Universidade Estadual de Campinas

Instituto de Biologia

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Estudos funcionais da proteína Reguladora Humana Ki-1/57 e seus parceiros de interação.

Tese a ser apresentada ao Instituto de Biologia da Universidade Estadual de Campinas para obtenção do Título de Doutor em Biologia Funcional e Molecular, na área de Bioquímica.

Orientador: Prof. Dr. Jörg Kobarg

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Aristóteles (384 a. C.– 322 a. C.)

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Lista de Abreviações e Siglas

AdOx: (*methyltransferase inhibitor adenosine dialdehyde)*;

CD: Dicroísmo dircular (*circular dicroism*);

cDNA: DNA complementar;

CD30: Receptor de citocina 30 (*cytokine receptor 30*);

EGFP: Proteína de Fluorescência Verde (*enhanced green fluorescence protein*);

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

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

CIRBP: Proteína que se liga ao RNA induzida por frio (*cold inducible rna binding prot*ein);

CTGF: Fator de crescimento de tecido conectivo (*connective tissue growth fa*ctor);

DAXX: Proteína associada à morte (*Death-associated pro*tein);

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

eIF4E: Fator de iniciação eucarioto 4E;

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

FXRP/ FMRP: (*Fragile X mental retardation protein*);

FXR1: Proteína relacionada com retardamento mental X-frágil 1;

FXR2: Proteína relacionada com retardamento mental X-frágil 2;

GST: glutationa-S-transferase;

6His: cauda de 6 histidinas em proteínas recombinantes;

hnRNPQ: Ribonucleoproteína heterogênea nuclear Q (*Heterogeneous Nuclear Ribonucleoprotein Q*);

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

kDa: Quilo Dalton (*kilo Dalton*);

Ki-1/57: Antígeno de 57 kDa do anticorpo Ki-1 (*Ki-1 antigen of 57 K*Da);

mg: miligrama (*milli gram*);

mRNA: RNA mensageiro (*messenger RNA*);

NSAP1: Proteína associada a NS1 (*NS1-associated protein*);

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

PAI-1: Inibidor do ativador do plasminogeno 1 (*plasminogen activator inhibitor 1*);

PAI-RBP1: Proteína que se liga ao mRNA de PAI-1 (*PAI-1 mRNA- binding protein*);

PKC: Proteina quinase C (*protein kinase C*);

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

PML: Proteína da leucemia promielocítica (*protein of promielocytic leukemia*);

PRMT1: Proteína arginina metiltransferase 1 (*protein Arginine Methyltransferase 1);*

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

RanGAP1: (*Ran GTPase-activating protein*);

RPM: rotações por minuto;

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

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

F2/p32: Fator de *splicing* 2/p32 (*splicing factor 2, subunit p32*);

SENPs: (*Sentrin –specific proteases*);

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

STAT: Sinalizador e ativador da transcrição;

SUMO: (*small ubiquitin-like modifier*);

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

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

WB: *Western blot*;

µg: Micrograma (*microgram*);

RGG-box: Motivo rico em argininas e glicinas;

3'UTR: (*Three-prime untranslated regions*)

Resumo

A proteína Ki-1/57 foi descoberta através da reação cruzada do anticorpo monoclonal Ki-1 em células do linfoma de Hodgkin. Estudos anteriores demonstraram que Ki-1/57 interage com a proteína RACK-1, sofre fosforilação por PKCs e metilação por PRMT1, uma arginino metiltransferase que modula diversas proteínas ligantes ao RNA. Estudos mostraram que Ki-1/57 é capaz de controlar o *splicing* do pré-mRNA do gene viral E1A. Análises por SAXS, gel filtração analítica e ultracentrifugação analítica indicaram uma estrutura bastante alongada e flexível para a construção C-terminal 6xhis-(122-413)Ki-1/57. Ensaios de proteólise limitada também mostraram uma baixa composição de núcleos hidrofóbicos estáveis e compactos, sugerindo que a proteína é intrinsecamente desordenada, o que pode explicar o elevado número de diferentes proteínas parceiras que ela é capaz de interagir. Neste trabalho foi identificada a interação de Ki-1/57 com proteínas da Família do X frágil, e sua localização no Perfil Ribossomal, além de ser capaz de ativar a tradução quando conectada ao gene repórter da Luciferase. Outra observação importante é que Ki-1/57 é sumoilada *in vitro* e *in vivo,* sendo as lisinas alvos desta sumoilação identificadas, e a proteína não sumoilada é incapaz de atuar no splicing do pré-mRNA do gene viral E1A. Mais experimentos foram feitos no sentido de caracterizar a interação RACK-Ki-1/57. A interação entre as duas proteínas é forte, possuindo uma constante de dissociação de 0,7 μM, seguindo uma estequiometria de 1:1 observada em experimentos de sedimentação em equilíbrio. Experimentos de ultracentrifugação analítica mostraram que a proteína RACK1 tem propriedades hidrodinâmicas similares ao seu modelo predito por homologia, e que em solução ela é encontrada em sua forma monomérica, possuindo uma leve tendência a agregação. A superexpressão de Ki-1/57 e CGI-55 provocou a alteração de 413 e 217 genes respectivamente, que em sua grande maioria foram regulados negativamente. A maioria dos genes, que foram inibidos pela superexpressão de Ki-1/57, estão envolvidos na proliferação celular, muitos sendo expressos em diferentes tumores. Os dados obtidos no teste do MTS confirmam que houve uma diminuição na proliferação celular em células superexpressando as proteínas Ki-1/57 e CGI-55. Com base nos resultados obtidos neste trabalho, novas funções da proteína Ki-1/57 foram descritas, tais como: a influência de Ki-1/57 na proliferação celular, na tradução proteica e o papel importante que a modificação pós traducional, como a sumoilação, representa para que a proteína Ki-1/57 desempenhe sua função.

Abstract

The protein Ki-1/57 was discovered through cross reactivity of the monoclonal antibody Ki-1 in cells of Hodgkin lymphoma. Previously studies demonstrated that Ki-1/57 interacts with RACK-1 protein, undergoes phosphorylation by PKCs and methylation by PRMT1, an arginine methyltransferase that modulates several RNA binding proteins. Studies have shown that Ki-1/57 is able to control the pre-mRNA splicing of the viral E1A gene. SAXS analysis, analytical gel filtration and analytical ultracentrifugation indicate a rather elongated and flexible structure to build C-terminal 6xhis-(122-413) Ki-1/57. Limited proteolysis assays also showed a low composition of stable and compact hydrophobic cores, suggesting that the protein is intrinsically unstructured, it could explain the wide array of protein partners with which it is able to interact. In this work we identified the interaction of Ki-1/57 proteins with Family fragile X, and its location on Ribosomal profile, besides being able to increase the translation when tethered to the reporter gene Luciferase. Another important observation is that Ki-1/57 is sumoylated *in vitro* and *in vivo,* the targets of this lysine sumoylated were identified, and the not somoylated protein is unable to act in the pre-mRNA splicing of E1A. More experiments were made to characterize the interaction of RACK1 with Ki-1/57. The interaction between the two proteins is strong, possessing a dissociation constant of 0.7 μM and follows the stoichiometry of 1:1 as observed by sedimentation equilibrium experiments. Analytical ultracentrifugation experiments showed that human RACK1 has similar hydrodynamic properties to that predicted to homology model, and that in solution it is found in its monomeric form, with a slight tendency to aggregation. Overexpression of Ki-1/57 and CGI-55 caused changes in 413 and 217 genes respectively, which were mostly negative regulated. Most genes that were inhibited by overexpression of Ki-1/57 are involved in cell proliferation, and expressed in different types tumors. The MTS data obtained confirm a decrease in cell proliferation through overexpressing of Ki-1/57 and CGI-55 protein. Based on the results of this study new functions for Ki-1/57 protein have been described such as: the influence of Ki-1/57 in cell proliferation, in protein translation and the important role that the post translational modification, such as sumoylation, represents so that the protein Ki-1/57 performs its function.

1-Introdução.

1.1-O Histórico e características da proteína Ki-1/57.

O anticorpo monoclonal Ki-1 foi o primeiro a ser utilizado para detectar células malignas do linfoma de Hodgkin (Schwab *et al.,* 1982). O Anticorpo Ki-1 reage com uma glicoproteína de membrana de 120 kDa chamada de CD30 e também detecta por reação cruzada uma proteína intracelular de 57 kDa que foi denominada de Ki-1/57 (Hansen *et al.,* 1989). Usando a porção C-terminal de Ki-1/57 foram produzidos dois novos anticorpos monoclonais (A26 e E320) que interagem de maneira específica com a proteína Ki-1/57, verificado pelas técnicas de Imunoprecipitação e *Western- bloting* (Kobarg *et al.,* 1997).

Para testar se Ki-1/57 poderia funcionar como marcador para doenças neoplásicas, Kobarg e colaboradores verificaram a expressão desta proteína em vários tecidos neoplásicos por imunohistoquímica. Foi observado que o anticorpo monoclonal A26 reage com células tumorais em linfoma de Hodgkin, linfoma de célula T- não-Hodgkin, leucemia linfática de célula B, carcinoma de próstata e de bexiga (Kobarg *et al.,* 1997).

Ki-1/57 é fosforilada em resíduos de serina e treonina, através de experimentos de microscopia eletrônica verificou- se que ela é encontrada não somente no citoplasma, mas também nos poros nucleares e no núcleo, associada ao nucléolo (Hansen *et al.,* 1990; Kobarg *et al.,* 1997; Rhode *et al.,* 1992).

A digestão tríptica de Ki-1/57 forneceu vários peptídeos que foram seqüenciados e as seqüências peptídicas resultantes usadas para a clonagem do cDNA parcial de Ki-1/57 que compreende aproximadamente 60% do C- terminal da proteína (Kobarg *et al.,* 1997). Inicialmente a tentativa de clonar a proteína inteira não foi bem sucedida, pois os 100 pares de base iniciais do cDNA de Ki-1/57 é rico em GC e dificultava a sua amplificação. O gene de Ki-1/57 foi localizado por FISH (*Fluorescence In Situ Hybridization*) e se encontra na banda 9q22.3-q31 no braço longo do cromossomo 9 humano (Kobarg *et al.,* 1997).

Na década de 90, a seqüência parcial de Ki-1/57 não revelou nenhuma homologia significativa com proteínas conhecidas do banco de dados, o que levou a crer que ela era uma nova proteína com função ainda desconhecida.

1.2-A proteína Ki-1/57 é intrinsecamente desenovelada.

Bressan e colaboradores (2008) mostaram que Ki-1/57 realmente possui uma seqüência de aminoácidos típica de proteínas intrinsecamente desestruturadas, rica em aminoácidos carregados e pobre em aminoácidos hidrofóbicos, isto faz com que a proteína tenha dificuldade em estabelecer um *core* hidrofóbico estável e compacto. Diversas abordagens experimentais foram utilizadas para confirmar que a porção C-terminal de Ki-1/57 é intrinsicamente desestruturada como: cromatografia analítica de filtração em gel (*analytical gel filtration chromatography*), Dicroismo Circular (*Circular Dichroism*), proteólise limitada, SAXS (*Small-Angle X-ray Scattering*) dentre outros (Bressan *et al.,* 2008). Proteínas alongadas ou não estruturadas apresentam uma massa molecular discrepante quando eluidas na coluna de cromatografia analítica de filtração em gel (Longhi, *et al*., 2003). Baseado no volume de eluição da região C-terminal da proteína Ki-1/57, que compreende dos aminoácidos 122 ao 413, ela deveria ter 101 kDa (massa experimental maior do que a predita pelo programa ProtParam), isto é característico de proteínas que não são globulares e possuem uma forma alongada. Os dados obtidos no Dicroismo Circular indicam a ausência de estrutura secundária regular, e mostram um sinal típico de proteínas desestruturadas. Ki-1/57 também demonstrou ser muito sensível a degradação proteolítica pela proteinase K, revelando uma exposição incomum de resíduos hidrofóbicos na cadeia polipeptídica, sugerindo uma baixa capacidade de formação de núcleos hidrofóbicos estáveis e compactos (Bressan *et al.,* 2008). Para finalizar estes estudos um modelo da proteína foi construído usando os dados obtidos com a técnica de SAXS (Figura 1).

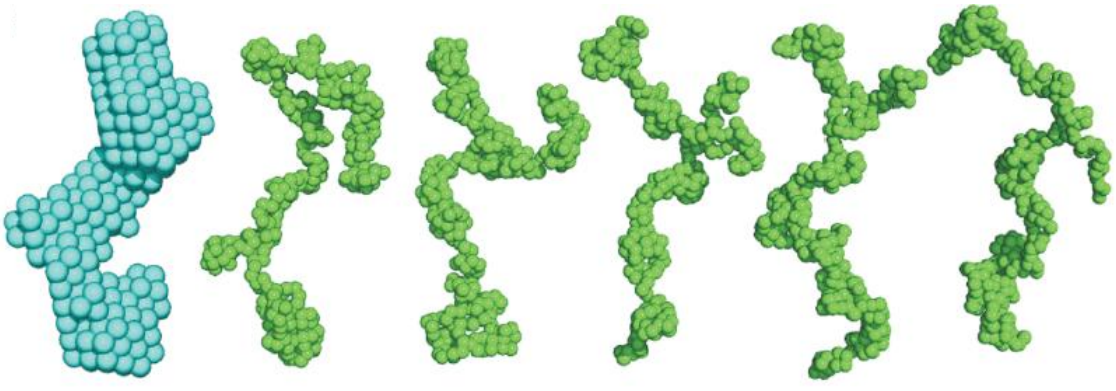

Figura 1- Ki-1/57 é uma proteína alongada e não estruturada. Modelos baseados em dados de SAXS da proteína 6His- Ki-1/57 (122-413) - região C-terminal. Modelo de baixa resolução DAMMIN (em azul)

seguido por cinco modelos representativos GASBOR (em verde). Os modelos foram gerados pelo programa DAMMIN e representados pelo programa PyMOL (DeLano *et al.,* 2002). Extraído de Bressan e colaboradores (2008).

Estes estudos demonstraram que a região C-terminal da proteína Ki-1/57, que compreende os aminoácidos 122 ao 413, é desestruturada, ou seja, possui um baixo conteúdo de estruturas secundárias regulares.

Na última década, o paradigma estrutura-função de proteínas tem sido reavaliado baseado na evidência crescente de que muitas proteínas são desenoveladas no seu estado funcional (Sánchez-Puig *et al.,* 2005). Tentando explicar tal questionamento, Dyson e Wright (2005) consideram que as metodologias bioquímicas clássicas de preparação de proteínas a partir de homogenatos de tecidos, seguidas de purificação, teste de atividade e determinação da estrutura tridimensional, automaticamente selecionam proteínas enoveladas, uma vez que esses homogenatos fatalmente liberam proteases às quais as proteínas desenoveladas são muito mais sensíveis.

Funcionalmente, a maioria das proteínas intrinsecamente desenoveladas estão freqüentemente envolvidas em processos de sinalização celular ou regulação, através de vias de interação não catalíticas com DNA, RNA ou outras proteínas (Wright e Dyson, 1999; Lakoucheva *et al.,* 2002). Em muitas dessas proteínas, as regiões desenoveladas se tornam enoveladas quando ligadas ao seu alvo de interação (Lakoucheva *et al.,* 2002; Sánchez-Puig *et al.,* 2005; Dyson e Wright, 2005).

O reconhecimento molecular de regiões desenoveladas possui duas características que fornecem importantes vantagens funcionais. Primeiro, regiões que possuem desordem intrínseca promovem diversidade de ligação, permitindo a interação com inúmeros parceiros. Segundo, regiões desenoveladas permitem a ligação a seus alvos com alta especificidade e relativamente baixa afinidade, características particularmente apropriadas para proteínas envolvidas em transdução de sinal, que precisam não apenas se associar especificamente para iniciar um processo de sinalização, mas também precisa ser capaz de se dissociar quando a sinalização é completada (Lakoucheva *et al.,* 2002; Dyson e Wright, 2005).

1.3-Duplo Hibrido - Uma busca sobre informações funcionais de Ki-1/57.

Para desvendar o papel funcional da proteína Ki-1/57 foram realizados ensaios de duplo hibrido de levedura utilizando como isca as regiões N- terminal (1-150) e C- terminal (122-413)

da proteína (Nery *et al*., 2004; Nery *et al*., 2006b). Foram encontradas diversas proteínas parceiras de interação as quais podem ser funcionalmente agrupadas em: Proteínas de sinalização (RACK1, PRMT1, EB-1, RIL, ALEX 2, CTGF, EPS8 e APLP1) de regulação da transcrição/ ligação ao DNA (CHD3, TOPORS, ZFP106, ZFP189, TIP60, BTBD2, YB-1/NSEP1, GADD34, DAXX, PIAS, p100 e HMG), metabolismo de RNA (CIRBP, YB-1/NSEP1, SFRS9, SF2/p32, RPL38, p100 e FXR1P) dentre outras (Nery *et al*., 2006).

Agrupando as proteínas encontradas no Duplo hibrido em classes funcionais, foi possível ter subsídios para direcionar a busca pela função de Ki-1/57.

1.3.1- RACK1

A interação de Ki-1/57 com a proteína RACK1(*receptor of activated kinase C-1*) foi a primeira a ser descrita, o mapeamento dos sítios de interação de Ki-1/57 com RACK1 revelou que a região C-terminal de Ki-1/57 interage com RACK1 *full-length* (Nery *et at.,* 2004).

Ensaios de fosforilação com PKC (*protein kinase C*) mostrou que Ki-1/57 é substrato para PKC isolada de células L540 ativadas por PMA (*Phorbol 12-Myristate 13-Acetate*), um ativador das vias de PKC. Esta quinase co-imunoprecipitou a proteína Ki-1/57, demonstrando, portanto, que PKC interage com Ki-1/57 após ativação celular. Além disso, nesta condição, Ki-1/57 fosforilada deixou de interagir com RACK1 e, ambas as proteínas, Ki-1/57 e RACK1, saíram do núcleo e foram para o citoplasma (Nery *et al*., 2004). Esses achados são coerentes com os dados encontrados na literatura de que a forma citoplasmática de Ki-1/57 é fosforilada e que está associada com a atividade de proteíno-quinases (Hansen *et al*., 1990; Kobarg *et al*., 1997).

Nery e colaboradores mostraram que a interação Ki-1/57-RACK1 parece envolver alguns triptofanos expostos na estrutura RACK1, predita, através de modelagem molecular, como sendo muito similar à subunidade β da proteína G (Nery *et al*., 2006).

Além disso, RACK1 é um componente dos ribossomos de células eucarióticas, associada a subunidade 40S (Nilsson *et al.,* 2004, Sengupta *et al.,* 2006). RACK1 recruta a proteína quinase C (PKC) até o ribossomo, que por sua vez fosforila o fator de iniciação 6 (eIF6) que leva a estimulação da tradução (Nilsson *et al.,* 2004).

1.3.2- SFRS9, PRMT1 e hnRNPQ1

No duplo hibrido realizado com a proteína Ki-1/57 foram identificadas proteínas que se ligam a RNA e que são funcionalmente associadas ao processo de *splicing* de RNA mensageiro

(Bressan *et al*., 2009). As proteínas de *splicing* SF2p32, YB-1 e SFRS9 foram encontradas interagindo com o N-terminal da proteína Ki-1/57 (Figura 2). SFRS9 é uma proteína encontrada não apenas em locais de armazenamento de fatores de *splicing* no núcleo, os *speckles*, mas também em regiões descritas como grânulos de estresse nucleares (do inglês *nuclear stress bodies*) (Biamonti, 2004; Denegri *et al*., 2001). É interessante frisar que Ki-1/57 sofre metilação por PRMT1 (Passos *et al.,* 2006) e que ambas, tanto Ki-1/57 como PRMT1, interagem com hnRNPQ1 (também chamada de NSAP1). Foi observado que Ki-1/57 possui regiões ricas em Arginina e Glicina (RGG-box) e a sua metilação influencia em sua localização subcelular, Ki-1/57 é direcionada para o núcleo quando as células são tratadas com inibidor de metilação, o Adox (Passos *et al.,* 2006).

A proteína hnRNPQ1 possui em seu N-terminal três domínios de ligação a RNA, e tem sido descrita associada aos polissomos, participa do processo de degradação de RNAs específicos, da tradução protéica e co-localiza com grânulos de stress (Quaresma *et al.,* 2009).

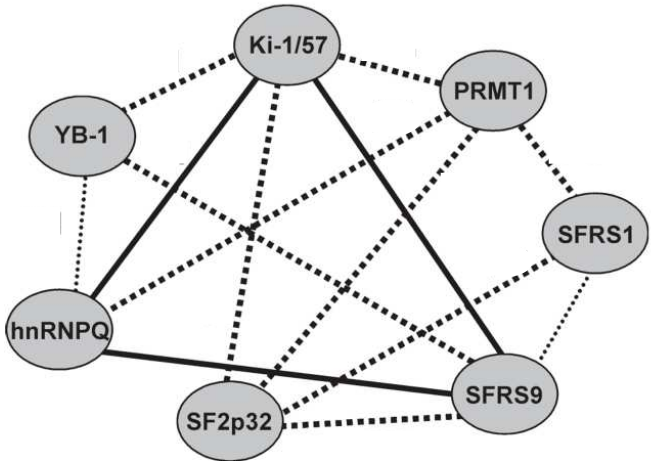

Figura 2- Interconexões funcionais de Ki-1/57 com proteínas reguladoras do splicing e seus parceiros de interação. PRMT1 é encontrada interagindo com SF2p32 (Yanagida *et al.,* 2004) e SFRS9 está no mesmo complexo protéico que SF2p32 e YB-1(Petersen-Mahrt *et al.,* 1999). (Extraído de Bressan e colaboradores (2009b).

A sequência de aminoácidos de Ki-1/57 tem vários motivos ricos em arginina e glicina (RGG-box), que são importantes para a interação de muitas proteínas de ligação ao RNA

(Kiledjian e Dreyfuss, 1992; Burd e Dreyfuss, 1994). A região C-terminal de Ki-1/57 possui dois grandes domínios RGG-box. Em ensaios de mudança de mobilidade eletroforética, foi encontrado que Ki-1/57-GST recombinante é capaz de se ligar a uma sonda de RNA poli-U marcada radioativamente, porém apenas a construção C-terminal de Ki-1/57 (122-413) se liga fortemente a esta sonda. Essas observações sugerem que a ligação de Ki-1/57 ao seu suposto mRNA alvo na célula deve envolver regiões ricas em U (Bressan *et al*., 2009b). Além disso, análises de microscopia de fluorescência e confocal revelaram a localização de Ki-1/57-GFP principalmente em corpúsculos nucleares envolvidos no processamento de RNA ou em agregados de ribonucleoproteína (Bressan *et al*., 2009b).

1.4-A proteína CGI-55 é um possível parálogo de Ki-1/57

A proteína humana de 55 KDa, CGI-55, divide 40,7% de identidade e 67,4% de similaridade com Ki-1/57, sugerindo que elas pudessem ser parálogas e ter funções similares ou redundantes nas células humanas. CGI-55 é também uma proteína presente no núcleo e citoplasma (Lemos e Kobarg, 2006) e foi descrita como uma proteína envolvida na estabilidade de mRNA, por ser capaz de se ligar a região 3´-UTR do mRNA que codifica o inibidor do ativador de plasminogênio tipo 1 (PAI, *plasminogen activator inhibitor*), tendo sido denominada PAI-RBP1 (*PAI RNA binding protein 1*) (Heaton *et al*., 2001). O sistema ativador de plasminogênio tem sido considerado um importante contribuidor em mecanismos de invasão e metástase de tumores sólidos, o que possibilitou a verificação da super-expressão de CGI-55 em cortes histológicos de câncer de ovário (Koensgen *et al*., 2007).

O sistema de duplo híbrido em levedura também foi realizado com CGI-55 e identificou-se que Ki-1/57 e CGI-55 possuem alguns parceiros de interação em comum. Essas parceiras de interação incluem CHD3 (proteína envolvida na regulação da transcrição e na remodelagem da cromatina), TOPORS (proteína que se liga a topoisomerase I), DAXX (proteína que se liga a Fas) e PIAS (inibidor de STAT ativada) e o fato de todas serem envolvidas direta ou indiretamente no controle da transcrição (Lemos e Kobarg, 2006), sugere Ki-1/57 e CGI-55 possam ter funções em comum: remodelagem da cromatina e regulação da transcrição.

Diversas proteínas que interagem com Ki-1/57 e com CGI-55 são proteínas nucleares funcionalmente relacionadas ao controle da transcrição e conhecidas por localizarem-se em corpúsculos nucleares PML (*promyelocytic leukemia nuclear bodies*) (Lemos e Kobarg, 2006).

Estes corpúsculos correspondem a um sub-compartimento nuclear, contendo várias proteínas não-histonas e sem a presença de cromatina ou RNA, sendo encontrados próximos a outras estruturas nucleares tais como cromatina e os corpúsculos de Cajal (Maul *et al*., 2000). Os corpúsculos PML estão envolvidos na supressão de tumores, regulação da expressão gênica, regulação do ciclo celular e transcrição, estrutura da cromatina, apoptose e na infecção viral (Seeler e Dejean, 1999). As evidências de que Ki-1/57 e CGI-55 interagem com proteínas presentes nos corpúsculos PML sugerem que elas podem estar envolvidas com a composição ou com a função destes corpúsculos nucleares.

1.5-Ki-1/57 participa do processo de splicing alternativo

Bressan e colaboradores mostram que Ki-1/57 interage com proteínas reguladoras do *splicing* através da sua região N-terminal (Bressan *et al.,* 2009b). Com base nessas interações foi investigado a capacidade de Ki-1/57 participar do processo de *splicing*. Ensaios de cotransfecção em células de mamíferos revelaram a influência de Ki-1/57 na seleção de sítios de *splicing* do gene adenoviral E1A. Estudos com formas truncadas para o N- e C-terminal mostraram que essas regiões precisam agir conjuntamente para uma eficiente atividade de *splicing*. Esses dados podem sugerir que tanto as atividades de interação a proteínas de *splicing* (N-terminal) quanto à ligação a RNA (C-terminal) sejam importantes para a modulação de processos de *splicing* por Ki-1/57 (Bressan *et al.,* 2009b).

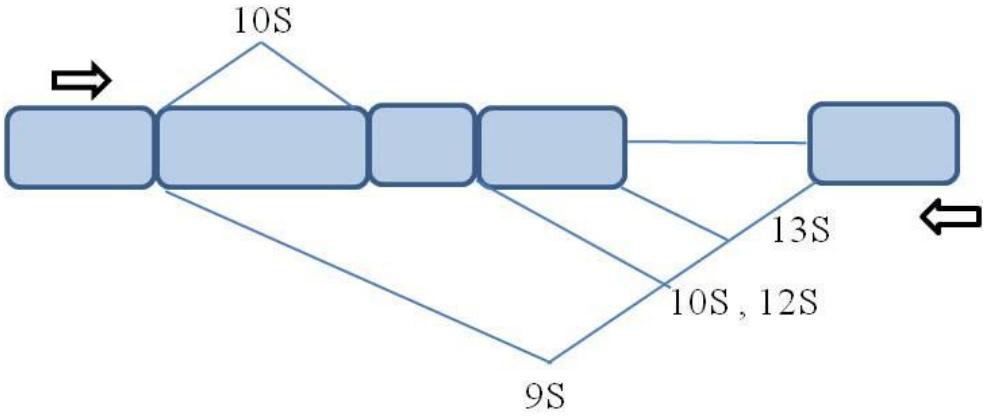

Figura 3- Ki-1/57 influencia no *splicing* do pré-mRNA do gene E1A. O diagrama mostra os eventos de *splicing* que geram o 13S, 12S, 10S e 9S RNA mensgeiros do gene repórter E1A (Stephens e Harlow 1987 e Caceres *et al*., 1994).

1.6-Sumoilação

A sumoilação é uma modificação pós-traducional reversível que regula as funções biológicas das proteínas. Neste processo, a proteína SUMO (*small ubiquitin-like modifier*) é ligada covalentemente a resíduos de lisina na sequência *Ψ*K*X*E (onde *Ψ* é um resíduo altamente hidrofóbico, K é uma lisina, *X* representa qualquer aminoácido e E é um ácido glutâmico) de uma proteína substrato, especialmente proteínas nucleares (Geiss-Friedlander e Melchior, 2007; Gill, 2004; Johnson, 2004). As proteínas SUMO possuem cerca de 10 KDa e, apesar de dividirem apenas 18% de identidade da sequência de aminoácidos com a ubiquitina, reúnem uma estrutura tridimensional próxima a da ubiquitina (Bayer *et al*., 1998).

Quatro homólogos de SUMO foram descritos em mamíferos: SUMO-1, SUMO-2, SUMO-3 e SUMO-4, que podem modificar substratos comuns e diferentes. Assim, alguns substratos podem ser simultaneamente modificados por SUMO-1 e SUMO-2/3, enquanto a RanGAP1, por exemplo, é predominantemente modificada por SUMO-1 e a Topoisomerase II é predominantemente modificada por SUMO-2/3 (Azuma *et al*., 2003; Saitoh e Hinchey, 2000). SUMO-2 e SUMO-3 são 97% idênticas, mas dividem apenas 50% de identidade com SUMO-1 (Geiss-Friedlander e Melchior, 2007; Gill, 2004). SUMO-1 e SUMO-2/3 são ubiquamente expressas em tecidos, enquanto SUMO-4 parece ser expressa principalmente no fígado, nodos linfáticos e rins (Guo *et al*., 2004). SUMO-2/3, mas não SUMO-1, são capazes de formar cadeias poliméricas através de sua lisina 11 (Tatham *et al*., 2001).

Todas as proteínas SUMO são expressas como formas imaturas, pois possuem uma região C-terminal de comprimento variável (2-11 aminoácidos) depois de um motivo invariante Glicina-Glicina (Figura 4). Antes da primeira conjugação, a proteína SUMO é proteoliticamente processada por isopeptidases específicas (*sentrin-specific proteases*; SENPs), que removem quatro aminoácidos da SUMO-1, onze da SUMO-2 e dois da SUMO-3. Ainda não é conhecido se SUMO-4 precisa ser processada para se tornar madura (Guo *et al*, 2004). As etapas seguintes da sumoilação requerem uma cascata enzimática que envolve três classes de enzimas: E1 (enzimas ativadoras), E2 (enzima conjugadora) e SUMO E3 Ligases (Geiss-Friedlander e Melchior, 2007; Gill, 2004; Johnson, 2004).

Após o processamento, a proteína SUMO é ativada pela enzima ativadora E1 (formada pelo heterodímero AOS1-UBA2) em uma reação dependente de ATP. Essa reação resulta em uma ligação tioéster entre a região C-terminal do resíduo de Glicina e o carbono 173 na UBA2. SUMO é então transferida para o resíduo catalítico de uma cisteína da enzima conjugadora E2 (UBC9). Por fim, uma ligação isopeptídica é formada entre o C-terminal do resíduo de glicina

de SUMO e o resíduo de lisina na proteína substrato. Nesta etapa, pode haver a participação de SUMO E3 ligases, que são enzimas que catalisam a transferência de SUMO da UBC9 para a proteína substrato (Geiss-Friedlander e Melchior, 2007; Gill, 2004; Johnson, 2004). As proteínas E3 ligases mais conhecidas e estudadas são a PIAS (*protein inhibitor of activated signal transducer*) e a RanGAP1 (*Ran GTPase-activating protein*).

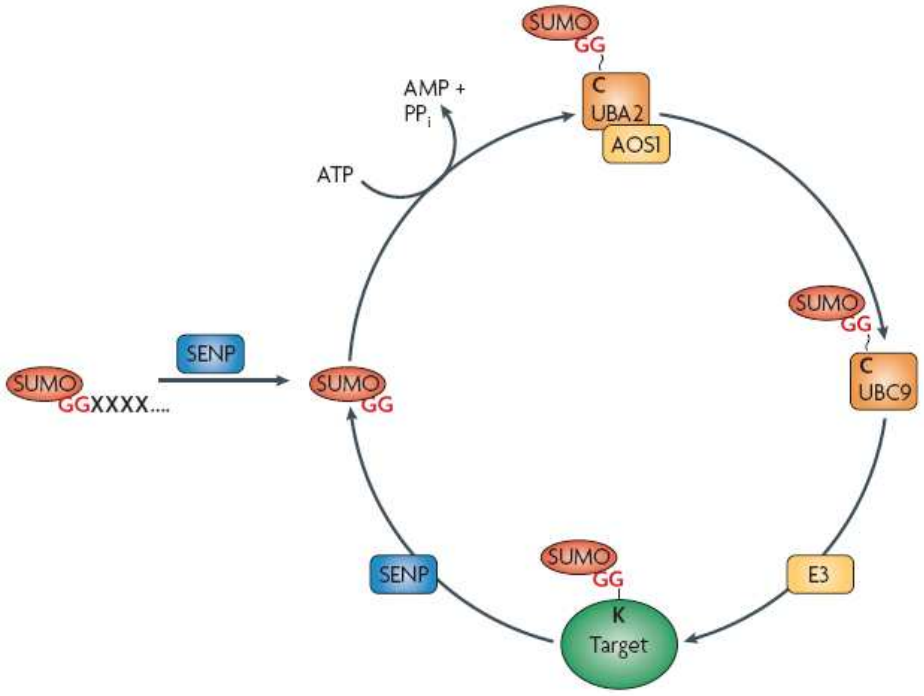

Figura 4- Esquema representando as etapas do processo de Sumoilação de uma proteína alvo. Extraído de Geiss-Friedlander e Melchior (2007).

Muitos dos alvos de sumoilação são proteínas nucleares com importantes papéis na regulação da transcrição, estrutura da cromatina e reparação de DNA (Gill, 2004; Wu *et al*., 2006). No entanto, os alvos de SUMO são também encontrados na membrana plasmática, retículo endoplasmático e citoplasma (Geiss-Friedlander e Melchior, 2007). A proteína RanGAP1 (*Ran GTPase-activating protein*), por exemplo, foi a primeira proteína caracterizada por se ligar covalentemente a SUMO-1 e a sua modificação modula o seu transporte entre o núcleo e citoplasma (Mahajan *et al*., 1997; Matunis *et al*. 1996).

A sumoilação de fatores de transcrição está freqüentemente associada com a repressão da transcrição (Gill, 2004; Hay, 2005). A modificação por SUMO mostrou afetar negativamente a

função de ativação transcricional de fatores de transcrição, como Elk-1 ou receptores de hormônio nuclear (Sapetschnig *et al*., 2002). Entretanto, a regulação positiva também já foi encontrada, como tem sido mostrado para as proteínas da família de coativadores de receptores de esteróides, em que a sumoilação de SRC-1 leva a ativação indireta da atividade transcricional de receptores de progesterona (Abdel-Hafiz *et al*., 2009).

Ainda não existe uma explicação molecular clara para o mecanismo pelo qual a adição de SUMO regula a atividade funcional de seu alvo. *In vivo*, a sumoilação deve influenciar em um aspecto simples de uma proteína alvo, como sua estabilidade, localização ou atividade. A nível molecular, a modificação com SUMO altera a superfície de proteínas alvo levando a modificações conformacionais e, portanto, influenciando a interação com outras macromoléculas (Geiss-Friedlander e Melchior, 2007; Wu *et al*., 2006). Conseqüentemente, a sumoilação pode promover ou inibir interações proteína-proteína, como já foi reportado que essa modificação pós-traducional, pode regular a formação de complexos transcricionais, alterar a localização celular de proteínas, inibir ou ativar enzimas, regular a atividade de canais ou ainda alterar a dinâmica mitocondrial (fusão e fissão mitocondrial) (Geiss-Friedlander e Melchior, 2007; Gill, 2004; Johnson, 2004).

2- Objetivos

2.1- Objetivo geral.

O objetivo geral deste trabalho foi estudar os aspectos funcionais da proteína Ki-1/57 em células humanas, através da análise de sua interação com seus possíveis ligantes protéicos.

2.2- Objetivos Específicos.

- Estudar a interação de Ki-1/57 com proteínas envolvidas na tradução protéica e tentar relacionar esses dados com uma possível função.

- Analisar a sequência primária de aminoácidos da proteína Ki-1/57 para identificar possíveis resíduos de lisina que sofrem sumoilação e contextualizar a importância desta modificação pós traducional na função da proteína.

- Estudar quais genes são regulados pela superexpressão das proteínas Ki-1/57 e CGI-55.

- Caracterizar a proteína RACK1 (parceira de interação de Ki-1/57) e a interação entre as duas através de estudos Biofísicos como espalhamento de raios-X a baixos ângulos (SAXS) e Ultracentrifugação Analítica.

3- Resultados

3.1- Artigo I

Evidence for the association of the human regulatory protein Ki-1/57 with the translational machinery

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Evidence for the association of the human regulatory protein Ki-1/57 with the translational machinery

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Keywords: regulatory protein, protein-protein interaction, translation

Abstract

Ki-1/57 is a cytoplasmic and nuclear protein of 57 kDa first identified in malignant cells from Hodgkin's lymphoma. Based on yeast-two hybrid protein interaction data we found Ki-1/57 interacting with adaptor protein RACK1 (receptor of activated kinase 1), CIRP (cold-inducible RNA-binding protein), RPL38 (ribosomal protein L38) and FXR1 (Fragile X mental retardation-related protein 1). Since these proteins are involved in the regulation of translation we suspected that Ki-1/57 may also have a role in it. We show by immunoprecipitation the association of Ki-1/57 with FMRP. Confocal microscopy revealed that Ki-1/57 colocalizes with FMRP/FXR1/2 to stress granules. Furthermore Ki-1/57 co-sediments with free ribosomal particles and enhances the

translation, when tethered to a reporter mRNA, suggesting that Ki-1/57 might be involved in translational regulation.

1. Introduction

Ki-1 was the first monoclonal antibody used in the specific detection of the malignant Hodgkin and Sternberg-Reed cells in Hodgkin lymphoma [1]. It has been demonstrated that Ki-1 recognizes the 120 kDa trans-membrane CD30 protein and a 57 kDa intracellular antigen, called Ki-1/57 [2-4]. Electron microscopy analyses revealed that Ki-1/57 is located in the cytoplasm, nuclear pores and nucleus [2]. When isolated from the Hodgkin's lymphoma analogous cell line L540, Ki-1/57 was shown to be phosphorylated on serine and threonine residues [5]. Several of the identified Ki-1/57-interacting proteins are involved in transcriptional control, such as CHD3, p53 and MEF2C [6-8].

Moreover, it has been found that Ki-1/57 contains multiple RGG/RXR box clusters commonly found as methylation targets for the methyl-arginine transferase PRMT1 [9]. These motifs may be involved in protein-RNA interaction in several RNA-binding proteins [10], suggesting that Ki-1/57 is involved in RNA metabolism. Indeed, recent studies showed that Ki-1/57 binds to U-rich RNA probes in gel shifty assays, associates with splicing proteins such as hnRNPQ and SFRS9 and modulates the pre-mRNA processing of an E1A gene construct in co-transfection assays [11, 12]. Furthermore, confocal microscopy analyses showed that Ki-1/57 can be found at distinct nuclear bodies, whose functions have been associated to RNA/pre-mRNA processing events. Inhibition of methylation affects the sub-nuclear localization of EGFPC-Ki-1/57 at nucleoli, speckles, GEMS and Cajal bodies, suggesting a relationship between arginine methylation activity, Ki-1/57 and processes of assembly, maturation and storage of splicing complexes in the nucleus [11].

Along with proteins involved in pre-mRNA splicing regulation, our previous yeast two-hybrid analyses also demonstrated that Ki-1/57 interacts with proteins involved in translational regulation, suggesting that its function in RNA metabolism would not be restricted to the nucleus. Among the translation regulatory proteins found as prey in our analyses, one of them was the FXR1P protein (fragile X-related protein 1), a member of fragile X protein family. This family includes three proteins highly conserved in amino acids sequence called FXR proteins: FMRP (fragile X mental retardation protein), FXR1P and FXR2P (fragile X-related protein 1 and 2) [13-15]. Limiting amounts of functional FMRP lead to the fragile X syndrome, which is characterized mainly by mental retardation and macro-orchidism [13, 16]. All three FXR proteins contain RNA-binding domains, such as two hnRNP K-homologous (KH) domains and one RGG box [17, 18, 19].

Although these proteins are detected predominantly in the cytoplasm, it was demonstrated that they contain a nuclear localization and nuclear export signals, that allow them to shuttle between cytoplasm and nucleus [18, 19]. In addition, FXR proteins are also associated with free ribosomes, predominantly with 60S ribosomal subunits, and polyribosomes, which suggested they may have functions in translation or mRNA stability [20-22].

Here we performed immunoprecipitation assays and co-localization analyses that showed an association of Ki-1/57 with FXR1P, FXR2P and FMRP in cultured human cells. Co-localization after cellular stress occurs in a punctuated fashion in the cytoplasm in foci known as stress-granules. Furthermore, we performed sucrose gradient fractionation experiments, which suggest that Ki-1/57 and its interacting proteins can be found associated to ribosomal particles. In addition, Ki-1/57 was able to alter the translation activity of a reporter gene construct in a tethering translation assay. Together, our findings suggest that Ki-1/57 might be involved in translation regulatory events.

2. Materials and Methods

Plasmid Constructions

Cloning of the complete cDNA encoding Ki-1/57 into pEGFPC vector has been described previously [11]. To obtain an N-terminal Flag-tagged Ki-1/57, we subcloned the Ki-1/57 cDNA into the pCDNA6 Myc/His vector (Invitrogen) between the BamHI and EcoRI restriction sites in frame with a Flag encoding sequence, which was previously inserted into this vector between HindIII and BamHI sites. Several sets of oligonucleotides were designed to allow subcloning of cDNAs encoding the human proteins FXR1, FXR2 and FMRP into pEGFPC vector (Life Technologies Corporation, Carlsbad, CA, USA). The bidirectional Renilla/Firefly luciferase constructs containing none or eight MS2-CP-binding sites were kindly provided by C. Gueydan (Bruxelles, Belgium) and are described elsewhere [23]. The MS2-CP (pCMS2) plasmid was also described previously [24]. The plasmid encoding Ki-1/57-MS2-CP, was obtained by inserting the full-length coding cDNA between BamHI and XhoI restriction sites of pCMS2 by direct subcloning.

Co-immunoprecipitation assays

Jurkat cells were cultivated in RPMI Media (GIBCO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO_2 atmosphere. Cells were collected and lysed in 1 ml of lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1% NP-40, 1 mM PMSF, 0.1% Triton X-100). After centrifugation (10,000

×g at 4°C) for 10 min the supernatant was incubated overnight with rabbit anti-FMRP (Abcam) or mouse anti Nek11 or rabbit anti Nek6 isotype matched control antibodies on G-Sepharose 4 fast flow beads (GE Healthcare)**.** The beads were recovered and washed 3 times with the lysis buffer. 30 uL of 4X SDS-PAGE sample buffer (250 mM Tris-HCl, pH 6.8, 0.8% SDS, 0.2% bromophenol blue, 45.5% glycerol and 20% 2-mercaptoetanol) were added to these beads, which were heated to 95°C for 15 minutes to recover the antibody-protein complexes. The supernatants were analyzed by Western blotting with PVDF membranes, using a hybridoma supernatant Ki-1/57 (A26) and polyclonal anti-FMRP (Abcam) antiserum. The membranes were incubated with the horseradish peroxidase conjugated secondary antibody (1:5000; Santa Cruz Biotechnology) for 1 h and washed again three times with TBS. The membranes were developed by chemi-luminescence using the reagent Luminol (Santa Cruz Biotechnology).

Cell culture and stress-induction assays

Cos-7 cells were cultivated in RPMI Media (GIBCO®) supplemented with 100 U/ml penicillin/G-streptomycin and 10% heat-inactivated fetal bovine serum (Biochrom) at 37 °C and 5% CO2. For stress-induced assays, cells were grown until a confluency of 50–70% on glass cover slips and treated with Arsenite (Sigma) (0.5 mM) for 30 min, with additional 30 min for recovery.

Microscopic analyses

After stress treatment, cells were washed with phosphate-buffered saline (PBS), cells were fixed in 2% paraformaldehyde in PBS for 30 min at 37 °C, and then permeabilized for 10 min in PBS+ Triton 0.3% at 25 °C. After three washes in PBS of 5 min each, cells were treated with PBS+100 mM of glycine for 5 min at 25 °C. After three washes, cells were incubated in blocking buffer (BSA 2% in PBS) for 1 h at 25 °C. Cells were then exposed, after five more washes, to primary antibodies. The primary polyclonal antibodies anti-FXR1, FXR2 or FMRP (Abcam), polyclonal anti-TIA1 (Santa Cruz Biotechnology) and monoclonal anti-Flag (Sigma) were incubated at room temperature in PBS 2% BSA followed by incubation with the secondary antibodies conjugated to the fluorophores Alexa-594, (Molecular Probes), FITC (Santa Cruz Biotechnology) or Rhodamine (Santa Cruz Biotechnology). As negative controls, Cos-7 cells were incubated with secondary antibodies only and no staining was observed. DAPI (Molecular Probes) was used to stain nuclei. Fluorsecence microscopy was performed on a Nikon fluorescence microscope (Fig 2A) and confocal microscopy analysis was performed on a Axioplan Carl Zeiss LSM 510 META microscope (Fig 2B).

Sucrose gradient and cell fractionation

Polysome profiles were analyzed on sucrose gradients as previously described [25]. Cells were cultivated up to 50% confluence. Following addition of 100 μg/ml cycloheximide, $5x10^7$ cells were collected and lyzed using 500 μl of polysome buffer (PB) containing 20 mM Tris–HCl pH 7.5, 100 mM NaCl, 10mM $MgCl_2$, 1mM DTT, 1% v/v Triton X-100 and 100 μg/ml cycloheximide. Extracts were clarified by centrifugation at 20,000 x g for 10 min at 4°C. Ribosomal subunits, monosomes and polysomes were separated by centrifugation at 40,000 rpm for 4 h at 4°C using a Beckman SW41 rotor. Protein precipitation and removal of sucrose for immunoblot analyses was performed as described [26]. Proteins were resolved by SDS–PAGE and transferred to PVDF membranes at 80 mA for 1 h in buffer containing 25 mM Tris–base, 200 mM glycine and 20% methanol. Subsequently, membranes were blocked for 2 h with TBST buffer: 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% v/v Tween 20, containing 5% low fat milk. The blots were incubated at room temperature for 2 h with primary antibodies and for 1 h with secondary antibodies. Rabbit polyclonal antibodies for RPS6, and GAPDH (Bethyl Laboratories) were used at a 1:5000 dilution. Anti-Ki-1⁄57 (A26) (mouse mAB, hybridoma supernatant, 1:2 dilution) and anti-RACK-1 monoclonal antibody (Transduction Laboratories, 1:2000 dilution) in 0.2% BSA, respectively. The secondary antibodies used were horseradish peroxidase-conjugated goat anti-mouse IgG (Calbiochem) and donkey anti-rabbit IgG (GE Healthcare) both at 1:5000 dilutions. The immunoblots were developed using the ECL western blotting analysis system (GE Healthcare).

Luciferase assay

Cell lysis and luciferase assays were performed by using the Promega Dual-Luciferase System Kit and a AB2200 luminometer (ATTO, Tokyo) essentially as recomended by the suppliers. Each assay was performed using three independent replicates of transfected cells. Statistical analyses (one way of variance ANOVA) were performed with the Oringin 6.0 software (Micrococal Software, Inc.).

Real-time quantitative PCR (RQ-PCR)

For RQ-PCR, total RNA was extracted from samples of cells using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol and quantified by spectrophotometrical methods. 1 ug of total RNA was treated with DNase (GE Healthcare) and then transcribed into first strand cDNA using First Strand cDNA Synthesis Kit (Amersham Biosciences/GE Healthcare). Briefly, RNA was treated with 20 U of DNase I (Amersham Biosciences/GE Healthcare) in Tris–HCl 40 mM pH 7.5, $MgCl_2$ 6 mM buffer. After 15 minutes DNase was inactivated by heating to

80°C for 10 minutes. For the cDNA synthesis, we followed the manufacturer's instructions, except using that 500 ng of random hexamers.

RQ-PCR assays were performed using the Applied Biosystems 7500 Systems (Applied Biosystems) and each sample was run in triplicate. All PCR reactions were carried out in a final volume of 25 μL containing 1X of SYBR Green PCR Master Mix (Applied Biosystems), a previously determined concentration of each gene specific primers, 1 μL cDNA, and sterile deionized water. The standard cycling conditions were 50 °C for 2 min, 90 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 1C for 1 min. Data were analyzed by relative quantification using beta-actin as endogenous control.

3. Results and Discussion

Protein–protein association analysis

In previous yeast two-hybrid analyses using Ki-1/57 as bait, we found proteins involved in the regulation of translation or associated with ribosomes/polysomes (Fig 1A). The proteins CIRP (cold-inducible RNA-binding protein), RPL38 (ribosomal protein L38) and FXR1 (Fragile X mental retardation-related protein 1) were found interacting with the N-terminal region of the protein Ki-1/57 (amino acids 1-122, unpublished observation). Previous studies had revealed the capacity of CIRP to down-regulate mRNA translation [27], RPL38 interacting with the translation machinery [28] and members of the fragile X related (FXR) protein family in association with polysomes and participating in translation regulation [29]. The protein FMRP has been shown to be both a negative [15, 30-31] and positive [32] regulator of translation depending on the specific target mRNA studied. Moreover, in another screen, now using the C-terminal region of Ki-1/57, we found that 54% of the identified clones represent RACK1, a receptor for activated PKC (protein kinase C) [33, 34]. Literature data show that RACK1 may affect gene expression through translation regulation and activation of ribosome assembly [35, 36]. Therefore, this set of protein-protein interaction data lead us to hypothesize the putative association of Ki-1/57 with translational regulatory events.

In order to obtain additional data that could strengthen such association, we first explored the potential physical association of endogenous Ki-1/57 with FMRP *in vivo*. For that, endogenous FMRP was immunoprecipitated from Jurkat cell lysates, and the co-precipitated proteins were validated by Western blot (Fig.1B). We can see that Ki-1/57 was co-precipitated in a specific fashion, thereby providing support for the hypothesis of a functional association between Ki-1/57 and FMRP, and consequently, for a possible functional involvement of Ki-1/57 in translation

regulatory events. For FXR1 we could not demonstrate a co-precipitation with endogenous proteins, most likely because of the very low expression level of FXR1 in Jurkat and several other cell lines we tested. We were not able to detect the protein in the cells lysates. However, when we performed the experiment with tagged recombinant over-expresssed proteins Flag-Ki-1/57 and EGFPC-FXR1/2 in Hek-293 cells we could detect a co-precipitation between these proteins, too (data not shown). Therefore Ki-1/57 seems to associate with members of the FXR protein family in cells.

Ki-1/57 co-localizes with stress granules under cellular stress conditions

To further study a possible co-localization of Ki-1/57 with FMRP and FXR1/2 proteins in human cells we performed microscopic studies. All of these proteins display a diffuse distribution in the cytoplasm and in the case of Ki-1/57 also in the nucleus (not shown). As this finding might be limited in supporting information regarding their co-localization, we sought for treatments that could result in co-localization of both proteins in specific regions inside the cells. Previous studies reported on the localization of several translation related proteins [37], including the putative Ki-1/57 paralogue CGI-55 [38], to specific cytoplasmic foci, called stress granules, after application of diverse types of cellular stress.

In order to first test whether Ki-1/57 would also localize to stress granules, we performed immune co-localization analyses between Ki-1/57 and the stress granule marker protein TIA1 [39] in cells treated with arsenite, a known stress granule inducer (Fig.2A). Indeed there was a clear superposition of the observed Ki-1/57 and TIA1 foci (merge) suggesting co-localization of these two proteins in the stress granules after arsenit treatment.

We tested next whether Ki-1/57 co-localizes also with FMRP and FXR1/2 in Cos-7 cells after arsenite treatment. Cells were transfected with plasmids encoding the fusion fluorescent protein GFP-Ki-1/57 and subsequently analyzed by confocal microscopy (Fig. 2B). Again a clear cut superposition of the observed foci was found, suggesting that the proteins co-localize in stress granules after arsenite treatment. Indeed FMRP, FXR1 and FXR2 proteins have previously been described as constitutive components of stress granules [37, 40, 41].

Through this association of Ki-1/57 with the stress granules a possible association to translational control mechanisms can be made, since the granules formed transiently in the cytoplasm of cells subjected to different stressors and are enriched in RNA binding proteins involved in controlling the translation and / or stability of mRNAs. The complex between stress granule marker protein TIA1 and TIA-1 related protein (TIAR) is able to inactivate the translation initiation complexes [39]. Stress granules can lead to general repression of protein synthesis from the majority of mRNAs [39]. Therefore, the localization of Ki-1/57 in stress granules along with

FMRP and possibly its others interacting proteins can reinforce the hypothesis that it would be associated to translational regulatory events.

Ribosomal particle sedimentation analysis

Based on findings about the physical interaction of Ki-1/57 with the translation regulatory protein FMRP, the cellular co-localization of both proteins to stress granules, previous results showing the interaction between Ki-1/57 and the translational regulator RACK1 [33, 34] and, that the finding that RACK1 and FMRP are also found in the same complex, interacting with polysomes and messenger RNA [35, 42], we decided to examine whether Ki-1/57 co-sediments with free ribosomal subunits, monosomes and/or polysomes.

To test the possible association of Ki-1/57 to ribosomes we performed by western blot analysis of polysomes fractionated on sucrose gradients, using a standard protocol to isolate polysomal extracts [26]. Ki-1/57 was found to co-sediment mainly with both small and large ribosomal subunits, while a small but significant fraction of Ki-1/57 was seen in the high molecular weight polysomes (Figure 3). RACK1 shows a sedimentation profile similar to the ribosomal protein RPS6, while Ki-1/57 is enriched in the fractions near the 40S sedimentation range. This profile resembles the sedimentation profile of translation initiation factors and of other proteins associated to the 43S and 48S translation pre-initiation complexes, which dissociate from these complexes following 60S subunit joining, as for example eIF6, a RACK1 interacting partner that functions in the activation of 60S ribosome subunits [36]. Interestingly, Ki-1/57 differs from the sedimentation of FMRP which is found in high molecular weight polysomes [43]. We also used sucrose gradient fractionation to compare cells super-expressing GFP-Ki-1/57 in contrast to cells super-expressing only GFP (control). Global polysome profiles were not perturbed and we did not observe difference in the amount of Ki-1/57 associated to polysomes fractions (data not shown).

Tethering translation assay

RNA-binding proteins are key components in the post-transcriptional regulation of gene expression. These proteins often contain conserved RNA-binding domains mediating RNA contact, subcellular targeting and domains involved in protein–protein interactions [44]. hnRNPs constantly shuttle between the nucleus and the cytoplasm and some of these proteins are involved in various aspects of RNA metabolism in the cytoplasm such as RNA stability, nuclear/cytoplasmic transport, RNA localization and translation [45]. Both Ki-1/57 and CIRP are proteins that fit in this classification [2, 11, 27]. Since it has been reported that the Ki-1/57 interacting protein CIRP inhibits translation [27] we decided to investigate the function of Ki-1/57 in mRNA metabolism by

using the same tethering assay. In this approach, the MS2-CP bacteriophage protein is a highly specific ligand of a defined hairpin MS2 RNA sequence. When a protein or protein domain is expressed in fusion with MS2-CP, it is addressed onto any RNA harboring the MS2 stem-loop (Fig 4A), allowing the function of this protein to be analyzed in the context of mRNA stability/translation activity [46]. DNA constructs encoding MS2-CP alone or in fusion with Ki-1/57 were co-transfected in HEK-293T cells with a reporter plasmid carrying the *Firefly luciferase* (Fluc) and *Renilla luciferase* (Rluc) genes under the control of the bidirectional CMV promoter, with the *Renilla luciferase* 3'UTR containing 8 or zero (control) regulatory repeats of the MS2 sequence (Fig 4A). The effect of Ki-1/57 MS2- CP tethering towards the reporter mRNA was evaluated by comparing Rluc activities obtained with constructs with or without MS2 repeats. The Rluc values were divided by the Fluc activity values to normalize transfection and recovery efficiencies. We observed that expression of Ki-1/57-MS2-CP leads to a ~10% increase in the Rluc/Fluc ratio for the 8 MS2-containing reporter gene as compared to the control lacking MS2. This effect is specific to Ki-1/57 MS2-CP, since we used this system with a protein called TTP (kindly provided by Dr. Cyril Gueydan) as a control, which is known to decrease the Rluc/Fluc ratio for the 8 MS2-containing reporter gene relative to the control lacking MS2. Our data further confirmed the data reported by De Leeuw and colaborators [27], who showed that CIRP causes a inhibition of translation. Thus, when tethered to a reporter mRNA, CIRP can repress translation while Ki-1/57 can enhance translation. To address at which level the increase in translation was exerted, we analyzed the effect of Ki-1/57-MS2-CP expression on Rluc mRNA accumulation. To normalize the transfection and recovery efficiencies, the accumulation of Fluc mRNA was measured in the same RNA samples. As shown in Fig. 4C, the steady-state levels of Rluc mRNAs containing the 8 MS2 binding sites seemed to be slightly higher but the observed differences are not statistically significant. This suggests that Ki-1/57 increases the translation of the Rluc mRNA not by stabilizing its mRNA but on another level, possibly at the initiation of translation.

Conclusions

Translational control plays a key role in regulation of gene expression. Previous studies in our group using the *yeast two-hybrid system* identified several proteins that interact with Ki-1/57 ([7] and unpublished data). Many of these proteins participate directly or indirectly in events related to translation as for example RACK1, CIRP, FXR1 and RPL38. Recently, Kondrashov and co-workers provided evidence that the ribosome is a regulatory machine per se, showing that RPL38, as part of the ribosome, facilitates 80S complex formation on selective mRNAs [47]. However, the mechanism by which RPL38 controls translation of specific targets is still unknown. We also have

shown that Ki-1/57 co-sediments with large molecular weight complexes of 43- 48S translation pre-initiation complexes and that Ki-1/57 can increases the expression level of reporter gene luciferase. Together these results suggest that Ki-1/57 might function in the context of translational regulation, thereby opening new avenues of investigation for this regulatory protein.

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5. References

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6 Figure legends

Figure 1: Functional interconnections of Ki-1 ⁄ 57 with translation proteins through direct physical interactions or participation in common protein complexes.

(A) Dotted lines: experiments described in this article (see Results for details). Solid lines: previously published findings; Ki-1/57 is found in the same complex with FXR1, and is associated with RACK-1 [33]. Thin bold lines: FXR1 and FXR2 are homologous and both interact with Ki-1/57 (this study); FXR1 and FXR2 are very similar in overall structure to FMRP (~60% amino acid identity) [37]. The interaction of PRMT1 with Ki-1/57 and CIRP [9] and between RACK1 and FMRP [49] have been described. Interaction of the possible Ki-1/57 orthologue VIG and the dFXR in Drosophila have been previously reported, too [48]. Interaction # has been published as an abstract (Twenty Second Annual Undergraduate Biology Research Conference, University of Arizona. available at http://ubrp.arizona.edu/conferences/11/abstract.cfm?id=1047) and interactions * represent unpublished data from our group. (B) Immunoprecipitation assays (IP) of endogenous proteins in Jurkat cells. Lysates of Jurkat were subjected to immunoprecipitation assays using a polyclonal antibody against the FMRP protein and endogenous Ki-1/57 was detected by Western blot. An aliquot of each whole cell lysate was also obtained to monitor the levels of Ki-1/57 or FMRP. Extracts were immunoprecipitated with G-Sepharose beads and the indicated antibodies. The obtained protein complexes were analyzed by

Western Blot (WB) as indicated in the figure panels. (C) Immunoprecipitation with the indicated isotype matched control antibodies is shown on the right side. IP with anti NEK 11 (antibody produced in mouse (IgG1) and IP with anti NEK 6 (antibody produced in rabbit, IgG1).

Figure 2- Microscopic analysis of the co-localization of Flag-Ki-1/57 with endogenous TIA-1 proteins and confocal microscopic analysis of EGFPC-Ki-1/57 co-localization with FMRP/FXR1/2 in COS-7 cells after arsenite stress. (A) Co-localization of Flag-Ki-1/57 and TIA-1 protein. COS-7 cells were grown on glass cover slips, tranfected and treated with arsenite (0.5 mM for 30 min, plus 30 min of recovery). After treatments, cells were fixed and immuno-stained as previously described. Endogenous TIA-1 was detected with primary antibody (polyclonal) and secondary antibody, coupled by Rhodamine (red). Flag Ki-1/57 was detected with anti-Flag primary antibody (monoclonal) and secondary anti-mouse antibody coupled with Fluorescein - FITC (green) Nuclei were stained with DAPI (blue). Merge of pictures was performed only with green and red staining. All the arrows show the partial co-localization, indicated by the few yellow spots (panels A). (B) EGFPC-Ki-1/57 co-localizes with human proteins FMRP and FXR1/FXR2. Confocal analysis of EGFPC-Ki-1/57 (green) in COS-7 cells indicates that Ki-1/57 co-localizes preferentially in the cytoplasm in specific points with FMRP and FXR1/2 proteins under stress conditions. The proteins were immunodetected with the following antibodies: FMRP/FXR1/FXR2 rabbit polyclonal antibodies and Alexa594-coupled secondary antibody (red). DAPI staining (blue) was used to counterstain the nuclei. Superimposing the two colors (merge) results in a yellow/orange signal.

Figure 3: Analysis of Ki-1/57 sedimentation on a sucrose density gradient. (A) Polysome profile of HEK-293T whole-cell polysomal extracts prepared according to Johannes and Sarnow. (B) Western blot analysis of Ki-1/57 sedimentation on a sucrose density gradient of whole-cell polysomal extracts. The proteins RACK1 and RPS6 are shown as references for ribosome and polysome sedimentation. GAPDH was used as reference for proteins not associated to ribosomes. Whole cell lysates derived from HEK-293T cells were applied to a sucrose density gradient and fractionated. The collected fractions were analyzed on a 4–12% gradient SDS-PAGE and subjected to Western analyses using the indicated antibodies.

Figure 4: Ki-1/57 induces translational activation when tethered to the 3′UTR of a reporter mRNA. (A) Structure of the constructs used in plasmid transfection experiments. A CMV bidirectional promoter controls the transcription of both Firefly and Renilla luciferases. In the transcribed mRNA, the Renilla coding sequence is followed by human b-globin 3'UTR without any supplementary sequence element (0), or a synthetic class II ARE (AUUU)8. Measurement of expression from the Firefly luciferase gene allows normalization between experiments. (B) The luciferase activities measured in the cell extracts are reported as the ratio of the Rluc to Fluc activities (mean±SD of three independent transfections). Blue bars: 0 MS2; red bars: 8 MS2. Differences between 0 and 8 MS2 activity ratios that are statistically significant are marked with asterisks ($p<0.05$). (C) Quantitative real time RT-PCR analysis of Rluc/Fluc in HEK293T cells, over-expressing Ki-1/57 fused with MS2-CP. Differences between 0 and 8 MS are not significant.

Figura 1

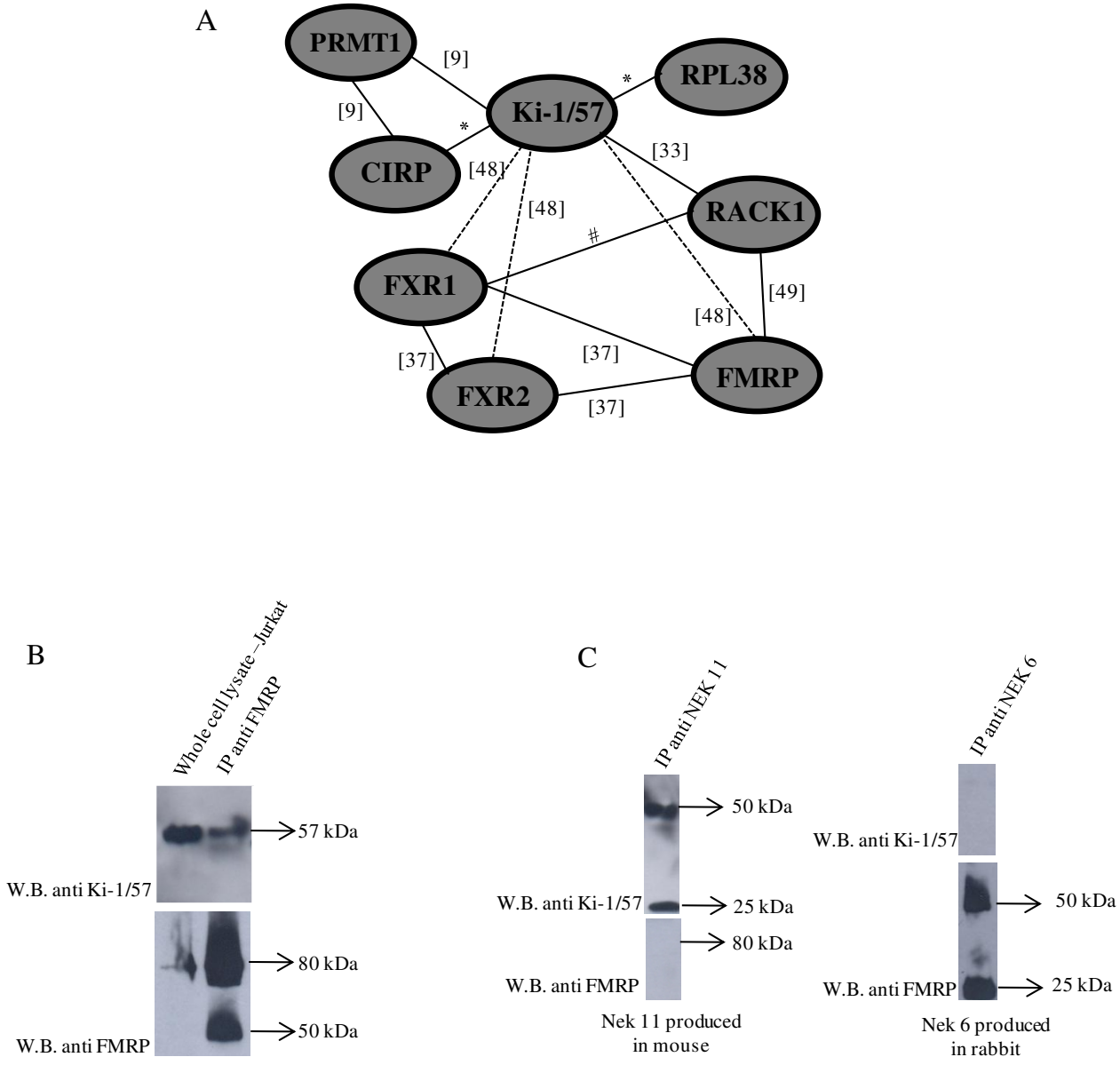

A
PRMT1
Ki-1/57
RPL38
CIRP
RACK1
FXR1
FXR2
FMRP
[9]
[9]
*
*
[33]
[48]
[48]
#
[49]
[48]
[37]
[37]
[37]
B
Whole cell lysate – Jurkat
IP anti FMRP
57 kDa
W.B. anti Ki-1/57
80 kDa
50 kDa
W.B. anti FMRP
C
IP anti NEK 11
50 kDa
W.B. anti Ki-1/57
25 kDa
80 kDa
W.B. anti FMRP
Nek 11 produced
in mouse
IP anti NEK 6
W.B. anti Ki-1/57
50 kDa
W.B. anti FMRP
25 kDa
Nek 6 produced
in rabbit

Figura 2

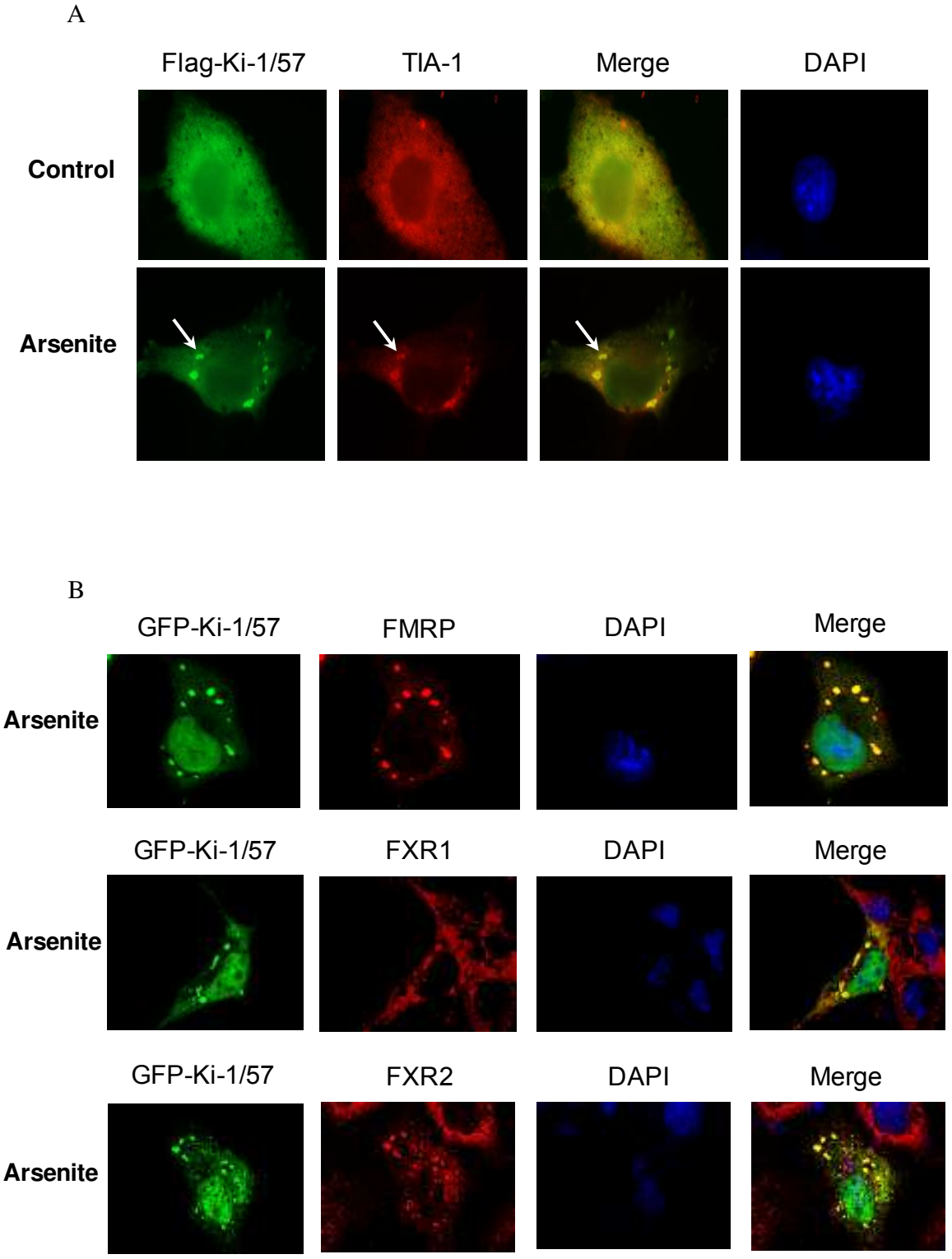

A
Flag-Ki-1/57
TIA-1
Merge
DAPI
Control
Arsenite
B
GFP-Ki-1/57
FMRP
DAPI
Merge
Arsenite
GFP-Ki-1/57
FXR1
DAPI
Merge
Arsenite
GFP-Ki-1/57
FXR2
DAPI
Merge
Arsenite

Figura 3

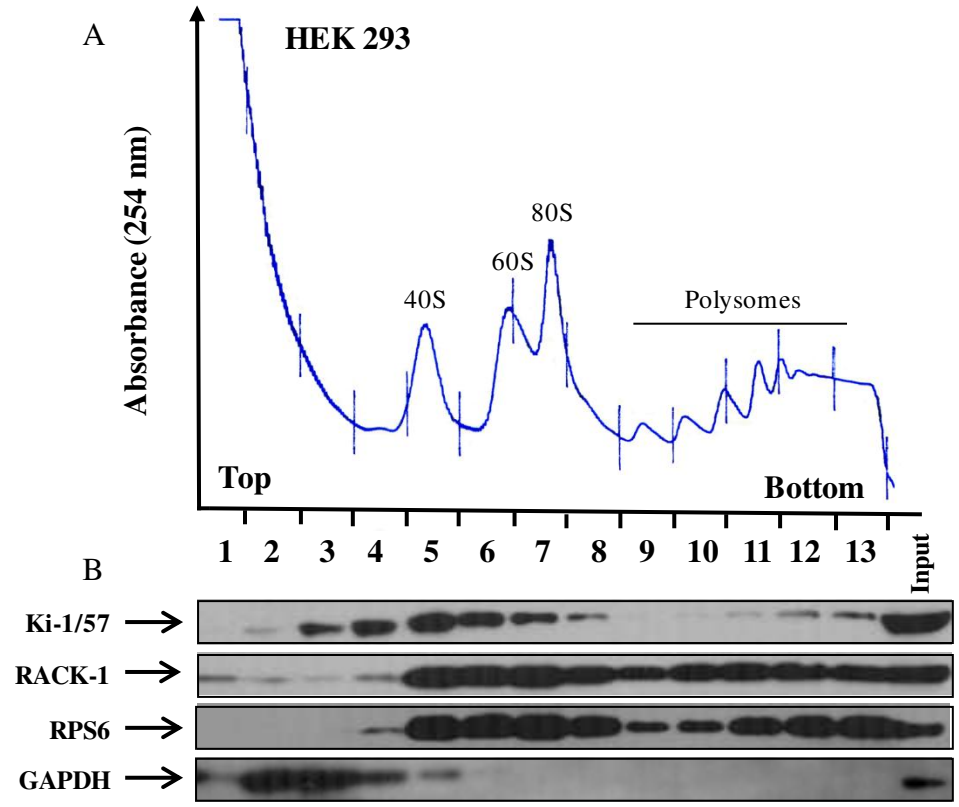
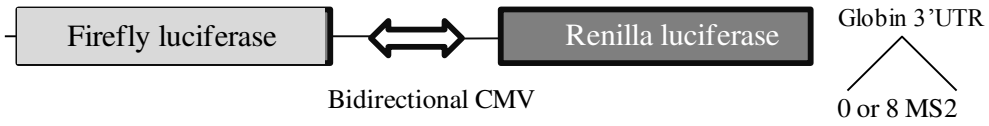
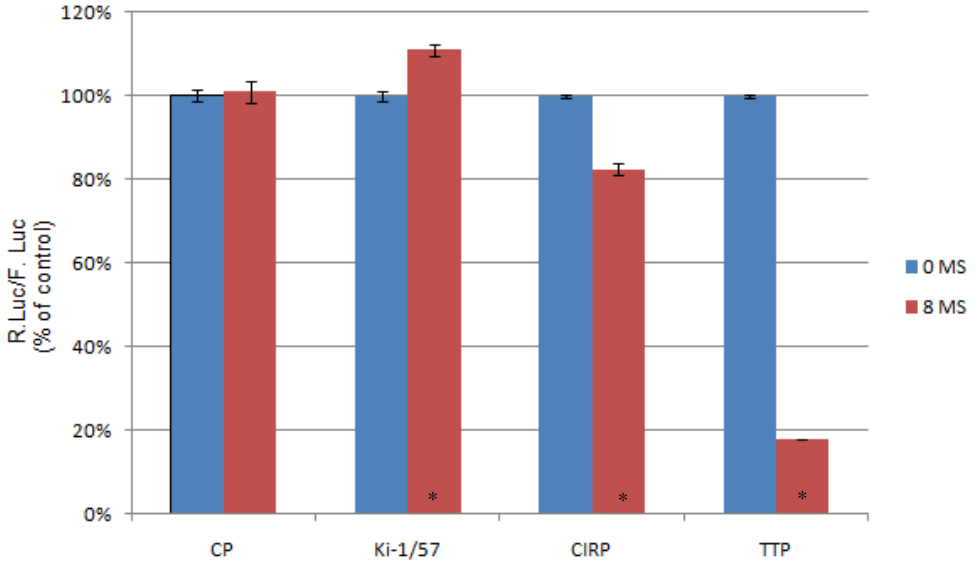

A
HEK 293
Absorbance (254 nm)
40S
60S
80S
Polysomes
Top
Bottom
1 2 3 4 5 6 7 8 9 10 11 12 13
Input
B
Ki-1/57
RACK-1
RPS6
GAPDH

Figura 4

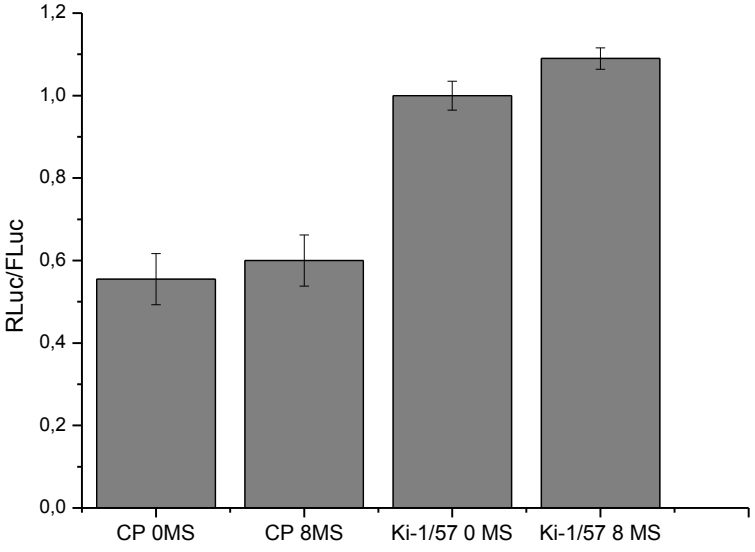
A


Firefly luciferase
Renilla luciferase
Globin 3'UTR
Bidirectional CMV
0 or 8 MS2

B


120%
100%
80%
60%
40%
20%
0%
R.Luc/F. Luc
(% of control)
CP
Ki-1/57
CIRP
TTP
0 MS
8 MS
*

C


1,2
1,0
0,8
0,6
0,4
0,2
0,0
RLuc/FLuc
CP 0MS
CP 8MS
Ki-1/57 0 MS
Ki-1/57 8 MS

3.2- Artigo II

Sumoylation of the human regulatory protein Ki-1/57.

Kaliandra de Almeida Gonçalves, Ângela Saito, Marcos Tadeu dos Santos, Gustavo Costa Bressan and Jörg Kobarg

Sumoylation of the human regulatory protein Ki-1/57

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Introduction

Ki-1/57 is a 57-kDa human regulatory protein first identified by the cross-reactivity of the CD30 antibody Ki-1 in malignant Hodgkin and Sternberg-Reed cells in Hodgkin lymphoma (Schwab *et al.,* 1982, Hansen *et al.,* 1989, Kobarg *et al.,* 1997). It has been demonstrated by electron microscopic analyses that Ki-1/57 is located to cytoplasm, nuclear envelope, chromatin structures and nucleoli (Rohde et al., 1992).

Spectroscopic and physical-chemical analyses demonstrated that Ki-1/57 has characteristics of an intrinsically unstructured protein, which may explain its pleiotropic function in the cell (Bressan et al., 2008). Ki-1/57 showed to be phosphorylated on the serine and threonine residues by protein kinase C PKC (Hansen et al., 1990, Nery et al., 2004) and methylated by methyl-arginine transferase PRMT1 (Passos et al., 2006). It interacts with proteins involved in chromatin remodeling and transcription regulation (CHD3), transcription factors (p53 and MEF2C) and with the receptor of activated kinase RACK1 (Lemos *et al.,* 2003, Nery *et al.,* 2004, Gonçalves *et al.,* 2010, Nery *et al.,* 2006 and Kobarg *et al.,* 2005). Ki-1/57 and RACK1 interaction is abolished upon activation of Hodgkin's lymphoma analogous cell line L540 with PKC-activator PMA (phorbol 12-myristate 13-acetate), resulting in Ki-1/57 translocation from nucleus to cytoplasm (Nery et al., 2006). Moreover, it has also been demonstrated that it contains multiple RGG/RXR box clusters and it is involved in RNA metabolism. Indeed, Ki-1/57 interacts with splicing proteins such as hnRNPQ and SFRS9 and modulates the splicing site selection of the E1A pre-mRNA (Bressan et al., 2009). In a yeast two-hybrid studies we also identified the interaction of Ki-1/57 with UBC9 and PIAS proteins that participate in the sumoylation machinery (unpublished data).

Several studies have identified sumoylation as an important post-translational modification that regulates the biological functions of a protein. In this process, SUMO (small ubiquitin-like modifier) is covalently attached to a lysine residue in a Ψ*KX*E sequence (where *Ψ* is a large hydrophobic residue and *X* represents any amino acid) of a specific target proteins (Matunis *et al.,* 1996, Mahajan *et al.,* 1997, Geiss-Friedlander *et al.,* 2007 and Johnson 2004). Sumoylation regulates diverse cellular processes, including transcription, DNA repair, chromatin structure, cell-cycle progression and intracellular trafficking. In many cases, SUMO modification alters the localization and/or activity of the substrate by providing a new protein-protein interaction interface (Geiss-Friedlander *et al.,* 2007, Johnson 2004, Gill 2005, Verger *et al.,* 2003 and Seeler & Dejean 2003). In mammals four members of the SUMO family have been described: SUMO-1 to 4. SUMO proteins are 11 KDa in size and, although they share only 18% of amino acids identity with ubiquitin, their three-dimensional structure are highly conserved (Bayer *et al.,* 1998). SUMO-2 and SUMO-3 are approximately 96% identical, but share only 50% of identity with SUMO-1. SUMO-4 is more similar to SUMO-2/3, it is however not clear whether SUMO-4 forms conjugates *in vivo* (Owerbach et al., 2005). SUMO-2/3, but not SUMO-1, are able to form polymeric chains through its lysine 11. The steps involved in the SUMO pathway are the ATP-dependent activation of mature SUMO protein by the SUMO E1 activating enzyme, SUMO transference to the E2 conjugation enzyme UBC9 and SUMO transference to the substrate protein by formation of an isopeptide bond between the C-terminal Gly residue of SUMO and a lysine site chain of the target. This last step can be accompanied by SUMO E3 ligase proteins that have been known to enhance the sumoylation by facilitating the transfer of SUMO from UBC9 to the target protein (Johnson 2004, Desterro *et al.,* 1997 and Lin *et al.,* 2002). Several SUMO E3 ligases, including PIAS and RAN-binding protein 2 (RANBP2), have been identified (Shuai et al., 2005).

In this work, we demonstrate that Ki-1/57 is a target of sumoylation both *in vitro* and *in vivo.* Moreover, we performed site directed mutagenesis to identify which of the seven potential the sites are modified by SUMO-1 in the Ki-1/57 amino acid sequence. We found that a Ki-1/57 triple mutant no longer is sumoylated by neither SUMO-1 nor SUMO-2. Interestingly, we found that Ki-1/57 sumoylation regulates the E1A pre-mRNA processing.

Materials and Methods

Plasmid Constructions

Cloning of the complete cDNA encoding Ki-1⁄57 or into pEGFPC vector or pCDNA6.1 N- terminal fusion Flag vector has been described previously (Bressan *et.al.,* 2009). The cDNAs encoding the seven other indicated mutations constructs of the Ki-1/57 were amplified and inserted into the pEGFPC or pCDNA6.1-Flag. Ki-1/57 mutants (K336R, K276R, K336/213,K213R/K276R/K336R,K336R/K213R/K276R/K313R,K336R/K213R/K276R/K313 R/K306R), were generated using site-directed mutagenesis according to the manufacturer's instructions (Stratagene, La Jolla, CA). The mutants were PCR-amplified and sub-cloned into pCDNA6.1 and pEGFPC. The SUMO-2 coding sequence was isolated from pACT-SUMO2 using the EcoRI/XhoI restriction sites and inserted into pmRFPL-SUMO2 was generated by transferring the EcoRI/BglII fragment from pACT-SUMO2 to pmRFPL. The vector pGEMT Easy with SUMO-1 was kindly provided by Dr. D.M. Trindade (LNBio- Brasil). The plasmid encoding SUMO-1, was obtained by inserting the full-length coding cDNA between BamHI and Hind III restriction sites of pEGFPC by direct sub-cloning.

In vitro SUMOylation

For *in vitro* SUMOylation assays of Ki-1/57 we used a SUMOylation Kit (BIOMOL) according to the manufacturer's instructions. We used 2 nM of RanGAP1-GST protein provided by the kit as well as 2 nM Ki-1/57-GST, which was expressed and purified as described above. GST (2 nM) was used as control. For controls all reactions were carried out in the absence of ATP. The reactions were analyzed by Western blot using rabbit polyclonal anti-SUMO1 (1:1000 – BIOMOL), rabbit polyclonal anti SUMO-2/3 (1:1000- BIOMOL) or mouse monoclonal anti-Ki-1/57 (A26) (Kobarg J. et al., 1997), followed by membrane stripping and analysis by mouse monoclonal anti-GST antibody (Assmann et al., 2006).

Protein Expression and Purification

Full length Ki-1/57 cDNA was cloned into the bacterial expression vector pGEX 2TK to allow its expression as a GST-tagged fusion protein. BL21 strain (Invitrogen) was transformed with recombinant vector and grown in LB medium with 25 ug/ml of ampicillin. When reaching the logarithmic growth phase the recombinant bacteria were induced for protein production with 0,5 mM of isopropyl-b-D-thiogalactoside (IPTG) at 37 ºC for 4 h. After harvest and lysis the resulting suspension was cleared by centrifugation. The obtained supernatant was

loaded onto a *GST-Trap* (Amersham) and eluted in buffer (50 mM Tris-HCl pH 8,0; 50 mM NaCl; 0,1 mM EDTA; 20 mM reduced glutathione) The obtained GST- affinity purified fractions were pooled and dialyzed against the buffer: 50 mM Tris-HCl pH 8,0; 50 mM NaCl; 0,1 mM EDTA. The concentration of the recombinant protein was determined spectroscopically using the calculated extinction coefficient for the denatured proteins as described (Sambrook et al., 1989).

Co-immunoprecipitation

HEK-293T cells were cultivated in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5% CO_2 atmosphere. Transient transfections were performed using the calcium phosphate method. Cells were collected and lysed in 1 ml of lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1% NP-40, 1 mM PMSF, 0.1% Triton X-100). After centrifugation (10,000×g at 4 °C) for 10 min the supernatant was incubated with a rabbit polyclonal anti-Ki-1/57 polyclonal or rabbit Anti SUMO-1(Abgent) or mouse monoclonal Anti Flag (Sigma) in G-Sepharose 4 fast flow beads (GE Healthcare) at overnight. The beads were recovered and washed 3 times with the lysis buffer. To these beads, 30 μL of SDS 4X buffer were added (Tris-HCl 250 mM pH 6,8, SDS 0,8%, bromofenol blue 0,2%, glycerol 45,5% e 2-mercaptoetanol 20%). This mix was heated to 95 °C for 15 minutes to recover the antibody-protein complexes. The eluates were analyzed by Western blot with PVDF membranes, using rabbit polyclonal anti-SUMO-2 (Sigma), polyclonal anti SUMO-1 (Abgent) and monoclonal anti Flag (Sigma). The membranes were incubated with the secondary horseradish peroxidase conjugate (1:5000; Santa Cruz Biotechnology) for 1 h and washed again three times with TBS. The membranes were developed by chemi-luminescence using the reagent Luminol (Santa Cruz Biotechnology).

Microscopic analyses

For the sub-cellular localization assays, COS-7 cells were grown on glass coverslips with DMEM (Dulbecco's modified Eagle's médium) culture + 10% FCS medium, 100 U/ml penicillin and 100 μg/ml streptomycin and transfections were performed by using the calcium phosphate method, using pEGFPC-Ki-1/57. Cells were fixed in a solution containing 2% paraformaldehyde, permeabilized with 0.3% of Triton X-100, and blocked with PBS 2% BSA

at room temperature for 30 min. For stress-induced assays, cells were incubated a 42 °C for 1hour, with additional 30 min for recovery. The primary antibodies anti-SUMO-1 (Abgent) or anti-SUMO-2, (Sigma) all polyclonal antibodies obtained from rabbits were incubated at room temperature in PBS 2% BSA followed by incubation with the Alexa-594 (Invitrogen) conjugated secondary antibody. As a control we used, a slide with COS-7 cells incubated only with the secondary antibody. DAPI (Molecular Probes) dye was used to stain the nuclei of cells. Confocal microscopy analysis was performed on a Axioplan Carl Zeiss LSM 510 META microscope.

In vivo splicing assay

For the in vivo splicing analysis, we transiently transfected COS-7 cells with the minigene E1A encoding plasmid pMTE1A (Zerler *et al.,*1986), in combination with crescent amounts of Ki-1⁄57 wild type or triple mutant. The DNA concentration in each transfection was kept constant by using the empty vector pEGFP (Life Technologies Corporation). After 48 h of transfection, the cells were re-suspended in 1 mL of TRizol reagent (Life Technologies Corporation) for total RNA extraction according to the manufacturer's protocol. cDNA synthesis was performed using oligo-dT primer (GE Healthcare, Waukesha, WI, USA) and the Moloney murine leukemia virus reverse transcriptase (Life Technologies Corporation). The PCRs were performed with the primers 5'-ATTATCTGCCACGGAAGGTGT-3' (sense) and 5'GGATAGCAGGCGCCATTTTA-3' (anti-sense), as previously described (Raffetseder *et al.,* 2003). After separation of the amplification products on 3% agarose gels containing ethidium bromide, the band intensities were calculated using the software image j (http://rsb.info.nih.gov/ij/index.html; National Institute of Mental Health, Bethesda, MD, USA). The intensities of all isoforms were summed, set as 100%, and used to normalize the intensity of each band.

Results and Discussion

Ki1-/57 is modified by SUMO-1 and SUMO-2/3 *in vitro*

Sumoylation is an important post-translational modification that regulates the biological functions of proteins in essential processes, such as: gene transcription, sub-nuclear organization, and cell cycle progression (Kerscher 2007). Results previously obtained by our

research group showed that Ki-1/57 interacts with proteins of the sumoylation machinery, including UBC9 e PIAS3 (data not shown). We therefore analyzed the amino acid sequence of Ki-1/57 for the presence of sumoylation motifs. We identified seven potential SUMO-1 attachment sites in the Ki-1/57 amino acid sequence, using the program SUMOplot™ web available tool from Abgent (http://www.abgent.com/doc/SUMOplot) (Fig 1A). This analysis showed that the lysines 213, 276 and 336 are the most likely sites of SUMOylation, with a score higher than 67%. By comparing the scores with those of other known SUMOylated proteins, we concluded that it is seemed likely that Ki-1/57 may be also a target of SUMOylation. To test this hypothesis we performed an *in vitro* SUMOylation assay using recombinant GST-Ki-1/57 as a target (Fig 1B). We observed that the Ki-1/57 protein is sumoylated by both SUMO-1 and SUMO-2/3.

Identification of sumoylation sites in Ki-1/57 sequence

Next we wanted to know if Ki-1/57 is also a sumoylated protein *in vivo*. For this purpose, we performed immunoprecipitation analyses of cellular extracts from HEK 293T cells using anti-SUMO-1 antibodies, followed by immunoblotting with anti-Flag or anti Ki-1/57. As shown in Fig. 2A, immunoprecipitated SUMO-1 could also be detected with an anti-Flag or anti-Ki-1/57 antibody, indicating that Ki-1/57 is a sumoylated protein *in vivo*. To examine whether the predicted sites indeed serve as SUMO-1 acceptors in Ki-1/57 in vivo we first generated two double (K213R / K276R and K276R / K336R) and on triple Ki-1/57 mutant (K213R / K276R / K336R), in which the corresponding lysine residues had been changed to arginines, in order to preserve the positive charge. Wild-type (wt) and the mutated Ki-1/57 were then transfected in HEK-293 cells with co-transfection of EGFP-SUMO-1 plasmid (Fig. 2B). The cell lysates were immunoprecipitated with an anti-Flag antibody followed by immunoprobing with an anti-SUMO-1 antibody. As shown in Fig. 2B, SUMO-1 was significantly weaker but could still be detected in the two double mutants (K213R / K276R and K276R / K336/R) of Ki-1/57. In the triple mutant of Ki-1/57 (K213R / K276R / K336R), SUMO-1 modification was no longer detectable. In summary these data suggest that the three lysines 213, 276 and 336 are all acceptor sites for SUMO conjugation in Ki-1/57. It is interesting to note that these three lysines were the ones that had the highest score in the analysis with the program SUMOplot™. The triple mutant Ki-1/57-K213R / K276R / K336R was also tested to see if it was still sumoylated by SUMO-2 *in vivo*. As show in Fig. 2C only

the wild type protein but not the triple mutant is sumoylated with SUMO-2, too, although the degree of modification with SUMO-2 seems to be lower than than that with SUMO-1 (Fig.2B).

Confocal analysis of the localization of Ki-1/57 with SUMO-1 and SUMO-2

It has been described previously that Sumoylation of proteins might be an important role for nuclear import /export or to address proteins to distinct cellular compartments and biological function in proteins (Barry *et al.,* 2010)**.** Numerous works have demonstrated that proteins are sumoylated in response to stress-inducible heat shock and that heat-shock response treatment enhances SUMO conjugation (Hietakangas *et al.,* 2003, Vertegaal *et al.,* 2006 and Pelisch *et al.,* 2010). Ki-1/57 co-localizes in a spot like fashion in the cytoplasm to TIA1 positive organeles called stress granules (Gonçalves *et al.,* 2011). Cytoplasmic stress granules are formed by RNA binding proteins, which are mainly involved in controlling the translation and stability of specific mRNAs. Stress granules can lead to a general repression of protein synthesis from the majority of mRNAs, except those that present regions of IRES (internal ribosome entry site) (Kedersha *et al*., 1999; Holcik *et al*., 2005).

Therefore, we performed immune-localization studies of Ki-1/57 wild type with SUMO-1 and SUMO-2 in COS-7 cells after and before thermal stress (42 °C). Ki-1/57 wild type has a diffuse distribution in the cytoplasm and nucleus (Fig. 3A). After heat shock we observed a co-localization of both proteins in specific regions inside the cells (stress granules) which may suggest that there is possible functional context for the sumoylation during the stress (Fig. 3B). We therefore tested next whether the triple Ki-1/57 mutant (K213R / K276R / K336R) has an altered cellular localization and observed that it is still diffusely distributed in the cell but after heat shock it still localizes to the stress granules as the wild type protein (data not show). This may suggest that the localization of Ki-1/57 to the stress granules is not dependent on its sumoylation status.

Influence of Ki-1/57 on E1A pre-mRNA splicing *in vivo*

The association of Ki-1/57 with splicing proteins pointed to a possible functional role in pre-mRNA splicing regulation. Bressan et al., 2009 showed that Ki-1/57 modulates the splicing site selection of the adenoviral E1A test minigene, previously explored for the Ki-1/57-interacting proteins SFRS9 (serine/arginine-rich splicing factor 9) and YB-1 (Raffetseder *et al.,* 2003). Pelisch and co-workers observation that SF2/ASF expression levels control the

sumoylation status of RNA metabolism-related proteins may suggest that SF2/ASF may be a putative molecular and functional link between the RNA processing and sumoylation machineries SF2/ASF belongs to the serine/arginine (SR)-rich family of pre-mRNA splicing factors, together with SFRS9 which interacts with Ki-1/57.

Depending on the 5' splice site selection, the E1A pre-mRNA may generate five isoforms: 13S, 12S, 11S, 10S, and 9S (Fig. 4A) (Stephens & Harlow 1987). These isoforms can be monitored by RT-PCR followed by agarose gel analysis, where the intensity of each band in the gel directly correlates with the splicing site selection, which, in turn, reflects the positive or negative influence of regulatory proteins (Stephens & Harlow 1987, Caceres *et al.,* 1994). We transiently co-transfected the encoding E1A minigene plasmid with increasing amounts of vectors expressing the recombinant enhanced green fluorescent protein (EGFP)–Ki-1⁄57 in COS7 cells (Control) or (EGFP)–Ki-1⁄57 triple mutant. We observed a significant effect of the EGFP–Ki-1⁄57 triple Lys to Arg mutant (K213R / K276R / K336R) in modifying the pattern of splicing of E1A mRNA in comparison with (EGFP)–Ki-1⁄57 wild type (Fig. 4B). With this experiment we could show one of the possible functional roles in sumoylation protein Ki-1/57. Sumoylation in Ki-1/57 protein seems to be essential for its splicing related activity.

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Figure legends

Figure 1. Ki-1/57 has seven potential SUMOylation motifs and is SUMOylated in vitro. (A) Identification of seven potential SUMO-1 attachment sites in the Ki-1/57 amino acid sequence, using the program SUMOplot^{TM} web available tool from Abgent. **(B)** *In vitro* SUMOylation. For *in vitro* SUMOylation assays of Ki-1/57 we used a SUMOylation Kit (BIOMOL) according to the manufacturer's instructions. We used 2 nM of RanGAP1-GST protein provided by the kit as well as 2 nM Ki-1/57-GST, which was expressed and purified as described above. GST (2 nM) was used as control. For control all reactions were carried out in the absence of ATP. The reactions were analyzed by Western blot using rabbit polyclonal anti-SUMO1, rabbit polyclonal anti SUMO-2 (1:1000 – BIOMOL), or mouse monoclonal anti-Ki-1/57 (A26) (Kobarg J. et al., 1997), followed by membrane stripping and analysis by mouse monoclonal anti-GST antibody (Assmann et al., 2006).

Figure 2. Ki-1/57 is modified by SUMO-1 *in vivo*. (A) HEK-293 cells were transiently transfected with pCDNA6 Flag Ki-1/57. Afterwards, cells were lyzed, and immunoprecipitated (*IP)* with *polyclonal rabbit anti S*UMO1. Immunoprecipitates were subjected to SDS-PAGE followed by Western immunoblot analysis using anti-Flag and anti Ki-1/57 antibodies. Immunoprecipitation with the indicated control antibody is shown on the right side (B) vectors expressing wild type Ki-1/57, or the consensus site single mutants, Ki-1/57 (K336R), Ki-1/57 (K276R), or double mutant Ki-1/57 (K336R,K213R) were transfected into HEK-293 cells.

SUMO-1 modification of wild type Ki-1/57 and the indicated Lys to Arg mutants were assessed by Western immune-blotting as described above. Transfection efficiencies were assessed by Western immune-blotting using anti-Flag monoclonal antibodies on whole cell lyzates. The arrows indicate the positions of SUMO-1 modified Ki-1/57 species. The positions of the molecular mass standards are indicated on the right.

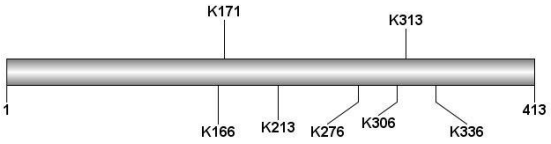
Figure 3- Co-localization of the Ki-1/57 with SUMO-1 and SUMO-2. Confocal analysis of EGFP-Ki-1/57 in COS-7 cells indicates that Ki-1/57 co-localizes with SUMO-1 and SUMO-2 proteins. COS-7 cells were grown on glass cover slips, transfected with pEGFP-Ki-1/57 and cells were incubated a 42 °C for 1 h, with additional 30 min for recovery. After treatments, cells were fixed and immuno-stained as previously described. Endogenous SUMO-1 or SUMO-2 were detected with primary antibody (polyclonal Abcam or Sigma respectively) and secondary antibody Alexa594-coupled *(red)*. Nuclei were stained with DAPI (blue). All the arrows show the partial co-localization, indicated by the few yellow spots (panels A-View the merge of the figures).

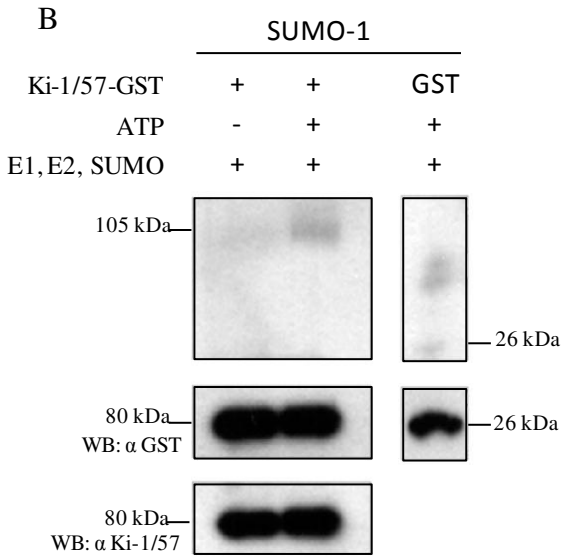
Figure 4- SUMOylation of Ki-1/57 is required for its splicing regulating activity (A) Diagram showing the splicing events that generate the 13S, 12S, 10S and 9S mRNAS of the E1A pre-mRNA reporter gene construct (Stephens & Harlow 1987, Caceres *et al.,* 1994). (B) In vivo splicing assays. COS7 cells were transiently co-transfected with an E1A minigene encoding plasmid, an empty pEGFP vector and increasing amounts (1X, 5µg; 2X, 10 µg; 3X, 15µg) of pEGFP-Ki-1/57 (full length) or pEGFP–Ki-1/57 MUT (K213R / K276R / K336R) vectors. The empty pEGFP vector was used to keep constant the DNA concentration in each transfection. Splicing activity quantitation was performed as described in Experimental procedures. The displayed figures are representative of three independent experiments. We achieved approximately 60% transfection efficiency in all experiments performed. M = marker.

Figura 1

A

No.	Pos.	Group	Score
1	K336	YRDDM VKDD YEDDS	0.93
2	K276	EEESP AKVP ELEVE	0.69
3	K213	AFDQR GKRE FERYG	0.67
4	K306	QEQTR PKPE FNIRK	0.61
5	K313	PEFNI RKPE STVPS	0.44
6	K166	ADFTA EKFP DEKPG	0.39
7	K171	EKFPD EKPG DRFDR	0.33





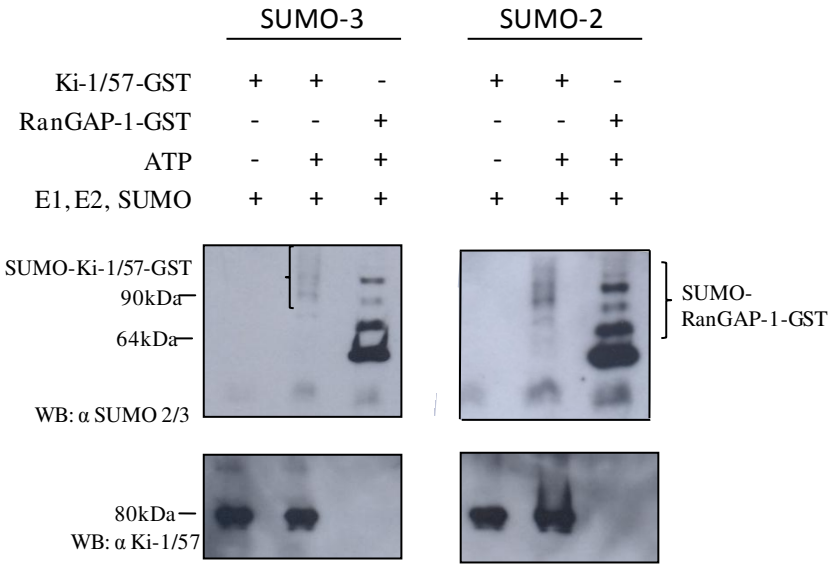


Figura 2

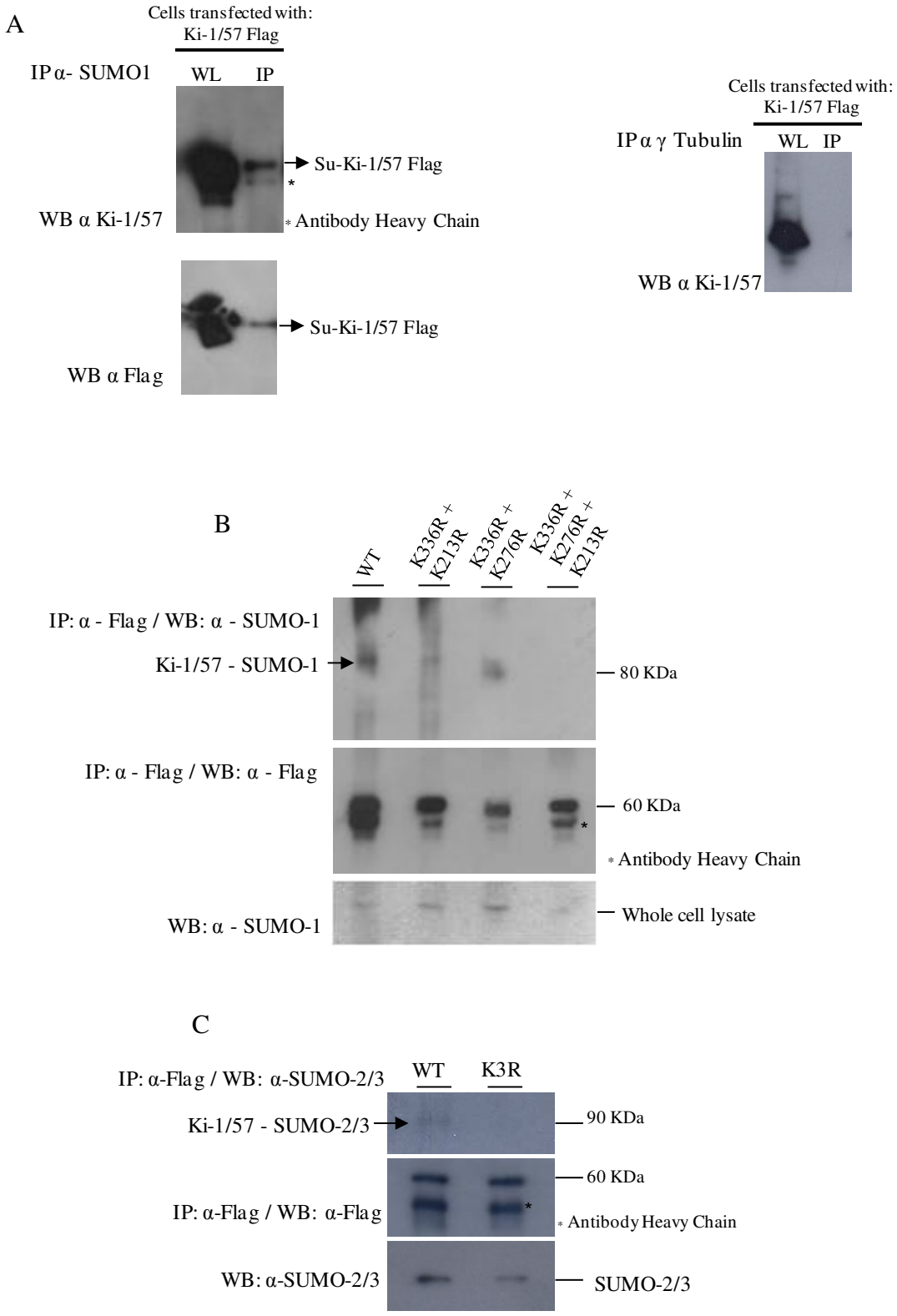

A
Cells transfected with:
Ki-1/57 Flag
IP α- SUMO1
WL
IP
Su-Ki-1/57 Flag
*
WB α Ki-1/57
* Antibody Heavy Chain
Su-Ki-1/57 Flag
WB α Flag
Cells transfected with:
Ki-1/57 Flag
IP α γ Tubulin
WL
IP
WB α Ki-1/57
B
WT
K336R + K213R
K336R + K276R
K336R + K276R + K213R
IP: α - Flag / WB: α - SUMO-1
Ki-1/57 - SUMO-1
80 KDa
IP: α - Flag / WB: α - Flag
60 KDa
*
* Antibody Heavy Chain
WB: α - SUMO-1
Whole cell lysate
C
IP: α-Flag / WB: α-SUMO-2/3
WT
K3R
Ki-1/57 - SUMO-2/3
90 KDa
60 KDa
IP: α-Flag / WB: α-Flag
*
* Antibody Heavy Chain
WB: α-SUMO-2/3
SUMO-2/3

Figura 3

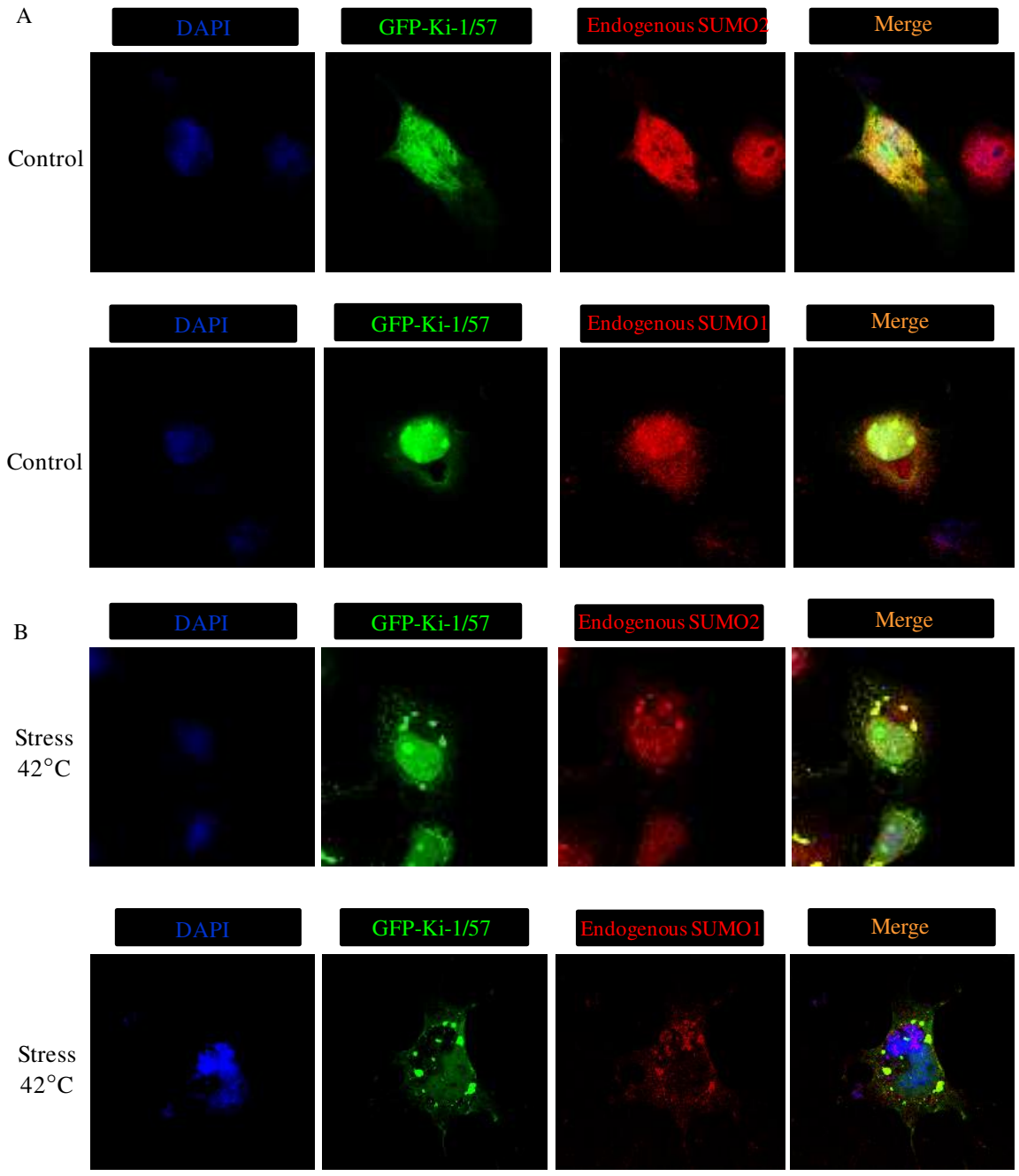
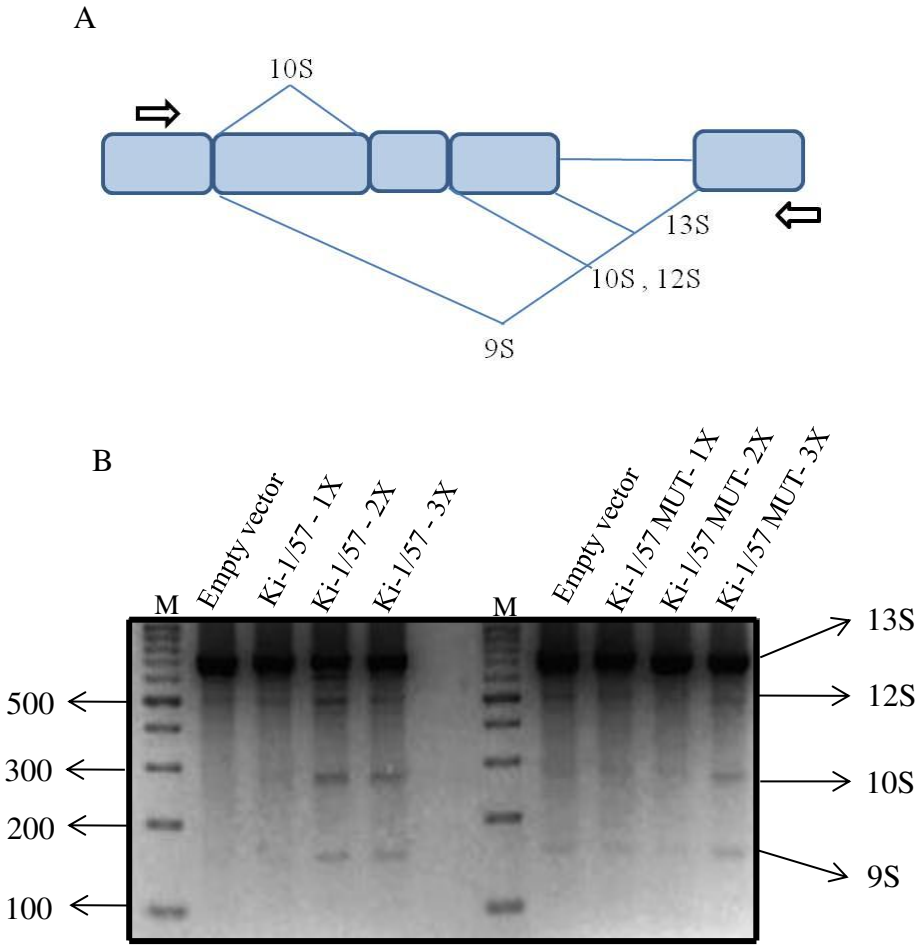

A
DAPI
GFP-Ki-1/57
Endogenous SUMO2
Merge
Control
DAPI
GFP-Ki-1/57
Endogenous SUMO1
Merge
Control
B
DAPI
GFP-Ki-1/57
Endogenous SUMO2
Merge
Stress
42°C
DAPI
GFP-Ki-1/57
Endogenous SUMO1
Merge
Stress
42°C

Figura 4


A
10S
13S
10S , 12S
9S
B
M
Empty vector
Ki-1/57 - 1X
Ki-1/57 - 2X
Ki-1/57 - 3X
M
Empty vector
Ki-1/57 MUT- 1X
Ki-1/57 MUT- 2X
Ki-1/57 MUT- 3X
500
300
200
100
13S
12S
10S
9S

3.3- Artigo III

Solution structure of the human signaling protein RACK1

Kaliandra A Goncalves, Julio C Borges, Julio C Silva, Priscila F Papa, Gustavo C Bressan, Iris L Torriani e Jorg Kobarg

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RESEARCH ARTICLE Open Access

Solution structure of the human signaling protein RACK1

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Abstract

Background: The adaptor protein RACK1 (receptor of activated kinase 1) was originally identified as an anchoring protein for protein kinase C. RACK1 is a 36 kDa protein, and is composed of seven WD repeats which mediate its protein-protein interactions. RACK1 is ubiquitously expressed and has been implicated in diverse cellular processes involving: protein translation regulation, neuropathological processes, cellular stress, and tissue development.

Results: In this study we performed a biophysical analysis of human RACK1 with the aim of obtaining low resolution structural information. Small angle X-ray scattering (SAXS) experiments demonstrated that human RACK1 is globular and monomeric in solution and its low resolution structure is strikingly similar to that of an homology model previously calculated by us and to the crystallographic structure of RACK1 isoform A from *Arabidopsis thaliana*. Both sedimentation velocity and sedimentation equilibrium analytical ultracentrifugation techniques showed that RACK1 is predominantly a monomer of around 37 kDa in solution, but also presents small amounts of oligomeric species. Moreover, hydrodynamic data suggested that RACK1 has a slightly asymmetric shape. The interaction of RACK1 and Ki-1/57 was tested by sedimentation equilibrium. The results suggested that the association between RACK1 and Ki-1/57(122-413) follows a stoichiometry of 1:1. The binding constant (KB) observed for RACK1-Ki-1/57(122-413) interaction was of around $(1.5 \pm 0.2) \times 10^6\ M^{-1}$ and resulted in a dissociation constant (KD) of $(0.7 \pm 0.1) \times 10^{-6}$ M. Moreover, the fluorescence data also suggests that the interaction may occur in a cooperative fashion.

Conclusion: Our SAXS and analytical ultracentrifugation experiments indicated that RACK1 is predominantly a monomer in solution. RACK1 and Ki-1/57(122-413) interact strongly under the tested conditions.

Background

The adaptor 36 kDa protein RACK1 (receptor of activated kinase 1) was originally identified as an anchoring protein for protein kinase C and contains seven WD repeats that mediate its protein-protein interactions [1,2]. It is also found to be up-regulated in human carcinomas and during tissue regeneration after ischemic renal injury [3,4]. Furthermore, RACK1 has been functionally implicated in the development of cardiac hypertrophy,[5] in the increase of focal adhesion[6] and regulation of cell adhesion [7]. Also, recent data suggest that RACK1 may affect gene expression through translation regulation and assembly activation of ribosomes [8-10]. Overall, these data suggest that RACK1 is a multi-purpose protein whose regulatory roles may impact various functional contexts.

It has been reported that RACK1 interacts with several molecules including β PKC β;[11] Src;[12] β integrins;[13] PDE4D5;[14] STAT1[15] and the regulatory protein Ki-1/57, [16,17] a cytoplasmic and nuclear protein initially identified in malignant cells from Hodgkin's lymphoma [18,19]. Recent studies showing its phosphorylation, arginine methylation and interaction with other regulatory proteins, suggest that the functional role of Ki-1/57 in human cells seems to be related to splicing regulation and other events in RNA metabolism [16,20,17,21].

RACK1 is a member of the family of β-propeller proteins and the first member of this protein family whose structure has been determined was the β-subunit of the mammalian heterotrimeric G protein [8,22]. Significant sequence similarity with the G protein β-subunit has led to the prediction that RACK1 may also adopt a seven-

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bladed β-sheet propeller [22,23]. This suggests the availability of multiple protein interaction surfaces. We previously modeled the three-dimensional structure of human RACK1[24] and the crystal structure of a RACK1 ortholog from *Arabidopsis thaliana* was recently elucidated with a resolution of 2.4 Å (PDB entry 3md0) [25].

Here, we analyzed the structural properties of human RACK1 in solution through a set of biophysical approaches. We showed that RACK1 is a predominantly monomeric protein in solution accordingly to data obtained from analytical ultracentrifugation (AUC) and small angle X ray scattering (SAXS) experiments. We restored the human RACK1 envelope from SAXS curves through *ab initio* approaches and compared it with previously obtained related structures. These results suggest a strong similarity between the structures of human RACK1 and RACK1 isoform A from *Arabidopsis thaliana*, in accordance with the high conservation between both amino acids sequences. The functional activity of RACK1 in solution was also accessed through its binding to Ki-1/57 protein. Fluorescence spectroscopy experiments after titration of Ki-1/57(122-413) in solution with RACK1 and sedimentation equilibrium experiments showed that this association is strong and might involve cooperative events.

Results and Discussion

Small Angle X-Ray Scattering results

Small-angle X-ray Scattering (SAXS) is an important technique to obtain shape information and dimensional parameters of biological macromolecules. Recently, the application of SAXS to predict low resolution protein structures gained even more importance with the advances in the X-ray sources and computational methods. Since efforts to crystallize the human RACK1 have been so far unsuccessful, an important step in the structural analysis of this protein is to perform X-ray scattering experiments to determine its conformation in solution. The obtained intensity SAXS curve, normalized by concentration, is presented in Figure 1A together with the regularization fit obtained from the indirect Fourier transform, which provided the pair distance distribution function p(r) as described in the "materials and methods" section. The resulting p(r) function is displayed in Figure 1B. In order to confirm the sample monodispersity, we inspected the Guinier [26] approximation in its validity region of the intensity curve. Guinier plots are displayed in the *inset* of Figure 1A. The linearity of this region confirmed its monodispersity. The radius of gyration (R_g) of the human RACK1 in solution was (20.8 ± 0.8) Å, obtained using the Guinier approximation, and (20.6 ± 0.4) Å when calculated from the normalized second moment of the p(r) function. Both R_g values are in very close agreement. The maximum dimension (D_{max}) of the human RACK1 in solution provided by the p(r) function was 60 Å. These data suggest that the protein is globular and slightly oblate. The Kratky plot shown in the *inset* of Figure 1B indicates that the protein conformation is quite compact. Using a bovine serum albumin (BSA) solution as a standard, the MM estimated for human RACK1 from the SAXS data was ~40 kDa, which is very close to the value of 37 kDa predicted from the amino acid sequence of the recombinant protein. Based on these results we conclude that the human RACK1 protein is a monomer in solution.

Amino acids sequence analysis and comparison of known structures of RACK1 with experimental SAXS data

Human RACK1 has significant amino acid sequence similarity to RACK1 isoform A from *Arabidopsis thaliana* with 66% amino acid sequence identity and 78% amino acid sequence similarity (Figure 2A). This comparison suggests that both proteins may have a very similar three dimensional structures. The alignment of both amino acid sequences shows that the main difference between the two proteins is located in their C-terminal region. RACK1 orthologs have also been discovered in unicellular eukaryotes, such as *Chlamydymonas,*[27] and are highly conserved in plants [28]. The high degree of sequence conservation likely reflects a high degree of functional conservation of the proteins in these species. Although mutations are acquired through evolution the preservation of the protein structure is usually higher than the conservation of the amino acid sequence, since the maintenance of the structure is pivotal for the proteins function [29].

The crystallographic structure of the RACK1 isoform A from *Arabdopsis thaliana* was recently solved and deposited in the Protein Data Bank under the code 3dm0 [25]. Furthermore, the homology model of the human RACK1 was previously published, [24] allowing to calculate the scattering intensity curve from the atomic coordinates of these two structures as described in the "material and methods" section. We compared these calculated curves with the experimental data obtained for the human RACK1 in solution. The results are shown in Figure 2B. Both calculated curves compare quite well with the experimental data, which indicates that the human RACK1 may have a very similar structure compared to both the homology model and the RACK1 isoform A from *Arabdopsis thaliana*, although the calculated intensity for the homology model (red curve) seems to be nearer to the experimental data for human RACK1.

Low resolution models obtained from SAXS data

One of the great advantages of the SAXS technique is that it offers the possibility of restoring the three dimensional

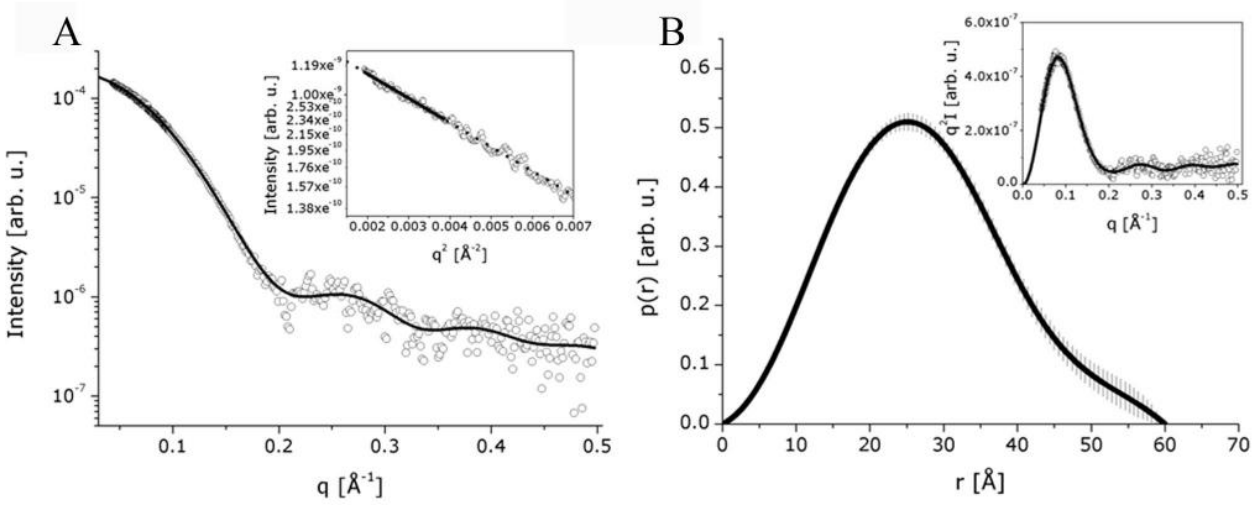

Figure 1 Small Angle X-ray Scattering (SAXS) results. (A) Experimental scattering curve of 6xHis-RACK1 (open circles) and the theoretical fitting (solid line) by using the program GNOM. *Inset*: Guinier Region and linear regression (solid line) for R_g evaluation. (B) Pair distance distribution function p(r). *Inset*: Kratky plot.

molecular envelope from the experimental data. This modeling can be improved when portions of the structure are already known, solving the possible ambiguities resulting from the calculation. This seems to be the case of the human RACK1, since both a homology model of human RACK1 as well as the crystal structure of RACK1 from *Arabidopsis thaliana*, are available. Since we studied the protein fused to a 6xHis tag at the N-terminal and we already observed that the homology modeled human RACK1 structure provided a calculated SAXS curve in agreement with the experimental SAXS data, we restored the human RACK1 molecular envelope using the method of addition of missing loops. Fixing the known part of the human homology model, we built dummy residues (DR) chains in the position attached to the proper amino acids in the N-terminal region of the structure. The number of DRs was known from the amino acid sequence. Both programs CHADD and GLOOPY provided very similar SAXS data based models for the human RACK1.

The resulting model is presented in Figure 2C (yellow spheres) superimposed to the *ab initio* homology model (ribbons representation). For the correct alignment of the two models, we used the program SUPCOMB [30,31]. To show the region of the *ab initio* model where the (6xHis tag) was attached to the protein, the dummy elements are shown as blue spheres. The intensity curve calculated from the model (derived from SAXS data) is displayed in the Figure 2B (black curve), showing, as expected, an even better fit to the experimental data. These results give a strong indication of the similarity of the structure of human RACK1 and RACK1 isoform A from *Arabdopsis thaliana* and confirms the structure predicted by the homology model of human of RACK1.

Analytical ultracentrifugation experiments

Aiming to gain further informations on the structural parameters of human RACK1 in solution through its hydrodynamic properties, we performed sedimentation velocity experiments in an analytical ultracentrifuge (AUC). All experiments were performed at 3 protein concentrations, 4 °C, and the data were analyzed by the SedFit software. Figure 3A show the continuous c(S) distribution which suggests that the protein present one predominant species and, at least, 3 other species in solution (Figure 3A, *inset*). RACK1 as monomer should be the main particle in solution (~90% of the total mass) as observed on the area under the c(S) curve ratio. RACK1 as dimer (~6%), tetramer (~2%) and a hexamer or octomer (~2%), could be also observed in the c(S) curve (Figure 3A, *inset*). Moreover, these data are in accordance which previous observations that RACK1 tends to oligomerize and/or aggregate in multi-oligomer species depending on the storage conditions, such as ionic strength, protein concentration and temperature [24]. This property also forced us to include an ultracentrifugation step before subjecting our samples to SAXS data acquisition in order to solve problems with polydispersity (for details see Materials and Methods section).

The predominant monomeric particle in solution observed for RACK1 sedimentation velocity experiments provided a $s^0_{20,w}$ of 3.00 ± 0.01 S as presented in Figure 3B. We estimated the diffusion coefficient by dynamic light scattering at 4 °C and corrected it to standard condition ($D_{20,w}$, Materials and Methods). Both $s^0_{20,w}$ and $D_{20,w}$ were used to calculate the RACK1 MM through the s/D ratio (Equation 1) and the result suggest that the predom-

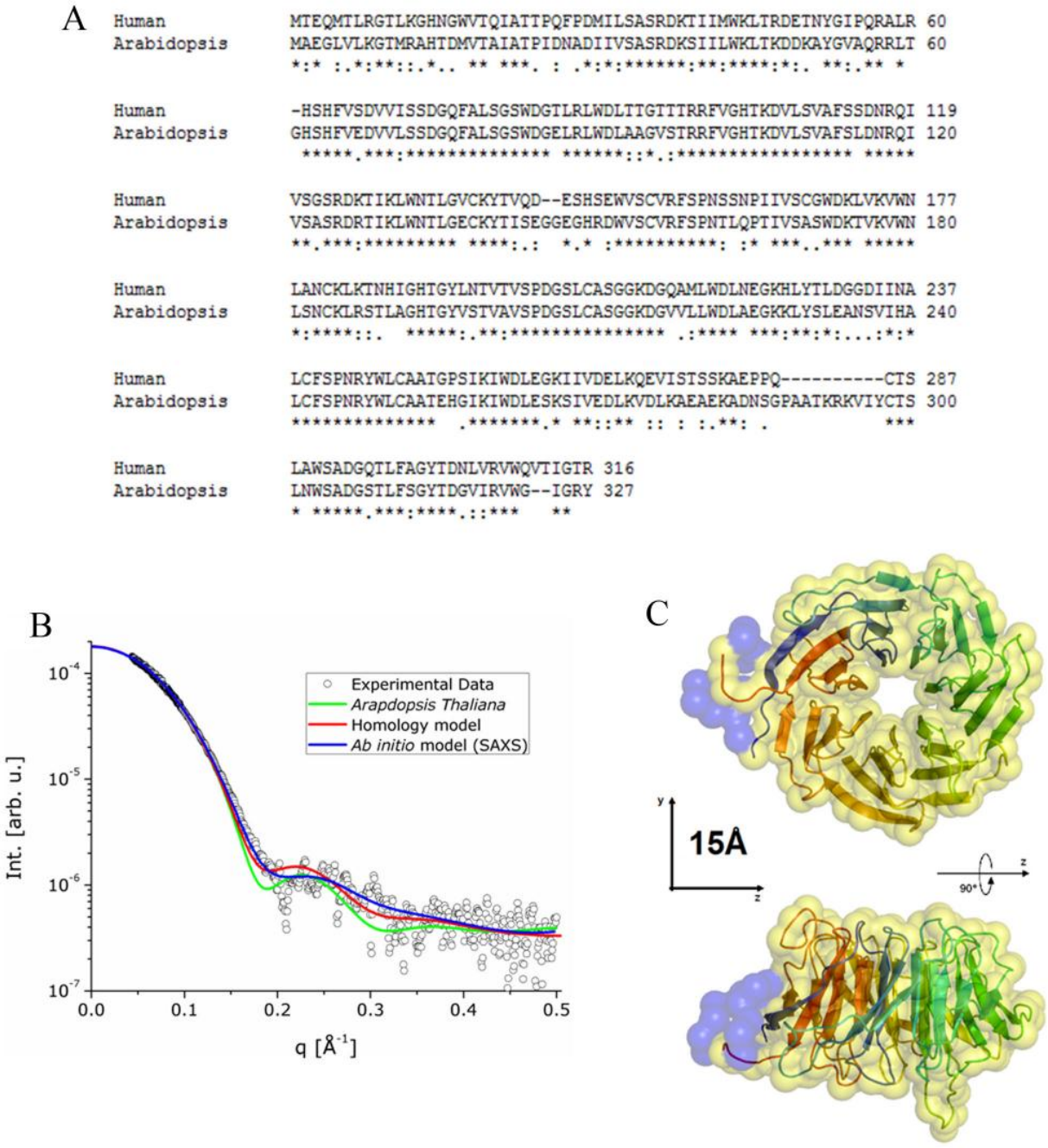

Figure 2 Sequence alignment and *ab initio* model for human 6xHis-RACK1 protein in solution. (A) Alignment between amino acid sequences of RACK1 from *Homo sapiens* (GI:83641897) versus RACK1A from *A. thaliana* (GI:30685669). The symbols: * showing identical residues, : residues with high similarity and residues with low similarity. (B) Experimental scattering curve of human 6xHis-RACK1 (open circles) and the calculated scattering intensity from the RACK1 from *A. thaliana* (solid green line) and *Homo sapiens* (solid red line) by using the program CRYSOL. The solid blue line represents the scattering intensity calculated from the *ab initio* model of 6xHis-RACK1. (C) Two orthogonal views of the homology model of human RACK1 (cartoon representation with secondary structure elements) superposed to the three dimensional low resolution *ab initio* model (semi-transparent yellow surface) of the human RACK1, obtained from processing the SAXS data.

inant particle in solution was a monomer of around 37 kDa (Table 1). In spite of differences in the buffers used in SAXS experiments (containing 20% of glycerol and 150mM of NaCl) and AUC experiments (3% of glycerol and 500 mM of NaCl), the results of both techniques suggest that RACK1 is a monomer in solution. Indeed, the

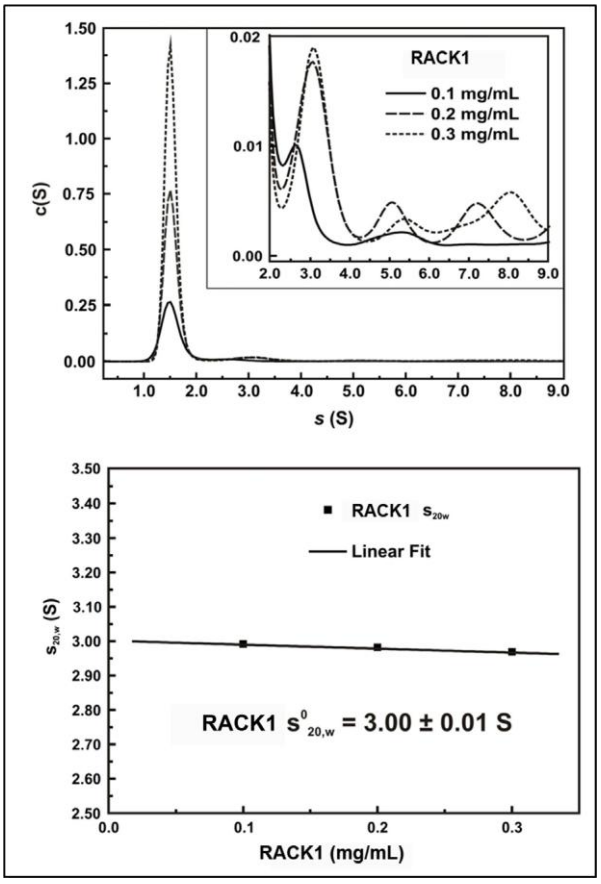

Figure 3 RACK1 sedimentation velocity experiments. (A) Sedimentation velocity experiments were carried out at 4°C, 40,000 rpm (AN-60Ti rotor), and with scan data acquisition at 272 nm. Data were fitted using SedFit software (Version 11.8). Figure displays the c(S) fitting for RACK1 at concentrations of 0.1, 0.2 and 0.3 mg/mL. The c(S) curves suggest that RACK1 present a predominant specie and three or four others species with higher sedimentation coefficients (*inset*) (B) Plots of $s_{20,\omega}$ (of the predominant specie) versus protein concentration fitted by linear regression to calculate the $s^0_{20,w}$: 3.00 ± 0.01 Svedberg.

polydispersity observed in the AUC experiments may be artifactual and may have been caused by the lower glycerol content in the AUC buffer, the larger incubation time in this experiment (in comparison with the SAXS experiments) and finally also the relatively higher protein concentration used here. All of these conditions may promote the observed protein aggregation in the AUC experiments.

Table 1 summarizes the RACK1 hydrodynamic properties calculated for a sphere of 37 kDa (RACK1's MM) and the experimental data determined by dynamic light scattering and sedimentation velocity. We also used the RACK1 homology model previously calculated [24] to obtain its hydrodynamic properties using HydroPro software (see Material and Methods) [32]. Both $D_{20,w}$ and $s^0_{20,w}$ determined experimentally were in accordance to the hydrodynamic properties calculated for the homology model, suggesting that it is a reasonable model for RACK1 in solution. All hydrodynamic data, together with the frictional ratio calculated by either SedFit software (~1.4) or estimated from the ratio of s_{sphere} to $s^0_{20,w}$ (~1.3), suggest that RACK1 is a slightly asymmetric or distorted particle.

Equilibrium sedimentation is an analytical ultracentrifugation method based on the equilibrium between the centrifugal and diffusional forces. It is a thermodynamic technique that allows to obtain the MM of a particle independently of its molecular shape and to perform protein association studies [33]. RACK1 equilibrium sedimentation experimental data fitted with a model of a single specie system (variable MM) and resulted in a MM of around 46 kDa (data not shown), which suggested that human RACK1 solutions contained monomers of 37 kDa and oligomers, as also observed in sedimentation velocity experiments. Thus, we fitted the equilibrium sedimentation data using several models of self-associating systems. Considering that the sedimentation velocity experiments presented above suggested the presence of a monomer-dimer-tetramer system (Equation 2), we used a model of self-association to fit the sedimentation equilibrium data, using the SedPhat software. The presence of the hexamer or octomer, however was not considered for this fitting analysis, because of their low molar concentration in the human RACK1 solution used. Figure 4A (upper panel) presents RACK1 at 0.3 mg/mL in equilibrium at 3 speeds in which the data were fitted with the Equation 3. For this, we fixed the monomer MM at 37.2 kDa. The lower panel in Figure 4A, shows a residuals fitting dispersed around zero, which points to a good fitting. As expected, human RACK1 formed dimers and tetramers with $KB_{M\text{-}D}$ of (2 ± 0.2) ± 10^5 M^{-1} and $KB_{D\text{-}T}$ of (6 ± 2) × 10^5 M^{-1}, respectively. Dissociation constant (KD) for monomer-dimer ($KD_{M\text{-}D}$) and dimer-tetramer ($KD_{D\text{-}T}$) equilibrium where of 51 ± 2 μM and 17 ± 9 μM, respectively.

Thus, our findings that RACK1 is predominantly a monomer in solution seem at first to contrast with some data found in the literature, that relate that human RACK1 can engage in dimerization interaction in *in vitro* pull-down assays and *in vivo*, using cross-linker experiments [34]. A more recent study however, demonstrates that such a RACK1 dimerization *in vivo* may depend crucially on its previous phosphorylation, while dephosphorylation of RACK1 causes the disintegration of the homodimeric complex [35] Altogether, these findings are in good agreement with our results, since our bacterially expressed RACK1 is not phosphorylated, whereas RACK1 in human cells is likely to be phosphorylated and regulated by phosphorylation. Therefore, the dephosphorylated RACK1 should present low KB while the phospho-

Table 1: RACK1 hydrodynamic properties.

RACK1 Hydrodynamic property	Predicted for a sphere	DLS*	AUC	HydroPro of homology model	SAXS
M (kDa)	37.2	36.4 ± 0.8[&]	36.4 ± 0.8[&]	-	40
s020,w (S)	4.01	-	3.00 ± 0.01	3.16 ± 0.02	-
D20,w (10^{-7}cm^2/seg)	9.6	7.7 ± 0.2	-	8.0 ± 0.1	-
Dmax (Å)	-	-	-	63 ± 1	60
Rg (Å)	-	-	-	20.0 ± 0.1	20.8 ± 0.8[$]
					20.6 ± 0.4[#]

[&]from s/D ratio (Equation 1)
$: from Guinier approximation
#: from the normalized second moment of the p(r) function
* Dynamic light scattering

rylated RACK1 may be able to dimerize with higher KB. However, we cannot exclude that the relative low KB_{M-D} and K_{D-T} described above may be an artifact induced by the experimental conditions tested.

RACK1 binding activity in solution

We also used sedimentation equilibrium methods to assess how functional RACK1 protein was in our experimental conditions. Previously, we described the interaction of RACK1 with the regulatory protein Ki-1/57 and analyzed their interaction through spectroscopic methods [17,24]. We then used here a truncated form of Ki-1/57 encompassing the amino acids 122-413 to obtain further thermodynamic details of this association. We analyzed the absorbance data by the SedPhat program as multi-speed equilibrium data and the results are presented in Figure 4B by using the Equation 3 presented above for a hetero-association system with a stoichiometry between RACK1 and Ki-1/57(122-413) of 1:1 (Equation 4). The lower panel (Figure 4B) shows the residuals fitting randomly dispersed around zero, which points to a good data fitting. The KB observed for RACK1-Ki-1/57(122-413) interaction was of around (1.5 ± 0.2) × 10^6 M^{-1} that resulted in a KD of 0.7 ± 0.1 μM. This data suggest that the equilibrium interaction between these two proteins is strong in the tested conditions. The KB for RACK1-Ki-1/57(122-413) interaction is 7.5 times higher than RACK1 KB_{M-D} suggesting that, in the protein concentration assayed, around 91% of the RACK1 is in the hetero-dimeric complex and that the concentration of RACK1 as a homo-dimer must be lower than 0.01 μM and could be neglected from this analysis. This highly productive association may explain why around 54% of clones restored from our yeast two-hybrid screenings using Ki-1/57 as bait represented the RACK1 protein [16].

Titration of Ki-1/57(122-413) to RACK1 solution was carried out at 25 °C in 20 mM Tris-HCl (pH 7.5) and followed by intrinsic fluorescence emission. However, we did not observe any induced changes in the maximum fluorescence emission wavelength upon Ki-1/57(122-413) titration (Figure 5A). On the other hand, the titration curve presented two well defined regions (Figure 5, *inset*) suggesting that the RACK1-Ki-1/57(122-413) interaction involves, at least, two effects: up to 0.2 μM of Ki-1/57(122-413), it is possible to observe an initial fluorescence decrease probably due to fluorescence quenching. At 0.3 μM of Ki-1/57(122-413), the emission fluorescence increased nearly twice suggesting that RACK1 and/or Ki-1/57(122-413) may undergo a conformational change upon Ki-1/57(122-413) binding (Figure 5, *inset*). We cannot explain this result, but we speculate that this intriguing effect may be a result of Ki-1/57(122-413) binding to the human RACK1 surface where at least part of the Trp residues seem to be exposed as our previous homology model of RACK1 suggested [24]. These Trp residues may be already quenched by the solvent and RACK1 association with Ki-1/57(122-413) could lead the tryptophans to a conformation able to show an increment in the total fluorescence intensity emission.

At concentrations higher than 0.3 μM the Ki-1/57 titration induced RACK1 fluorescence emission quenching which follows a sigmoidal curve (Figure 5, *inset*), that represents another effect due to protein association. Figure 5B represents the difference of fluorescence intensity at 334 nm (F_0 - F = θ) as function of Ki-1/57 concentration range from 0.3 μM up to 1.4 μM. Adjusting this titration curve with a modified Hill equation (Equation 5) we could estimate the KD for the interaction between Ki-1/57 and RACK1 as about 0.60 μM and the number of cooperative sites n as 8 ± 1. This points to an apparent cooperativity of the interaction, since the stoichiometry

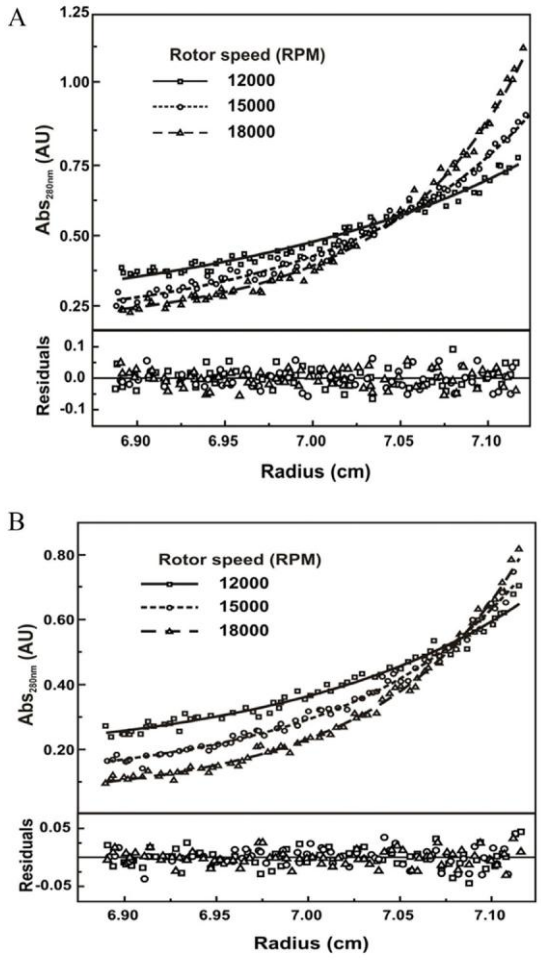

Figure 4 RACK1 Sedimentation equilibrium experiments. (A) The figure shows the best fits of experimental data for 300 µg/mL of RACK1 (4 °C) at 12000, 15000, and 18000 rpm with the self-association methods (SedPhat program - Material and Methods). The random distribution of the residuals (bottom panel) indicates that the fit is satisfactory. Sedimentation equilibrium data of RACK1 agree with a monomer structure with 37.2 kDa in equilibrium with of dimers and tetramers (see Results and Discussion for details). (B) Sedimentation equilibrium interaction between RACK1 and KI-1/57(122-413) was tested at 300 µg/mL of both proteins at 12,000, 15,000, and 18,000 rpm and 4 °C. The data were fitted by self-association method (SedPhat program - Material and Methods) and resulted in random distribution of the residuals (bottom panel) which indicates that the fit is satisfactory. The fitting data suggest that RACK1 and Ki-1/57(122-413) interacts with an affinity constant of $(1.5 \pm 0.2) \times 10^6\ M^{-1}$.

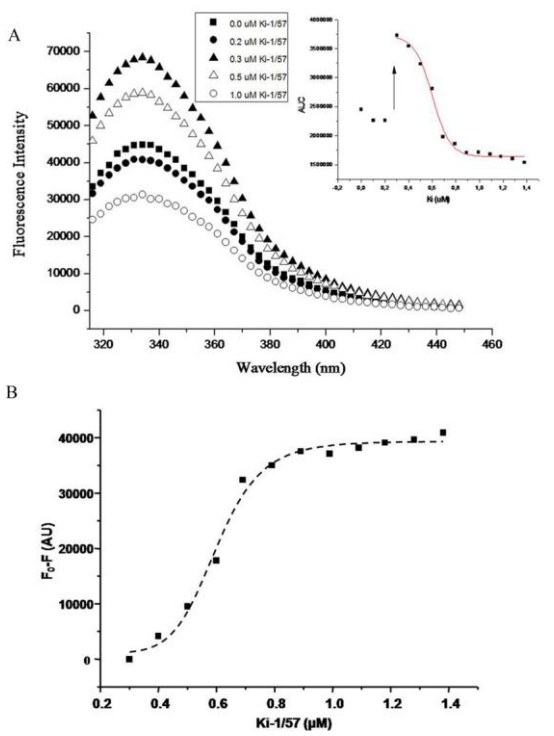

Figure 5 Titration of Ki-1/57(122-413) into RACK1 solution monitored by fluorescence. (A) Fluorescence emission spectra titration of purified 6xHis RACK1 protein (1 µM) with titration of Ki-1/57 at 0.1 µM (black, filled square), 0.2 µM (black, filled circle), 0.3 µM (black, filled triangle), 0.5 µM (open, white triangle) and 1 µM (open, white circle). The excitation wavelength was 295 nm and spectra were background corrected. *Inset*: Area under curve (AUC) of RACK1 fluorescence emission spectrum in function of Ki-1/57(122-413) concentration. The arrow indicates the increase on the RACK1 fluorescence signal at Ki-1/57(122-413) 0.3 uM. (**B**) The titration curve was represented as difference of fluorescence intensity at 334 nm θ (black, filled square) as function of Ki-1/57(122-413) concentration in the range from 0.3 µM and 1.4 µM and fitted with the modified Hill equation (Equation 5). The dashed line represents the fitting curve. The KD between RACK1 and Ki-1/57(122-413) was of around 0.60 µM which corresponds to the dissociation constant of RACK1\Ki-1/57(122-413) observed in sedimentation equilibrium experiments. The number of cooperative sites n was 8 ± 1.

determined by sedimentation equilibrium was 1:1. It is also interesting to note that this KD corresponds to the KD of RACK1\Ki-1/57(122-413) observed in sedimentation equilibrium experiments. These data emphasize that the interaction between human RACK1 and Ki-1/57 was strong in the experimental conditions tested.

The human RACK1 protein contains thirteen tryptophan residues, which yield strong signal fluorescence emission intensities but also increase the complexity of fluorescence emission spectra. Despite the high number of Trp residues in the RACK1 structure, the homology model developed for RACK1 suggests the presence of, at least, two kinds of Tryptophans on RACK1: 1) the exposed Trps on the RACK1 surface and 2) the buried or partially buried Trps [24]. Thus, these different classes of Trps may suffer distinct effects upon Ki-1/57(122-413) binding: while some of them may be more protected, others may be rather quenched. However, we cannot exclude the participation of the 3 Trps on the Ki-1/57(122-413) recombinant protein. Ki-1/57(122-413) has been described as an intrinsically unfolded protein [36] and therefore it could bind to RACK1 by several contact

points, possibly inducing conformational changes on either protein. The sigmoidal titration curve observed may be explained by possible cooperative effects in the binding of the two proteins. However, more detailed emission fluorescence or other spectroscopic experiments are required to address this newly raised issue.

Conclusion

Human RACK1 has significant amino acid sequence and structurally similarity to RACK1 isoform A from *Arabidopsis thaliana*. Analytical ultracentrifugation experiments showed that human RACK1 has similar hydrodynamic properties to that predicted to homology model previously developed. Moreover, the SAXS data demonstrated that the human RACK1 in solution may have the same or very similar molecular envelope as that of the RACK1 isoform A from *Arabidopsis thaliana*. Both techniques also showed that the homology model previously proposed by shows a good agreement with the experimental data presented here [24].

The hydrodynamic properties of the human RACK1 were established by both sedimentation velocity and dynamic light scattering. The samples presented an apparent aggregation in the hydrodynamic experiment and they showed polydisperse behavior. Although the equilibrium sedimentation analytical ultracentrifugation results indicated a tendency of the protein for di- and tetramerization, the data showed that human RACK1 is predominantly a monomer in solution (over 90%). SAXS data also confirmed the human RACK1 monomeric conformation in solution, although we previously adjusted the glycerol content in the buffer and centrifuged the sample to assure its monodispersity. This condition was checked by DLS before the SAXS experiments (data not shown).

The interaction of RACK1 with the Ki-1/57(122-413) is strong and follows the stoichiometry of 1:1 as observed by sedimentation equilibrium experiments. RACK1 is a Trp-rich protein and its fluorescence emission spectrum presents a maximum of around 334 nm suggesting that some of these Trp are exposed to the solvent. The interaction of RACK1 with the Ki-1/57(122-413) monitored by fluorescence emission spectroscopy agreed with the interaction strength observed in the sedimentation equilibrium experiments. Furthermore, the titration curve resembles a sigmoidal curve suggesting a cooperative effect in the interaction of RACK1 with the Ki-1/57(122-413). Considering that Ki-1/57(122-413) is an intrinsically unfolded protein,[36] its interaction with RACK1 may involve several binding sites and seems to induced conformational changes upon binding.

Methods

Plasmid constructions

Cloning of the cDNA coding for RACK1 full-length into the pET-28a plasmid (Novagen/EMD Biosciences, San Diego, CA) was performed as described previously [16]. The recombinant vector pET-28a-RACK1 expresses a construct containing 24 additional amino acids at the N-terminal of RACK1. These extra residues encopass the 6 × His-tag and other poly-linker encoded amino acids. cDNA encoding human Ki-1/57 protein fragments was amplified by PCR and then directionally cloned into the bacterial expression pPROEx (Invitrogen, Carlsbad, USA), as previously described [36].

Expression and purification of recombinant proteins

BL21(Δ SlyD) strain (Invitrogen) was transformed with recombinant pET28a- RACK1 vector and grown in LB medium with 30 ug/ml of kanamycin. When reaching the logarithmic growth phase the recombinant bacteria were induced for protein production with 1.7 mM of isopropyl-b-D-thiogalactoside (IPTG) at 30 °C for 4 h. After harvesting (6000 × *g*, 10 min), the bacterial cells were resuspended and incubated for 40 min at 4 °C in lysis buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 20% glycerol, 1 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride, and 0.05 mg/ml DNase. Additional pellet disruption was performed by 10 cycles of sonication in an ice bath, followed by centrifugation at 18 000 × *g*, 4 °C for 30 min. The obtained supernatant was loaded onto a HiTrap Chelating HP column (Ge Healthcare) and eluted by a gradient of 0-400 mM imidazole. The obtained Ni^{2+}-affinity purified fractions were pooled and dialyzed against the buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 20% glycerol.

To obtain protein preparations free of degradation and up to 95% of purity, Ki-1/57(122-413) was purified in two steps as follows as described previously [36]. The concentration of the recombinant protein was spectroscopically determined using the calculated extinction coefficient for the denatured proteins [37].

SAXS experiments

The SAXS experiments were performed at the D02A-SAXS2 beamline of the Laboratório Nacional de Luz Síncrotron (LNLS, Campinas, Brazil). Before the analysis, the samples of human RACK1 were centrifuged at 356,000 × g for 30 min at 18 °C to remove any possible aggregates. The composition of the buffer solution was adjusted to avoid aggregation, [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 20% glycerol]. Dynamic light scattering (DLS) was used to test the monodispersity of the solution. Measurements were performed with a monochro-

matic X-ray beam with a wavelength of λ = 1.488 Å and the X-ray scattering patterns were recorded using a two-dimensional position-sensitive MARCCD detector. The sample-to-detector distances were set at 506.8 mm and 1722.5 mm, corresponding to the scattering vector range of $0.01 < q < 0.50 Å^{-1}$ where q is the magnitude of the ***q***-vector defined by $q = (4\pi/\lambda)\sin\theta$ (where 2θ is the scattering angle). Three successive frames of 300 s each were recorded for each sample. The scattering data from the buffer were recorded before and after the measurement of each sample. The experiments were performed with two different sample concentrations: 1.95 and 2.54 mg/mL. The sample and buffer intensity curves were individually corrected for detector response and scaled by the incident beam integrated intensity and the sample absorption. Subsequently, the buffer scattering was subtracted from the corresponding sample scattering. The resulting curves were inspected for radiation-induced damage, but no such effect was observed. A 5 mg/ml BSA (66 kDa) solution in the same sample buffer was used as MM standard sample to estimate the MM of RACK1. This value was inferred from the ratio of the extrapolated values of the intensity at the origin, *I(0)*, from both sample and BSA solutions scattering [38,39].

The initial analyses of the SAXS data were performed following the standard procedures. The radius of gyration and the forward scattering intensity *I(0)* were obtained from the Guinier approximation [26,40,41] where we assumed that the intensity can be represented as $I(q) = I(0)\exp(-(qR_g)^2/3)$ at very small values of q ($qR_g < 1$). Thereupon, the pair distance distribution function p(r) was calculated by indirect Fourier transform of the scattering intensity through a regularization method implemented in the program GNOM [42]. This p(r) function also provided the *I(0)* and R_g values. These parameters proved to be in agreement with those obtained from the Guinier approximation. This function also allows determination of the maximum dimension of the protein in solution since $p(r > Dmax) = 0$.

We compared the experimental scattering curve from the human RACK1 protein with its calculated scattering curve from the homology model from the human RACK1 and with the calculated curve from the crystallographic structure of the RACK1 isoform A from *Arabdopsis thaliana*. The calculation of the scattering intensity from the atomic coordinates of the latter structures was performed using the program CRYSOL [43]. Since the sample we studied was in a solution with 20% glycerol, we calculated the electron density of the solvent for this condition (0.3497 electrons/$Å^3$). The contribution of the other components of the buffer solution to the the electron density of the water (0.3344 electrons/$Å^3$) was considered negligible, due to their low concentration.

Ab initio modeling from the SAXS data

The *ab initio* models of the human RACK1 in solution were calculated from the SAXS data using the homology model previously obtained. We used a modeling method that is an extension of the original Dummy-Residues (DR) approach [31]. It can be used when parts of the protein are known and the location of the interface of the known and missing parts is also known [44]. Here, as we studied the protein fused to a 6xHis tag at the N-terminal, the homology model of the human RACK1 is used as the known part. The 6xHis tag is represented as a chain of DRs attached to the proper residues (known from the sequence) in this structure. We performed random modification in this chain of DRs using the simulated annealing method for global minimization of the scoring function to provide the best fit to the experimental scattering curve. This method is implemented in two programs: [44] CHADD and GLOOPY. Both programs are similar but the second one takes into account the primary structure (amino acid sequence) of the protein.

Analytical ultracentrifugation

Sedimentation velocity and equilibrium sedimentation experiments were performed using a Beckman Optima XL-A analytical ultracentrifuge and analyzed as previously described [45]. Briefly, sedimentation velocity experiments were carried out at 4°C and 40,000 rpm (AN-60Ti rotor) in buffer Tris-HCl 20 mM (pH 7.5), NaCl 500 mM and Glicerol 3% (0.404 M). Human RACK1 was tested at 0.1, 0.2 and 0.3 mg/ml and absorbance data acquisition was at 272 nm. The fitting of absorbance *versus* cell radius data was done using SedFit (Version 11.8), which solves the Lamm equation in order to discriminate the spreading of the sedimentation boundary from diffusion [46,47]. The maximum of peaks of the c(S) curves gave the apparent sedimentation coefficient (*s*). The frictional ratio (f/f_0) was the regularization parameter in fitting routine. The relative amount of each particle in c(S) curves was estimated by the integral of the area under curve of each peak in the c(S) curve using SedFit software (Version 11.8) and OriginPro® (Version 8). The software Sednterp http://www.jphilo.mailway.com/download.htm was used to estimate RACK1 partial specific volume at 20°C (*Vbar*= 0.7288 mL/g), buffer density at 4 °C (ρ = 1.02976 g/mL), buffer viscosity at 4 °C (η= 0.017934 poise), and also the s_{sphere} and the D_{sphere} for a globular protein of about 37.2 kDa (Table 1). This software was also used to estimate the standard sedimentation coefficient ($s_{20,w}$) for each protein concentration in order to estimate the $s_{20,w}$ at 0 mg/mL of protein concentration ($s^0_{20,w}$) by linear extrapolation [45]. This procedure minimizes interferences caused by temperature, viscosity and molecular crowd [48]. The diffusion coefficient parame-

ter, D, was obtained from dynamic light scattering with a DynaPro-MS/X device (Protein Solutions) at 4°C. The $D_{20,w}$ was calculated as described [49] in similar way that described above to $s_{20,w}$. The MM can be estimated from the ratio of the s to D as indicated by the equation 1:

$$MM = \frac{sRT}{D(1-Vbar\rho)} \quad (1)$$

where R is the gas constant, T is the absolute temperature.

The HydroPro software [32] was applied to estimate the standard diffusion coefficient ($D_{20,w}$), standard sedimentation coefficient ($s_{20,w}$) and hydrodynamic maximum distance ($D_{max,h}$) from the RACK1 model previously developed [24] in order to compare to the experimentally hydrodynamic data.

Sedimentation equilibrium experiments were carried out at 4°C and at three speeds as follow: 12,000, 15,000 and 18,000 rpm (AN-60Ti rotor). Scan data acquisitions were taken at 280 nm after 12 h of centrifugation at each speed, in which the equilibrium was checked by subsequent scans. Sedimentation equilibrium analysis involved fitting of absorbance *versus* cell radius data using nonlinear regression routine implemented by SedPhap software (Version 6.5). The Self-Association method was used to analyze the sedimentation equilibrium experiments using several models of association for RACK1. As observed in the sedimentation velocity experiments, RACK1 tends to oligomerize, thus the self-association model described by the equation 2 applied, where $KB_{M\text{-}D}$ and $KB_{D\text{-}T}$ are the binding constant of monomer-dimer and dimer-tetramer, respectively (see Results and Discussion section for details).

$$4M \underset{}{\overset{KB_{M-D}}{\rightleftharpoons}} 2D \overset{KB_{D-T}}{\rightleftharpoons} 1T \quad (2)$$

Distribution of the protein along the cell, obtained in the equilibrium sedimentation experiments, was fitted with the following equation for associating system.

$$C = C_{monomer,r_0} e\left[\frac{M(1-V_{bar}\rho)\omega^2\left(r^2-{r_0}^2\right)}{2RT}\right] + KB * \left(C_{monomer,r_0}\right)^n e\left[\frac{M(1-V_{bar}\rho)\omega^2\left(r^2-{r_0}^2\right)}{2RT}\right] \quad (3)$$

where C is the protein concentration at radial position r, $C_{mononer,r0}$ is the protein concentration at radial position r_0 (initial radial position), ω is the centrifugal angular velocity, KB is the binding constant and n is the stoichiometry. Equation 3 can be developed for equilibrium systems with more species.

Interaction between RACK1 (37.2 kDa) and the C-terminal of Ki-1/57(122-413) (37.3 kDa) [36] was also tested by equilibrium sedimentation. Both proteins were prepared in the buffer Tris-HCl 20 mM (pH 7.5), NaCl 500 mM and Glicerol 3% (0.404 M) at 300 ug/mL (~8.0 μM) and submitted to centrifugation in the same conditions presented above. SedPhat software (Version 6.5) [50] was used to fitting the hetero-association model following the Equation 4 where KB is the binding constant between RACK1 and the C-terminal of Ki-1/57(122-413). Again, Equation 3 was used for fitting protein distribution along the cell.

$$1A + 1B \underset{}{\overset{KB}{\rightleftharpoons}} 1C \quad (4)$$

The software Sednterp was used to estimate the molar absorbance of the folded protein at 280 nm for RACK1 (80.940 $mol/L^{-1}.cm^{-1}$) and the C-terminal of Ki-1/57(264-413) (32.890 $mol/L^{-1}.cm^{-1}$). These parameters were used in SedPhat software for hetero-association fitting.

Fluorescence spectroscopy

The experiments were performed with an Aminco BowmanR Series 2 (SLM-Aminco) spectrofluorimeter equipped with a 450W lamp. Experiments were carried out at 25 °C in 20 mM Tris-HCl (pH 7.5). The recombinant proteins were analyzed at concentration of 1 μM. The intrinsic Tryptophan fluorescence was investigated with an excitation wavelength of 295 nm using a spectral band pass of 4 nm for both excitation and emission. The titration curve of RACK1 and Ki-1/57 was used to fit with a modified Hill equation [51] to determine the KD of the interaction as described above:

$$(F_0 - F) = \theta = \theta_0 + (\theta_{max} - \theta_0)\frac{[Ki\text{–}1/57]^n}{KD + [Ki\text{–}1/57]^n} \quad (5)$$

where F_0 is the fluorescence intensity at 0.3 μM of Ki-1/57, F is the fluorescence intensity at a given Ki-1/57 concentration (in μM), θ is the difference of fluorescence intensity, θ_0 is θ at 0.3 μM of Ki-1/57; θ_{max} is θ at Ki-1/57 saturating concentration, and n is the number of cooperative sites. The software OriginPro® (Version 8) was used to this fitting routine.

Authors' contributions

KAG and JK conceived and designed the experiments, analyzed the data and wrote the manuscript. KAG performed or participated in all experiments. JCB participated in analytical ultracentrifugation, spectroscopic experiments and performed their respective data interpretation. PFP helped with protein expression and purification optimization. JCS performed SAXS experiments and interpreted them together with ICLT. GCB helped in the designing of experiments and performed protein expression and purification. JK supervised the project. All authors read and approved the final version of the manuscript.

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3.4- Artigo IV

Over-expression and microarray analysis of the human regulatory protein Ki-1/57 as well as of its paralogue CGI-55 causes broad transcriptional repression and a decrease in cellular proliferation.

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Resultados complementares (Artigo IV em preparação).

Análise do efeito da superexpressão da proteína Ki-1/57 por microarranjos de DNA

A capacidade da proteína Ki-1/57 de interagir com proteínas envolvidas na regulação da transcrição tais como p53, Daxx, PIAS, YB-1 e PRMT1 sugere que Ki-1/57 possa atuar neste contexto funcional (Imagawa *et al*., 2009; Rytinki *et al*., 2009; Gupta *et al*., 2009; Finkbeiner *et al*., 2009; Kleinschmidt *et al*., 2008), esta proteína também pode estar envolvida em atividades relacionadas ao metabolismo de RNA e com mecanismos de controle da transcrição (Nery *et al*., 2006b).

Em busca de novas pistas capazes de ajudar na compreensão da função de Ki-1/57, foram realizadas análises da expressão gênica global (DNA *microarrays*, Affymetrix) de células superexpressando essa proteína e seu possível parálogo em células de mamíferos, a proteína CGI-55. A similaridade de seqüência de aminoácidos Ki-1/57 e CGI-55 ultrapassa 65% e pode sugerir, juntamente com dados de interação proteína-proteína também obtidos para CGI-55, que ambas possam desempenhar funções redundantes ou relacionadas dentro da célula (Bressan *et al*., 2009b).

Após o tratamento e análise dos dados, foi gerada uma lista final contendo os genes que foram modificados por mais de 2 vezes em relação ao controle, a um nível de significância superior a $p = 0,01$ (dados não mostrados). Seguindo esses critérios, foram observados 413 genes que tiveram a sua expressão alterada pela superexpressão de Ki-1/57, 88% desses genes apresentaram uma diminuição nos níveis de mRNA (Figura 5), sugerindo que Ki-1/57 possivelmente tenha um efeito predominantemente repressor na expressão gênica global.

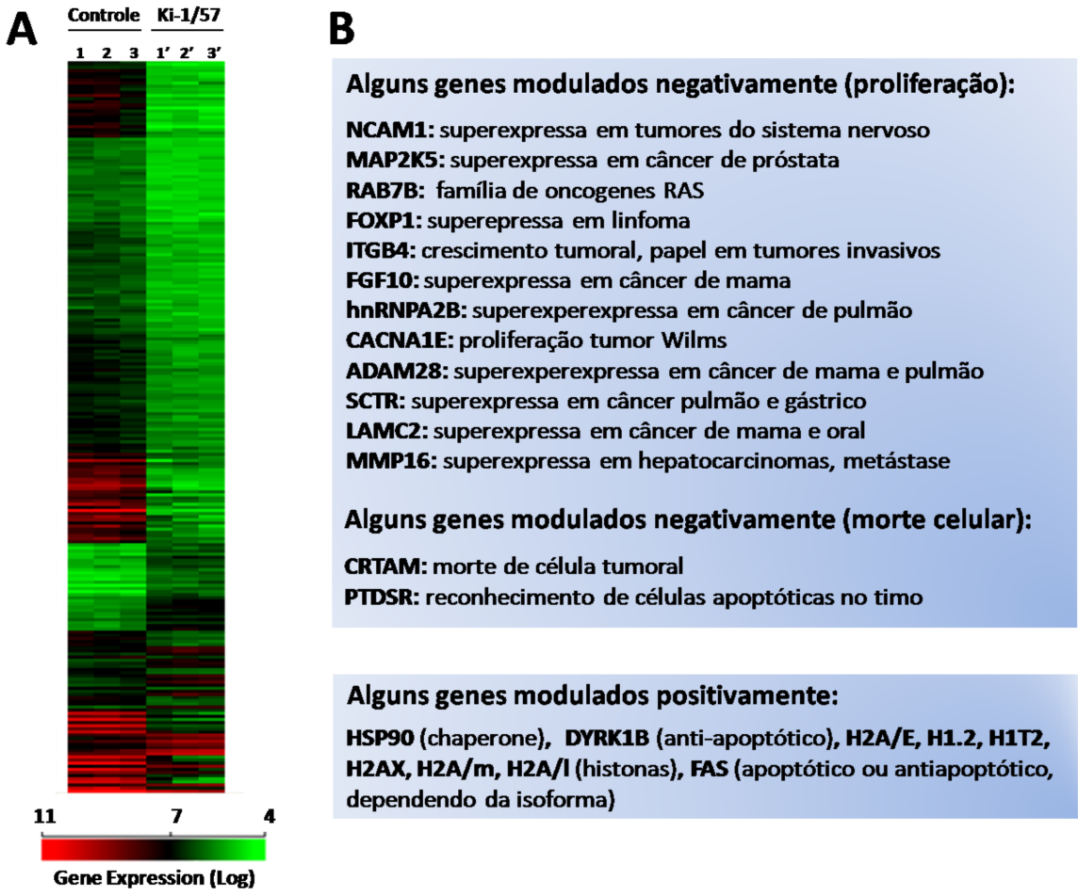


Figura 5: Análise do efeito da superexpressão de Ki-1/57 na expressão global de genes por microarranjos de DNA. Células HEK-293 foram transfectadas transientemente pelo método de fosfato de cálcio com o vetor pCDNA6 myc/his expressando ou não (pCDNA6 vazio) uma contrução de Ki-1/57 fusionada a -Flag no seu N-terminal (pCDNA6-Flag-Ki-1/57). Foram realizadas 6 transfecções independentes, 3 com o vetor pCDNA6 vazio e 3 com vetor pCDNA6-Flag-Ki-1/57, as quais foram coletadas após 40 horas para a extração de seu RNA total pelo método do reagente TRIZOL (Gibco-BRL). Em seguida, a fração de RNAs mensageiros (mRNA) foi obtida pelo *kit Oligotex mRNA Purification System* (Quiagen). Os experimentos de microarranjos foram realizados com 1 µg da fração de mRNA através do sistema Affymetrix (*GeneChip Human Genome U133 plus 2.0 Arrays*) para a síntese do cRNA biotinilado, sua hibridização, marcação e escaneamento. Após análise e processamento iniciais das imagens obtidas através do software GCOS (*Gene Chip Operating Software version 1.4*), o conjunto "bruto" de dados de expressão gênica foi submetido ao programa ArrayAssist (*ArrayAssist x.5 package*, Stratagene), possibilitando a realização das normalizações e tratamentos estatísticos adequados para os procedimentos de clusterização e geração de uma tabela final contendo todos os genes modificados acima de 2 vezes (positivamente ou negativamente) e com um grau de confiança acima de p=0,01 (dados não mostrados). **A-** Representação gráfica da clusterização dos 413 genes diferencialmente expressos obtidos através do programa ArrayAssist. Cada linha mostra o perfl da expressão de um único gene (vide legenda parte inferior) em cada uma das amostras analisadas (colunas: 1, 2, 3, 1', 2,

3'). O efeito repressor de Ki-1/57 pode ser claramente notado pela predominância da cor verde no lado direito da figura (colunas 1', 2' e 3'), significando a diminuição da expressão gênica em relação ao controle (colunas 1, 2, e 3, lado esquerdo). **B-** Análise preliminar de alguns genes modificados pela superexpressão de Flag-Ki-1/57.

Foi possível observar que a superexpressão de Ki-1/57 diminuiu a expressão de vários genes envolvidos com a proliferação celular, muitos dos quais são encontrados alterados em diferentes tumores ou já foram associados com mecanismos oncogênicos (Figura 5B). Esses resultados sugerem que Ki-1/57 pode induzir a expressão de genes desencadeadores de morte celular ou apoptose. Porém, também foi observado a repressão de genes envolvidos com a apoptose, sugerindo que o efeito de Ki-1/57 possa não somente ser o de prevenir a proliferação, mas também a morte celular (Figura 5B). É provável que Ki-1/57 esteja envolvida em respostas celulares a estímulos ambientais danosos, já que um período de latência sob determinadas condições pode representar um período de "recuperação" importante, principalmente considerando os mecanismos celulares de reparo e sobrevida frente a situações que podem ser momentâneas.

Análise do efeito da superexpressão da proteína CGI-55 por microarranjos de DNA

De maneira semelhante o mesmo experimento foi realizado com a proteína GCI-55 para buscarmos pistas funcionais, semelhanças e diferenças existentes entre as proteínas GCI-55 e Ki-1/57. Foi realizada uma análise de expressão gênica de células HEK-293 superexpressando o seu cDNA fusionado a –Flag no N-terminal.

Foram encontrados 217 genes com expressão alterada nas células superexpressando CGI-55, 90% deles tendo sido regulados negativamente (Figura 6A). Semelhante ao observado para Ki-1/57, os resultados sugerem que CGI-55 possa ter uma atividade primariamente repressora no controle da expressão gênica. Embora a análise preliminar desses genes não permita uma visualização muito clara do efeito que pode estar ocorrendo, cerca de 20 genes foram comuns ao encontrado para Ki-1/57, a maioria deles está envolvida em processos proliferativos ou tumorigênicos e todos tiveram a sua expressão diminuída (Figura 6B).

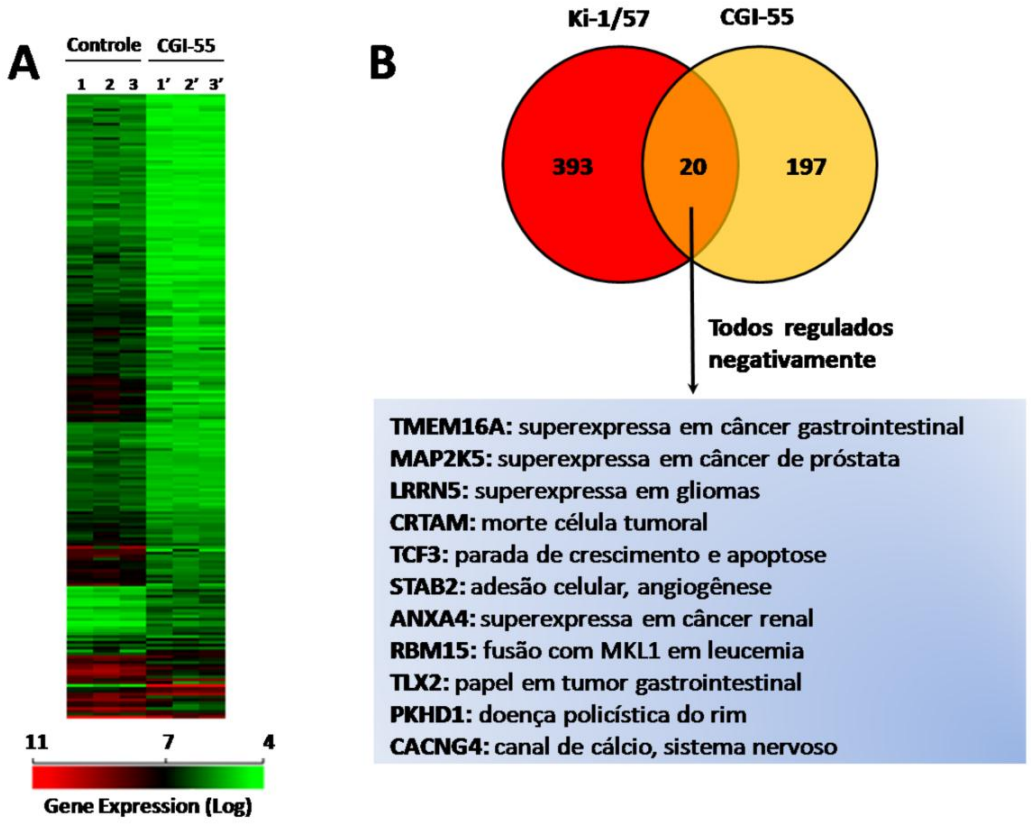


Figura 6: Análise do efeito da superexpressão de CGI-55 na expressão global de genes por microarranjos de DNA. De forma semelhante ao realizado para Ki-1/57, foram feitas 6 transfecções independentes em células HEK-293, 3 delas com o vetor pCDNA6 vazio e 3 com vetor pCDNA6-Flag-CGI-55, coletadas após 40 horas para a extração de seu RNA total pelo método do reagente TRIZOL (Gibco-BRL). Os procedimentos subseqüentes de isolamento da fração de RNAs mensageiros e a análise da expressão gênica por microarranjos foram realizados como descrito na legenda da Figura 5. **A-** Representação gráfica da clusterização dos 217 genes diferencialmente expressos através do programa ArrayAssist. Cada linha mostra o perfil da expressão de um único gene (vide legenda parte inferior) em cada uma das amostras analisadas (colunas: 1, 2, 3, 1', 2', 3'). **B-** Diagrama de Venn realçando o número de genes modificados pela superexpressão de Ki-1/57 e CGI-55 (acima) e análise preliminar de 11 desses genes (tabela, abaixo). Os demais 9 genes co-regulados não possuem trabalhos publicados na base de dados do pubmed e não estão mostrados.

O número de genes modificados por Ki-1/57 (413 genes) foi 52% maior do que aqueles modificados por CGI-55 (217 genes). Isso possivelmente é devido ao fato de que a superexpressão de Flag-Ki-1/57 foi mais eficiente do que a de Flag-CGI-55 quando comparada aos seus respectivos controles (Figura 7) e devido ao fato de no duplo hibrido Ki-1/57 ter interagido com uma maior quantidade de proteínas com funções diferentes quando comparado

com CGI-55. É possível que haja mais genes em comum alterados por essas duas proteínas, de qualquer forma, esses dados sugerem que Ki-1/57 e CGI-55 podem estar envolvidas em processos celulares semelhantes, ou até mesmo redundantes; em conformidade com os dados de duplo híbrido previamente obtidos.

O conjunto de dados de *microarrays* obtidos revelou que essas proteínas podem estar envolvidas principalmente em mecanismos repressores da expressão gênica, uma vez que grande parte dos genes modificados sofreu diminuição em seus níveis de RNA mensageiros. Dentre aqueles genes encontrados reprimidos, muitos são descritos como envolvidos tanto na promoção da proliferação quanto no desencadeamento da apoptose. A hipótese de trabalho para esses resultados aparentemente destoantes seria a provável atuação dessas proteínas em mecanismos de proteção celular durante situações de injúria. Durante essas condições, Ki-1/57 e CGI-55 possivelmente sofreriam ativação e contribuiriam para a diminuição da proliferação e apoptose, permitindo um período de latência pelo qual a célula estaria submetida a seus diferentes mecanismos de reparo e sobrevivência.

Os dados de expressão gênica para Ki-1/57 e para CGI-55 foram confirmados em novos experimentos de superexpressão transiente seguido de análise por PCR em Tempo Real (Figura 7). Em seguida, foram realizados ensaios de proliferação celular em células superexpressando Ki-1/57 e CGI-55 e o resultado obtido está em conformidade com o esperado: uma significativa queda na proliferação de células HEK-293 (Figura 8). Experimentos de citometria de fluxo foram realizados para analisar se estava ocorrendo uma parada no ciclo celular (Figura 9), porém o que foi observado é que as células não estavam acumulando em uma determinada fase do ciclo, o que sugere uma maior lentidão no ciclo celular. Para confirmar esta hipótese o próximo experimento a ser realizado é o de análise da proliferação por incorporação de BrdU. A bromodesoxiuridina (BrdU o 5-bromo-2-desoxiuridina) é um nucleotideo sintético análogo a timidina, cuja a analogía a timidina permite uma substituição quase total (entre 99,8 e 100%) dos nucleotídeos de timidina em uma célula na fase de síntese de DNA. Desta maneira poderemos analizar se as células transfectadas com Ki-1/57 ou CGI-55 estão completando o ciclo celular mais lentamente do que as células transfectadas com o vetor vazio.

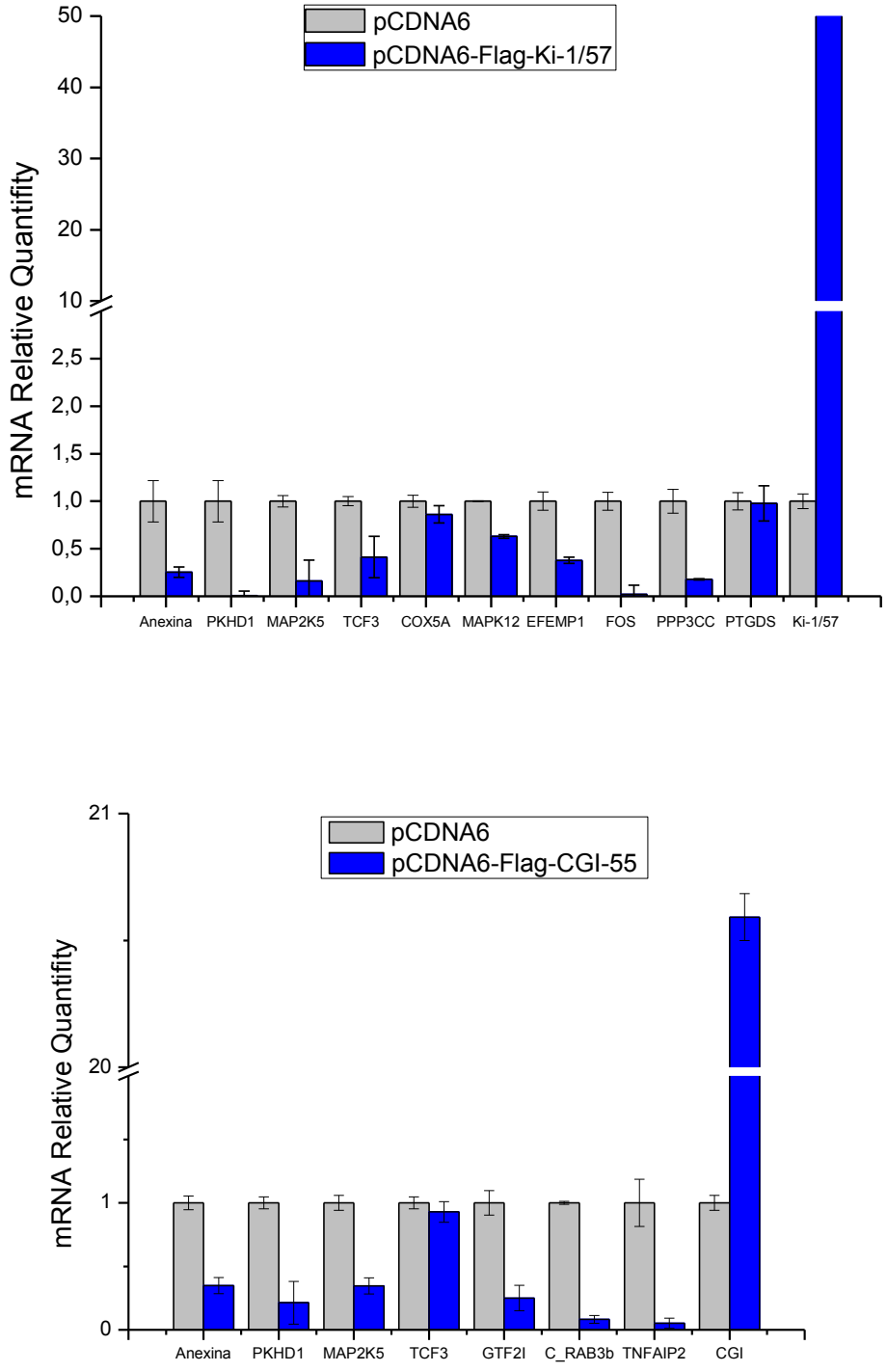


Figura 7. Ensaios de confirmação dos dados de microarrays por PCR em Tempo Real (qRT-PCR). Os genes indicados no eixo "x" dos gráficos foram aleatoriamente selecionados para atestar a fidelidade dos ensaios de microarrays de células superexpressando transientemente Flag-Ki-1/57 ou Flag-CGI-55. Em uma nova rodada

de experimentos independentes, 6 transfecções transientes, utilizando o agente lipofectamine (Invitrogen) de acordo com as recomendações do fabricante, foram realizadas de forma a contrastar 3 réplicas biológicas para a transfecção do vetor pCDNA6-Flag-Ki-1/57 e 3 réplicas biológicas para a transfecção do vetor vazio pCDNA6, utilizado como controle. Após ~40 horas de transfecção, as células foram ressuspendidas no Reagente Trizol para a extração da fração de RNA total, a qual foi utilizada para a síntese dos cDNAs utilizados nos ensaios de qRT-PCR. Os ensaios foram realizados utilizando o reagente SYBRGreen para a quantificação relativa pelo método ΔΔCt. A superexpressão de Flag-Ki-1/57 e Flag-CGI-55 nas amostras foi controlada/confirmada e também está mostrada nos gráficos. A maioria dos genes testados foram confirmados, indicando, portanto, que o conjunto de dados de *microarrays* obtidos são relevantes.

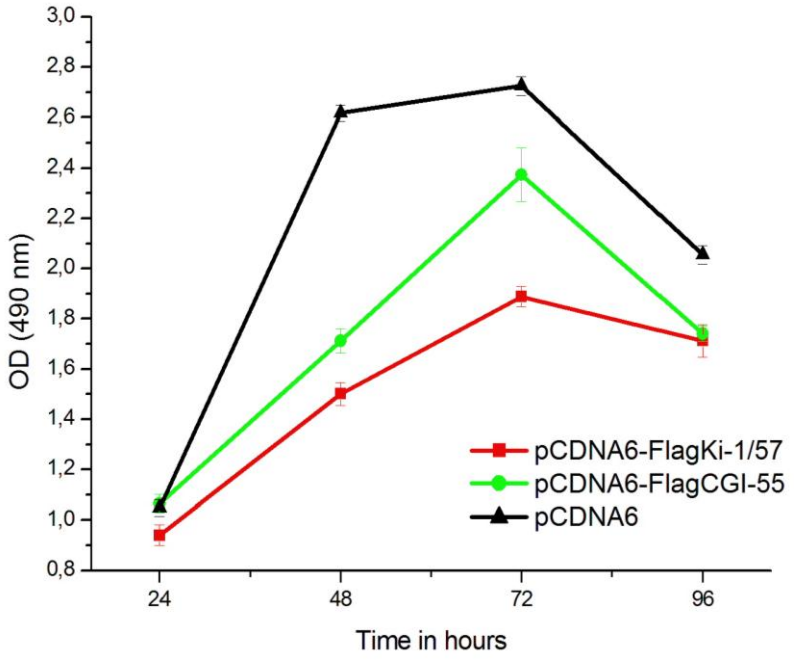
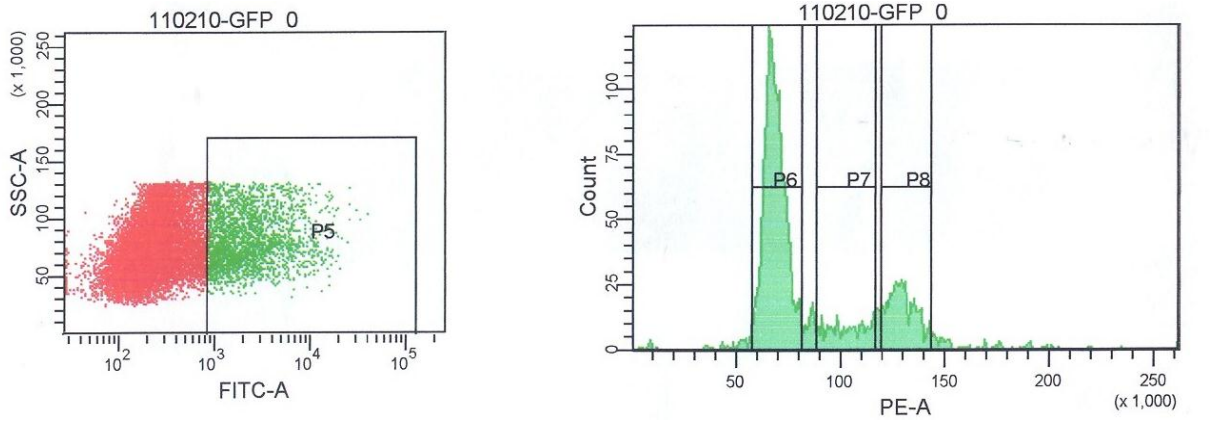
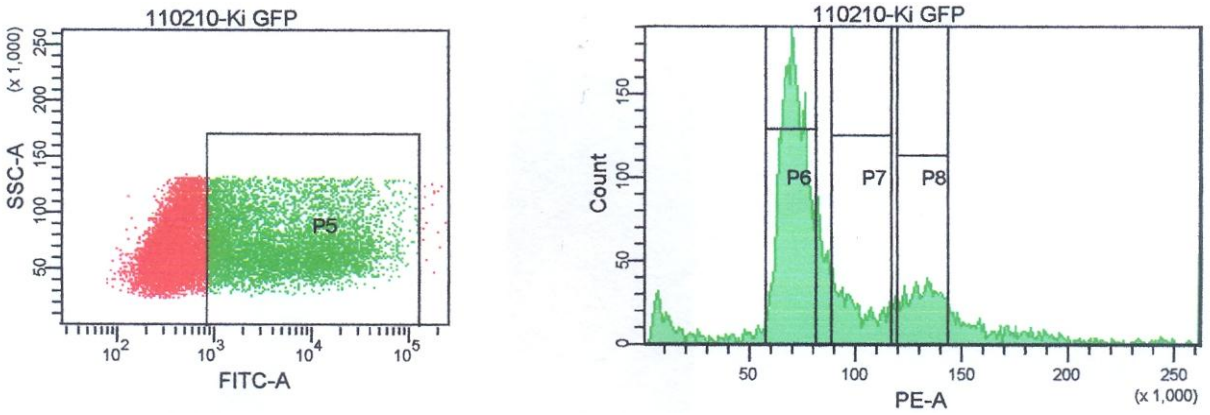


Figura 8. Ensaios de proliferação. As taxas de proliferação de células superexpressando Flag-Ki-1/57 e Flag-CGI-55 foram determinadas através do reagente MTS (Promega), seguindo todas as recomendações do fabricante. Para a realização do ensaio, realizado em quadriplicata, células HEK-293 foram plaqueadas em placas de 96 poços por 48 horas antes das transfecções, realizadas através do agente lipofectamine (Invitrogen), de acordo com as especificações do fabricante, e os plasmídeos: pCDNA6-Flag-Ki-1/57, pCDNA6-Flag-CGI-55 e pCDNA6 (vazio). Após a remoção do meio de transfecção, foi adicionado um novo meio de cultura não suplementado com soro fetal bovino, de forma a evitar o mascaramento do efeito dessas proteínas pelos potenciais agentes mitogênicos constituintes dessa suplementação. Nos tempos indicados, as células foram então ensaiadas pelo reagente MTS e os valores de densidade óptica determinados em leitor de placas de ELISA e plotados no gráfico apresentado.



Population	#Events	%Parent	PE-A Mean
P5	2,236	14.6	88,332
P6	1,328	59.4	68,194
P7	239	10.7	103,572
P8	398	17.8	129,854



Population	#Events	%Parent	PE-A Mean
P5	5,672	40.2	97,893
P6	2,651	46.7	70,408
P7	624	11.0	100,792
P8	704	12.4	131,800

Figura 9. Citometria de Fluxo. Para a análise de citometria de fluxo, as células HEK-293 foram transientemente transfectadas utilizando o agente lipofectamine (Invitrogen), de acordo com as especificações do fabricante com os plasmídeos pEGFPC-Ki-1/57 ou pEGFPC vazio, as células foram coletadas 72 horas após a transfecção. No momento da remoção do meio de transfecção, foi adicionado um novo meio de cultura não suplementado com soro fetal bovino, de forma a evitar o mascaramento do efeito dessas proteínas pelos potenciais agentes mitogênicos constituintes dessa suplementação. As células foram fixadas em etanol gelado 70% e armazenadas a -20 °C. As amostras foram lavadas uma vez em PBS e ressuspendidas em uma solução

de iodeto de propídio (5 g / ml) e RNase A (0,5 mg / ml) em PBS. A suspensão de células foram analisadas em um FACSCalibur (Becton Dickinson, Nova Jersey, EUA.

4- Discussão

4.1- Parceiros de interação da proteína Ki-1/57

Desde que a proteína Ki-1/57 foi identificada nas células do linfoma de Hodgkin inúmeros estudos tem sido feitos para a caracterização desta nova molécula. Um perfil de interação proteína-proteína foi traçado através da técnica de Duplo Hibrido de leveduras. Percebeu-se que vários dos parceiros de Ki-1/57, estavam funcionalmente interconectados entre si através de interação física (proteína-proteína) ou presentes num mesmo complexo no contexto do controle da tradução.

A interação de Ki-1/57 com proteínas que regulam a tradução ou estão relacionadas de alguma maneira com a tradução chamou bastante atenção. As proteínas CIRBP, RPL38 e FXR1 foram encontradas interagindo com o N-terminal da proteína Ki-1/57. Experimentos revelaram a capacidade de CIRBP regular negativamente a tradução de RNAs mensageiros (De Leeuw *et al.,* 2007), RPL28 interage com proteínas da maquinaria de tradução (Kenmochi *et al.,*1998) e as proteínas conhecidas como sendo da família do X frágil (que são as FXR1, FXR2 e FMRP) são encontradas em associação com os polissomos (Darnell *et.al.* 2009) e participando do processo de regulação da tradução (Vasudevan *et al.,* 2007).

Em 54% dos clones encontrados no duplo hibrido por interagirem com a região C-terminal de Ki-1/57 estava a proteína RACK1. Alguns estudos mostram que RACK1 pode afetar a expressão gênica através da regulação da tradução e ativação da montagem dos ribossomos (Angenstein *et al*., 2002; Ceci *et al*., 2003).

Seguindo essa lógica, neste trabalho foram apresentadas diversas evidências experimentais comprovando a interação de Ki-1/57 com proteínas da tradução proteica, bem como a sua capacidade de alterar a tradução do gene repórter da luciferase (Artigo I). Além disso, foi mostrada a associação de Ki-1/57 com os ribossomos e polissomos através do perfil ribossomal, todas essas evidencias nos levam a crer que Ki-1/57 regula de alguma maneira a tradução protéica.

A proteína FMRP tem sido descrita na literatura como um positivo ou um negativo regulador traducional dependendo do RNA mensageiro alvo em que ela se liga (Ceman *et al.,*

1999; Laggerbauer *et al.,* 2001; Narayanan *et al.,* 2008; Bechara *et al.,* 2009). Os nossos dados sugerem que Ki-1/57 também é uma proteína regulatória específica, que tem um efeito de aumentar ou diminuir a tradução protéica dependendo do RNA mensageiro alvo que ela se liga.

4.2- A relevância da Sumoilação de Ki-1/57 para o seu contexto funcional.

A sumoilação é uma importante modificação pós traducional que regula as funções biológicas das proteínas e é essencial para alguns processos biológicos como a transcrição gênica e a progressão do ciclo celular (Kerscher 2007). A maioria das proteínas que são alvos de sumoilação estão envolvidas em eventos nucleares, como a transcrição, reparo de DNA e transporte entre o núcleo e o citoplasma (Johnson, 2004; Hay, 2005; Geiss-Friedlander and Melchior, 2007). A sumoilação regula diversos aspectos da expressão gênica incluindo a transcrição de DNA, *splicing* de RNA mensageiro e a poliadenilação. (Johnson, 2004; Geiss-Friedlander & Melchior, 2007; Vethantham *et al.*, 2007, 2008).

Xu e colaboradores mostraram que eIF4E (*eukaryotic initiation factor 4E*) é modificado por SUMO-1 e que a sumoilação não afeta a capacidade desta proteína se ligar ao cap do RNA mensageiro e sim promove a dissociação de eIF4E e 4E-BP1 reforçando assim a tradução cap dependente de eIF4F. A não sumoilação de eIF4E anula a sua propriedade anti apoptótica e inibe a transformação de fibroblastos de rato (Xu *et al.,* 2010).

A função da sumoilação em certos alvos proteicos foi descrita como sendo repressora transcricional de muitos fatores e co fatores da transcrição (Gill, 2004; Hay, 2005). Estudos recentes sobre o co-ativador transcricional p300 revelou que a sumoilação recruta a histona HDAC6 levando a repressão da transcrição (Girdwood *et al*., 2003; Yang e Sharrocks, 2004).

Em nossos estudos de duplo hibrido com a proteína Ki-1/57 foi verificado que ela possui a capacidade de interagir com proteínas que participam do processo de sumoilação, como as proteínas UBC9 e PIAS (Figura 10). PIAS foi pescada com a região C-terminal de Ki-1/57 (122-413) e UBC9 com a região N-terminal (1-150) da proteína (Tabela 1).

Região da proteína usada como isca	Proteínas que interagiram com Ki-1/57	Processo biológico que participam.
Ki-1/57- C-terminal (122-413)	**RACK-1**	Liga-se à PKC ativada, aos ribossomos e ao RNA (Stebbins *et al.,* 2001 e Angenstein *et al.,* 2002).
	Topors	Proteína que se liga a topoisomerase I rica em arginina e serina - ativação transcricional (Haluska *et al.,*1999).
	DAXX	Participa da regulação da transcrição (Li *et al.,* 2000).
	PIAS	SUMO E3 ligase (Shuai *et al.,* 2005).
	RPL38	Ligação à fosfatidil-etanolamina / liga-se a lipídeos, interage com a maquinaria de tradução protéica (Kenmochi *et al.,* 1998).
Ki-1/57 – N-terminal (1–150)	**UBC9**	Proteína que participa do processo de Sumoilação (Shuai *et al.,* 2005).
	CIRBP	Proteína que se liga ao RNA induzida por frio (cold inducible RNA binding protein) - Regulação de *splicing* alternativo e tradução (Nishiya *et al.,* 1998 e De Leeuw *et al.,* 2007).
	FXR1P	Proteína relacionada com retardamento mental X-frágil- Associa-se com os polissomos (60 S) e participa do processo de tradução (Vasudevan *et al.,* 2007).
	YB-1 ou NSEP1	Domínio de ligação ao DNA- RNA, interação com elementos regulatórios no DNA. Ativador de p53 (Didier et al., 1988).
	SFRS9	Interage com NSEP 1, controle de hnRNP A1 por *splicing* alternativo, funciona como repressor do sitio 3'de *splicing* (Simarde e Chabot, 2002).

Tabela 1: Proteínas parceiras de interação de Ki-1/57 identificadas através de técnica de duplo híbrido em leveduras.

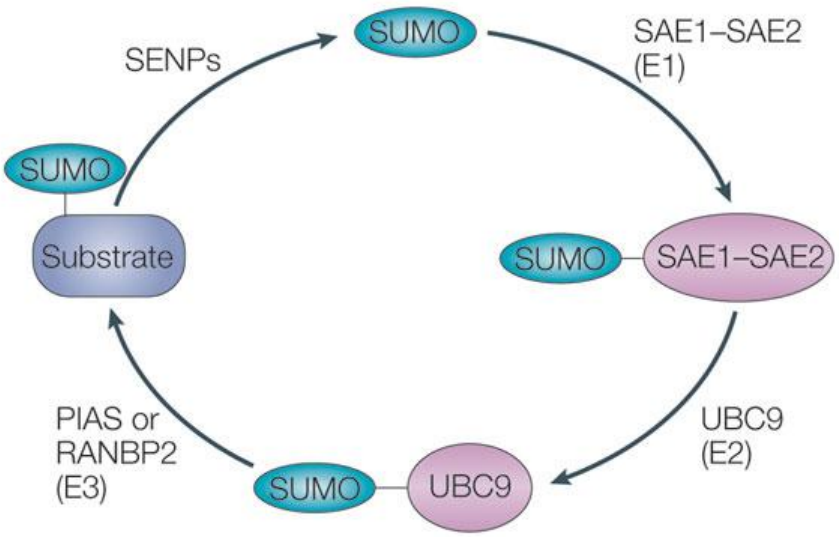


Figura 10- Enzimas envolvidas no processo de sumoilação. A Enzima E1 consome ATP para fazer a clivagem do C-terminal de SUMO, deixando os resíduos de glicina expostos, uma ligação tioéster é então formada entre o resíduo de SUMO e um resíduo de cisteína na proteína SAE2. Em seguida, SUMO é transferida de E1 para o resíduo de cisteína da enzima E2 (enzima ubiquitina-conjugando 9 - UBC9). A SUMO E3 ligase (E3), em seguida, promove a transferência de SUMO de E2 para o substrato alvo. Várias SUMO E3 ligases, incluindo PIAS (*protein inhibitor of activated signal transducer*), RANBP2 (*RAN-binding protein 2*) tem sido identificadas. Extraído de Shuai *et al.,* (2005).

Foi verificado que a proteína Ki-1/57 possui 7 sítios preditos para sumoilação em sua seqüência de aminoácidos. A análise feita através do programa SUMOplot™ mostrou que as lisinas 213, 276 e 336 tinham uma alta probabilidade de serem sumoiladas. Comparando a seqüência da proteína Ki-1/57 com outras proteínas que já se sabia que eram sumoiladas foi levantada a hipótese de que Ki-/57 poderia ser um alvo de sumoilação. Para confirmar esta hipótese, testes de sumoilação *in vitro* e *in vivo* foram realizados, e foi constado que a proteína é realmente sumoilada. Através dos mutantes construídos conseguiu-se identificar as lisinas que são sumoiladas, e em seguida realizou-se uma análise sobre qual é a importância da sumoilação na função da proteína Ki-1/57.

Bressan e colaboradores (2009) descreveram a proteína Ki-1/57 influenciando os eventos do *splicing* alternativo do mini gene E1A, e neste trabalho foi mostrado a capacidade de Ki-1/57 inibir a expressão de genes relacionados com a proliferação celular. O clone da proteína Ki-1/57 com as três lisinas mutadas foi usado para repetir o experimento de *splicing* do

mini gene E1A e será usado para verificar se a superexpressão deste clone ainda terá a capacidade de inibir os genes relacionados com a proliferação celular, sendo capaz desta maneira de analisar a importância da sumoilação nestes dois processos.

Os dados obtidos mostram que quando a proteína Ki-1/57 não é mais sumoilada por SUMO ela perde a capacidade de modular as isoformas de *splicing* no mini gene E1A (dados mostrados no artigo I).

4.3- A localização da proteína Ki-1/57 em situações de stress.

Os dados obtidos com os experimentos de microarrays revelaram que as proteínas Ki-1/57 e CGI-55 podem estar envolvidas principalmente em mecanismos repressores da expressão gênica, pois grande parte dos genes modificados sofreu diminuição em seus níveis de RNA mensageiros. Analisando os genes que foram encontrados reprimidos, muitos são descritos como envolvidos tanto na promoção da proliferação quanto no desencadeamento da apoptose.

Diversas analises de localização subcelular em células submetidas a diferentes condições estressantes foram realizadas e observado que em todas as situações testadas a proteína Ki-1/57 sofreu relocalização para regiões puntiformes proeminentes no citoplasma (Artigo I Fig. 2 e Artigo II Fig 3). Em ensaios de co-localização com proteínas marcadoras, esses corpúsculos citoplasmáticos foram mostrados tratarem-se de regiões denominadas "grânulos de estresse" do inglês *stress granules* (Artigo I Fig. 2).

Essas subestruturas formadas transientemente no citoplasma de células submetidas a diferentes agentes estressantes são enriquecidas em proteínas ligadoras a RNAs envolvidas no controle da tradução (tais como FMRP, FXR1 e FXR2) e estabilidade de mRNAs (Dolzhanskaya *et al.,* 2006; Goodier *et al.,* 2007 e Denegri *et al*., 2001). Acredita-se que os fatores de *splicing* tais como SFRS9, SF2/ASF e Sam68 são encontrados em regiões granulares no núcleo (chamadas de *nuclear stress bodies*) e que essas regiões alteram o *splicing* de genes específicos que possam ser relevantes para a resposta a injúria (Biamonti, 2004).

Sabe-se que após o stress esses mRNAs são capazes de retornar aos poliribossomos ativos para serem traduzidos (Brengues *et al.,* 2005; Teixeira *et al.,* 2005). Os corpúsculos conhecidos como grânulos de estresse podem levar à repressão generalizada da síntese de proteínas a partir da maioria dos mRNAs, com exceção daqueles que apresentam regiões de IRES (*internal ribosome entry site*) (Kedersha *et al.,* 1999; Holcik *et al.,* 2005). As regiões

IRES são encontradas em mRNAs que codificam para proteínas protetoras, como da chaperona BiP, que não tem a sua tradução inibida, o que permite a célula a se adaptar à condição danosa (López-Lastra *et al*., 2005).

Portanto, considerando os dados de localização subcelular, ensaios de proliferação e de expressão gênica obtidos, a hipótese de que tanto Ki-1/57 quanto CGI-55 possam atuar em mecanismos de proteção celular a situações estressantes parecem ser adequadas. Em termos de implicações, esses resultados levantam outras hipóteses relativas à participação dessas proteínas em vias de sobrevivência de células tumorais, principalmente aquelas resistentes a agentes quimioterápicos. Considerando o grande número de oncogenes modificados nos ensaios de *microarrays*, é válido ser averiguado se a ativação anormal de Ki-1/57 e CGI-55 durante o estresse quimioterápico não seja contribuidor em mecanismos de sobrevivência que resultem em resistência ao tratamento. Experimentos adicionais estão sendo realizados no grupo com o objetivo de pelo menos confirmar o efeito protetor dessas proteínas em situações de indução da apoptose.

5- Conclusões

O presente estudo obteve diferentes dados experimentais que comprovam a relação funcional de Ki-1/57 com mecanismos de tradução proteica, por interagir e co-localizar com as proteínas da Familia do X frágil, estar associada aos polissomos e ribossomos, interagir com RACK1(uma proteína também encontrada nos ribossomos) e finalmente por influenciar os níveis de tradução de gene repórter da Luciferase. Informações estruturais que atestam a natureza intrinsecamente desestruturada de Ki-1/57, explicam a sua plasticidade funcional, sugerida pelos seus diversos parceiros de interação identificados previamente. Considerando essas informações, alguns resultados publicados, a classe funcional de suas proteínas parceiras e ainda os dados de expressão gênica obtidos, supõe-se que Ki-1/57 seja uma proteína regulatória envolvida em diferentes níveis de controle da expressão gênica através do metabolismo de RNAs, passando pela transcrição, *splicing* e tradução. De forma mais específica pode ser concluído:

Artigo I

- O ensaio de imunoprecipitação em células humanas (Jurkat) com proteínas endógenas, mostrou que Ki-1/57 interage in vivo com a proteína FMRP (Fragile X mental retardation protein), sendo mais um idicio de seu possível papel na ativação/inibição da tradução protéica.

- Imagens de microscopia de Fluorescência mostram que Ki-1/57 sob condições estressantes, co-localiza com a proteína TIA-1, que é uma proteína marcadora de grânulos de stress.

- Imagens feitas utilizando um microscópio confocal revelaram que Ki-1/57 co-localiza em células COS-7 com as proteínas FMRP, FXR1 and FXR2, predominantemente no citoplasma em granulos de stress (células stressada com Arsenico).

- Ki-1/57 além de interagir com RACK1, uma proteína que já foi descrita na literatura por estar associada a subunidade menor dos ribossomos, ela também foi encontrada associada aos polissomos.

Através do sistema Dual Luciferase Assay foi constatado que a proteína Ki-1/57 tem a capacidade de aumentar a tradução do gene repórter da Luciferase. Este teste foi feito utilizando células COS-7.

Artigo II

- Uma análise de Bioinformática mostrou que a proteína Ki-1/57 tem 7 sítios preditos de sumoilação (baseado no programa SUMOplot^{TM}), nos quais as 3 lisinas 213, 276 e 336 possuem uma alta probabilidade de serem sumoiladas.

- Ensaios de Sumoilação *in vitro* e *in vivo* mostraram que a proteína Ki-1/57 realmente é encontrada sumoilada nas células tanto por SUMO-1 quanto por SUMO-2/3.

- Mutações sitio-dirigidas foram feitas na proteína Ki-1/57 no sentido de identificar quais lisinas eram sumoiladas. Imunoprecipitações com os diferentes mutantes nos permitiram concluir que as lisinas sumoiladas por SUMO-1 eram realmente as três que possuíam o maior *score* segundo o programa SUMOplot^{TM}.

- Em paralelo o mutante triplo foi testado para saber se o mesmo deixava de ser sumoilado por SUMO-2, e foi observado que sim.

- Ensaios de co-transfecção em células COS-7 revelaram que a sumoilação é importante para que Ki-1/57 mantenha a influência na seleção de sítios de *splicing* do mini gene adenoviral E1A.

Artigo III

- Os dados de SAXS demonstraram que a proteína RACK1 humana além de ter sua seqüência de aminoácidos muito similar a proteína RACK1 isoforma A de *Arabidopsis thaliana*, em solução possui um envelope molecular muito similar também.

- A ultracentrifugação analítica, através dos resultados obtidos com a sedimentação em equilíbrio, mostrou que a proteína RACK1 humana é predominantemente monomérica em solução (aproximadamente 90%), este dado também foi confirmado pela técnica de SAXS.

- A interação de RACK1 com o C-terminal de Ki-1/57 (122-413) é bem forte, tendo uma constante de dissociação baixa, de aproximadamente 0.7 μM, e a estequiometria de reação é de 1:1.

- RACK1 é uma proteína rica em triptofano e seu espectro de emissão de fluorescência apresenta um máximo em cerca de 334 nm, sugerindo que alguns destes Trp estão expostos ao solvente.

Artigo IV

- A superexpressão da proteína Ki-1/57 modificou a expressão de 413 genes, dos quais 88% apresentaram uma diminuição nos seus níveis de mRNA, sugerindo que Ki-1/57 possivelmente tenha um efeito predominantemente repressor na expressão gênica.

- A maioria dos genes que foram inibidos pela superexpressão de Ki-1/57 estão envolvidos na proliferação celular, muitos dos quais são encontrados alterados em diferentes tumores.

- Foi observado também a repressão dos genes que induzem a apoptose. Por esse lado, Ki-1/57 talvez esteja envolvida em respostas celulares a estímulos ambientais danosos, já que um período de latência sob determinadas condições pode representar um período de "recuperação" importante, principalmente considerando os mecanismos celulares de reparo e sobrevida frente a situações que podem ser momentâneas.

- Foram encontrados 217 genes com a expressão alterada em células superexpressando CGI-55, dois quais 55% deles tendo sido regulados negativamente. Esses dados são similares aos observados para Ki-1/57, tal achado sugere que CGI-55 possa ter uma atividade primariamente repressora no controle da expressão gênica. Embora a análise preliminar desses genes não permita uma visualização muito clara do efeito que pode estar ocorrendo, cerca de 20 genes foram comuns ao encontrado para Ki-1/57, a maioria deles está envolvida em processos proliferativos ou tumorigênicos e todos tiveram a sua expressão diminuída.

- Células superexpressando as proteínas Ki-1/57 e CGI-55 tiveram a sua proliferação celular diminuída entre 48 a 72 horas após a transfecção em relação as células controles, transfectadas com o vetor vazio.

- Experimentos de citometria de fluxo sugerem que a superexpressão da proteína Ki-1/57 não está causando uma parada no ciclo celular (o que poderia ser observado com um acumulo de células em uma determinada fase do ciclo).

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Uso exclusivo da CIBio:

Número de projeto / processo: JK 05.02

Formulário de encaminhamento de projetos de pesquisa para análise pela CIBio - Comissão Interna de Biossegurança da ABTLuS – Associação Brasileira de Tecnologia de Luz Síncrotron

Título do projeto: Geração e expressão de mutantes delecionais e pontuais da proteína reguladora humana Ki-1/57 para estudos funcionais e estruturais

Pesquisador responsável: **Jörg Kobarg**
Experimentador: **Kaliandra de Almeida Gonçalves**

Nível do treinamento do experimentador: [ ]-Iniciação científica, [ ]-mestrado, [ ]-doutorado, [x]-doutorado direto, [ ]-pós-doutorado, [ ]-nível técnico, [ ]-outro, especifique:_____

Resumo do projeto:

O antígeno Ki-1/57 foi identificado por um anticorpo monoclonal, Ki-1, que detectou especificamente células malignas de Hodgkin e células Sternberg-Reed no linfoma de Hodgkin. Este anticorpo detectou além de Ki-1/57, uma proteína intracelular de 57 kDa, um outro antígeno: uma glicoproteína de superfície de membrana de 120 kDa chamada CD30. O antígeno Ki-1/57 é associado com atividade de quinase e mostrou-se fosforilado em resíduos de serina e treonina. Análises de microscopia eletrônica demonstraram que o antígeno Ki-1/57 não está localizado apenas no citoplasma, mas também nos poros nucleares e no núcleo, associado ao nucléolo. O antígeno Ki-1/57 é expresso em células de uma variedade de tipos de câncer (ex. linfoma de células T, adenocarcinoma, carcinoma de próstata, carcinoma de bexiga), sendo também expresso em leucócitos do sangue humano ativados com mitógenos, mas não em células não ativadas. Em estudos de doplo híbrido em levedura recentemente realizados no nosso grupo identificamos várias proteínas que interagem com a proteína Ki-1/57 que na sua maioria representam proteínas funcionalmente associadas á regulação da transcrição. Duas delas são a proteína adaptadora RACK-1 e a proteína p53 que está relacionada com o mecanismo de verificação de mutações na seqüência de DNA das células. O objetivo deste projeto é o estudo funcional e estrutural da proteína Ki-1/57. Os estudos funcionais envolvem a mutagenese através da técnica de "Duplo Híbrido Reverso que permitirá saber qual ou quais aminoácidos específicos estão envolvidos na interação de Ki-1/57 com as proteínas RACK-1 e p53. Além disso iremos fazer microarray para analise de expressão gênica em células superexpressando a proteína Ki-1/57. Na parte estrutural serão feitos ensaios de proteólise limitada com a proteína 6xHis-Ki-1/57 expressa na bactéria ou no sistema de Báculo vírus para achar um domínio / núcleo estável da proteína, caracterização e utilização deste núcleo através de espectrometria de massas. Novas proteínas delecionais da Ki-1/57 serão posteriormente empregadas em ensaios de cristalização e para outros ensaios in vitro.

A CIBio analisou este projeto em reunião realizada no dia: 16.4.2007 .

Parecer final: [X]-projeto aprovado, [ ]-projeto recusado, [ ]-projeto com deficiências, favor comentários abaixo: _____

Jörg Kobarg
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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação de Mestrado/tese de Doutorado intitulada Estudos funcionais da proteína Reguladora Humana Ki-1/57 e seus parceiros de interação.

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

(x) CIBio – Comissão Interna de Biossegurança , projeto No: JK 05.02, Instituição: Associação Brasileira de Tecnologia de luz Sincrotron- Laboratório Nacional de Biociências.

( ) CEUA – Comissão de Ética no Uso de Animais , projeto No. _____, Instituição: _____.

( ) CEP - Comissão de Ética em Pesquisa, protocolo No. _____, Instituição: _____

** Caso a Comissão seja externa ao IB/UNICAMP, anexar o comprovante de autorização dada ao trabalho. Se a autorização não tiver sido dada diretamente ao trabalho de tese ou dissertação, deverá ser anexado também um comprovante do vínculo do trabalho do aluno com o que constar no documento de autorização apresentado.*

Kaliandra A. Gonçalves
Aluno: (Kaliandra de Almeida Gonçalves)

Jörg Kobarg
Orientador: (Jörg Kobarg)

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Carimbo e assinatura

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Para uso da Comissão ou Comitê pertinente:
( ) Deferido ( ) Indeferido

_____
Carimbo e assinatura