9 cells were infected with wild type BV (BV-WT) or recombinant BV (BV-6×His-HA-CHD3(1839–2000), BV-6×His-CGI55, BV-Ki-1/57) at a multiplicity of infection >20. Two days after infection, cells were lysed in 1 ml Tris–HCl (pH 8.5) containing protease inhibitors. Lysate was treated with DNase (Promega) and cleared at 14000×*g* for 15 min. Next 20 μl protein A Sepharose beads (Pharmacia) were loaded with the indicated antibodies (anti-HA mAb: Clontech), washed (Tris–HCl, pH 8.5) and incubated with the indicated lysates for 2 h at 4°C. After further two washes with the same buffer the beads were resuspended in SDS–PAGE loading buffer, boiled and analyzed by SDS–PAGE and Western blot using different mAb.

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

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

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

3. Results

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

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

A

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

huKi-1/57 KDEAAAAAGAGPRGGRSPAGASGHRAGAGGRRESQKERKSLPAP---VAHRPD--SPGGG 112
huCGI-55  AGGGGVGGPGAKSAAQAAAQTNSNAAGKQLRKESQKDRKNPLPPSVGVVDKKEETQPPVA 100
CeCGI-55  EEKSVKPAQPVKPAAAPVAATKTDGAAGRGRGGRGRGGAGRP---RDGERVSNENGDR  98

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

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

huKi-1/57 DNMGGCGVRTWGSGKDT-SDVEPTAPMEEPTVVEESQGTPEEESPAKVPELEVEEETQVQ 289
huCGI-55  DKRGGSGSHNWGTVKDELTDLDQSNVTEETPEGEEHHPVADTEN--KENEVEEVKEEGPK 265
CeCGI-55  DRKDGHGKGNWGDQKDE---LAGETENTAPEGAESTEPEVPREKTAEELAYEAELAVLAK 251

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

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

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

B

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

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

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

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

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

3.3. Yeast two-hybrid screens

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

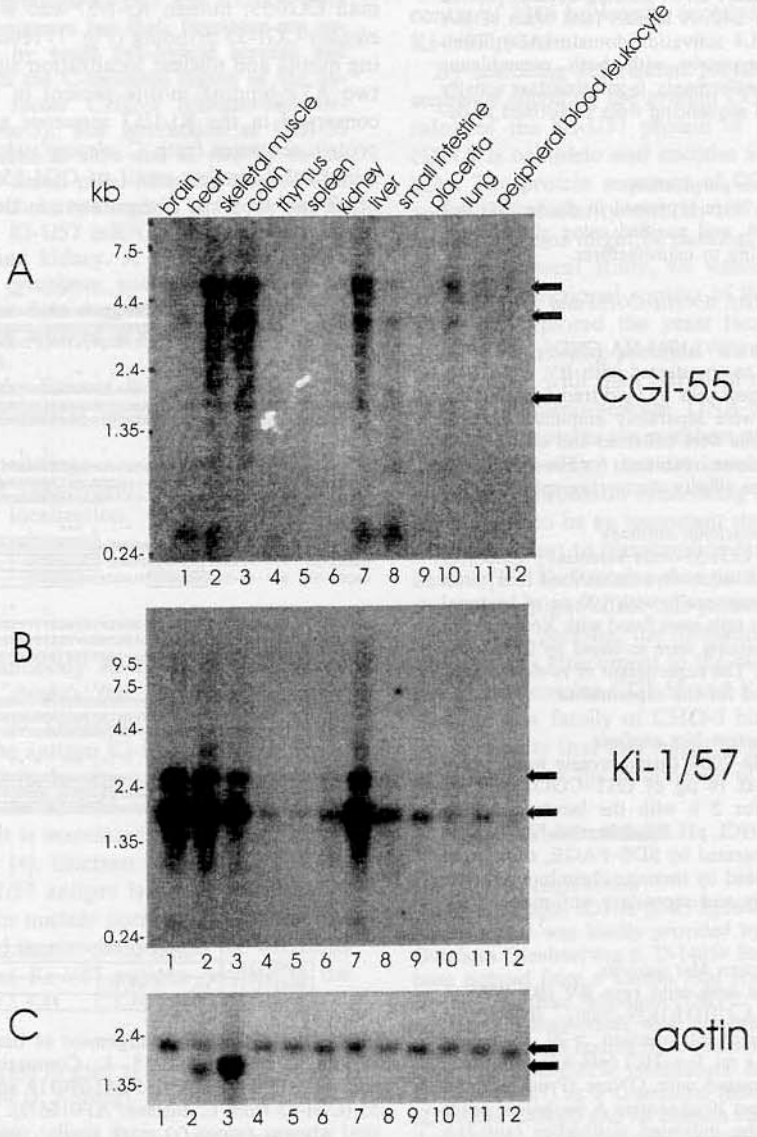

Fig. 2. Differential expression of CGI-55 and Ki-1/57 mRNAs in human tissues. A Northern blot of poly(A)$^+$ RNA isolated from several human tissues (Clonetech) was hybridized with human CGI-55 (A), Ki-1/57 (B) and β-actin (C) cDNA probes.

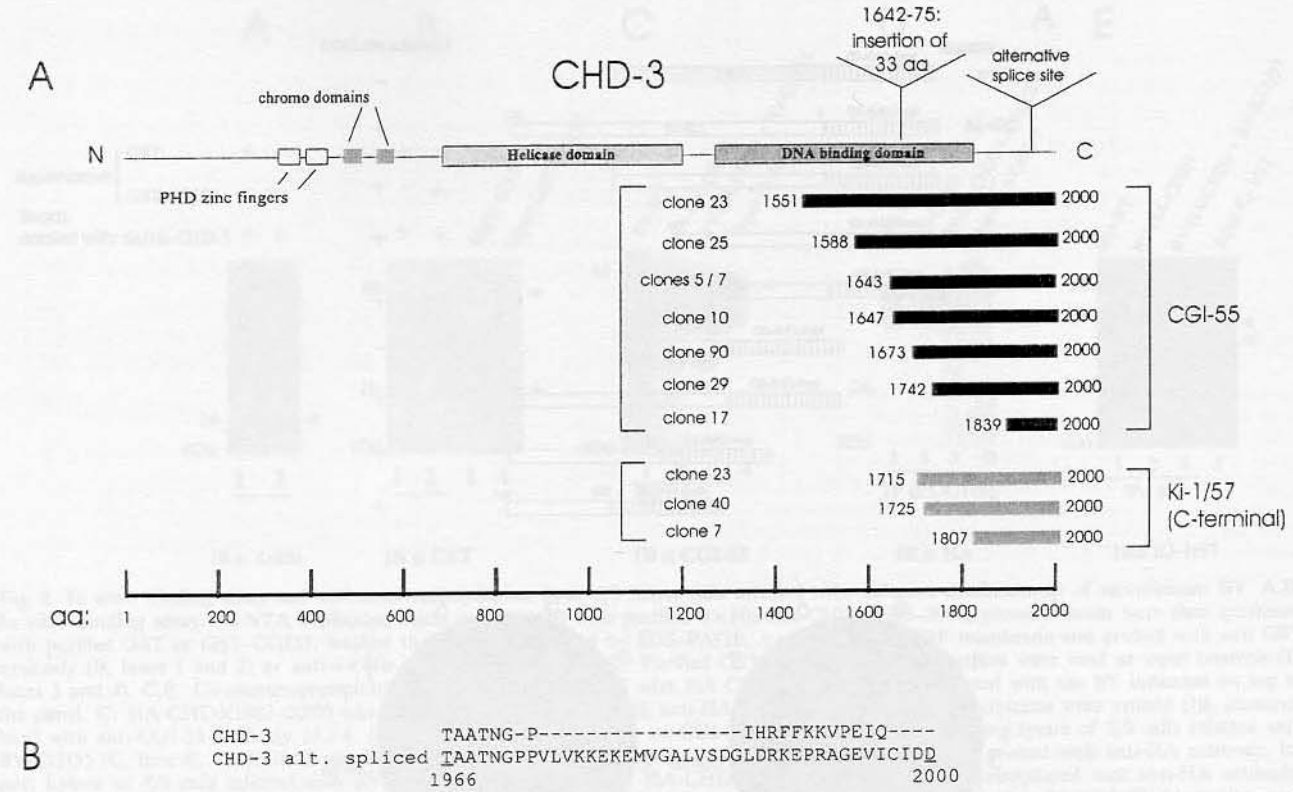

Fig. 3. CGI-55 and Ki-1/57 interact with the C-terminal region of CHD-3. A: Schematic representation of the domain organization of the human protein CHD-3. The position and of the identified CGI-55 interacting clones is shown along the full-length CHD-3 protein. The ruler indicates the length of CHD-3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

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

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

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

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

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

3.6. Co-immunoprecipitation from BV infected Sf9 insect cells

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

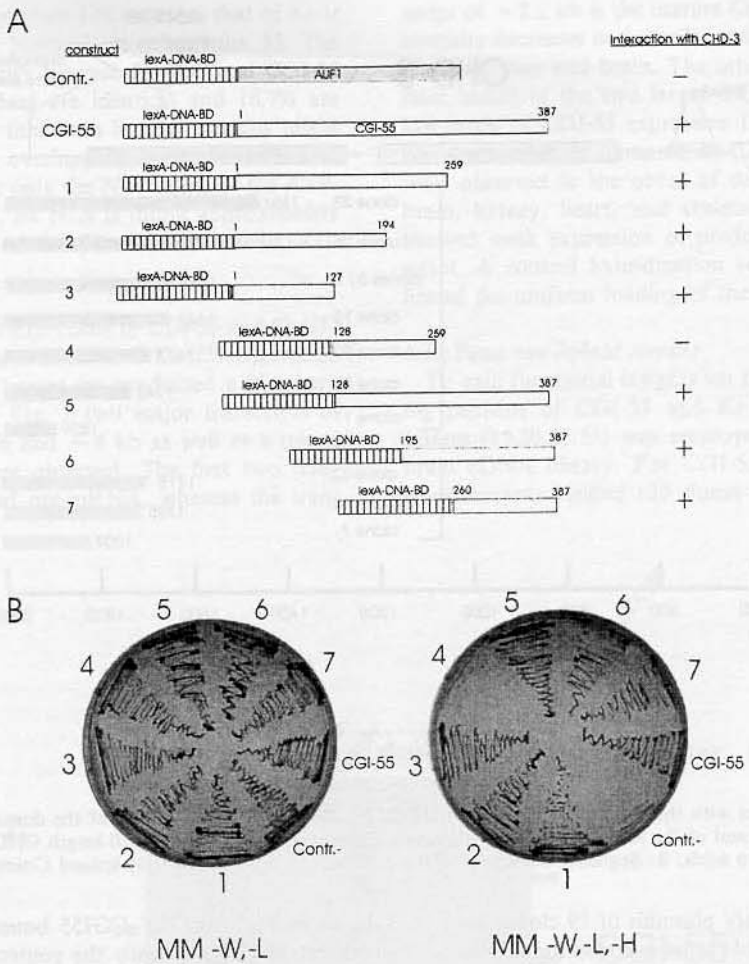

Fig. 4. Mapping of the regions of CGI-55 that interact with CHD-3. A: Various N- and C-terminal truncations of human CGI-55 were fused in frame to the DNA-binding domain of lexA in plasmid pACT2 and transformed into yeast L40 together with the fusion CHD3-Gal4-AD cloned in pBTM116. B: Interaction was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

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

3.7. Subcellular localization of CGI-55

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

4. Discussion

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

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

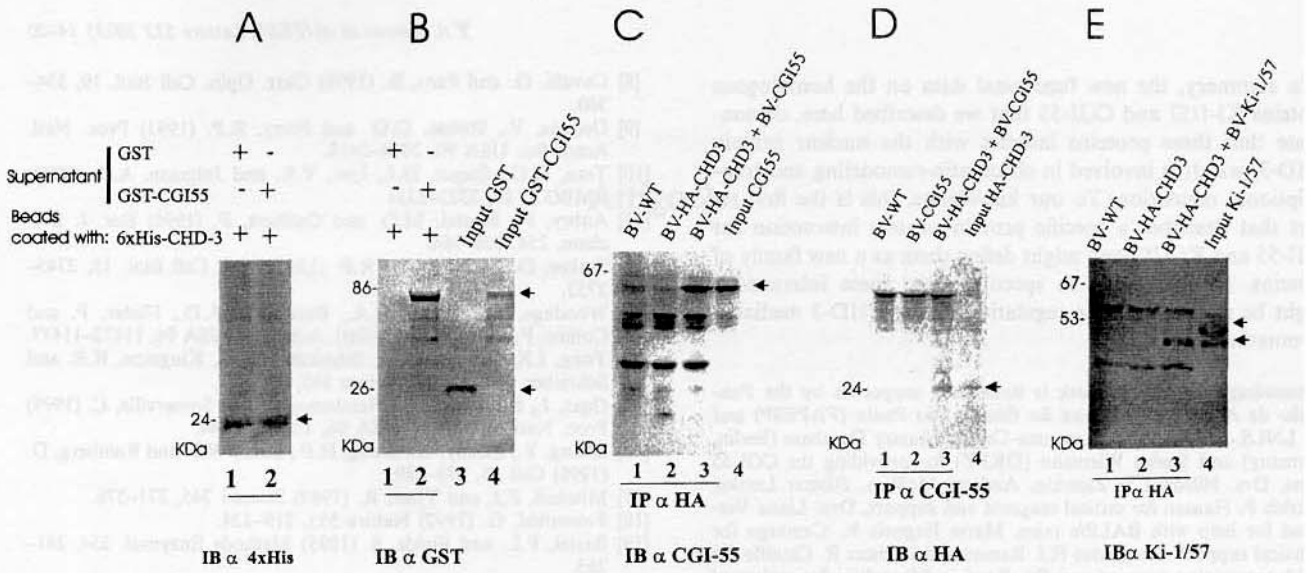

Fig. 5. In vitro binding assay and co-immunoprecipitation from *Sf*9 insect cells infected with different combinations of recombinant BV. A,B: In vitro binding assay. Ni-NTA Sepharose beads were loaded with purified 6×His-HA-CHD3(1983–2000) protein. Beads were then incubated with purified GST or GST–CGI55,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

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

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

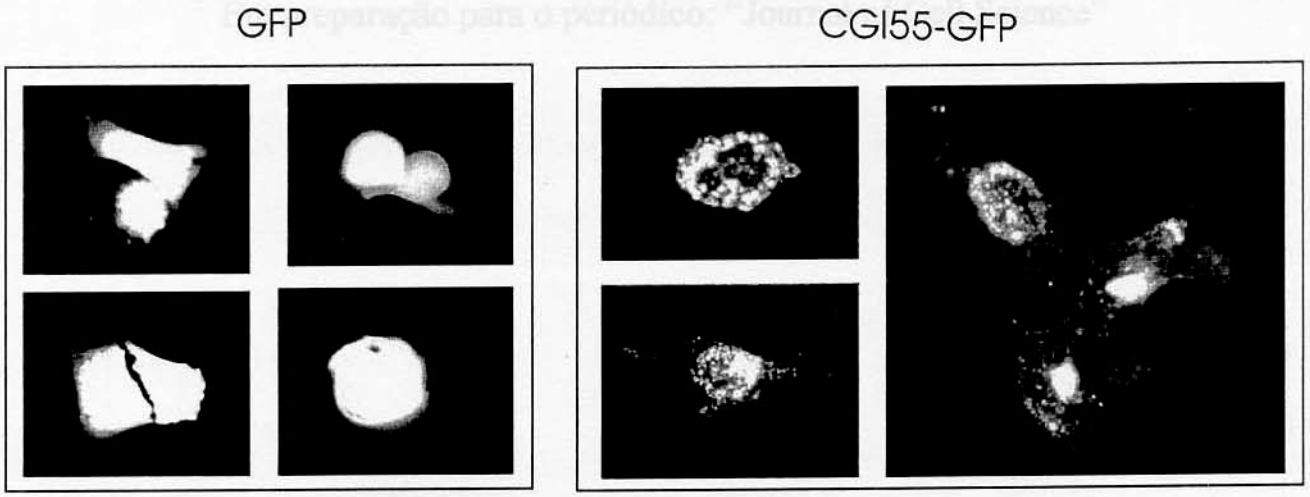

Fig. 6. Cellular Localization of CGI55–EGFP. HeLa cells were transiently transfected with expression vector pEGFP or recombinant expression vector CGI55–pEGFP-N1. After 24 h transfected cells were examined with a Nikon microscope.

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

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Artigo 2

CGI-55 interacts with proteins that are associated with PML nuclear bodies and co-localizes with the nucleolus and coiled bodies

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Em preparação para o periódico: "Journal of Cell Science"

CGI-55 interacts with proteins associated with PML nuclear bodies and co-localizes with the nucleolus and coiled bodies

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Keywords: PML-NBs, coiled bodies, protein-protein interaction, two-hybrid, domain mapping, immunolocalization

Abbreviations: GFP, green fluorescence protein, MM, minimal medium, PBS, phosphate buffered saline, NLS, Nuclear Localization Sequence.

Abstract:

The human protein CGI-55 has been described previously as a CHD-3 interacting protein (Chromo-Helicase-DNA-binding domain protein-3). A product of alternative splicing of the CGI-55 mRNA has been termed PAI-RBP and was found to interact with the 3'-region of the PAI mRNA. Here, we used CGI-55 as a bait in a yeast two-hybrid screen and identified eight new proteins that interact with CGI-55: Daxx, Topors, HPC2, UBA2, TDG and PIAS-1, -2 and-y. Several of these proteins are structurally or functionally associated with PML-nuclear bodies, protein sumoylation and the regulation of transcription. The interactions of CGI-55 with Daxx, Topors, PIASy and UBA2 could be confirmed by *in vivo* co-localization experiments in Hela cells, utilizing GFP and Red fluorescence fusion proteins. A mapping study of the CGI-55 binding site for these proteins revealed three distinct patterns of interaction. The fact that CGI-55-GFP has been localized in the cytoplasm and nucleus in a speckled fashion, together with its interaction with several proteins associated to PML-nuclear bodies, suggested that CGI-55 might be functionally associated to PML nuclear bodies. We found that CGI-55 as well as its interacting proteins co-localize with PML but that the PML-nuclear bodies only partially co-localize with the CGI-55 nuclear speckles. CGI-55 however co-localizes with the nucleolus and with p80-coilin containing nuclear coiled bodies. In summary our data are consistent with the idea that CGI-55 might be involved in important nuclear functions like the regulation of transcription or the processing of RNA.

Introduction

In the majority of patients with APL (acute promyelocytic leukemia), a distinct subtype of myeloid leukemia, the PML gene (on chromosome 15) fuses to the RARα gene (on chromosome 17) as a reciprocal and balanced chromosomal translocation (Melnick and Licht, 1999). The majority of PML protein is found in a large multiprotein complex referred to as PML-NBs (nuclear bodies), ND10 (nuclear body 10), Kremer bodies or PODS (PML oncogenic domains) (Matera, 1999). Electromicroscopy analysis reveals that the PML-NBs is a macromolecular structure of doughnut shape with 0.2 -1 micrometer in size. Cells typically contain 10-30 of these macromolecular structures, although their number and size may vary depending on cell type, cell cycle and response to external stimuli. The PML-NBs are disrupted in APL cells revealing the oncogenic potential of the PML-RARα fusion protein and underscoring the importance of the disruption of PML and PML-NB function in leukemogenesis (Lin et al. 2001, Salomoni and Pandolfi, 2002). Certain features of PML are required for association with NBs and subsequently for its physiological functions. PML contains a cystein-rich zinc finger domain (RING), two B-boxes and an adjacent leucine coiled-coil forming the RBCC motif. The RBCC motif mediates protein-protein interaction, is responsible for PML multimerization, localization to PML-NB, and heterodimerization with PML-RARα. However, does not confer DNA-binding capability (Salomoni and Pandolfi, 2002). Although the majority of PML forms NBs, some is located in cytoplasmic bodies and some remains soluble in the nucleus. Furthermore, there are several PML isoforms that vary in subcellular distribution (Borden, 2002). To date, several additional proteic components of the nuclear body have been identified, including SUMO-1, Sp100, sp140, CBP, BLM, Daxx, pBR and p53 (Zhong et

al., 2000). Involvement in various biological functions has been attributed to PML-NBs, including: tumor suppression (Salomoni and Pandolfi, 2002), cell cycle regulation (Everett et al., 1999), transcription regulation (Zhong et al., 2000), viral infection (Maul, 1998), and DNA replication and repair (Borden, 2002).

The ubiquitin-like protein SUMO-1 can bind covalently to PML at three lysine residues. Unmodified PML is associated with the soluble nucleoplasmic fraction, whereas the sumoylation leads PML to be associated with the nuclear body, suggesting that PML needs to be sumoylated in order to localize to the nuclear body. Other components of PML-NBs such as Sp100, Daxx and p53 also must be sumoylated to co-localize to nuclear bodies (Zhong et al. 2000).

Besides the PML nuclear bodies higher-eukaryotic nuclei can contain numerous distinct sub-structures that are refered to as nuclear bodies (Matera, 1999). Beside the PML nuclear bodies the other predominant nuclear bodies are termed "coiled bodies" and occur in zero to six copies per nucleus, with the most frequent beeing two (Andrade et al., 1993). The exact function of these coiled bodies (CBs) is still unclear but they are highly enriched in several classes of small ribonuclearproteins, cell-cycle control proteins and basal transcription factors, with the protein p80-coilin beeing their only unambiguous molecular marker component (Matera, 1999, Andrade et al., 1993). The protein composition of the CBs suggests that they might be important for the processing of RNA and/or transcriptional regulation (Matera et al., 1999).

The protein called CGI-55 (PAI-RBP1) has high sequences similarity with Ki-1/57 (Kobarg et al., 1997) and its homolog is also found in *C. elegans* (Lemos et al., 2003). Recently, it has been shown that CGI-55 interacts *in vivo* and *in vitro* with the chromatin-

remodeling factor CHD-3 (Lemos et al., 2003). An alternative splice variant of CGI-55 has been termed PAI-RBP1 and was identified as a PAI-1 (plasminogen activator inhibitor 1) mRNA binding protein (Heaton et al., 2001). This protein is identical with CGI-55, with the exception of a 6 amino acid deletion near residue 202 (Heaton et al. 2001). The cDNA that encodes CGI-55 has been isolated from a human fetal brain cDNA library and contains two putative ATP-binding sites, one NLS, two putative coiled-coil motifs, five candidate lysine residues that might be modified by SUMO-1, an arginine-rich region, a glycine-rich region and a glutamic acid-rich region. The C-terminal of CGI-55 contain several hyaluronan or mRNA binding motifs (Huang et al., 2000). Here, we used CGI-55 as " bait" in a yeast two-hybrid screen to identify interacting protein patterns. In addition to CHD-3, which we have described earlier elsewhere (Lemos et al., 2003), we identified another eight proteins that interacted with CGI-55: Daxx, Topors, HPC2, PIAS-1,3 and y, TDG and UBA2. Most of these proteins have been previously described as permanent or transient components of PML nuclear bodies. Our immunolocalization data demonstrate that these proteins co-localize with both CGI-55 and PML and that CGI-55 co-localizes with both the nucleolus and nuclear coiled bodies. In summary, our data suggest the functional association of CGI-55 with PML-NB and CBs and solidify the previous notion that CGI-55 engages in specific protein-protein interactions that are envolved with important nuclear functions such as transcriptional regulation and RNA processing.

Materials and Methods

Plasmid constructions

To perform the two hybrid screening, human CGI-55 (GenBank Accession number AL080119.1) was PCR amplified from a full-length cDNA clone (DKFZp564M2423Q3) and sub-cloned in frame to the lexA-DNA binding domain using the *Eco*RI and *Bam*HI sites of vector pBTM116 (Fields and Song, 1989), as described previously (Lemos et al., 2003). The CGI-55 cDNA clone has been provided by the Resource Center/Primary Database (Heubnerweg 6, D-14059 Berlin, Germany). This clone has been isolated from a human fetal brain cDNA library (DKFZhfb2) created by Stefan Wiemann (DKFZ, Heidelberg, Alemanha). For the mapping of the CGI-55 interaction site seven deletion constructs were used (Fig. 1A), which has been described previously (Lemos et al., 2003). In a similar fashion CGI-55 was cloned in frame into pEGFP-N1 to express the GFP-CGI-55 fusion protein (Lemos et al., 2003). The cDNAs encoding the HA-tag fusions HA-Topors (aa 360-1045), HA-DAXX (aa 368-740), HA-PIASy (aa 255-510 and HA-UBA2 (aa 368-640) were amplified from pACT2 and sub-cloned in frame into pdSRedC1 to express the RED-HA-DAXX, RED-HA-PIASy, RED-HA-Topors and RED-HA-UBA2 fusion proteins, respectively.

Yeast two-hybrid system screen

CGI-55 fused to the LexA DNA-binding domain and a human fetal brain cDNA library (Clontech) cloned in frame to the Gal4 activation domain (vector pACT2) were used for the screening. The two-hybrid screen was performed by a sequential transformation of

bait and then library plasmids in *Saccharomyces cereviisae* strain L40, carrying the genomically integrated reporters *LexA-HIS3* and *LexA-LacZ*. After transformation, yeast cells were plated on selective minimal medium (MM,-W-L,-H) (Vojtek and Hollenberg, 1995) and incubated at 30°C until transformants with interaction phenotype appeared. Transformants were re-streacted in duplicate in selective medium (MM,-W-L-H) and tested for β-galactosidase expression. The plasmid DNA of blue clones was isolated and amplified in *E. coli* HB101. Plasmid DNA of positive clones was sequenced and analyzed by similarity searche in databases (BLAST).

Mapping the interaction site of CGI-55

Seven N- and C-terninal deletion constructs of CGI-55 fused to LexA DNA-binding domain were co-transformed in *Saccharomyces cerevisiae* strain L40 with the "bait"-plasmid DNAs isolated from the two-hybrid screening. Complete CGI-55 was used as a positive control and an unrelated pACT-AUF1 construct (Moraes et al., 2003) was used as a negative control. After transformation, yeast clones were streak on MM,-W,-L,-H for testing their growth capacity under interaction-selective conditions. The presence of both types of plasmids was controled by growth on plates with MM, -W,-L (Vojtek and Hollenberg, 1995).

Cell culture, transient and permanent transfection and fluorescence microscopy

HeLa cells were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). For transient tranfection cells were cultured on

glass coverslips for 24 h and then transfected with recombinant vector CGI-55-pEGFP-N1 using the lipid transfection method (Lipofectamine, Invitrogen). For permanent transfection cells were grown to about 60% confluency on 24 wells plate and then transfected for 48 h with recombinant vector HA-Daxx-pDSRed-C1, HA-Topors-pDSRed-C1, HA-UBA2-pDSRed-C1 and HA-PIASy-pDSRed-C1 using the lipid transfection method (Lipofectamine, Invitrogen). After 48 h, transformed cells were incubated with DMEM containing 1 mg/ml G418 for one week. Positive clones were identified by fluorescence microscopy, isolated and maintained in DMEM with 1 mg/ml G418. Cells were washed with PBS and mounted in 80% glycerol/10 mM Tris-HCl (pH 7.5) on coverglasses and analyzed with a fluorescence microscope (Eclipse E600, Nikon). In all cases, cell nuclei were counter-stained with DAPI (2.5 μg/ml).

Immunofluorescence and antibodies

The transiently and permanently transfected cells grown on glass coverslips were washed with PBS and fixed in 100% methanol for 3 min at -20 °C. After 30 min incubation in PBS with 3% bovine serum albumin (BSA), cells were incubated overnight with primary antibodies: monoclonal mouse anti-CGI-55 (Lemos et al., 2003), polyclonal rabbit anti-HA (Y-11), monoclonal mouse anti-HA (F-7) or polyclonal goat anti-PML (A-20) (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA), as indicated in the figure legends. The antibodies were diluted 1:200 in PBS with 1% BSA, except for anti-CGI-55, which was used as hybridoma culture supernatant. After washing in PBS, 1% BSA, 0,2% Tween 20, the cells were incubated for 1 h with the secondary antibodies FITC-conjugated anti-mouse, rhodamine-conjugated anti-rabbit or rhodamine-conjugated anti-goat (Santa Cruz

Biotechnology Inc., Santa Cruz, CA), diluted 1:1000 in PBS with 1% BSA. After additional washes, cells were analyzed by fluorescence microscopy as above. Rhodamine was detected using a rhodamine filter set G-2E/C TRIC (excitation and emission at 528-553 nm and >565 nm, respectively), whereas FITC fluorescence was detected using fluorescein filter set B-2E/C FITC (excitation and emission at 465-485 nm and >505 nm, respectively).

For co-localization of CGI-55 to coiled bodies the transiently transfected, CGI-55-GFP expressing HeLa cells were incubated for 2 hours at 25°C with primary polyclonal antibody rabbit anti-human p80-coilin R288 (Andrade et al., 1993) diluted 1:100 in PBS with 1% BSA. This antibody had been kindly provided by Dr. L.E.C. Andrade (Fleury, São Paulo). As secondary antibody we used rhodamine-conjugated anti-rabbit antibody. CGI-55 was detected and cells were analyzed as described above.

Results

Identification of proteins that interact with CGI-55

CGI-55 is a new protein of unknown function. We performed a yeast two-hybrid system screen, in order to identify proteins interacting with CGI-55 and therefore get a first hint on the functional context of the protein. 5 x 10^6 tranformants were tested, of which 582 grew in minimal medium without histidine, 125 were of blue color in the β-galactosidase assays and DNA sequences of over 20 clones were retrieved. The 40% of clones represented the chromatin remodelling factor CHD-3. This interaction could be confirmed by *in vivo* and *in vitro* tests and has been published previously (Lemos et al., 2003). HPC2

(human polycomb homolog 2) that is associates with the modulation of the chromatin structure such as CHD-3 was identified interacting with CGI-55. The other proteins that were identified as CGI-55 interacting partners are summarized in Table 1. Seven of these proteins have previously been described to localize to PML-NBs and/or to be involved in the SUMO modification process.

The CGI-55-interacting proteins involved in SUMO-1 modification are: three members of the PIAS family of proteins. PIAS1 and PIASy are inhibitors of activated STAT1 and PIAS 3 is inhibitor of activated STAT3. In addition to this STAT inhibition activity PIAS family proteins were described as sumoylation catalyzing enzymes or SUMO-ligases (Kotaja et al., 2002). Furthermore the SUMO-1 activating enzyme subunit 2 (UBA2) was identified as an CGI-55 interacting protein.

The second group of proteins that interact with CGI-55 were associated with PML-NBs: Topors: Topoisomerase I binging RS protein (Topors), which is also known as p53-binding protein and Daxx. There is relationship between these two groups of proteins, since most proteins found in PML-NB, including PML itself are sumoylated.

Three patterns of interaction for CGI-55

To delineate the region(s) of CGI-55 that engage in interactions with the proteins identified in the yeast two-hybrid assays, various deletion constructs of CGI-55 were generated as shown in Figure 1A. We were able to identify three basic patterns of interaction as shown in Table 2: proteins that interact just with full length CGI-55 (group A), the protein UBA-2 which interacts only with the C-terminal region of CGI-55 ("group" B) and finally proteins that interact with both the C- and N- terminal regions of CGI-55, but

fail to interact with the deletion mutant 4 which represents the central region of CGI-55 (group C).

Since the N-terminal region of CGI-55 contains a putative coiled-coil motif, we also analyzed the interacting proteins for the presence of such coiled-coil regions. The interacting proteins can be separated in three groups: i) proteins with several coiled-coil regions throughout the whole protein sequence (Daxx), ii) proteins with a single coiled-coil at the C-terminal (Topors, PIASy, UBA2 and TDG) and iii) proteins without any coiled-coil (HPC2, PIAS-1 and -3). Prediction for coiled-coil structures for CGI-55 and for the CGI-55-interacting proteins was performed by using the COILS-software available at the web site www.ch.embnet.org/software/ COILS_form.html of the Swiss Institute for Experimental Cancer Research, using windows of 14 and 21 amino acids.

These data indicate, that the interactions of CGI-55 and the proteins identified by the two hybrid screen do not seem to depend on the presence of coiled-coil structures.

Cellular localization of CGI-55

We have recently shown that GFP-CGI-55 appears in a speckled pattern in the cytoplasm, perinuclear region and nucleus of Hela cells (Lemos et al. 2003). Such a speckled pattern has been observed previously for other proteins, including several proteins that we found to interact with CGI-55: PIAS-1, 3 and y (Valdez et al., 1997, Miyauchi et al., 2002), Topors (Haluska et al., 1999, Zhou et al., 1999, Rechsteiner and Rogers,1996, Rasheed et al., 2002) and Daxx (Torii et al., 1999, Ishov et al., 1999).

HeLa cells were permanently transfected with HA-Daxx, HA-Topors, HA-PIASy, HA-UBA2 or transiently transfected with CGI-55-GFP as indicated in the legend of Fig. 2, counter-stained with DAPI and analyzed by fluorescence microscopy.

The overexpressed CGI-55-GFP fusion showed the speckled pattern with the same distribution as described above. However, the endogenous CGI-55, which was detected by indirect immunofluorescence, using a specific anti-CGI-55 monoclonal antibody, showed a slightly different staining pattern, with a dotted but more difuse overall staining and a marked labelling of the perinuclear region, the nueleoli and nuclear speckles (Figs. 2 and 5, first columns)

The RED-HA-Daxx(368-750) fusion protein showed a diffuse, speckled pattern in the cytoplasm, perinuclear and nuclear regions (Fig. 2, 4). Lin et al. (2003) had previously observed that HA-Daxx (aa 1-625) was cytoplasmic, whereas a C-terminal fragment of HA-Daxx(501-740) was nuclear. This suggests that the C-terminal region of Daxx (aa501-740) is required for the nuclear localization.

RED-HA-Topors(360-1045) expressed in Hela cells exhibits a pattern of cytoplasmic, perinuclear and nuclear dots (Fig. 2, 4). Although GFP-Topors has been described as nuclear dots associated to PML-NBs (Haluska et al., 1999, Zhou et al., 1999, Rechsteiner et al., 1997, Rasheed et al., 2002), GFP-Topors lacking residues 540-704 and 705-1045 localized diffusely in nuclei (Rasheed et al., 2002).

Cytoplasmatic (Fig. 2) and nuclear dots (Fig. 4) were observed when Red-HA-PIASy(255-510) was expressed in Hela cells. PIAS has been described as located to nuclear dots that are associated with SUMO-1 previously (Valdez et al., 1997, Miyauchi et al., 2002).

The RED-HA-UBA2(368-640) localized diffusely in the cytoplasm and nuclei (Figs. 2, 4). The yeast homolog of UBA2p is also located in the nucleus (Dohmen et al., 1995), but Shih et al., 2002 found the *Drosophila melanogaster* homolog *Dm*Uba2 at the nucleus, organized in the form of caps corresponding to the cortical actin caps that form over the nucleus, but also in the deeper cytoplasm, depending on the cell cycle period.

CGI-55-GFP co-localizes *in vivo* with its interacting proteins

In Hela cells transiently transfected with CGI-55-GFP and permanently transfected with RED-HA-Daxx, both proteins show co-localization, as clearly demonstrated by the merge of the DAXX and CGI-55 images (Fig. 3). Indeed the localization of Daxx in PML-NBs (Torri et al., 1999, Li et al., 2000, Ishov et al., 1999) has been demonstrated when Daxx and ASK1 were co-overexpressed and Daxx localization was changed from the nucleus to the cytoplasm (Ko et al., 2001). The overexpression of the nucleolar protein MSP58 alleviates the transcriptional repression elicited by Daxx, correlating with the sequestration of Daxx to the nucleolus via Daxx/MSP58 interaction (Lin et al., 2003). This result suggests that a CGI-55/Daxx complex might co-translocate from the nucleus to the perinuclear region. CGI-55-GFP also co-localizes with RED-HA-Topors (Fig. 3), and RED-HA-PIASy (Fig. 3), at perinuclear region suggesting these proteins might be sequestered to the perinuclear region by protein-protein interactions with CGI-55. RED-HA-UBA2 has been shown in perinuclear region and cytoplasm also co-localizes with CGI-55 (Fig. 3).

Co-localization of CGI-55 and its interacting proteins with PML

Hela cells were transfected with the RED-HA-Daxx, RED-HA-Topors, RED-HA-PIAS and RED-HA-UBA2 and then co-immunostained with anti-PML and anti-HA antibodies. HA-Daxx appears as dots, diffusely in the nucleus and cytoplasm, and interestingly also at the nucleoli (Fig. 4), in a similar pattern as seen with endogenous CGI-55 (Fig. 2). Most of the Daxx dots coincide with the PML spots, and only a few dots do not overlap. This confirms the known finding, that Daxx has been described as associated with PML (Torri et al. 1999, Li et al, 2000, Ishov et al. 1999). Other data show, that Daxx when associated with Ask1 is found in the cytoplasm (Ko et al. 2001), and when interacting with MSP58 at the nucleoli (Lin et al. 2003). Although several dots coincide, Topors does not co-localized with PML (Fig. 4) to the same extent as Daxx. Rasheed and co-workers (2001) already have described that in some cells GFP-Topors dots do not have corresponding PML dots, and suggest that GFP-Topors may also localize to nuclear bodies distinct from PML-NBs. Besides, Topors was found in the cytoplasm and nucleoli, like CGI-55 (Fig. 2).

The HA-PIASy nuclear dots colocalize only partially with those of the PML-NB spots (Fig. 4). Such a co-localization has not been described previously for PIASy, although PIAS 1 and 3 have been described as SUMO-1 ligases and colocalized with SUMO at nuclear dots (Kotaja et al. 2002). Some proteins such as Daxx are located at PML-NBs only after SUMO-1 modification (Li et al. 2000). The co-localization of PIASy and PML may suggests that PIAS could function as a SUMO-1 ligase and might cause the translocation of sumolated proteins such as PML and DAXX to the PML-NBs.

In contrast to the finding, that PML localizes to the nucleus only after its modification by SUMO-1 (Muller et al., 1998), we only found a partial co-localization of

PML with HA-UBA2 (Fig. 4). PML shows a dotted pattern in both cytoplasm and nucleus, whereas UBA2 has a more diffuse staining predominantly in the cytoplasm. *Drosophila melanogaster* Uba2 has been found in the nucleus, perinuclear region and also in the deeper cytoplasm, depending on the cell cycle period (Shih et al., 2002).

CGI-55-GFP and endogenous CGI-55 showed a dotted pattern both in the cytoplasm and in the nucleus, with a prominent staining of the nucleolus in the latter (Fig. 5, 2). Some of the CGI-55-GFP speckles co-localized with PML-NB in the nucleus (Fig. 5), although we cannot rule out that these speckles also co-localize with non-PML nulear bodies. Endogenous CGI-55 however, showed a lesser degree of co-localization with PML, suggesting that CGI-55 co-localizes with PML only when overexpressed as a CGI-55-GFP fusion protein or that endogemous CGI-55 is expressed at low levels.

CGI-55 co-localizes with nuclear coiled bodies

Since we did not observe a lot of co-localization of the CGI-55 positive nuclear speckles with the PML-NB, we speculated that they might more clearly co-localize with other nuclear bodies, such as coiled bodies. To test this hypothesis we performed immuno co-localization studies of CGI-55 with the only unambiguous coiled body protein marker p80-coilin (Matera, 1999, Andrade et al., 1993). For a stronger CGI-55 signal we again overexpressed CGI-55-GFP by transiently transfecting HeLa cells and detected it with antibody 10.5.6, whereas we used antiserum R228 (Andrade et al., 1993) to detect p80-coilin (Fig. 6). We found that the p80 positive nuclear bodies were also labeled by our anti-CGI-55 monoclonal antibody 10.5.6 (Fig. 6, white arrows). Again, we observed additional smaller nuclear spots in the anti-CGI-55 labelled cells (Fig. 6, CGI-55 upper panel), and

also labelling of the nucleolus (Fig. 6, CGI-55, lower panel). These results clearly demonstrate that CGI-55 co-localizes with p80-coilin positive nuclear coiled bodies. Furthermore, they suggest that CGI-55 might traffic between the nucleolus, coiled bodies and other not yet characterized nuclear sub-domains including PML-NB. Such a traffic between distinct intranuclear compartments has been proposed for other nuclear proteins such as the small nucleoproteins (Matera, 1999).

Discussion

CGI-55 is a 55 kDa protein which has been described to interact with the chromatin remodeling factor CHD-3 (Lemos et al., 2003), however its exact function remains to be elucidated. The mRNA of CGI-55 is expressed in all tissues analyzed but highly expressed in heart, skeletal muscle, kidney and placenta (Lemos et al., 2003). A variant of CGI-55, which is probably generated by alternative splicing, lacks six amino acids and has been termed PAI-RBP1 (Heaton et al., 2001). Heaton et al. identified PAI-RBP1 as a PAI-1 mRNA-binding protein. The fact that PAI-RBP1 has an RGG box, RG-rich and Arg-rich motifs, might place it in the general category of RNA-binding proteins (Heaton et al., 2001). In addition CGI-55 has two putative ATP-binding sites, a nuclear localization signal, two putative coiled coil regions and regions rich, glutamic acid and five lysine residues that could covalently bind SUMO-1. CGI-55, like its related molecule Ki-1/57 (IHABP4) contains several putative hyaluronic acid binding motif (Huang et al., 2000).

We performed a yeast two-hybrid screen with CGI-55 and identified besides CHD-3 (Lemos et al., 2003) eight proteins that interact with CGI-55: Daxx, Topors, PIAS-1, -3 and

-y, HPC2, TDG and UBA2. The identification of these interactions suggests that CGI-55 might not only interact with negatively charged macromolecules such as RNA or hyaluronate but also engages in highly specific protein-protein interaction, which might be important for its modification and function and allows to speculate about its functional cellular context in the regulation of gene expression, apoptosis and oncogenesis.

Three clones coding the protein Topors were identified. Two of them encompass both the topoisomerase-binding and p53-binding domains at its C-terminal, the third one encompasses the DNA-binding and ZFC3HC4 domains from the N-terminal region. Topors has been identified independently in yeast two-hybrid screens as Topoisomerase I binging RS protein (Haluska et al., 1999) and a also as the p53-binding protein 3 (p53BP3) (Zhou et al., 1999). Chu et al. (2001) showed that Topors as a protein that is highly expressed in the lung and gave it a third name LUN. Topors contains a RING-type zinc finger domain, a bipartite nuclear localization signal and a region rich in arginines and serines (RS domain). In addition, Topors features five streches of amino acids enriched in proline, glutamine, serine and threonine (PEST sequences), which are characteristic of several rapidly degraded proteins (Rechsteiner and Rogers, 1996). The region encompassing residues 51-375 contains the ring finger motif, leucine zipper and coiled-coil regions and binds to DNA (Chu et al., 2001). The presence of RING finger and RS domains may suggest that topors is involved in RNA polymerase II-mediated transcription and mRNA processing. The localization of GFP-Topors fusion protein in punctuated nuclear sub-domains is consistent with these hypotheses (Haluska et al. 1999).

Daxx was first identified in a yeast two-hybrid screen for cDNAs encoding proteins capable of binding to the cytosolic domain of the Fas receptor, an apoptosis-inducing

member of the tumor necrosis factor (TNF) receptor family (Yang et al., 1997). In our screen with CGI-55, two Daxx clones were identified, one missing only 59 aa at its N-terminal and the other representing aproximately the C-terminal half of Daxx. Lin et al. (2003) showed that Daxx, when missing its C-terminal region (aa 501-740), failed to interact with the glucocorticoid receptor (GR). They further showed that the Daxx C-terminal region not only interacts with GR but also represses its transcriptional activity. Lin et al. suggested that Daxx C-terminal contains a major docking domain for protein-protein interactions because these region has been reported to interact with several transcriptional factors, such as Pax3, Pax5 and ETS1 (Torri et al. 1999, Lin et al., 2003). Emelyanov et al. 2002) showed that Daxx binds as well as a wide variety of other molecules, including Fas receptor, PML, centrometric protein CENP-C, DNA methyltransferase I, HSP27, Glut4, Ubc9 and SUMO-1.

HPC2 is a member of the polycomb protein family and was identified in our two-hybrid screen as a CGI-55-interacting protein. HPC2 and other members of the Drosophila polycomb (PC) gene family are part of a cellular system that is responsible for the inheritance of gene activity by progeny cells (Satijn et al., 1997). HPC2 shares a homologous domain known as the chromodomain with the Drosophila heterochromatin-binding protein, HP1. Using immunofluorescence, Satijn et al. (1997) found that human HPC2 co-localizes with another human PC homolog, CBX2, in interphase nuclei, suggesting that these proteins are part of a larger complex. Based on the results of studies with mutant proteins and overexpression of wildtype protein, these authors suggested, that human PC2 is a repressor of proto-oncogene activity and that interference with human PC2

function could lead to derepression of protoncogene transcription and possibly to cellular transformation.

Three members of the PIAS (protein inhibitor of activated STAT) family of proteins were identified to interact with CGI-55: PIAS-1, -3 and -y. Two of them contain the complete Zinc finger domain Zf-Miz and the clone encoding PIAS1 contains the C-terminal half of this domain. PIAS1 was first identified using a yeast two-hybrid screen with a portion of STAT1 as a bait (Liu et al., 1998). Functionally PIAS1 inhibited STAT1-mediated gene activation in response to interferon and co-immunoprecipited specifically with STAT1 *in vivo* (Liu et al., 1998). Furthermore PIAS1, but not the other analyzed PIAS proteins, inhibited the DNA binding activity of STAT1 *in vitro*. These results suggested that PIAS1 is a specific inhibitor of STAT1-mediated gene activation and that this inhibition is mediated through the blocking of the STAT-DNA interaction (Liu et al., 1998). On the other hand, Valdez et al., (1997) identified PIAS1 as an Gu-binding protein (GuBP) in yeast two-hybrid studies. Gu is an RNA helicase II, which belongs to the DEAD box family of proteins (Valdez et al, 1997, Gorbalenya and Koonin, 1991). Using immunofluorescence, Valdez and colaborators found that epitope-tagged GuBP (=PIAS1) localized to the nucleus in a speckled, diffuse pattern. PIAS1 was also found associated with SUMO-1, p53 and UBC9 (Kotaja et al., 2002, Kahyo et al., 2001, Schmidt and Muller, 2002). Mutations in its RING finger-like domain still allowed binding of p53 and SUMO1, but not of UBC9 (Kotaja et al., 2002).

PIAS-3 (inhibitor of activated STAT3) was first identified using PIAS1 in EST database searches (Liu et al., 1998). Ueki et al. (1999) independently identified a PIAS-3 cDNA encoding a deduced 619-amino acid protein that has a sequence identity of 56% with

PIAS1 and 39% with PIASy. PIAS-3 binds to microphthalmia-associated transcription factor (MITF), a DNA-binding protein in rat basophilic leukemia cells and mouse melanocytes (Levy et al., 2002). Levy et al.(2002) observed that PIAS3 can block MITF DNA-binding activity in vitro, and that co-transfection of MITF and PIAS3 in NIH-3T3 cells inhibits MITF-driven transcription activity.

Recent data suggest that PIAS proteins can function as SUMO ligases, or possibly as a tightly bound regulators of sumoylation (Kahyo et al., 2001, Kotaja et al., 2002, Schmidt and Muller, 2002, Jackson, 2001). PIAS1, for example, catalyzed the sumoylation of p53 both in U2OS cells and *in vitro* in a domain-dependent manner (Kotaja et al., 2002)

Most interestingly and in agreement with the finding that PIAS proteins might act as SUMO ligases, we also found the SUMO-1 activating enzyme subunit 2 (UBA2) as an CGI-55 interacting protein in our two-hybrid screen. The CGI-55-interacting UBA2 clone contains the UBACT domain. SUMO activating enzyme is a heterodimeric enzyme that consists of Aos1 (Sua1, SAE1) and UBA2 (SAE2) (Desterro et al., 1999, Gong et al., 1999, Okuma et al., 1999). Aos1 and UBA2 form thioesters bonds with SUMO-1, -2 and -3 (Gong et al. 1999), and are required for the SUMO-1 modification of IκBα (Desterro et al., 1999), p53 (Rodriguez et al., 1999) and RanGAP1 (Okuma et al., 1999).

Finally, we identified TDG (Thymine DNA-glycosylase) as an CGI-55 interacting protein in the yeast two-hybrid screen. TDG was first identified by Neddermann et al. (1996) and mediates the repair of G/T and G/U mismatches, which are commonly associated with CpG islands, by removing the thymine and uracil moieties. G/U and G/T mismatches are generated by either misincorporations during replication or by spontaneous hydrolytic deaminations of the cytosine or 5-methyl cytosine bases (Lindahl, 1982). In

addition, TDG has been described to be associated with the transcriptional co-activators CBP and p300 and the resulting complexes are competent for both the excision step of the DNA repair as well as for histone acetylation (Tini et al., 2002). TDG stimulates CBP transcriptional activity in transfected cells and also serves as a substrate for protein acetylation by CBP/p300. A acetylation of TDG triggers the release of CBP from the DNA and also regulates the recruitment of the repair endonuclease APE.

Most of the CGI-55 interacting proteins are either structurally (Daxx, Topors) or functionally (PIAS-1, -3 and -y, UBA2) associated with PML-nuclear bodies (NBs) and with the process of protein sumoylation (PIAS-1, -3 and –y, UBA2). PML-NBs are important nuclear sub-domains that are involved in transcriptional regulation, apoptosis, cell cycle control. Because of its association to these proteins, CGI-55 might be involved in the same processes. Our fluorescence microscopy analysis showed that Daxx, Topors, PIASy and UBA2 co-localized with GFP-CGI-55, suggesting that these proteins also interact with CGI-55 *in vivo* in human cells. Daxx (Torri et al., 1999, Li et al., 2000, Shih et al., 2002), Topors (Rasheed et al., 2002) and SUMO-1 (Muller et al., 1998, Boddy et al., 1996) were described to localize to PML-NBs, so CGI-55 could be localized at these structures, too. Immunolocalization showed that CGI-55-GFP co-localizes only partially with PML-NBs but principally with other, non PML-containg nuclear bodies, including p80-coilin positive coiled bodies.

The association of CGI-55 to PML and coiled bodies may depend on its SUMO modification. This has been described for several other proteins, including PML itself as well as Daxx, p53 and Sp100 (Gong et al., 1999). Zhong and co-workers (2000) proposed a

model where PML needs first to be sumoylated and then recruits others components, also sumoylated, to start the formation of PML nuclear bodies.

We have found that CGI-55 interacts with the SUMO activating enzyme subunit 2 UBA2 (Desterro et al., 1999) and a with the SUMO ligases PIAS1 and -3 (Kahyo et al., 2001, Kotaja et al., 2002, Schmidt and Muller, 2002, Jackson, 2001), indicating that CGI-55 might be a substrate for sumoylation. This hypothesis is supported by the observation that CGI-55 has five possible sumoylation sites in the central region. Mapping CGI-55 binding sites to its interacting proteins, we found that none of the proteins interacted with the central region of CGI-55, suggesting that it could be blocked by SUMO.

In summary, GCI-55 interacts and co-localized with proteins associated with PML-NBs and with proteins that are either targets of sumoylation or itself involved in the process of sumoylation. Furhtermore CGI-55 co-localizes with the nucleolus and nuclear bodies coiled bodies. These data are suggestive of the idea that CGI-55 might be involved in nuclear functions like transcriptional reguation or RNA processing and that it might be a target of sumoylation. Future studies will have to address if CGI-55 is sumoylated and what are the functional consequences of such a modification.

Acknowledgements

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Figure Legends

Fig. 1. Mapping of the regions of CGI-55 that are involved in the interaction with the proteins identified in yeas two-hybrid screen. (A) Various N- and C-terminal truncations of human CGI-55 were fused in frame to the DNA-binding domain of *Lex*A in plasmid pBTM116 and transformed into yeast strain L40 together with the fusions: Topors-Gal4-AD, PIAS1-Gal4-AD and UBA2-Gal4-AD cloned in pACT2. (B) Interaction was determined by the ability of the co-transformed cells to grow on MM,-W,-L,-H (lower panels). Presence of "bait" and "prey" plasmids in the co-transformed cells was controlled by growth on MM-W,-L (upper panels).

Fig. 2. Cellular Localization of CGI-55-GFP, endogenous CGI-55, Red-HA-Daxx, Red-HA-Topors, Red-HA-PIASy and Red-HA-UBA2. HeLa cells were transiently transfected with the expression vector CGI-55-pEGFP-N1 or permanently transfected with vectors pDSRed1-C1-Daxx, pDSRed1-C1-Topors, pDSRed1-C1-PIASy or pDSRed1-C1-UBA2. Cells were grown on glass coverslips and double-stained with DAPI. Endogenous CGI-55 was detected by immunostaining via primary mouse monoclonal antibody 10.5.6 (Lemos et al., 2003) and fluorescein-labeled secondary anti-mouse antibody. Cells were examined with a Nikon microscope. DAPI staining revealed the position of the nucleus. Immunolabelled CGI-55 protein or transfected proteins are visualized with the respective colors as indicated in the upper right corners of the images. The bottom row of images shows the superimposition of the protein and DAPI stainings.

Fig. 3. Immuno co-localization of CGI-55-GFP and Red-HA-Daxx, Red-HA-Topors, Red-HA-PIASy and Red-HA-UBA2. HeLa cells permanently transfected with pDSRed1-C1-HA-Daxx, pDSRed1-C1-HA-Topors, pDSRed1-C1-HA-PIASy and pDSRed1-C1-HA-UBA2 were transiently transfected with recombinant expression vector CGI-55-pEGFP-N1. These cells were grown on glass coverslips, fixed in 100% methanol and immunostained with primary mouse monoclonal antibody anti-CGI-55 (10.5.6) and rabbit polyclonal anti-HA (Y-11). As secondary antibodies were used fluorescein anti-mouse and rhodamine conjugated anti-rabbit. All cells were also stained with DAPI and examined with a Nikon microscope. Immunolabeled proteins are indicated in the right upper corners of the individual panels. Superimposition of the green and red fluorescence colours (Merge,

fourth column) was visualized as yellow. The fifth column shows the merged images of CGI-55 and DAPI.

Fig. 4. Immuno co-localization of Red-HA-Daxx, Red-HA-Topors, Red-HA-PIASy, Red-HA-UBA2 with PML. HeLa cells permanently transfected with pDSRed1-C1-Daxx, pDSRed1-C1-Topors, pDSRed1-C1-PIASy or pDSRed1-C1-UBA2 were grown on glass coverslips, fixed in 100% methanol and immunostained with primary mouse monoclonal antibody anti-HA (F-7) and goat polyclonal anti-PML (A-20). The secondary antibodies were: fluorescein-labeled anti-mouse and rhodamine-conjugated anti-goat. Cell nuclei were stained with DAPI and cells were examined with a Nikon microscope. Immunolabeled proteins are indicated in the upper right corners of the panels. Superimposition of the green and red fluorescence colors (Merge, fourth column) was visualized as yellow. The fifth column shows the merged images of the indicated proteins and DAPI.

Fig. 5. Immuno co-localization of CGI-55-GFP or endogenous CGI-55 with PML. HeLa cells were (first row) or were not (second row) transiently transfected with recombinant expression vector CGI-55-pEGFP-N1, fixed in 100% methanol and immunostained with primary monoclonal antibody mouse monoclonal anti-CGI-55 and goat polyclonal anti-PML (A-20). The secondary antibodies were: fluorescein-labeled anti-mouse and rhodamine-conjugated anti-goat. Cell nuclei were stained with DAPI and cells were examined with a Nikon microscope. Immunolabeled proteins are indicated in the right upper corners of the panels. Superimposition of the green and red fluorescence colors

(Merge, fourth column) was visualized as yellow. The fifth column shows the merged images of CGI-55 and DAPI.

Fig. 6. Immuno co-localization of CGI-55-GFP with coiled bodies. HeLa cells were transiently transfected with recombinant expression vector CGI-55-pEGFP-N1, fixed in 100% methanol and immunostained with primary monoclonal mouse antibody 10.5.6 (anti-CGI-55) and rabbit polyclonal anti-R288 anti-p80-colin antibody (Andrade et al., 1993). The secondary antibodies were: fluorescein-labeled anti-mouse and rhodamine-conjugated anti-rabbit. Cell nuclei were stained with DAPI and cells were examined with a Nikon microscope. Immunolabeled proteins and stainings are indicated in the upper regions of the panels. Superimposition of the green and red fluorescence colors (Merge, fourth column) was visualized as yellow. The white arrows indicate the co-stained nuclear coiled bodies. The fifth column shows the merged images of CGI-55 and DAPI.

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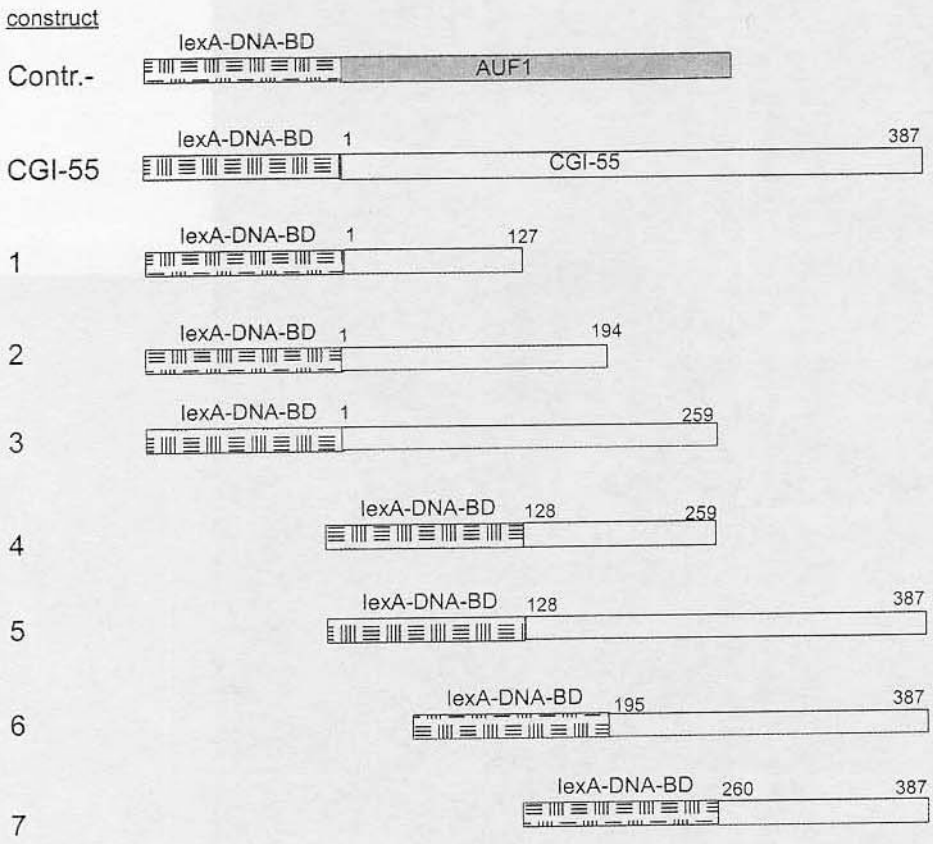
Table 1: Characteristics of the CGI-55 interacting proteins as identified in the yeast two-hybrid screen.

Proteins that interact with CGI-55	Coded protein residues (retrieved/full-size)	Domain composition	Function	References
DAXX	60-740/740 368-740/740	Glutamic acid rich region, JNK activator motif	Apoptose, transcription regulation	Torii et al.,(1999)
Topors	360-1045/1045 360-820/1045 100-600,1045	DNA-binding domain, topoisomarase I binding domain, p53 binding domain, Zf-C3HC4 motif	RNA polymerase II- mediated transcription and/ or transcriptional processing	Haluska et al.,(1999)
PIAS1	360-655/655	SAP domain, Zf-MIZ motif	Protein inhibitor of activated STAT 1	Liu et al., (1998)
PIAS3	204-619/619	Zf-MIZ motif	Protein inhibitor of activated STAT 3	Chung et al., (1997)
PIASy	255-510/510	SAP domain, Zf-MIZ motif	Protein inhibitor of activated STAT 1	Liu and Shuai, (2001)
HPC2	63-420/550	Chromo domain	derepression of proto-oncogene, regulation of transcription , cellular transformation	Satijn et al., (1997)
TDG	180-410/410	DNA-Glycosylase domain	DNA repair	Neddermann et al., (1996)
UBA 2	368-640/640	Thif domain, UBACT domain	activates SUMO-1	Okuma et. al., (1999)
CHD3	1551-2000/2000 1807-2000/2000	2 PHD, 2 chromo domain, 2 helicase domain, DNA-binding domain	Chromatin- remodeling, Transcription regulation	Lemos et al., (2003)

Table 2: Mapping of the binding sites of CGI-55 with interacting proteins as identified by the yeast two-hybrid screen.

Protein	Positive control CGI-55	Negative control AUF-1	Deletions 1	2	3	4	5	6	7	Group
Daxx	+	-	-	-	-	-	-	-	-	A
Topors	+	-	-	-	-	-	-	-	-	
UBA-2	+	-	-	-	-	-	-	+	-	B
PIASy	+	-	-	+	+	-	+	+	-	
PIAS-1	+	-	-	+	+	-	+	+	+	
PIAS-3	+	-	-	+	+	-	+	+	+	C
HPC2	+	-	-	+	-	-	+	+	+	
TDG	+	-	-	+	-	-	+	+	+	
CHD3	+	-	+	+	+	-	+	+	+	

A


construct
Contr.-
lexA-DNA-BD
AUF1
CGI-55
lexA-DNA-BD 1 387
CGI-55
1
lexA-DNA-BD 1 127
2
lexA-DNA-BD 1 194
3
lexA-DNA-BD 1 259
4
lexA-DNA-BD 128 259
5
lexA-DNA-BD 128 387
6
lexA-DNA-BD 195 387
7
lexA-DNA-BD 260 387

B

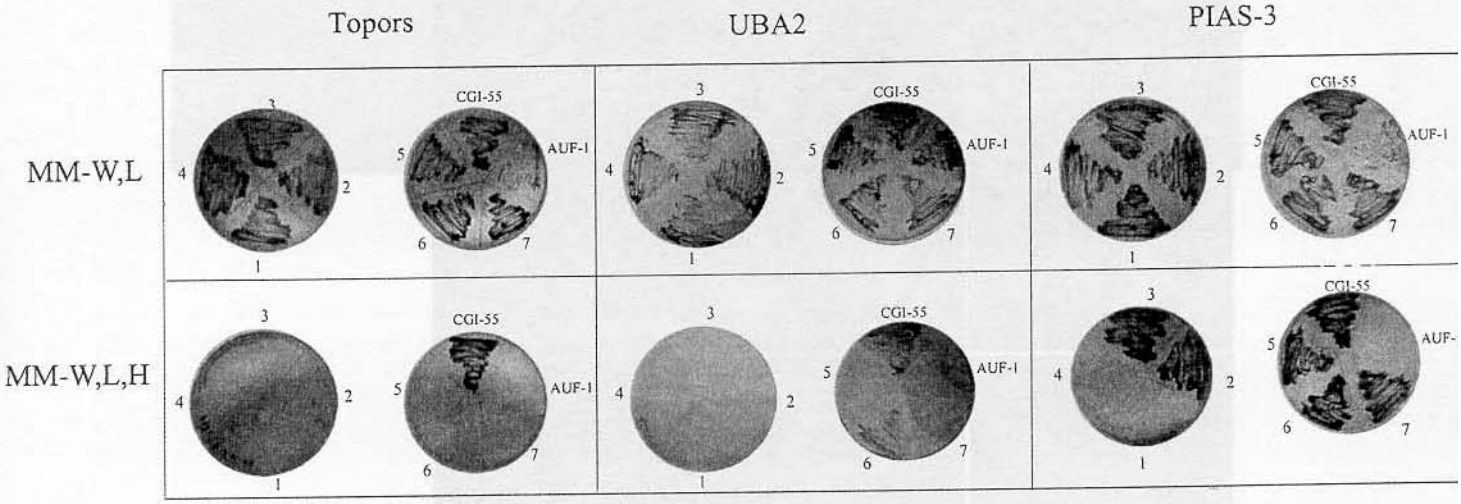
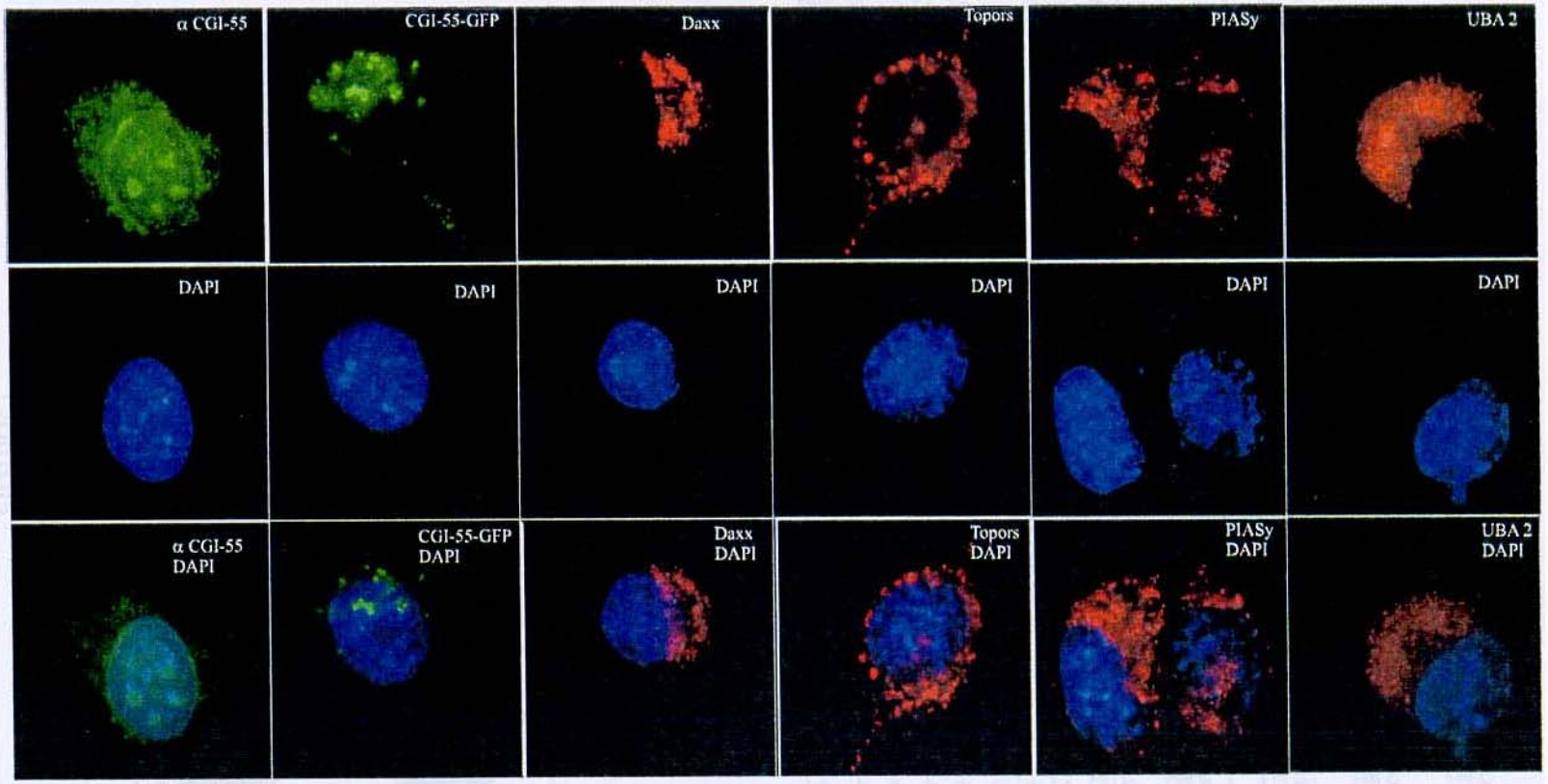

Topors
UBA2
PIAS-3
MM-W,L
MM-W,L,H
CGI-55
AUF-1
1
2
3
4
5
6
7

FIGURE 1


α CGI-55
CGI-55-GFP
Daxx
Topors
PIASy
UBA 2
DAPI
DAPI
DAPI
DAPI
DAPI
DAPI
α CGI-55
DAPI
CGI-55-GFP
DAPI
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DAPI
PIASy
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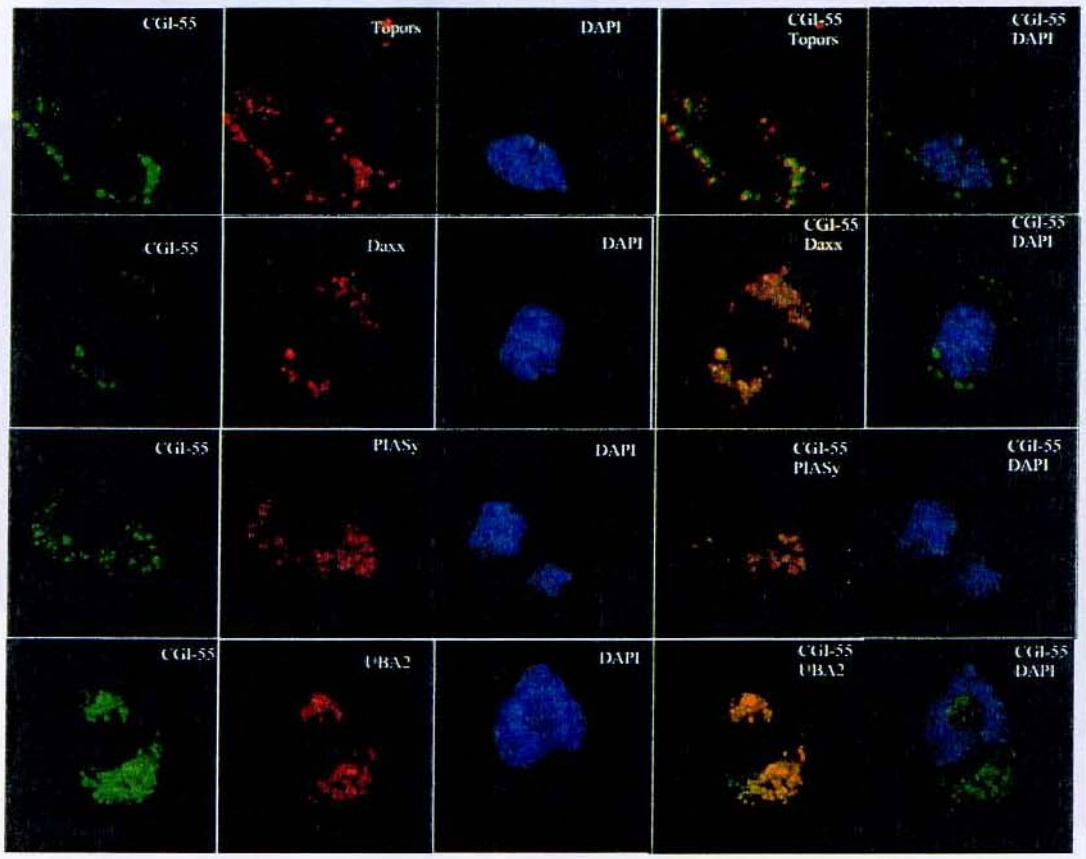
UBA 2
DAPI

FIGURE 2


CGI-55
Topors
DAPI
CGI-55
Topors
CGI-55
DAPI
CGI-55
Daxx
DAPI
CGI-55
Daxx
CGI-55
DAPI
CGI-55
PIASy
DAPI
CGI-55
PIASy
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DAPI
CGI-55
UBA2
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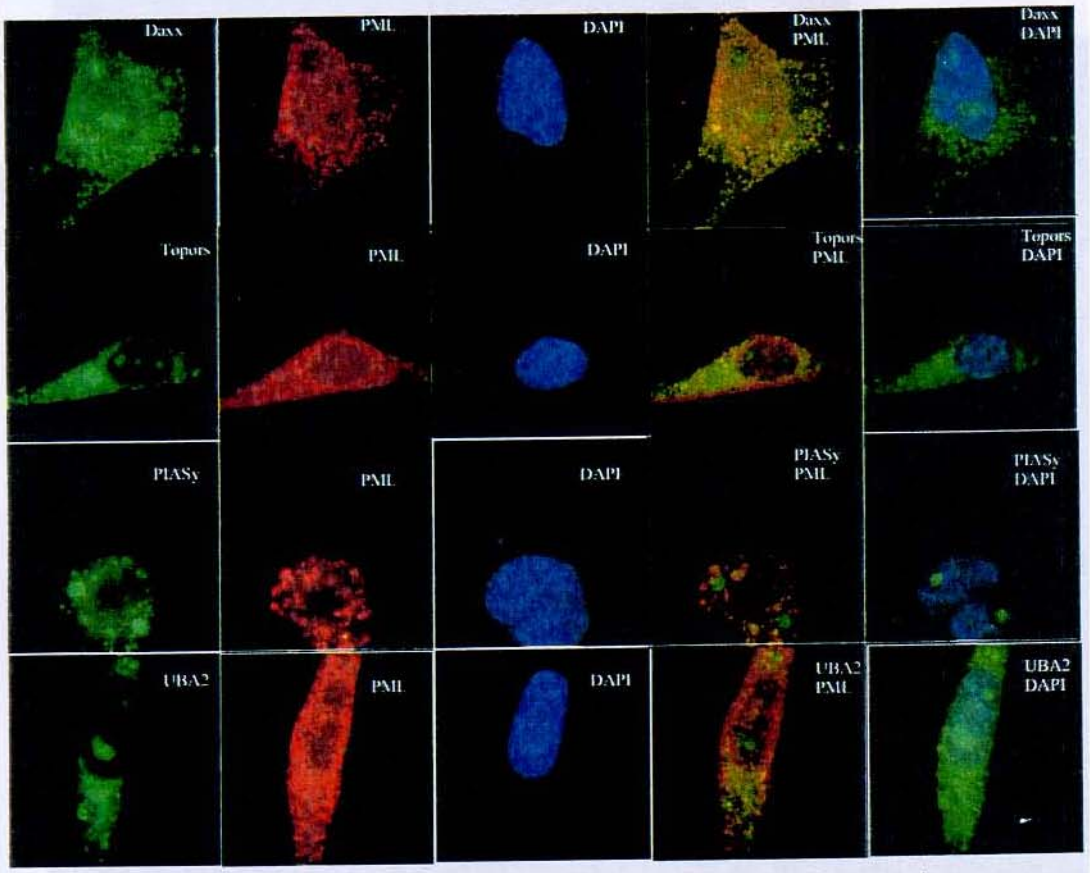
CGI-55
UBA2
CGI-55
DAPI

FIGURE 3


Daxx
PML
DAPI
Daxx
PML
Daxx
DAPI
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PML
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Topors
PML
Topors
DAPI
PIASy
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UBA2
DAPI

FIGURE 4

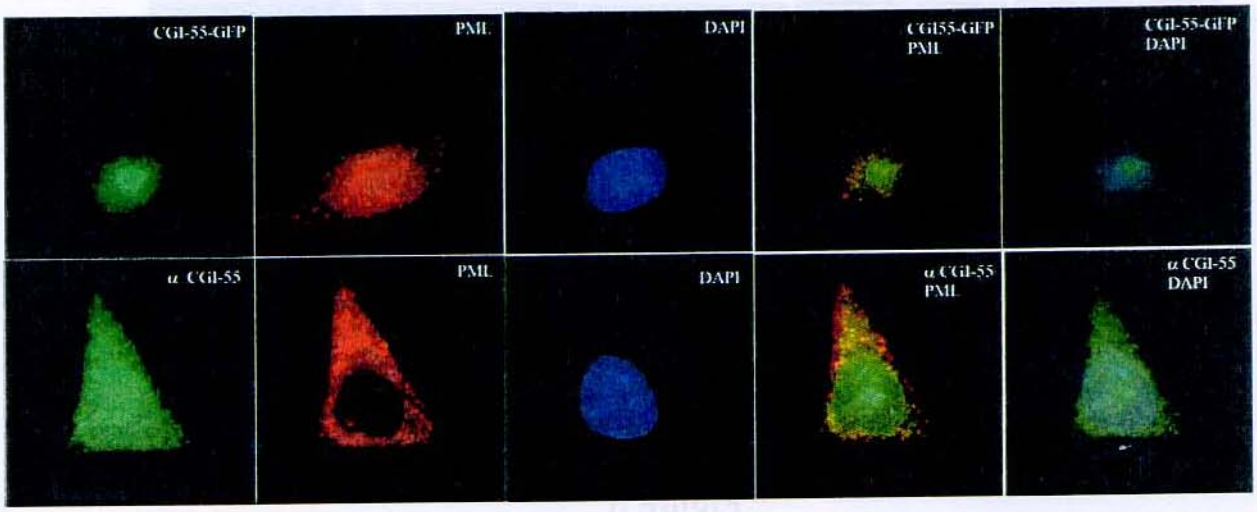
CGI-55-GFP
PML
DAPI
CGI55-GFP
PML
CGI-55-GFP
DAPI
α CGI-55
PML
DAPI
α CGI-55
PML
α CGI-55
DAPI

FIGURE 5

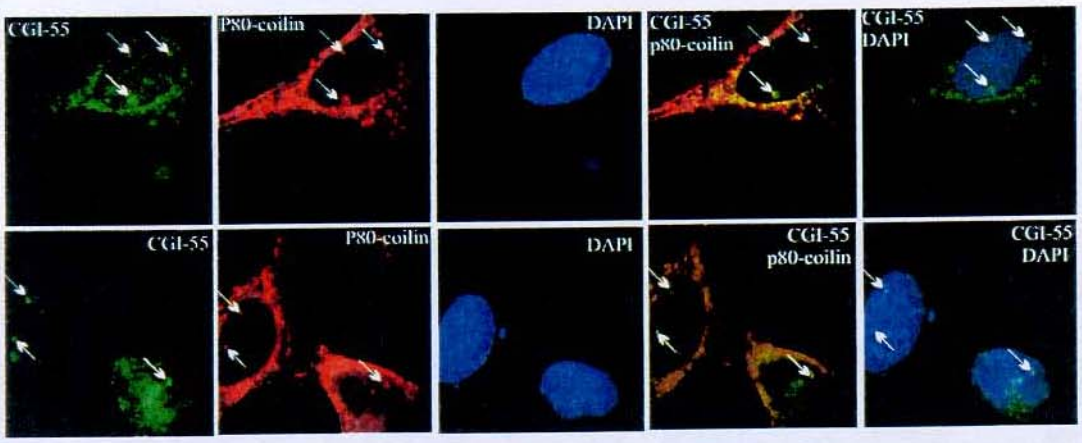
CGI-55
P80-coilin
DAPI
CGI-55
p80-coilin
CGI-55
DAPI
CGI-55
P80-coilin
DAPI
CGI-55
p80-coilin
CGI-55
DAPI

Figure 6

DISCUSSÃO

A identificação das proteínas que interagem com CGI-55 é um passo importante no caminho para a elucidação do seu papel celular, visto não haver nenhum indício sobre sua função. Até o ínicio de nossos estudo sabia-se apenas que CGI-55 exercia possivelmente a função de proteína reguladora ou oncoproteína. A proteína CGI-55 possui uma alta similaridade com o antígeno Ki-1/57, que apresenta várias características de uma oncoproteína, tais como: i) Ki-1/57 foi primeiramente descrito como um antígeno reconhecido pelo anticorpo Ki-1. Este anticorpo é utilizado para identificar células malignas de Hodgkin e células de Sternberg-Reed no linfoma de Hodgkin (Schwab et al., 1982; Hansen et al.,1989); ii) análises imuno-histoquímicas revelaram que esta proteína é expressa em vários tipos de câncer, tais como linfoma de células T, adenocarcinoma , carcinoma de próstata, carcinoma de bexiga e é fosforilada em leucócitos ativados por mitógenos (Kobarg et al., 1997); iii) o gene que codifica esta proteína está localizado no braço longo do cromossomo 9 (9q22.3-q31) (Kobarg et al., 1997). Esta região é freqüentemente afetada por deleções secundárias em leucemia mielóide aguda do tipo M2 e do tipo M3 (Heim e Mitelman, 1995); iv) Ki-1/57 é fosforilada nos resíduos serina e treonina e migra do citoplasma para o núcleo, associando-se ao nucléolo (Hansen et al.,1989, Rhode et al.,1992).

A identificação das proteínas que interagem com CGI-55 foi realizada através do método de duplo-híbrido de levedura descrito por Fields e Song (1989). Através deste método, identificamos nove proteínas que interagem com CGI-55. Este trabalho resultou em dois artigos. O artigo 1 anexado a tese foi publicado na revista "*FEBS Letters*" e o artigo 2 está sendo preparado para o periódico "*Journal of Cell Science*". Neste capítulo,

serão discutidos de forma geral os principais resultados apresentados no dois artigos e sugerido um possível modelo da função de CGI-55.

A proteína mais freqüentemente encontrada interagindo com CGI-55, no sistema de duplo híbrido foi a CHD-3, tendo sua seqüência codificadora aparecido em 42% dos clones analisados. A CHD-3 foi primeiramente isolada por Ge et. al. (1995) utilizando o anticorpo Anti-Mi2 que está associado a dermatomioses. Woodage et al. (1997) caracterizaram a proteína CHD-3 que possui 2000 aminoácidos (aa), e que inclui dois domínios "PHD *Zinc-finger*" e dois domínios reguladores da cromatina na porção N-terminal, um domínio "*Helicase/ATPase*" na porção central e domínio ligação ao DNA na porção C-terminal.

O domínio de ligação ao DNA da proteína CHD-1 homóloga à CHD-3 foi estudado por Strokes e Perry (1995) e parece estar envolvido na ligação com seqüências de DNA ricas em A+T. No domínio de ligação de DNA da proteína CHD-1, existem dois motivos contendo as seqüências RFRPKKR e RGRPR, que também são encontrados em outras proteínas, tais como HMG-I/Y e H1 que estão envolvidas nos processos de compactação e organização da cromatina e regulação da expressão gênica. A interação de CGI-55 com a região de CHD-3 (Fig.3A artigo 1) que compreende o domínio de ligação ao DNA, indica que CGI-55 pode estar associada ao remodelamento da cromatina.

A CGI-55 também interagiu com a HPC2, um homólogo humano do "polycomb" de *Drosophila.* Esta proteína, tal como a CHD-3, também está associada à regulação da estrutura da cromatina e da expressão gênica, visto que as proteínas da família Pc formam complexos protéicos, que quando associados à cromatina, reprimem a expressão de determinados genes. O gene que codifica a proteína HPC2 foi isolado de uma biblioteca

de cDNA de cérebro fetal humano e possui 1674pb que codificam 558aa, o que gera uma proteína de ~61kDa. Assim como a proteína CHD-3, a HPC2 possui um domínio regulador da cromatina na porção N-terminal (aa 8-48). No entanto, a função deste domínio na. HPC2 ainda não foi identificado. A HPC2 é expressa em todos os tecidos analisados e em várias linhagens de células humanas tumorais, com maior expressão na linhagem de célula tumoral Raji. Esta proteína está localizada no núcleo sob a forma de grânulos a sua superexpressão em células humanas faz com que estas sofram alterações morfológicas. A superexpressão da proteína mutada ΔHPC2, que possui a porção C-terminal deletada, resultou na perda da sua capacidade de reprimir a expressão de certos genes, tais como o proto-oncogene *c-myc* (Santij et al.,1997). O clone de HPC2 que interagiu com CGI-55 compreende a porção central e C-terminal desta proteína (aa 66-558). A associação da CGI-55 com HPC2 pode significar o seu envolvimento na regulação da expressão de certos proto-oncogenes, tais como *c-myc*.

A Topors ("*Topoisomerase I binding protein RS rich*"; "*p53 binding protein*") possui de 1045 aa equivalente à massa molecular de ~115kDa. A análise de sua seqüência identificou a presença do motivo "*Ring-Finger*" (aa 103-141). Este motivo possui um padrão de cisteínas e histidinas conservado (C3HC4) encontrado em várias proteínas de diferentes origens e funções. A porção N-terminal (aa 51-374) possui domínio de ligação ao DNA. Na porção central, observa-se o domínio de ligação à p53 (aa 456-731), e o motivo de ligação à topoisomerase I (aa 456-883) (Chu et al., 2001; Zhou et al., 1999; Haluska et al., 1999). Foram identificados três clones contendo a seqüência codificadora de topors que interagiram com CGI-55, o clone 56 (aa 100-600) compreende o domínio de ligação ao DNA; o clone 33 (aa 360-820) contem o domínio de

ligação à p53; e o clone 42 (aa 360-1045) compreende os domínios de ligação a p53 e à topoisomerase I. Recentemente, Rasheed et al. (2002) observaram que a Topors está associada aos corpúsculos nucleares PML. Estes corpúsculos nucleares PML apresentam várias funções, entre elas, regulação da transcrição e a manutenção da estrutura da cromatina, funções estas que já haviam sido propostas para Topors por Haluska et al. (1999). Topors possui dois sítios de ligação à SUMO-1, sugerindo sua modificação por SUMO-1, tal como vários outros componentes dos corpúsculos nucleares PML (Chu et al., 2001). Muller e Dejean (1999) verificaram que estes corpúsculos são afetados por infecções virais. Recentemente, Weger et al. (2002) identificaram que Topors está envolvida na regulação da expressão gênica do gene AVV2 (*Adeno-Associated Virus 2*) de adenovírus. O fato de CGI-55 interagir com Topors corrobora a hipótese de que CGI-55 está de alguma forma envolvida com a regulação da estrutura da cromatina e da expressão gênica. Além disso, a interação de CGI-55 com Topors sugere sua associação aos corpúsculos nucleares PML. Tal como Topors, CGI-55 também possui sítios de ligação à SUMO-1 e apresenta o padrão granular de localização sub-celular.

Três membros da família de proteínas PIAS (*Protein Inhibitor of Activated STAT*") interagem com CGI-55. As proteínas da família PIAS são inibidores de atividade de STAT (*Signal Transducer and Activator of Transcription*), que são por sua vez proteínas tradutoras de sinais e ativadoras de transcrição. As STATs são citoplasmáticas e, quando ativadas através das vias de sinalização de citocinas, são translocadas para o núcleo, onde iniciam o processo de transcrição de genes específicos. O clone 4 (aa 360-650) codifica a porção C-terminal da PIAS-1, e o clone 67 (aa 255-510) compreende a porção C-terminal da PIAS-y. Estas duas proteínas são inibidoras de atividade da STAT-

1 (Sturm et al., 2001; Valdez et al., 1997; Liu et al., 1998; Liu et al., 2001). O clone 51 (aa 204-619) codifica a porção C-terminal da proteína PIAS-3 (Ueki et al., 1999). Os três clones possuem o domínio Zf-MIZ, onde encontra-se o sítio específico de ligação ao DNA. Além disso, o domínio Zf-MIZ é encontrado em fatores de transcrição. Liu et al. (1998) sugeriram que as proteínas da família PIAS inibem a atividade das proteínas STATs através do bloqueio da ligação STAT-DNA. PIAS1, 3 e y estão envolvidas na regulação da transcrição do gene do receptor de andrógenos (AR) em células cancerígenas de próstata. É interessante ressaltar que PIAS-1 e 3 estimulam a transcrição deste gene, no entanto PIASy reprime a transcrição deste gene (Gross et al., 2001). As proteínas da família PIAS foram recentemente associadas à atividade de E3 ligase de SUMO-1, independentemente, por vários pesquisadores (Miyauchi et al., 2002; Schmidt e Muller, 2002; Jackson, 2001). A interação de CGI-55 com estas proteínas corrobora a hipótese de que CGI-55 possa ser modificada através da sumolização e ter sua localização celular regulada por esta modificação.

Dois clones que codificam Daxx, um a porção C-terminal (clone 31, aa 368-740) e outro a proteína Daxx quase completa (clone 22, aa 60-740), foram identificados pelo duplo-híbrido de levedura. Os clones englobam o domínio de ligação a JNK (aa 501-625) e os motivos ricos em ácido-glutâmico (aa 493-510; aa 510-514; aa 527-534). Daxx é uma proteína de 740aa e se liga à Fas, um indutor da via de apoptose. Daxx é uma proteína predominantemente nuclear, e está associada aos corpúsculos nucleares PML juntamente com outras proteínas tais como p53, Sp100, quando modificadas por SUMO-1 (Ishov et al, 1999). No entanto, quando não modificada por SUMO-1 Daxx pode ser encontrada no citoplasma ou difusa no núcleo. Zhong et al. (2000) demonstraram que a

presença de Daxx é essencial para a função de apoptose dos corpúsculos PML e sugeriram que a via de apoptose Daxx-PML-NBs é independente da via de tradução de sinal Fas-Daxx-JNK-jun. Daxx possui atividade de repressora quando interage com os fatores de transcrição Pax3 e ETS1 (Hollenbach et al., 1999, Li et al., 2000). Emelyanov et al. (2002) descobriram que Daxx age como repressor e/ou ativador da atividade de Pax 5. A proteína Daxx também esta associada à histona deacetilase II (HDAC II) e componentes da cromatina tais como: histonas H2A, H2B, H3, H4 e a proteína associada à cromatina Dek (Emelyanov et al., 2002). A interação de Daxx com CGI-55 indica que além dos processos de transcrição e regulação da estrutura da cromatina, CGI-55 possa estar envolvida no processo apoptótico.

A subunidade 2 da enzima ativadora de SUMO-1 (*small ubiquitin-related modifier 1*) (UBA2 ou SAE2) interagiu com CGI-55. A proteína SUMO inativa (SUMO-XXX) é hidrolisada por isopeptidases (Ulp1, Ulp2, Susp1, Semp1) e expõem os resíduos gly-gly às enzima ativadora de SUMO (E1). A enzima E1 se liga a proção C-terminal de SUMO nos resíduos gly-gly. Em seguida, SUMO se liga à enzima conjugadora E2 (Ubc9). Em alguns casos, é necessário a presença de ligases de SUMO, tais como PIAS1, 3 e y para o reconhecimento do substrato e sua posterior modificação (Alarcon-Vargas e Ronai, 2002). A proteína SUMO-1 está relacionada ao transporte das proteínas (PML, SP100, Daxx, p53 etc) para os corpúsculos nucleares PML (Melchior, 2000). SUMO-1 também participa na regulação do ciclo celular, na apoptose (p53) (Rodriguez et al., 1999; Zhong et al., 2000; Li e Hochstrasser, 1999) e no reparo de danos causados no DNA (Hardeland et al, 2002). O clone 104 (aa 368-640) compreende a porção C-terminal da enzima ativadora de SUMO-1 e inclui a maior parte do domínio UBACT (aa 345-

404). Este domínio é comumente encontrado nas proteínas da via de ubiquitinas e certas proteinoquinase (Dieckmann et al., 1998).

A última proteína que foi encontrada interagindo com CGI-55 foi à TDG (*Thymine DNA Glycosylase*). A TDG foi primeiramente descrita por Neddermann et al. (1996) e consiste em 410aa o que representa uma proteína de 46kDa. A TDG corrige as trocas G/T causadas pelo processo de deaminação hidrolítica espontânea das bases do DNA. O clone 94 (aa 180-410) codifica a porção C-terminal da TDG, a qual possui parte do domínio DNA-Glicosilase (aa 126-273). Recentemente, Hardeland et al. (2002) demonstraram que TDG é modificada por SUMO-1 e SUMO-3. Mutações nos resíduos de lisina, normalmente utilizados para o acoplamento à SUMO, causa à perda da atividade de timina DNA-glicosilase (Hardeland et al, 2002). TDG não se localiza nos corpúsculos PML, apesar deles também estarem envolvidos no reparo a danos no DNA e estarem associados à SUMO-1. O fato de CGI-55 ter sido encontrado interagindo com TDG sugere sua possível participação no reparo de danos no DNA.

A análise da interação de CGI-55 com outras proteínas através do sistema duplo-híbrido levedura, indica que sua provável função está associada à regulação da expressão gênica e estrutura da cromatina, processos apoptóticos e possivelmente ao reparo de danos no DNA. O fato de interagir com a UBA2 e apresentar sítios de acoplamento de SUMO na sua seqüência de aminoácidos sugere que CGI-55 possa ser alvo de modificações por SUMO-1. Além disso, CGI-55 está associado à várias proteínas modificadas por SUMO e à proteínas da família PIAS, as quais agem como ligases de SUMO (E3) (Fig. 7)

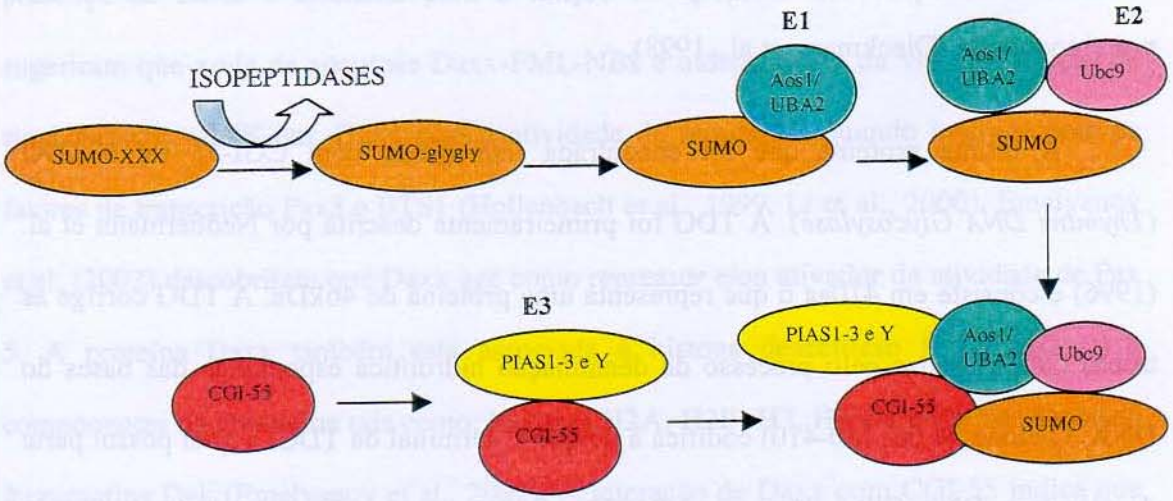

Figura 7: Hipótese da modificação de CGI-55 por SUMO-1. Após SUMO ser ativada por uma série de reações enzimáticas, ela se liga à CGI-55 através do reconhecimento da SUMO ligase PIAS que está ligada à CGI-55

A análise da localização celular demonstrou que CGI-55-GFP apresenta um padrão granular encontrado no citoplasma, no núcleo e na região perinuclear. Este padrão granular de localização celular foi observado nas proteínas associadas ao CGI-55, tais como Topors, Daxx e proteínas da família PIAS. A distribuição granular destas proteínas pode estar associada aos corpúsculos PML ou a outros corpúsculos nucleares. Nossos resultados condizem àqueles obtidos por outros pesquisadores (Torii et al., 1999; Li et al., 2000; Ishov et al., 1999; Rasheed et al.,2002), de que as proteínas Daxx e Topors colocalizam-se com a proteína PML e as proteínas PIASy, UBA2 e CGI-55 colocalizam-se parcialmente com a proteína PML. Estes dados sugerem que CGI-55 possa estar associada aos PML-NBs ou a outros corpúsculos nucleares e, provavelmente, às funções exercidas por estes corpúsculos. Estes resultados nos permitem sugerir um modelo das prováveis funções de CGI-55 (Fig. 8).

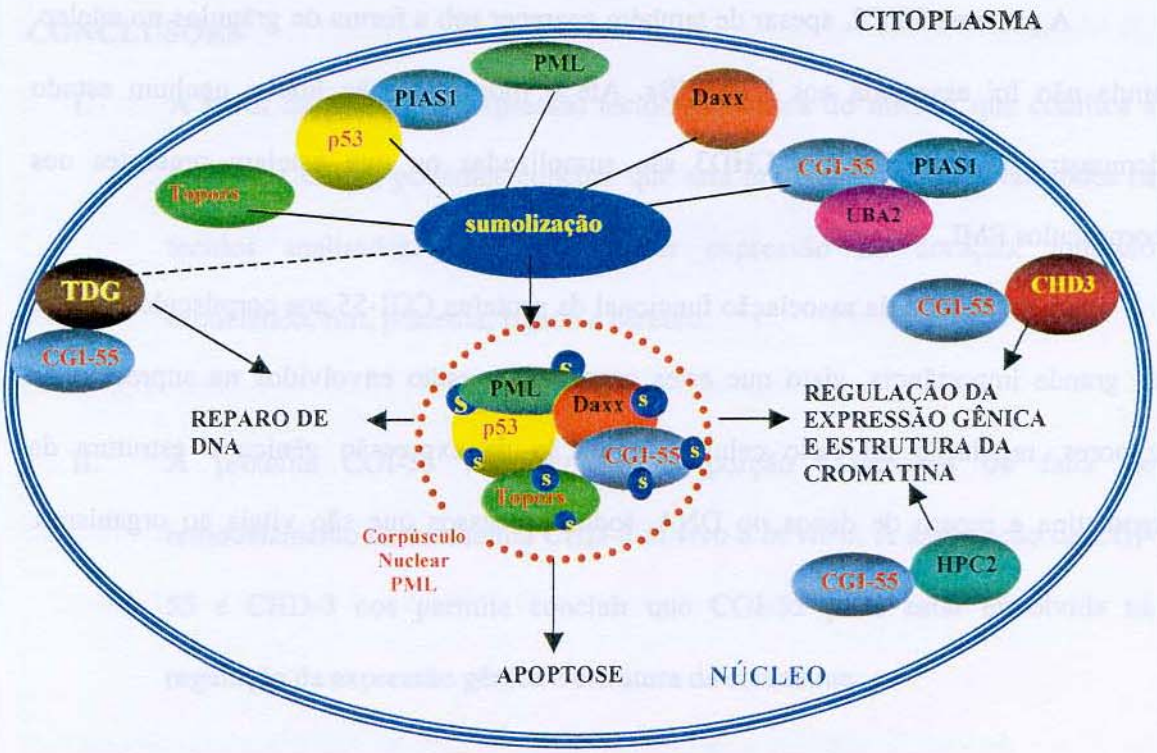

Figura 8: Modelo proposto para as possíveis funções da proteína CGI-55. A CGI-55, tal como Daxx, p53, Topors e PML, é sumolizada e, então, transportada para os corpúsculos PML. Estes corpúsculos estão associados à apoptose, regulação da expressão gênica e estrutura da cromatina e reparo de DNA. Além disso CGI-55 pode participar da regulação da estrutura da cromatina e expressão gênica através da associação à CHD-3 e HPC2. CGI-55 também pode estar envolvida no reparo de danos no DNA através da associação à TDG.

De acordo com Zhong et al., (2000) a proteína PML é sumolizada. Posteriormente, os outros componentes dos corpúsculos nucleares PML (PML-NBs) também são modificados por SUMO, tais como p53, Daxx, Topors e, possivelmente, CGI-55. A proteína PML, juntamente com os outros componentes sumolizados, irá formar os corpúsculos nucleares, os quais, como descrito na introdução, estão envolvidos na regulação da expressão gênica e estrutura da cromatina, reparo de danos no DNA e apoptose. A proteína TDG é sumolizada, porém não está associada aos corpúsculos PML.

A proteína HPC2, apesar de também aparecer sob a forma de grânulos no núcleo, ainda não foi associada aos PML-NBs. Até o momento, não houve nenhum estudo demonstrando que HPC2 e CHD3 são sumolizadas ou que estejam presentes nos corpúsculos PML.

A descoberta da associação funcional da proteína CGI-55 aos corpúsculos PML é de grande importância, visto que estes corpúsculos estão envolvidos na supressão de tumores, regulação do ciclo celular, regulação da expressão gênica e estrutura da cromatina e reparo de danos no DNA, todos processos que são vitais ao organismo.

CONCLUSÕES

I. A partir da análise da expressão tecido-específica do mRNA que codifica a proteína CGI-55, podemos concluir que esta proteína é expressa em todos os tecidos analisados, com uma maior expressão no coração, músculo esquelético, rim, placenta, fígado e cérebro.

II. A proteína CGI-55 interage com a porção C-terminal do fator de remodelamento de cromatina CHD-3 *in vivo* e *in vitro*. A associação de CGI-55 e CHD-3 nos permite concluir que CGI-55 pode estar envolvida na regulação da expressão gênica e estrutura da cromatina.

III. A CGI-55 também foi identificada interagindo com Daxx, Topors, PIAS 1, 3 e y, UBA2, HPC2 e TDG no sistema de duplo híbrido de levedura. Daxx, Topors, PIAS 1, 3 e y e UBA2 estão estruturalmente e funcionalmente associadas aos corpúsculos PML e ao processo de sumolização de proteínas. Assim como a CHD3, HPC2 está envolvida na regulação da expressão gênica e TDG no reparo de danos ao DNA. Os PML-NBs estão envolvidos na regulação da expressão gênica e apoptose. A interação de CGI-55 com as proteínas associadas aos PML-NBs sugere um possível envolvimento de CGI-55 nestes processos regulatórios.

IV. CGI-55 colocaliza-se *in vivo* com as proteínas Daxx, Topors, PIASy e UBA-2 em células HeLa, demonstrando que estas proteínas podem interagir não só em células de levedura, mas também co-localizam em células humanas.

V. O mapeamento dos sítios de interação de CGI-55 com as proteínas identificadas no duplo-híbrido resultou em três padrões de interação: proteínas que interagem apenas com o CGI-55 completo (Daxx e Topors), proteínas que interagem apenas com a porção C-terminal (UBA2) e proteínas que interagem com as porções C- e N-terminais (CHD-3, PIAS 1, 3 e y, TDG e HPC2).

VI. Análise da seqüência de aminoácidos de CGI-55 identificou cinco possíveis sítios de modificação por SUMO-1, tal como encontrado nas proteínas Daxx, Topors e TDG, sugerindo que CGI-55 pode ser modificada por SUMO-1.

VII. A análise de localização subcelular através de imunofluorescência e expressão de CGI-55 fusionada à GFP indicou que CGI-55 encontra-se distribuída no citoplasma e, principalmente, no núcleo e na região perinuclear na forma de grânulos.

VIII. As proteínas Daxx e Topors colocalizam-se com a proteína PML, enquanto as proteínas PIASy, UBA2 e CGI-55 colocalizam-se parcialmente com a proteína PML. Concluimos que CGI-55 está localizado em corpúsculos

nucleares os quais podem incluir os PML-NBs. Porém, CGI-55 também pode estar associada à outros corpúsculos nucleares.

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