Universidade Estadual de Campinas

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ESTUDOS FUNCIONAIS E ESTRUTURAIS DA PROTEÍNA HUMANA hnRNP Q/NSAP1

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"O homem de bem exige tudo de si próprio; o homem medíocre espera tudo dos outros".

(William Shakespeare)

"Dificuldades e obstáculos são fontes valiosas de saúde e força para qualquer sociedade".

(Albert Einstein)

"A mais bela experiência que podemos ter é a do mistério. É a emoção fundamental existente na origem da verdadeira arte e ciência. Aquele que não a conhece e não pode se maravilhar com ela está praticamente morto..."

(Albert Einstein)

"Se você tem uma laranja e troca com outra pessoa que também tem uma laranja, cada um fica com uma laranja. Mas se você tem uma idéia e troca com outra pessoa que também tem uma idéia, cada um fica com duas"

(Confúcio - Filósofo Chinês)

"Se seus sonhos estiverem nas nuvens, não se preocupe, pois eles estão no lugar certo; agora construa os alicerces"

(William Shakespeare)

"A convicção absoluta é inimiga mais perigosa da verdade do que a mentira".

(Friedrich Wilhelm Nietzsche)

"Aos que não sabem, ensinem-lhes tudo que puderem; a sociedade, cujo dever seria dar educação gratuita aos famintos de ciência, é a única responsável pelas trevas que produz"

(Vítor Hugo).

Dedico esse trabalho....

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José Maria do Vale Quaresma & Emília Adelaide Christino Quaresma

Ao meu querido irmão,

José Maria do Vale Quaresma Júnior

À minha querida e paciente companheira,

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Lista geral de abreviações e siglas

3AT	3-aminotriazol
AD	domínio de ativação transcricional da proteína Gal4 (*Activation domain*)
aDMA	Dimetilarginina assimétrica (*Asymmetric Dimethylarginine*)
AdoMet (**SAM**)	S-adenosilmetionina (*S-Adenosylmethionine*)
AdoHcy	S-Adenosilhomocisteína (*S-Adenosylhomocysteine*)
ANS	8-anilino naftaleno-1sulfonato
APS	Persulfato de Amônio (*Ammonium Persulfate*)
AR	Autoradiograma (*Autoradiogram*)
ATP	Trifosfato de adenosina (*Adenosine tryphosphate*)
BD	domínio de ligação ao DNA da proteína Gal4 (*Binding domain*)
BCFH	Biblioteca de Cérebro Fetal Humano (*Human fetal Brain Library*)
BSA	Albumina Sérica Bovina (*Bovine Serum Albumin*)
CD	Dicroísmo Circular (Circular Dichroism)
cDNA	DNA complementar
DOTAP	N-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethylammonium methyl-sulfate
DTT	1,4 ditiotreitol
EDTA	ácido etilenodiaminotetraacético
EMSA	Ensaio de Retardamento da Mobilidade Eletroforética (*Eletrophoretic Mobility Shift Assay*)
FPLC	Cromatografia Líquida de Alta Pressão (*Fast Performance Liquid Chromatography*)
GSH	glutationa reduzida
GST	glutationa-S-transferase
hnRNP Q	Ribonucleoproteína Heterogênea nuclear Q (Heterogeneous Nuclear Ribonucleoprotein Q)
hnRNP D	Ribonucleoproteína Heterogênea nuclear Q (Heterogeneous Nuclear Ribonucleoprotein D)
IPTG	Isopropil beta-D-galactosídeo;
kDa	quilo Dalton (k*ilo Dalton*)
Kg	Quilograma (*Kilo gram*)
LB	(meio) Luria Bertani
MCS	sítio de clonagem múltipla *(Multiple cloning site*)
MMA	Monometilarginina (*Monomethylarginine*)
mg	miligrama (*milli gram*)
mRNA	RNA mensageiro
ng	nanograma (*nono gram*)
Ni-NTA	ácido níquel-nitrilotriacético
NSAP1	Proteína associada a NS1 (NS1-associated protein)

NSEP1	proteína de ligação ao elemento sensível a nuclease 1 (*nuclease sensitive element binding protein 1*)
pb	pares de bases (*base pairs*)
PCR	reação da polimerase em cadeia (*Polimerase Chain Reaction*)
PDB	banco de dados de estruturas protéicas (*Protein Data Bank*)
PMA	forbol 12-miristato 13-acetato (*phorbol 12-myristate 13-acetate*)
PMSF	fluoreto de fenil metil sulfonila
PRMT1	Proteína Arginina Metiltransferase 1 (Protein Arginine Methyltransferase 1)
rpm	rotações por minuto
SDS	dodecil sulfato de sódio
SDS-PAGE	Eletroforese de Gel Poliacrilamida SDS (*SDS polyacrylamide gel electrophoresis*)
SMA	Atrofia Muscular Espinhal (*Muscular Atrophy Spinal*)
SMN	Neurônio motor de sobrevivência (*Survival Motor Neurons*)
Tris	tris-hidroximetil aminometano
Tween 20	polyoxyethylene sorbitanmonolaurate
UV	ultravioleta
WB	Western blot
µg	micrograma (*microgram*)
RRM	Motivo de Reconhecimento a RNA (*RNA Recognition Motif*)
RGG-box	Motivo rico em argininas e glicinas
snRNP	Ribonucleoproteínas pequenas nucleares (*Small nuclear ribonucleoproteins*)
SDHL	Sistema de Duplo Híbrido em Levedura (*Yeast two Hybrid System*)
GFP	Proteína de Fluorescência Verde (*Green Fluorescence Protein*)
RBD	*Domínio de ligação a RNA (RNA binding domain*)
KH	Domínio Homólogo K (K homology domain)
sDMA	Dimetilarginina simétrica (*Symmetric Dimethylarginine*)
X-gal	5-bromo-4cloro-3-indol beta-D-galactopiranosídeo (*5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside*);

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Resumo

Os membros da família de proteínas chamada hnRNPs (*heterogenous nuclear ribonuclein proteins*) apresentam importantes papeis no controle da expressão gênica e no metabolismo dos mRNAs. Os membros hnRNPD (AUF1) e hnRNPQ (NSAP1) foram alvos deste estudo. AUF1 apresenta dois domínios de ligação à RNA do tipo RRM (*RNA recognition motif*) e participa ativamente no processo de desestabilização de uma classe de mRNAs que apresentam um motivo rico em AU na região 3´ não traduzida. Demonstramos, através do sistema de duplo híbrido em levedura, que a isoforma p37 de AUF1 interagiu com as proteínas hnRNPQ, IMP-2, NSEP1 (YB-1) e UBC9. Além disso, a proteína hnRNPQ também foi pescada num outro ensaio de duplo híbrido em levedura, que utilizou como isca a proteína humana arginina metiltransferase (PRMT1). hnRNPQ apresenta, na sua região C-terminal, um "motivo rico em argininas e glicinas" (*RGG box*). Demonstramos que ela é alvo de metilação pela PRMT1 *in vitro* e *in viv*o. Funcionalmente, sua metilação é importante para sua localização nuclear. NSAP1 têm uma constituição modular com um domínio ácido (AcD) no seu N-terminal, seguido por três domínios de ligação à RNA do tipo RRM e o já mencionado *RGG box* no seu C-terminal. Funcionalmente hnRNPQ está envolvido em vários aspectos do metabolismo de RNA, incluindo a edição do mRNA da proteína humana ApoB. Para isso, ela interage não somente com o mRNA de ApoB, mas com a enzima efetora da edição Apobec1 e com a proteína que ativadora do Apobec1 (ACF1). Mostramos que o domínio ácido, de NSAP1 é capaz de interagir com Apobec1 e que sua fosforilação *in vitro* pela PKC inibe esta interação. Ainda identificamos que hnRNPQ interage com proteínas da família *heat shock* (incluindo HSP70 e BiP), e vimos que hnRNPQ é um alvo de fosforilação principalmente pela PKCδ, *in vitro*. A localização sub-celular de hnRNPQ é modificada pela ativação *in vivo* das PKCs. Em conseqüência desta ativação ou da aplicação de estresse oxidativo, térmico ou indução de estresse do reticulo endoplasmático (tratamento com tapsigargina) hnRNPQ se desloca do núcleo para o citoplasma aonde se encontra em vesículas/corpúsculos definidas. Em resumo, nossos dados sugerem que as diversas funções da hnRNPQ relacionadas ao metabolismo de mRNAs, sofrem diferentes regulações, mediadas por modificações pós-traducionais (fosforilação e metilação), que interferem tanto na sua localização celular quanto na sua afinidade por determinados proteínas parceiras.

Abstract

The members of the hnRNPs family (heterogenous nuclear ribonuclein proteins) play important roles in gene expression control and mRNAs metabolism. The proteins hnRNPD (AUF1) and hnRNPQ (NSAP1) were the main targets of this study. AUF1 has two RNA recognition motifs (RRM) and participates in the process of destabilization of a class of mRNAs that contain AU-rich sequences in their 3' untranslated regions (3´-UTR). We found, using the "yeast two-hybrid system" (Y2HS), that the isoform p37 of AUF1 (AUF1p37) interacts with the proteins: hnRNPQ, IMP-2, NSEP1 (YB-1) and UBC9. Moreover, the protein hnRNPQ was also identified as a prey protein in another Y2HS screen, which used as bait the human protein Arginine methyltransferase (PRMT1). HnRNPQ presents, in its C-terminal region, an "Arginine/Glicine-rich sequence" (RGG box). We are able to show that this RGG box is a target for methylation by PRMT1 *in vitro* and is methylated *in vivo*. Functionally, this methylation is important for its nuclear localization. hnRNPQ has a modular organization with an acid domain (AcD) in its N-terminal, followed by three RNA-binding domains (RRM) and the previously mentioned RGG box in its C-terminal. Functionally, hnRNPQ is involved in diverse aspects of RNA metabolism, including editing of the mRNA encoding the human protein ApoB. It has been shown previously to interact with the mRNA of ApoB, and also with the editing enzyme Apobec1 and the Apobec1 activation protein (ACF1). Here we show that the acid domain of hnRNPQ mediates the interaction with Apobec1 and that its *in vitro* phosphorylation (by PKC) inhibits this interaction. Furthermore, we found that hnRNPQ interacts with members the heat shock family of proteins (including HSP70 and BiP), and demonstrated that hnRNPQ can be *in vitro* phosphorylated by PKCδ. Finally, we discovered that the sub-cellular localization of hnRNPQ undergoes modification after activation of PKC pathways. This also occurs after application of endoplasmic reticulum stress (using tarpsigargin), oxidative or heat stress. Under all of these conditions hnRNPQ translocated from the nucleus to the cytoplasm, where it is found at defined vesicles or granules. In summary, our data suggest that the diverse functions of hnRNPQ in the context of mRNA metabolism, may suffer specific regulations, by post-translational modifications, including phosphorylation and methylation, which modify both the proteins sub-cellular localizations as well as its affinity to interacting protein partners.

1. Introdução

1.1. O controle da expressão gênica em eucariotos

A expressão gênica pode ser regulada em vários níveis: transcrição, *splicing*, poliadenilação e *capping* do pré-mRNA, transporte e localização do mRNA dentro da célula, estabilidade do mRNA, tradução, processamento das proteínas produzidas e a estabilidade das proteínas formadas durante a tradução (Lewin, B *et al*. 2000). Durante muitos anos o controle da transcrição, tradução e os mecanismos que envolvem os processos de *splicing* e *capping* (na extremidade 5´- do pré-mRNA) foram extensivamente estudados. Entretanto, somente há alguns anos, a função da estabilidade do mRNA, no contexto da expressão genética, começou a receber a atenção devida. Seqüências específicas (como elementos de ação *cis*) na região 3´ não traduzida (3´ - UTR) do mRNA, em conjunto com determinadas proteínas (fatores de ação *trans*), determinam a estabilidade da molécula de mRNA e, conseqüentemente, o nível de expressão de diversas proteínas. Alterações na estabilidade do mRNA podem ocorrer durante o desenvolvimento ou durante determinada doença (Becket, B 2001).

1.1.a. O papel da região 3´-UTR na degradação de mRNA

A maioria das moléculas de mRNAs apresentam uma cauda poli A na extremidade 3´. Geralmente a deadenilação (remoção da cauda) dessa molécula de mRNA é o primeiro passo na degradação desse mRNA (Beelman, C.A. *et al* 1995, Mitchel, P. *et al* 2000, Ross, J. *et al* 1995 e Xu, N. *et al* 1997). Após a deadenilação, a degradação pode ocorrer na mesma molécula de mRNA (degradação processiva) ou pode ocorrer em diferentes mRNA ao mesmo tempo (degradação distributiva) (Mitchel, P. *et al* 2000, Xu, N. *et al* 1997 e Peng, S.S. *et al* 1996). Por exemplo, há dois mRNAs que já foram extensivamente estudados: GM-CSF(*"granulocyte macrophage-colony stimulating factor"*) e o mRNA de c-fos. O mRNA de GM-CSF apresenta deadenilação com subseqüente degradação processiva, já o mRNA de c-fos apresenta deadenilação com degradação distributiva. A complexidade na escolha da via surge a partir das características intrínsecas de cada molécula de mRNA. Para ajuda na compreensão do presente, mRNAs foram organizados em três categorias baseado em características que afetam a sua decadência. A classe I de mRNA compreende moléculas que apresentam elementos de ação *cis* dispersos na seqüência. Podemos citar como exemplo os elementos ricos em AU, que mostram cinética de degradação distributiva (como é o caso de c-fos). Na Classe II de mRNA, o elemento *cis* determinante é caracterizado por um ou dois motivos seqüenciais do tipo U (U / A) (U / A) UUU (U / A) (U / A) U. Conseqüentemente, o mRNA mostra cinética de degradação processiva (como é o caso de GM-CSF). A Classe III, compreende os mRNAs que não apresentam regiões (elementos) ricas em AU e mostram cinética

de degradação distributiva (como em c-jun). Ainda há processos menos compreendidos que podem estar envolvidos na degradação do mRNA. Podemos citar uma delas que é a clivagem por endonucleases que reconhecem seqüências específicas, mas estes não serão discutidos aqui (Beelman, C.A. *et al* 1995, Xu, N. *et al* 1997 e Peng, S.S. *et al* 1996).

1.1.b. A importância dos elementos (motivos) na degradação do mRNA

Elementos de função *cis* ou seqüências presentes na molécula de mRNA apresentam fundamental papel na ligação entre esses mRNAs e proteínas. Embora muitos desses elementos sejam encontrados na região 3´-UTR do mRNA, em alguns casos é possível encontrar elementos tanto na região codificante, quanto na região 5´-UTR. A ligação de algumas proteínas na molécula de mRNA e a possibilidade de formação de estruturas secundárias podem permitir que esses elementos, presentes em determinadas moléculas, sejam capazes de interagir com outra região, também presente na molécula de mRNA (Misquitta, C.M *et al* 2001).

1.1.c. Seqüências específicas

O exemplo mais estudado de elementos de ação do tipo *cis* é o motivo rico em AU, geralmente presente na região 3´-UTR de alguns mRNA. Geralmente esse elemento é constituído por repetições AUUUA, as mesmas que participam na desestabilização dos mRNAs de GM-CSF; c-myc, e c-fos (Aharon, T. *et al* 1993, Malter, J.S. 1989 e Ross, J. *et al* 1995). Além disso, a seqüência, U (U / A) (U / A) UUU (U / A) (U / A) U também foi encontrada participando da desestabilização de determinados mRNAs (Akashi, M. *et al* 1994, Bonnieu, A. *et al* 1990, Chen, C.Y *et al* 1994 e Zubiaga, A.M. *et al* 1995). Seqüências que não são ricas em AU podem atuar como elementos desestabilizadores, por exemplo, repetições do tipo GUUUG na região 3´-UTR, são alvos de proteínas que desestabilizam o mRNA de c-jun. Um exemplo interessante pode ser encontrado nos mRNAs das diferentes isoformas da enzima fosfoenolpiruvato carboxiquinase. Os mRNAs das isoformas apresentam diferentes vias de degradação. Isso acontece porque cada isoforma apresenta diferentes elementos na região 3´-UTR. Por exemplo, uma isoforma possui seqüências ricas em AU, já as outras ou apresentam elementos ricos em CU, ou estruturas secundárias do tipo stem-loops, que são reconhecidos por proteínas que participam na desestabilização e conseqüente degradação do mRNA (Hajarnis, S. *et al* 2005). Em alguns casos, temos vários elementos agindo. Por exemplo, a região 3´-UTR do mRNA da proteína bcl-2 contém um elemento de desestabilização rico em AU e uma repetição rica em CA de 30 nucleotídeos (Lee, J.H. *et al* 2004).

1.1.d. O papel das estruturas secundárias do mRNA

Há pelo menos duas possibilidades para o papel de uma estrutura secundária, como um *stem-loop* durante o processo de degradação do mRNA. A primeira possibilidade seria de impedir o acesso ou progressão de exonucleases, facilitando assim a estabilização do mRNA. A segunda possibilidade seria fornecer sítios de ligação para interações com diferentes proteínas ou atuar como sítio na formação de um possível complexo, promovendo assim uma estabilização ou degradação - dependendo dos fatores envolvidos. Evidencias para ambas as funções já foram mostradas. Podemos citar como exemplo a mudança conformacional da região 3´-UTR, induzida por Mg^{+2} que afeta a interação entre a proteína AUF1 e a região rica em AU do mRNA (Wilson, G.M *et al* 2001[a,b]). Outro exemplo é a região rica em AU do fator de necrose tumoral (TNF) que forma uma estrutura do tipo *hairpin* que também regula a interação entre o mRNA e a proteína humana AUF1 (Fialcowitz, E.J et al 2005).

1.1.e. Proteínas envolvidas na degradação de mRNA

Proteínas que se ligam a mRNA desempenham um papel importante na degradação ou na estabilização desse ácido nucléico. Podemos citar como exemplo a proteína que se liga à cauda poli A (PABP). Quando a proteína PABP se liga, a cauda fica protegida contra o processo de deadenilação (Lewin, B *et al*. 2000, Beelman, C.A. *et al* 1995, Mitchel, P. *et al* 2000, Ross, J. *et al* 1995, Xu, N. *et al* 1997 e Peng, S.S. *et al* 1996). Outras proteínas podem agir afetando a função do exossomo, um complexo de exoribonucleases que degrada determinados mRNAs no sentido 3´-5´ (Tran, H. et al 2004). Várias outras proteínas, participantes desse processo, já foram caracterizadas, dentre elas a família das hnRNPs, que apresenta grande parte dos seus membros envolvidos na desestabilização de mRNAs, e a família Hu que tem como principal característica estabilizar determinados mRNAs (Ponka, P. *et al* 1998,1999[a,b]).

1.2. O papel das Ribonucleoproteínas (hnRNPs) (*"heterogeneous nuclear ribonucleoprotein*")

As heterogêneas ribonucleoproteínas nucleares (do inglês "*Heterogeneous nuclear ribonucleoproteins* - hnRNPs") foram primeiramente descritas como um grupo de proteínas que se ligavam à moléculas de RNA (Dreyfuss, G. *et al* 1993, Swanson, M.S. *et al* 1995). As primeiras evidências mostravam que um "core", contendo seis proteínas, ligava-se rapidamente a nascente molécula de mRNA, transcrito pela enzima RNA polimerase II e logo formava um complexo com diferentes ("*heterogeneous"*) moléculas de RNA, presentes no núcleo (hnRNAs), formando assim uma partícula que continha diferentes hnRNAs e diferentes proteínas hnRNP (McAfee, J.G. *et al* 1997). Um grande salto para caracterizar essa partícula foi quando esse complexo foi imunoprecipitado, com subseqüente identificação de muitas outras proteínas presentes na partícula.

Após vários estudos, inclusive estruturais, ficou demonstrado que as proteínas que formavam esse complexo (proteínas do complexo hnRNP) continham diversos domínios de ligação à RNA, com diversos RNAs-alvo. (Dreyfuss, G. *et al* 1993, Swanson, M.S. *et al* 1995).

Em seres humanos, aproximadamente 30 diferentes proteínas já foram identificadas do complexo de hnRNP e as estruturas de muitas delas já foram resolvidas, tanto por ressonância nuclear magnética (RNM), quanto por cristalografia. (Tabela 1). Na tabela 1 temos uma sumarização com as principais proteínas que fazem parte da família das hnRNPs. Além disso, podemos ver a constituição modular -presente em todos os membros dessa família, bem como suas principais funções e se há estrutura resolvida, da proteína completa, de algum domínio ou de algum complexo. Uma das principais características dos membros desse complexo é a presença de domínios utilizados para interações do tipo proteína-RNA, bem como para interações do tipo proteína-proteína – grande parte dessas interações do tipo proteína-proteína ocorrem entre os membros dessa família (Dreyfuss, G. *et al* 1993, Swanson, M.S. *et al* 1995 e Cartegni L. *et al* 1996). Outra importante característica dos membros desse complexo é a existência de isoformas, originadas por *splicing* alternativo, e modificações pós-traducional, tais como glicosilação, fosforilação e metilação.

A complexidade aumenta com a possibilidade de haver proteínas que poderiam ser membros dessa família, mas que ainda não foram caracterizados ou que já tenham sido agrupadas em outras famílias.

As proteínas hnRNPs estão envolvidas em muitos processos biológicos do metabolismo de RNAs como: transcrição, processamento do pré-RNA, transporte de mRNA para o citoplasma e tradução. A proteína hnRNP K, por exemplo, encontra-se no núcleo, no citoplasma e até mesmo na mitocôndria e é um ótimo exemplo da versatilidade dessa família de proteína, pois realiza diversos processos em cada um dos compartimentos celulares citados acima. Sabe-se também que ela é capaz de se ligar a algumas quinases, mediando assim algumas interações, reguladas por uma cascata de sinalização (Bomsztyk, K *et al* 2004).

Algumas hnRNPs são estritamente nucleares, já outras transitam entre o núcleo e o citoplasma, e a exportação destas proteínas provenientes do núcleo pode ser mediada por seqüências sinalizadoras conhecidas como NESs (*nuclear export sequences)* (Pinol-Roma, S. 1997 e Kim, J.H *et al* 2000), como é o caso da isoforma p37 da hnRNP D/AUF1, que possui um domínio de sinalização para transitar entre o núcleo e o citoplasma (Chen, C.Y et al 2004). Pesquisadores encontraram evidências que hnRNPs podem ser além de tudo reguladoras da expressão de genes através de ligação direta com DNA ou da interação com outras proteínas (Lau, J.S. *et al* 2000).

Tabela 1. Caracterização de algumas proteínas presentes no complexo hnRNP.

Principais hnRNPs	Domínios	Funções propostas	Estrutura resolvida
A1	2x RBD, RGG	Splicing, transporte de mRNA, biogênese do telômero, grânulos de estresse	SIM
A2/B1	2x RBD, RGG	Splicing, transporte de mRNA em grânulos	SIM
C1/C2	1x RBD	Splicing, empacotamento do transcrito, controle de qualidade de mRNAs - retenção nuclear	SIM
D1 (AUF1p37-45)	2x RBD	Transcrição, recombinação, mRNA degradação (NMD)	SIM
E1/E2 (PCBP1/PCBP2)	3x KH	Regula tradução, inibe a tradução de mRNAs ligados à hnRNP A2	SIM
F	3x RBD	Splicing, interage com proteínas ligadoras à 5´-cap, liga a regiões ricas em guanidina	SIM
G	1x RBD, RGG	Splicing, alvo de glicosilação	Não
G-T	1x RBD	Interage com mRNA, fundamental na espermiogênese	Não
H/H´	3x RBD	Splicing / poliadenilação	SIM
I (PTB)	4x RBD	Splicing (repressor), poliadenilação	SIM
K	3x KH, RGG	Transcrição, tradução	SIM
L	4x RBD	Estabilidade e exportação do mRNA	Não
M	3x RBD	Splicing, resposta a estresse térmico	SIM
P2 (TSL/FUS)	1x RBD, RGG	Oncogênese (liposarcoma)	Não
Q (Q1-3)	AcD, 3xRBD, RGG	Slicing, transporte de mRNA	SIM
R	3x RBD, RGG	Slicing, transporte de mRNA	SIM
U (SAF, p120)	1x RGG	Retenção nuclear, interage DNA, RNA	Não
Outras hnRNPs			
A0	2x RBD, RGG	Splicing	SIM
CUG-BP	3x RBD	Splicing, tradução	SIM
HuR	3x RBD	Estabilidade de determinados mRNA, transporte de mRNA	SIM

Diferentes funções são particulares de algumas isoformas, como acontece com as hnRNPs, C1, C2 e D, que são capazes de interagir tanto com o complexo de regulação responsável pelo encurtamento dos telômeros, quanto com a própria telomerase. Essas isoformas também podem interagir com as hnRNPs: A1, A2/B1, D e E1 que também possuem a capacidade de interagir com os telômeros (Ford, L.P. et al 2002). Sabe-se que a proteína hnRNP B1 está intimamente relacionada com a regulação pós-traducional que ocorre em células presentes no córtex temporal inferior de pessoas afetadas pelo mal de Alzheimer (Ishikawa, M. Et al 2004).

Outras hnRNPs como a G-T, quando modificadas, afetam tanto o *splicing* quanto a transdução alguns de sinais. A mutação hnRNP G-T_{R100H}, por exemplo, causa a troca de uma arginina altamente conservada e desse modo afeta o local de metilação, necessário para a ligação ao mRNA. Essa mutação, quando ocorre nos testículos, por exemplo, pode causar infertilidade. Interessantemente essa mutação é passada aos filhos pela mãe. O gene da proteína hnRNP G-T é super-expresso em espermatócitos (Westerveld, G.H. et al 2004).

1.3. O papel da proteína hnRNPD/AUF1

Os membros da família das hnRNPs constituem os principais fatores de desestabilização de determinados mRNAs (Carpenter, B., *et al* 2005). Podemos citar, como exemplo a proteína hnRNPA1 que apresenta duas funções primordiais na expressão gênica. Quando hnRNPA1 está localizada no núcleo, ela é capaz de interagir com seqüências intrônicas, regulando assim o processo de *splicing* alternativo de alguns genes (Hamilton, B.J *et al* 1997, Henics, T. *et al* 1994 e Nakamaki, T *et al* 1995). Entretanto, quando presente no citoplasma, hnRNPA1 se liga a seqüências ricas em AU e poliU. Essa interação ocorre principalmente em linfócitos T e tem como principal objetivo a estabilização do mRNA da proteína GM-CSF. Outras hnRNPs - como hnRNPA2/B1 e hnRNPC - também já foram caracterizadas com fundamental importância na regulação da degradação de determinados mRNAs (Kamma, H. *et al* 1999, Rajagopalan, L.E *et al* 1998, Gorlach, M. 1992).

AUF1 apresenta uma estrutura típica de uma proteína capaz de interagir com moléculas de mRNA. Para isso ela apresenta uma estrutura modular com a presença de dois domínios RBD (*RNA binding domain*) do tipo RRM (*RNA recognition motif*) e uma seqüência rica em glutamina na região C-terminal (Figura 1B) (DeMaria, C.T et al 1997 [a,b]). Os dois domínios de AUF1 podem interagir especificamente com seqüências ricas em AU ou ainda com seqüências de DNA simples fita, encontradas na região telomérica em humanos (Katahira, M. et al 2001). Esses domínios RRMs apresentam uma estrutura tridimensional similar com uma folha antiparalela do tipo β e duas α-hélices. A ligação entre o domínio RRM1 (N-terminal) de AUF1 e uma molécula de RNA forma uma

estrutura compacta. Essa interação ocorre justamente com a sua folha β - formada por quatro fitas antiparalelas (Nagata, T. *et al* 1999). Quando o domínio RRM2 (C-terminal) interage com uma molécula de RNA, essa interação também ocorre via ligação na folha β do domínio RRM – aqui formada por cinco fitas antiparalelas (Katahira, M. et al 2001).

O transcrito primário de AUF1 apresenta 10 exons que sofrem *splicing* alternativo, originando assim quarto isoformas: p37, p40, p42 e p45 (Sarkar, B *et al* 2003 e Wagner, B.J. *et al* 1998). Tanto a expressão quanto a localização celular dessas isoformas depende de diversos fatores: tipo celular, estágio do desenvolvimento da célula e da resposta a determinado estímulo (Wagner, B.J. *et al* 1998). A seqüência rica em alanina ("A"), na região N-terminal, está presente em todas as quatro isoformas e é fundamental na ligação entre os domínios RRMs e seqüências ricas em AU, presentes em determinados mRNAs (Figura 1B).

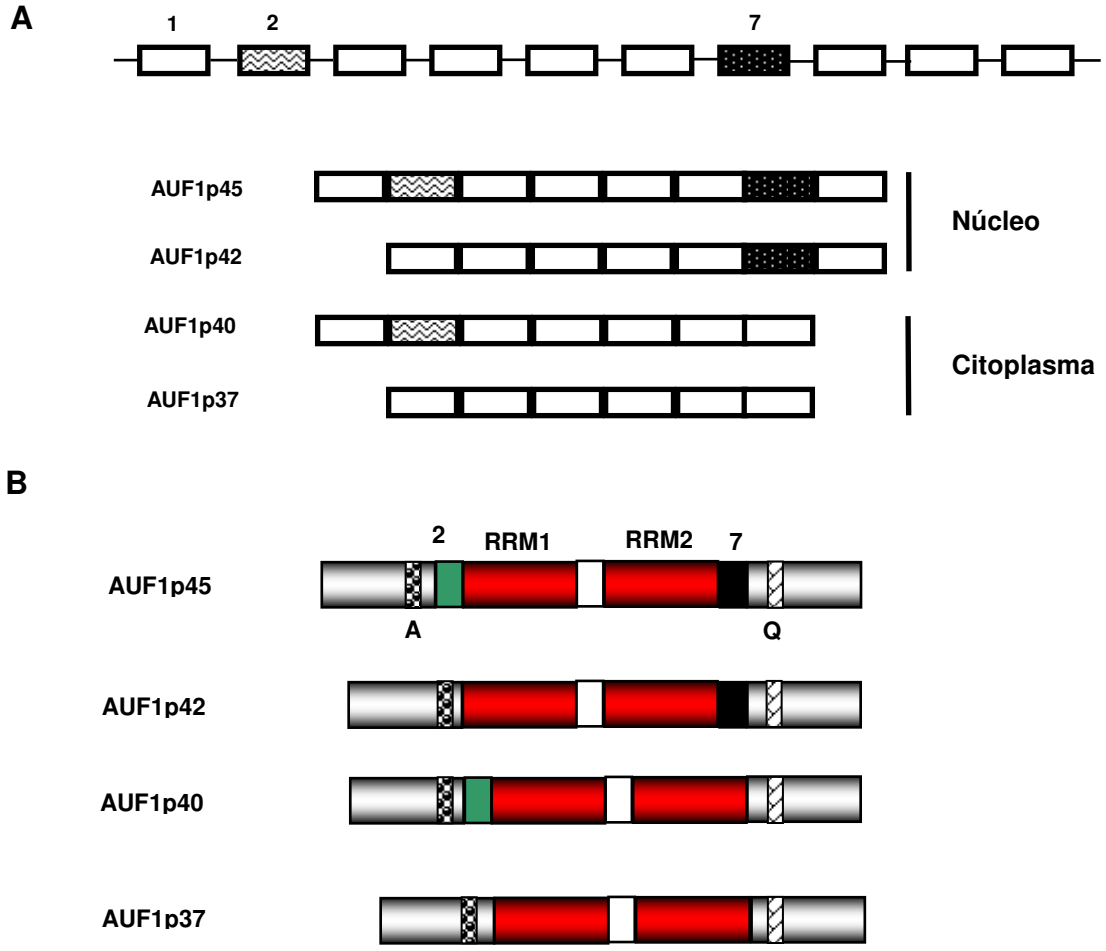

Figura1. Esquema geral da organização estrutural do gene da proteína humana hnRNP D/AUF1. (A) O splincing alternative que ocorre no gene da proteína AUF1. Acima organização intro/exon do gene. Abaixo a organização das diferentes isoformas com a presença dos exons 2 e 7, em destaque. (B) Estrutura geral das isoformas em nível da proteína. Os números 2 e 7 referem-se a presença ou não dos exons correspondentes. Já as regiões "A" e "Q" referem-se às regiões ricas em alanina e glutamina (respectivamente), presente em todas as isoformas. Em vermelho temos os domínios RRM1 (que apresenta uma folha do tipo β com 4 fitas antiparalelas) e o RRM2 (que apresenta uma folha do tipo β com 5 fitas antiparalelas).

Essa região também permite a formação de homo- ou heterodímeros entre as isoformas de AUF1(DeMaria, C.T *et al* 1997[a] e Wilson, G.M. *et al* 1999). As isoformas p37 e p42 – que não possuem o segmento codificado pelo exon 2 - apresentam maior afinidade entre si do que com as isoformas p42 e p45 (DeMaria, C.T *et al* 1996, 1997[a] e Loflin, P *et al* 1999). Assim, tanto a região rica em alanina ("A") quanto o exon 2 regulam a interação entre os membros dessa subfamília e regiões ricas em AU (Figura 1). Outro importante exon para diferenciar as quatro diferentes isoformas de AUF1 é o exon 7, responsável pela localização celular. As isoformas p42 e p45 apresentam esse exon e por isso ficam retidas no núcleo (Arão, Y. *et al* 2000). A combinação entre a capacidade de interagir com motivos ricos em AU e a sua localização celular é determinante no processo de desestabilização de mRNAs. Podemos citar como exemplo, quando há a ausência do exon 2 e a presença do exon 7 temos uma sensível redução na capacidade de interagir com motives ricos em AU. Portanto, AUF1p37 liga com maior afinidade a seqüências ricas em AU do que a isoforma p40. Por outro lado, a presença dos exons 2 e 7 aumenta a afinidade, o que resulta em AUF1p45 ter maior afinidade por seqüências ricas em AU do que a isoforma p40. Interessantemente, a isoforma AUFp40 liga dez vezes mais forte ao sítio de *splicing* do pré-mRNA (UUAG/G) do que a seqüências ricas em AU (Mitchell, P. *et al* 2000 e Wilson, G.M *et al* 1999).

Muitos mRNAs já foram descritos como alvos de AUF1. Dentre eles temos os que codificam: receptores adrenérgicos, receptores do hormônio luteizante, GM-CSF, bcl-2, SERCA2a, histona H4, c-fos, c-jun, c-myc, egr-1, algumas interleucinas, HSP70, catalase, cyclin D1 e cdc25 (Loflin, P *et al* 1999, Buzby, J.S. *et al* 1999, Blum, J.L *et al* 2005, Lapucci, A *et al* 2002, Pende, A. *et al* 1996, Ross, J. *et al* 1986). AUF1 pode interagir com mRNAs que apresentem regiões ricas em AU (Katahira, M., *et al* 2001, Wagner, B.J., *et al* 1998, Wilson, G.M., *et al* 1999 e Bhattacharya, S., *et al* 1999). Sabe-se que AUF1 se liga a repetições do tipo AUUUA, por conseqüência quanto maior o número de repetições maior será a instabilidade do mRNA. Ainda, inserções de guanina ou citocina, no meio dessas repetições podem aumentar drasticamente a estabilidade do mRNA (Katahira, M., *et al* 2001, DeMaria, C.T. *et al* 1996 e Bhattacharya, S., *et al* 1999). Outros trabalhos foram capazes de predizer que a interação entre AUF1 e os motivos ricos em AU ocorre mediante interações do tipo de empilhamento (do inglês *stacking*) (Moraes, K.C.M. *et al* 2003).

AUF1 também participa no processo de transporte de mRNA do núcleo para o citoplasma, através dos complexos de poro. Assim, a hipótese mais aceita é que AUF1 escolhe o mRNA a ser degradado ainda no núcleo, mesmo que a degradação da molécula de mRNA ocorra no citoplasma (Sarkar, B *et al* 2003). Dentre as isoformas de AUF1, apenas as isoformas p37 e p40 apresentam uma seqüência de importação nuclear, localizada no C-terminal das duas isoformas. Similarmente, um sinal de exportação nuclear é encontrado nas duas isoformas de maior peso molecular (p42 e p45).

AUF1, assim como a maioria das hnRNPs é alvo de modificações pós traducionais, incluindo, metilação, assim como hnRNPQ, fosforilação e glicosilação (Blum, J.L *et al* 2005, Wilson, G.M. *et al* 2003, Passos, D., *et al* 2006, Quaresma, A.J.C. *et al* 2006, Soulard, M. *et al* 1993). Essas modificações podem afetar sua afinidade e especificidade pelos motivos ricos em AU, assim como a sua localização celular e interações com outras proteínas (Loflin, P *et al* 1999). A fosforilação, por exemplo, é comum a todas quatro isoformas de AUF1 e pode ser influenciada pelo tratamento com PMA (Hamilton, B.J *et al* 1997, Blum, J.L *et al* 2005, Zhang, W. *et al* 1993, Wilson, G.M. *et al* 2003). A fosforilação da isoforma p40, por exemplo, influencia a sua afinidade por regiões ricas em AU, graças a mudanças conformacionais na interação proteína-RNA. Acredita-se que esse tipo de regulação pode tanto regular interações do tipo AUF1-RNA quanto AUF1-proteínaX (Wilson, G.M. *et al* 2003).

1.4. As diversas funções da proteína hnRNPQ/NSAP1 (“*Non structural associated protein1”/* hnRNP Q)

Outra importante hnRNP Q que apresenta fundamental importância em diversos processos durante o metabolismo de mRNA e hnRNPQ/NSAP1. Estudos utilizando a técnica de duplo híbrido de levedura encontraram a proteína NSAP1 como uma proteína que interage com a proteína NS1 de parvovírus. Além de ser uma proteína multifuncional responsável por vários aspectos da replicação viral, NSAP1 foi - alguns anos depois - re-caracterizada como membro da família das hnRNPs (*“heterogeneous nuclear ribonucleoproteins*”). Apesar de possuir 80% de identidade com a hnRNP R (Harris, C.E. *et al* 1999), classifica-se hoje esta proteína como uma das três “*splice variants”* da nova família de hnRNPs, denominada hnRNPQ (Figura 2).

Figura 2. Representação esquemática da proteína humana hnRNPQ/NSAP1.

Estruturalmente NSAP1 apresenta-se como uma proteína modular, apresentando um domínio rico em aminoácidos ácidos na região N-terminal, seguido por três domínios RRMs (“*mRNA recognition motifs”*). Já na região C-terminal temos um domínio RGG box (Mourelatos, Z. *et al* 2001). Esta proteína está envolvida na regulação do “*splicing”* de alguns mRNAs e no transporte dessas moléculas (Harris, C.E. *et al* 1999) . Ainda, alguns trabalhos conseguiram demonstrar que hnRNPQ interage com o domínio phosphoCTD (*“hyperphosphorylated C-terminal repeat domain*”) da RNA

polimerase II (Carty SM et al 2002), o que demonstra a sua importância na regulação da transcrição de diversos genes.

Através da técnica do duplo híbrido, vimos que hnRNPQ interage com AUF1 e mostramos que há uma seletividade dos domínios RRMs, de hnRNPQ, envolvidos na interação. A construção com o domínio ácido-RRM1 não interage com AUF1, entretanto as duplas dos domínios RRMs 1-2 e 2-3 interagem. Isso demonstra a seletividade na interação entre os domínios das proteínas e que esses domínios parecem atuar em cooperação não somente em interações do tipo proteína-proteína quanto as do tipo proteína-RNA (Moraes, K.C.M. *et al* 2003).

Além disso, a proteína humana hnRNPQ interage com o complexo de edição do mRNA da proteína ApoB (Blanc, V. *et al* 2001). A edição do mRNA de ApoB ocorre mediante a formação do complexo (editiossomo), formado pelas proteínas Apobec1 (enzima – citidina deaminase), ACF1 (*Apobec1 complementation factor1*) e hnRNPQ. Apobec1 e ACF1 são necessários e suficientes para que ocorra a edição do mRNA de ApoB. Entretanto, células transfectadas para superexpressara proteína hnRNPQ apresentaram inibição da edição do mRNA de ApoB (Blanc, V. *et al* 2001, Powell, L.M. *et al* 1987, Shah, R.R. *et al* 1991). Isso pode indicar que NSAP1, além de ligado ao editiossomo, pode funcionar como um regulador da reação de edição do mRNA. Nossos experimentos recentes *in vitro* mostraram que o domínio N-terminal (AcD) de hnRNPQ é capaz de interagir com Apobec1 e que a fosforilação de AcD inibe esta interação (Quaresma A.J.C. *et al* 2006, Artigo 3, p. 20).

A proteína hnRNP Q pode também interagir com polissomos (Hresko, R.C. *et al* 2002) e evita a deadenilação de mRNAs instáveis (Chen, C.Y. et al 2003, Grosset, C. *et al* 2000 e Kim, T.D. et al 2005). Ainda, tanto hnRNP Q quanto hnRNP R são componentes de grânulos citoplasmáticos, responsáveis pelo transporte de determinados mRNAs ao longo dos dendritos de células nervosas (Bannai, H. *et al* 2004 e Rossoll, W.S. *et al* 2003). Estudos recentes demonstraram que essa proteína está intimamente envolvida na síntese do RNA viral, responsável pela hepatite C em ratos (Choi, K. S. *et al* 2004). Essa diversidade de processos em que hnRNP Q participa, reflete a sua plasticidade e capacidade de interagir com diferentes parceiros.

1.5. Principais funções da proteína Bip/HSP70

Muitos dos conhecimentos adquiridos sobre o controle da tradução, induzida por determinado estresse, surgiram com o estudo sobre estresse induzido por calor (Duncan, R. F. 1996). Os dois principais eventos envolvidos no controle da tradução sob estresse térmico são: primeiro, repressão da tradução devido a fosforilação em alguns fatores de iniciação da tradução em eucariotos (Rhoads, R. E. *et al* 1995) e devido ao seqüestro de muitos fatores, necessários à tradução, em

grânulos de estresse (Kedersha, N. *et al* 2002). Segundo, manutenção (ou até aumento) da síntese de importantes proteínas necessárias para a sobrevivência celular sob condições extremas (estresse), incluindo "*heat shock proteins*" (HSPs) como BiP.

A proteína BiP foi identificada como uma proteína ligadora à cadeia pesada de algumas imunoglobulinas (Bole, D. G. *et al* 1986 e Haas, I.G. *et al* 1983) e que também ligava transientemente a muitas proteínas de membrana e da via secretora recém traduzidas e permanentemente a proteínas mal enoveladas ou desenoveladas que se acumulavam no interior do retículo endoplasmático (ER). Muitas funções foram propostas para BiP, dentre elas estão: auxílio no correto enovelamento de proteínas recém traduzidas e a retirada de proteínas desenoveladas do interior do ER (Pelham, H. R. *et al* 1986 e Munro, S. *et al* 1986). BiP é também conhecida como "*glucose-regulated protein 78*" (GRP78) e é membro da família das HSP70 (Lee, A.S. et al 1984). A expressão de BiP é regulada ao nível da transcrição e sua síntese pode ser induzida por muitos tipos de estresse, bem como falta de glicose, tratamento com ligadores de cálcio, como EGTA (Welch, W. J. Et al 1983, Wu, F.S. et al 1981, Olden, K.R. et al 1979) e tunicamicina ou glicosamina (ambos bloqueiam a glicosilação celular) (Stoeckle, M. Y. et al 1988). A transcrição do mRNA de BiP também é induzida por infecção viral como paramixovirus (Sarnow, P. et al 1989). Além disso, muitos trabalhos sugerem que a expressão de BiP é também regulada ao nível da tradução (Ulatowski, L. M. Et al 1993 e Yang, Q. Et al 1997). A tradução do mRNA de BiP é aumentada em células infectadas com poliovírus onde a tradução dos outros mRNAs da célula hospedeira é inibida (30). A região 5´-UTR do mRNA de BiP contem um elemento IRES ("*internal ribosome entry site*") que é alvo de regulação da proteína PTB ("*polypyrimidine tract-binding protein*") (Kim, Y. *et al* 2001). Interessantemente, hnRNP Q também é capaz de ligar à região IRES do mRNA de BiP e de aumentar a expressão de BiP. Estudos recentes também sugerem que, sob condições de estresse térmico, há um aumento da ligação de hnRNP Q na região IRES do mRNA de BiP. Além disso, o "*knockout*" de hnRNP Q diminui drasticamente a ativação de IRES e a expressão do mRNA de BiP, o que sugere que hnRNPQ apresenta um papel importante na modulação da tradução do mRNA de BiP (Cho, S. *et al* 2007).

1.6. Funções dos Grânulos de Estresse

Células eucarióticas respondem a diferentes condições de estresse, encontradas no ambiente, tais como estresse oxidativo, choque térmico, UV, estresse do retículo (ER) e algumas infecções virais. Essas condições alteram a maquinaria da tradução em eucariotos (Kedersha, N.L. et al 2002). A expressão de proteínas responsáveis por reparar o dano causado por condições de estresse cresce, enquanto a tradução de proteínas constitutivas é interrompida pelo re-direcionamento desses mRNAs dos polissomos para discretos pontos citoplasmáticos, conhecidos

como grânulos de estresse (**SG**) para transiente estocagem (Kedersha, N.L. et al 1999). Muitas proteínas celulares, incluindo "*T cell intracellular antigen-1*" (TIA-1), "*TIA-1 related protein*" (TIAR) e "*Ras-Gap-SH3 domain biding protein*" (G3BP) estão envolvidas na montagem dos SG (Tourriere, H. *et al* 2003).

Para que haja a tradução, é necessário a fosforilação da subunidade α do fator de iniciação da tradução em eucariotos (eIF2α). A subunidade α é alvo de quinases da família das serina/treonina, como: PKR, PERK-PEK, GCN2, HRI, dentre outras. Cada uma dessas quinases fosforilam eIF2α mediada pela ativação de diferentes vias de estresse. PKR é um sensor de temperatura e fosforila eIF2α caso haja algum choque térmico, bem como danos causados por UV, infecção viral e estresse oxidativo (Williams, B.R. 2001). Já PERK-PEK detecta localmente estresses que ocorram no retículo endoplasmático (ER) (Harding, H.P. *et al* 2000). GCN2 fosforila eIF2α quando há falta de aminoácidos no meio (Kimball, S.R. 2001) e HRI monitora mudanças na viabilidade do grupamento heme durante a diferenciação dos eritrócitos (Han, A.P. *et al* 2001 e Lu, L. *et al* 2001). A fosforilação de eIF2α em eucariotos impede a montagem do complexo ternário eIF2-GTP-tRNA^{met} e inibe o início da tradução e a montagem dos polissomos (Anderson, P. et al 2006). Estudos com quinases modificadas (inativas) e mutantes de eIF2α (não-fosforiláveis) mostram inibição da apoptose e indução da transformação celular. Isso confirma que a via PKR/PERK/GNC2/HRI-eIF2 apresenta um papel crítico na regulação tanto da tradução quanto da sobrevivência celular (Barber, G.N. et al 2001). TIA-1 e TIAR ligam-se a esse complexo ternário inativo bem como a mRNA poli(A)+, agregando-se e promovendo a montagem dos SG (Kim, J.H *et al* 2000).

1.7. Funções dos corpos de processamento ("*P-Bodies*")

"*Processing bodies*" (P-bodies) representam um outro tipo de pontos citoplasmáticos, identificados em células eucarióticas (Eulalio, A. et al 2007). Os P-bodies apresentam componentes da via de degradação de mRNAs, bem como silenciamento de miRNA dependente da proteína GW182 (Eystathioy, T. et al 2002). P-bodies não necessitam da fosforilação de eIF2α para sua formação (Kedersha, N.L. et al 2005). Embora SG e P-bodies apresentem diferenças de tamanho e forma, bem como em seus mecanismos de formação, esses dois tipos de pontos citoplasmáticos são encontrados juntos em células de mamíferos com aparente interação física e funcional. Além disso, já foi demonstrado que P-bodies apresentam maior mobilidade do que SG. Curiosamente, acredita-se que os SG podem enviar para os P-bodies, determinados mRNAs para que sejam degradados (Beck, A.R. et al 1996).

TIA-1 e TIAR são duas proteínas multifuncionais que migração núcleo-citoplasma (”*shuttling”*). Ambas são expressas em diversos tecidos (Jin, K. *et al* 2000 e Anderson, P. 1995) e apresentam 3 domínios RRMs (N-terminal) e uma região rica em glutamina, no C-terminal, denominada “*prion-related domain*” (PRD) (Gilks, N., et al 2004), que demonstra ter semelhança estrutural e funcional com o domínio de agregação, presente em prions de mamíferos e células de levedura (Anderson, P. 1995). Um recombinante clone sem os e RRMs foi incapaz de ligar à mRNAs poli(A)+ e recrutá-lo ao SG (Kedersha, N.L. et al 1999). A deleção do domínio PRD inibiu a sua agregação e a montagem do SG. A agregação de PRD é regulada pela chaperone “*Heat-shock protein 70*” (HSP70) e a superexpressão de HSP70 impede a agregação do domínio PRD (Gilks, N., et al 2004). Além disso, TIA-1 e TIAR regulam a tradução de alguns mRNAs, ricos em AU na região 5´-UTR. Elas impedem a tradução desses mRNAs ligando-se à região rica em AU (Anderson, P. et al 2002).

2. Objetivos

O presente trabalho teve como objetivo geral o estudo detalhado da função e aspectos estruturais da proteína humana hnRNP Q/NSAP1.

Objetivos específicos:

- identificar proteínas que interagem com AUF1

- Caracterizar a interação entre hnRNPQ e PRMT1, analisando a importância da metilação de hnRNPQ para sua localização celular.

- Caracterizar a interação do domínio AcD de hnRNPQ com Apobec1 e obter informações estruturais sobre AcD através de estudos espectroscópicos, de modelagem molecular e dinâmica molecular

- Compreender a importância funcional da fosforilação do domínio AcD para a interação com Apobec1.

- Estudar a fosforilação de hnRNPQ e as implicações funcionais dessa modificação, com ênfase na localização celular de hnRNPQ.

3. Resultados

Artigo I

Identification and Characterization of Proteins That Selectively Interact with Isoforms of the mRNA Binding Protein AUF1 (hnRNP D)

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Identification and Characterization of Proteins That Selectively Interact with Isoforms of the mRNA Binding Protein AUF1 (hnRNP D)

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The mRNAs that encode certain cytokines and proto-oncogenes frequently contain a typical AU-rich motif that is located in their 3'-untranslated region. The protein AUF1 is the first factor identified that binds to AU-rich regions and mediates the fast degradation of the target mRNAs. AUF1 exists as four different isoforms (p37, p40, p42 and p45) that are generated by alternative splicing. The fact that AUF1 does not degrade mRNA itself had led to the suggestion that other AUF1 interacting proteins might be involved in the process of selective mRNA degradation. Here we used the yeast two-hybrid system in order to identify proteins that bind to AUF1. We detected AUF1 itself, as well as the ubiquitin-conjugating enzyme E2I and three RNA binding proteins: NSEP-1, NSAP-1 and IMP-2, as AUF1 interacting proteins. We confirmed all interactions *in vitro* and mapped the protein domains that are involved in the interaction with AUF1. Gel-shift assays with the recombinant purified proteins suggest that the interacting proteins and AUF1 can bind simultaneously to an AU-rich RNA oligonucleotide. Most interestingly, the AUF1 interacting protein NSEP-1 showed an endoribonuclease activity *in vitro*. These data suggest the possibility that the identified AUF1 interacting proteins might be involved in the regulation of mRNA stability mediated by AUF1.

Key words: Domain mapping / mRNA degradation / Protein-protein interactions / Ribonuclease / RNA binding domains / RNA binding proteins.

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Introduction

The cytoplasmic concentration of mRNA is a critical factor for the final outcome of gene expression, and the mRNA degradation rate critically influences the expression of many gene products. mRNA decay in eukaryotes is defined either by constitutive mRNA decay rates or in response to changes in the cellular environment (Bernstein *et al.*, 1989, 1992; Ross, 1995).

Rapidly degraded mRNAs, such as those encoding oncoproteins, cytokines or inflammatory mediators, frequently contain one or more AU-rich elements (ARE) based on the motif AUUUA in their 3´-untranslated regions (3´-UTR). AU-rich elements mediate the deadenylation and subsequent cleavage of the mRNA chain. Several groups set out to clone and characterize specific proteins that bind to these critical AU-rich regions. The proteins that were discovered include: HuR (Fan and Steitz, 1998), Elav (Robinow *et al.*, 1988), AU-A (Katz *et al.*, 1994), AU-B (Bohjanen *et al.*, 1992), AUH (Nakagawa *et al.*, 1995), hnRNP A1 and C (Hamilton *et al.*, 1993) and AUF1 (Zhang *et al.*, 1993). All these proteins are part of a cellular degrading machinery, which is responsive to AU-rich elements (Holtmann *et al.*, 1999; Winzen *et al.*, 1999). Some of these proteins (*e.g.* HuR) stabilize AU-rich elements containing mRNAs, while others are involved with the mRNA degradation process. The protein AUF1 has been reported to be associated with an accelerated degradation of mRNAs (Bristow *et al.*, 1993; Buzby *et al.*, 1996; DeMaria and Brewer, 1996). The mechanisms that link the association of proteins like AUF1 with the process of accelerated decay are still unknown. However, a few reports also contribute a stabilizing effect to AUF1 (Kiledjian *et al.*, 1997; Xu *et al.*, 2001).

AUF1 is the best studied ARE-binding protein and was discovered by an *in vitro* mRNA decay system designed to identify factors that are involved in the degradation of the *c-myc* mRNA (Brewer, 1991). *c-myc* is involved in the control of cell growth, division, differentiation, and transformation, and its expression rate is tightly controlled at all levels of regulation. The loss of the degradation of the *c-myc* mRNA mediated by AU-rich elements has been shown to be associated with transforming phenotypes (Lee *et al.*, 1988).

Since its initial discovery several lines of evidence have demonstrated that the binding of AUF1 to the AREs of an mRNA is associated with its accelerated turnover. First, polysomal *c-myc* mRNA can be destabilized *in vitro* by

the addition of AUF1 protein (Brewer, 1991, 1999, 2000). Second, AUF1 not only binds directly to AREs, but the relative binding affinity of AUF1 to different AREs also correlates with the potential of these AREs to destabilize the mRNA (DeMaria and Brewer, 1996, 1997). Third, an increase in AUF1 protein levels is followed by decreased levels of β_1-adrenergic receptor mRNA (Bristow *et al.*, 1993; Pende *et al.*, 1996). Finally, the mRNA turnover mechanism is compromised in cells that express low levels of endogenous AUF1 (Buzby *et al.*, 1996; Ross, 1995).

The AUF1 gene encodes four protein isoforms that arise by alternative splicing of the AUF1 pre-mRNA (Wagner *et al.*, 1998). According to their corresponding molecular weights the four isoforms have been denoted as p37, p40, p42 and p45. The four isoforms differ in the presence or absence of two regions, 'a' and 'b', that flank the two central RNA recognition motives (RRMs, Figure 1A). The specific role of the four different AUF1 isoforms in the regulation of the mRNA turnover of cytokine or proto-oncogene mRNAs has not been determined yet. It seems likely that the different AUF1 isoforms have distinct mRNA targets or that they might link the mRNAs that they recognize to different degradation pathways. We speculated that the unique and isoform-defining N- and C-terminal domains of AUF1 might be regions involved in the interactions with proteins that are associated with the AU-rich mRNA degradation mechanism.

Here we used AUF1p37 as 'bait' in a yeast two-hybrid screen to identify interacting protein partners. We identified AUF1 itself and four other proteins that interacted with AUF1: NSAP-1, NSEP-1, IMP-2 and UBCE2I. We demonstrate here not only that all four proteins interact specifically *in vivo* and *in vitro* with AUF1, but also that they bind to an AU-rich RNA oligonucleotide. This suggests that these proteins could assemble into larger AUF1-containing protein complexes on the AU-rich RNA target. Furthermore, we found that the AUF1-interacting protein, NSEP-1, has endoribonuclease activity *in vitro*. In summary, our data suggest the possibility that the identified AUF1 interacting proteins might be involved in the mechanism of mRNA destabilization mediated by AUF1.

Results

Identification of Proteins That Interact with AUF1

It has been suggested that AUF1 is a protein that initiates mRNA degradation and that the four protein isoforms of AUF1 might bind distinct mRNA targets or mediate different mRNA degradation pathways. AUF1 might therefore interact with other proteins that determine the route of mRNA degradation. In order to identify proteins that interact with AUF1, we employed the yeast two-hybrid system (Hollenberg *et al.*, 1995; Vojtek and Hollenberg, 1995) and screened a human fetal brain cDNA library. A total of ~2×10^6 transformants were plated and 380 clones grew out on plates with minimal medium without histidine. About half of these colonies showed a strong blue color in the ß-galactosidase filter assay. Library plasmids were sequenced by automated sequencing. The sequence analysis revealed that 3 clones contain in-frame the cDNA encoding the mRNA binding protein NSAP-1 (Table 1), 3 clones have the NSEP-1 insert, 3 clones the AUF1 cDNA, 2 clones contain the cDNA of the ubiquitin-conjugating enzyme E2I (UBCE2I), and 1 clone contains the cDNA of IMP-2. Table 1 summarizes the domain organization and functional characteristics of the proteins found to interact with AUF1. The other sequenced clones represented either molecules not cloned in frame with the Gal4AD into the cloning site of pACT2 or will be described and characterized elsewhere.

Differential Interaction of AUF1 Isoforms with AUF1p37, NSAP-1, NSEP-1 and IMP-2

We found several AUF1 interacting clones whose plasmids contained inserts encoding AUF1 itself. This is in accord with the described fact that AUF1 can form dimers or even higher order oligomers (Wilson *et al.*, 1999). We were interested in exploring the yeast two-hybrid system to analyze whether the AUF1-p37 can interact with all of the four isoforms (Figure 1A and B). Our analysis revealed that AUF1p37 only interacts with the AUF1 isoforms p37 and p40 but not with p42 or p45 (Figure 1B). This result suggests that the presence of the inserted region 'b' (Figure 1A) in the AUF1 isoforms p42 and p45 prevents the dimerization. Maybe this region 'b', which consists of 49 amino acids, is located in a region of AUF1 that is important for the dimerization.

In a similar approach we tested whether the proteins identified to interact with AUF1 could interact with all of the four AUF1 protein isoforms or if they interact in an isoform-specific manner with AUF1. Co-transformation experiments of the yeast strain L40 with pBTM116-AUF1-p37, -p40, -p42 and -p45 and pACT2-NSEP-1, pACT2-NSAP-1 and pACT2-IMP-2 (Figure 1C) demonstrated that the three RNA binding proteins NSEP-1, NSAP-1 and IMP-2 all interact only with AUF1p37 and p40 but not with p42 or p45. In contrast, protein UBCE2I interacted with all four isoforms.

In vitro Analysis of the Interaction between AUF1 and Its Interacting Proteins

Next we performed *in vitro* pull-down assays to verify the observed interactions between AUF1 and the proteins NSAP-1, NSEP-2, IMP-2 and UBCE2I. To this end the interacting proteins were expressed in *E. coli* either as GST fusion proteins (NSEP-1, IMP-2, UBCE2I, GST as control) or as 6xHis fusion proteins [AUF1p37, NSAP-1, RACK (receptor of activated kinase C) as control] (Croze *et al.*, 2000). The purified proteins were immobilized on appropriate resins and then incubated with the purified 6xHis-AUF1p37 (or unfused AUF1p37 in the case of NSAP-1) fusion protein. After washing, the bound 6xHis-AUF1p37

Table 1 Characteristics of the AUF1-p37 Interacting Proteins as Identified by the Yeast Two-Hybrid System.

Protein interacting with AUF1	Insert length (bp)	Protein residues	Domain composition (native protein)	Function	References
AUF1 (hnRNP-D0)	1300	1 – 300	2 RRM domains	selective mRNA degradation	(Zhang *et al.*, 1993)
NSAP-1	1600	1 – 370	4 RRM domains	regulation of mRNA splicing and transport	(Harris *et al.*, 1999b)
NSEP-1	1500	80 – 323	C domain[a], 4 'basic/aromatic' – islands	mRNA binding (transcription and translation regulation)	(Murray *et al.*, 1992) (Kolluri and Kinniburgh, 1991)
IMP-2	3500	194 – 557	2 RRM[b], 4 KH domains	mRNA binding, translation repression	(Zhang *et al.*, 1999; Nielsen *et al.*, 1999)
Ubiquitin-conjugating enzyme UBCE2I	1200	9 – 159	–	protein degradation	(Wang *et al.*, 1996)

[a]The NSEP-1 clone found to interact with AUF1 is missing approximately the N-terminal half of the C-domain (residues 1 – 79).
[b]The IMP-2 clone found to interact with AUF1 does not contain the two N-terminal RRMs (residues 1 – 193).

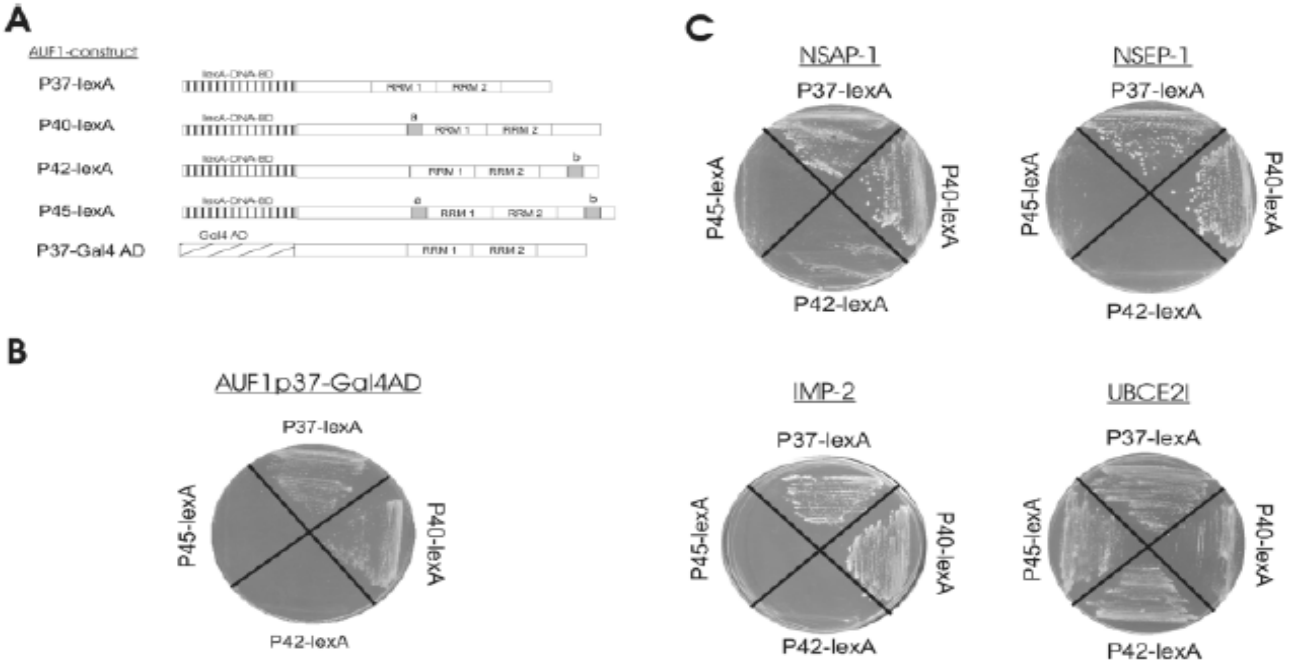

Fig. 1 Differential Interactions of the Four AUF1 Isoforms with the Proteins AUF1p37, NSAP-1, NSEP-1, IMP-2 and UBCE2I. (A) Schematic representation of human AUF1 isoforms fused either in frame to the activating domain of Gal4 in plasmid pACT2 ('prey'; p37) or fused to the lexA DNA-binding domain in plasmid pBTM116 ('bait'; p37, p40, p42, p45). (B) Interaction between AUF1 isoforms. Bait and prey AUF1-plasmids were co-transformed into yeast strain L40 and protein-protein interactions were evaluated by the ability of the cells to grow on minimal medium (MM) lacking H. (C) Test of interaction of the four AUF1 isoforms with the identified AUF1p37 interacting proteins. The identified AUF1 interacting proteins NSEP-1, NSAP-1, IMP-2 and UBCE2I are fused in frame to the Gal4 DNA-binding domain in plasmid pACT2 ('prey'-vector). AUF1 isoform bait plasmids and prey plasmids were co-transformed into yeast strain L40 and protein-protein interactions were evaluated by the ability of the co-transformed cells to grow on MM agar plates that are selective for the interaction (-W, -L, -H). The presence of both plasmids in the L40 cells was verified by growth on plates that contain H but lack W and L (not shown).

protein was detected by Western blotting using an anti-AUF1 antiserum (Figure 2). All four proteins interacted *in vitro* with AUF1p37. The interactions were specific since the control proteins GST and 6xHis-RACK did not interact with AUF1p37 *in vitro*. The immunodetection of 6xHis-AUF1p37 by the anti-AUF1 antiserum was specif-

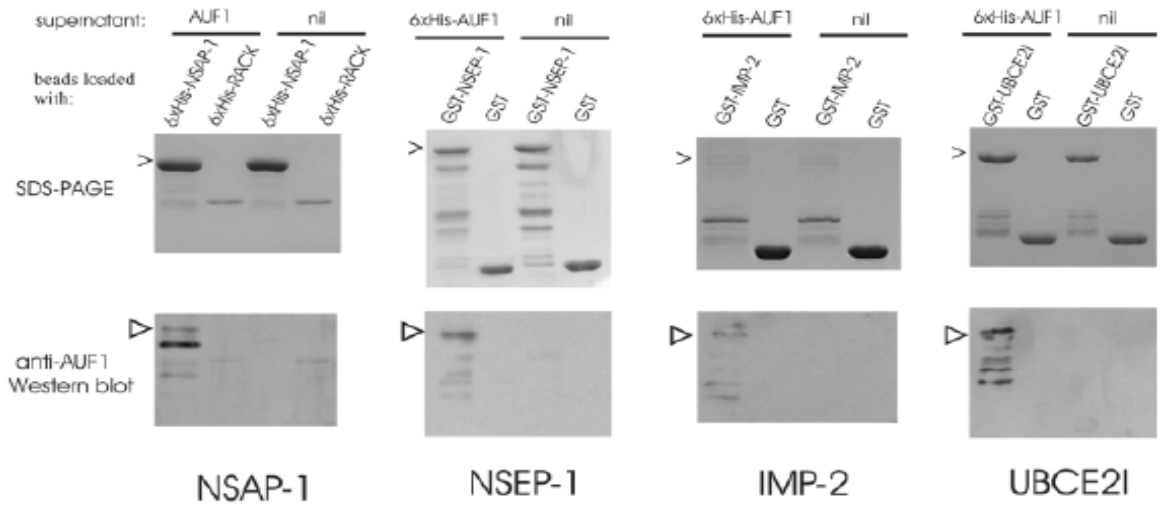

Fig. 2 *In vitro* Pull-Down Assay.
Proteins were expressed as GST or 6xHis fusions in *E. coli* and purified. GST fusion proteins were bound to glutathione Sepharose (NSEP-1, IMP-2, UBEC2I) and 6xHis-NSAP1 and 6xHis-RACK (control) to Ni-NTA-Sepharose. After washing, samples were or were not incubated with 6xHis-AUF1, or unfused AUF1 in the case of the 6xHis-NSAP-1. Free GST and 6xHis-RACK, respectively, served as controls. After washing, resin-bound proteins were resolved on an SDS/10% polyacrylamide gel and subsequently transferred to a PVDF membrane. Western blotting was performed with anti-AUF1 serum. The corresponding SDS gels in the upper panels demonstrate comparable protein loading of the lanes.

ic, since no protein bands were detected on the Western blot when the incubation step with the 6xHis-AUF1 protein was omitted (Figure 2, nil lanes).

Mapping of Protein Domains Involved in AUF1 Binding

The three RNA-binding proteins that were identified to interact with AUF1 *in vivo* and *in vitro* are multidomain proteins (Table 1). NSAP-1 has about ~80% amino acid sequence identity with the protein hnRNP R (Hassfeld *et al.*, 1998; Harris *et al.*,1999) and contains four RNA recognition motifs (RRMs). We wanted to determine which of these four RRMs are involved in the interaction with AUF1 and prepared a series of cDNA constructs encoding different domains of the NSAP-1 protein (Figure 3, Table 2). The deletions were generated using the vector pACT2, and the yeast two-hybrid method was used to identify the RRM domains that are required for the binding to AUF1. Our results show that the construct that encompasses the RRM domains 3 and 4, as well as that which contains domains 2 and 3, both interact with AUF1p37, whereas a construct containing domains 1 and 2 did not interact. The constructs that only contain the individual domains 2 or 3, however, failed to interact with AUF1. These results show that at least two domains of NSAP1 are required for the interaction with AUF1p37 and that domain 3 alone is not sufficient for the interaction, although it is present in the two interacting constructs NSAP 2–3 and NSAP 3–4. Interestingly, it had been reported previously that the two proteins AUF1 (=hnRNP D) and NSAP-1 are also functionally coupled *in vivo* (Grosset *et al.*, 2000). These authors demonstrated that AUF1 and NSAP-1 are part of a multiprotein complex that is associated with the major coding region determinant (mCRD) of instability of the *c-fos* mRNA.

NSEP-1 is another RNA binding protein and was described in *Xenopus* (Murray *et al.*, 1992) and humans (Kolluri and Kinniburgh, 1991). It contains at its N-terminus a C domain ('cold-shock domain') homologous to the major *E. coli* cold stress-response protein. The C-terminus of NSEP-1 contains four arginine-rich 'basic/aromatic islands' (B/A islands) that are similar to the RNA-binding domains found for example in the Tat protein of HIV (Murray *et al.*, 1992). All of the NSEP-1 clones that we identified to interact with AUF1 in our yeast two-hybrid screen lack the N-terminal ~60% of the C domain. This indicates that this region of the NSEP-1 protein is not required for the interaction with AUF1. We generated a series of cDNA constructs containing different fragments of the NSEP-1 protein (Figure 3). However, none of the three truncated fragments of NSEP-1, which contain two adjacent B/A islands, interacted with AUF1p37. These results suggest that more than two of the B/A islands are required for the interaction with AUF1. Two constructs that encompass three B/A islands (1-2-3 and 2-3-4) were both able to interact with AUF1p37.

IMP-2 is the third RNA binding protein we found to interact with AUF1. IMP-2 is a protein that has been described to bind to the 5′-UTR of the mRNA of insulin-like growth factor II (IGF II; Nielsen *et al.*, 1999). IMP-2 contains two RRM domains at its N-terminus and four KH domains (hn RNP K homology domains) further C-terminally. The IMP-2 clone we found to interact with AUF1

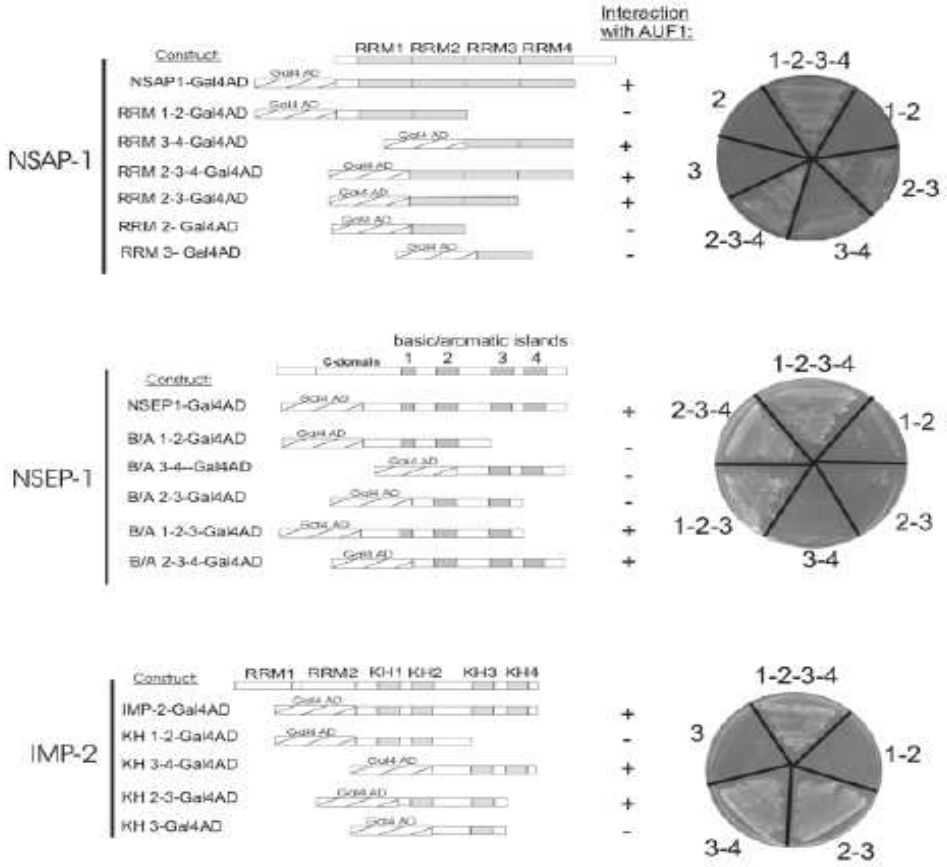

Fig. 3 Mapping of Domains of the RNA Binding Proteins Involved in AUF1 Binding.
AUF1p37 cDNA fused in frame to the lexA DNA-binding domain in plasmid pBTM116 ('bait', see Figure 1A) and the indicated deletion mutants of the AUF1 interacting proteins NSAP-1, NSEP-1 and IMP-2 were fused in frame to the Gal4 DNA-activating domain in plasmid pACT2 ('prey'). Bait and prey plasmids were co-transformed into yeast strain L40 and protein-protein interactions were evaluated by the ability of the co-transformed cells to grow on plates containing minimal medium selective for interaction (MM -W, -L, -H). The presence of both plasmids in the cells was verified by growth on plates that contain H but lack W and L (not shown). The constructs that have been evaluated with 'no growth' (interaction with AUF1: '–') showed no signs of growth.

lacks the two N-terminal RRM domains but contains the four KH domains. This suggests that the KH domains are involved in the interaction with AUF1. We generated a series of truncation constructs for IMP-2 (Figure 3) and observed that the two constructs containing the KH domains 2 and 3 or 3 and 4 were able to interact with AUF1. However, a construct that consists only of domain 3 (present in constructs 2–3 and 3–4) failed to interact with AUF1p37. This suggests that at least two KH domains are necessary for interaction with the two RRM domains of AUF1. The construct with KH domains 1 and 2 does not interact with AUF1.

Interactions of AUF1 and Its Interacting Proteins with an AU-Rich RNA Oligonucleotide

Next we tested whether the proteins we found to interact with AUF1 could bind to an AU-rich target RNA. We chose the well-studied AU-rich region (ARE) of the 3′-UTR from the TNFα mRNA (38-mer) and performed gel mobility shift assays (Figure 4) with decreasing protein concentrations. We observed that like AUF1 (Figure 4A), the proteins NSAP-1, NSEP-1, IMP-2 and UBCE2I all bound to the ARE oligonucleotide (Figure 4B-E). According to Carey (1991) the binding affinity of the proteins to the target RNA can be estimated from the protein concentration that is required to bind half of the nucleic acid in the gel-shift assay if the nucleic acid concentration is negligible in comparison to the protein concentration. Using this method we were able to compare the relative affinities of the five analyzed mRNA binding proteins. We determined that the relative affinity of the proteins to the AU-rich RNA oligonucleotide increases in the following order: GST-UBCE2I (~7400 nM protein was required to shift about half of the oligonucleotide) < GST-NSAP1 (~500 nM) < 6xHis-AUF-1 (~312 nM) ≅ GST-NSEP-1 (~250 nM) << GST-IMP-2 (2.7 nM). Free GST protein did not cause any detectable shift of the RNA oligonucleotide

Table 2 PCR Primer Sets Used to Generate NSAP-1, NSEP-1 and IMP-2 Truncations.

Protein, domain range (amino acid range)	PCR primer sets
NSAP-1, 2–3–4 (121–398)	T AGA ATT CAG GCA AAA ATT AAG GCA CTC TT
	TGT CTC GAG CCT TTT CTG ATC TGG TGG CTT GGC
NSAP-1, 1–2 (1–233)	TGA ATT CAG AAG AAT AAC CCC AGG AAG TAC
	GTT CTC GAG ATG TTT TCC AGA ACG AAT TTC ATG
NSAP-1, 2–3 (121–324)	T AGA ATT CAG GCA AAA ATT AAG GCA CTC TT
	GTT CTC GAG ATC AGC CCA TTC AAC AGT TCC
NSAP-1, 3–4 (216–398)	T AGA ATT CCA GCT CAG GAG GCT GTT AAA C
	TGT CTC GAG CCT TTT CTG ATC TGG TGG CTT GGC
NSAP-1, 2 (121–233)	T AGA ATT CAG GCA AAA ATT AAG GCA CTC TT
	GTT CTC GAG ATG TTT TCC AGA ACG AAT TTC ATG
NSAP-1, 3 (216–324)	T AGA ATT CCA GCT CAG GAG GCT GTT AAA C
	GTT CTC GAG ATC AGC CCA TTC AAC AGT TCC
NSEP-1, 1–2 (92–237)	TGA ATT CAG AAG AAT AAC CCC AGG AAG TAC
	TGT CTC GAG CCC GAT ACA TAT CTGCCT C
NSEP-1, 3–4 (208–322)	T AGG ATC CCC AAC CCT CCT GTG CAG GG
	GGT CTC GAG CTC AGC CCC GCC CTG C
NSEP-1, 2–3 (153–274)	A TGA AAT CGT CCT CCA CGC AAT TAC CAG
	GGT CTC GAG TGG CTG CTG ACC TTG GGT C
NSEP-1, 2–4 (153–322)	A TGA AAT CGT CCT CCA CGC AAT TAC CAG
	GGT CTC GAG CTC AGC CCC GCC CTG C
NSEP-1, 1–3 (92–274)	TGA ATT CAG AAG AAT AAC CCC AGG AAG TAC
	GGT CTC GAG TGG CTG CTG ACC TTG GGT C
IMP-2, 1–2 (216–386)	T AGA ATT CCC ATA AAG AAC ATC ACT AAG CA
	GCT CTC GAG AAT CTC CTG CTG CTC TGG ATA AG
IMP-2, 3–4 (331–556)	T AGA ATT CAG GTT GAG GCC TGT GCC AG
	CCT CTC GAG CTT GCT GCG CTG TGA GGC
IMP-2, 2–3 (248–467)	T AGA ATT CCC CCA GAG GGG ACT TCT G
	GCT CTC GAG CAG CTT CAC TTC TTC TTT GG
IMP-2, 2 (248–386)	T AGA ATT CCC CCA GAG GGG ACT TCT G
	GCT CTC GAG AAT CTC CTG CTG CTC TGG ATA AG
IMP-2, 3 (331–467)	T AGA ATT CAG GTT GAG GCC TGT GCC AG
	GCT CTC GAG CAG CTT CAC TTC TTC TTT GG

(data not shown). Our estimation of the relative affinity of the protein NSEP-1 is limited by the fact that we observed a rapid degradation of the RNA oligonucleotide due to the ribonuclease activity of NSEP-1 (see below). These results demonstrate that AUF1, NSAP-1 and NSEP-1 have affinities to the AU-rich oligonucleotide that lie in the same range of magnitude. In contrast, the affinity of GST-UBCE2I is more than about one order of magnitude lower, and the affinity of GST-IMP-2 is about two orders of magnitude higher, than that of AUF1, NSAP1 and NSEP1. It must be pointed out that the comparison of these relative affinities is limited by the fact that the proteins are composed by different types and numbers of RNA binding domains (Table 1, Figures 1 and 4).

We also performed the gel-shift assays of the AUF1 binding proteins in the presence of a constant amount of the protein AUF1. We observed that the simultaneous incubation of the interacting proteins with AUF1 caused an intensified signal of the shifted oligonucleotide band at the lower protein concentrations analyzed (Figure 4 B-E). This suggests that AUF1 bound simultaneously to the 38-mer RNA oligonucleotide at the lower NSAP-1, NSEP-1, IMP-2 and UBCE2I concentrations, thereby causing an increase in the amount of shifted oligonucleotide. However, we did not detect qualitative differences (*e.g.* supershifts) when comparing the shifts caused by the interacting proteins alone or those that occur in the presence of AUF1.

When we tested whether the protein NSEP-1 could interact with the AU-rich RNA oligonucleotide (Figure 4C), we observed that the labeled oligonucleotide disappeared and gave rise to a fraction of fast-migrating degradation products that appeared at the bottom of the gel. This suggested that the purified recombinant AUF1-interacting protein NSEP-1 has an RNA degrading activity. We tested a series of other nucleic acid substrates and found that NSEP-1 reproducibly degrades 25-mer poly-A, poly-C and poly-U RNA oligonucleotides (data not

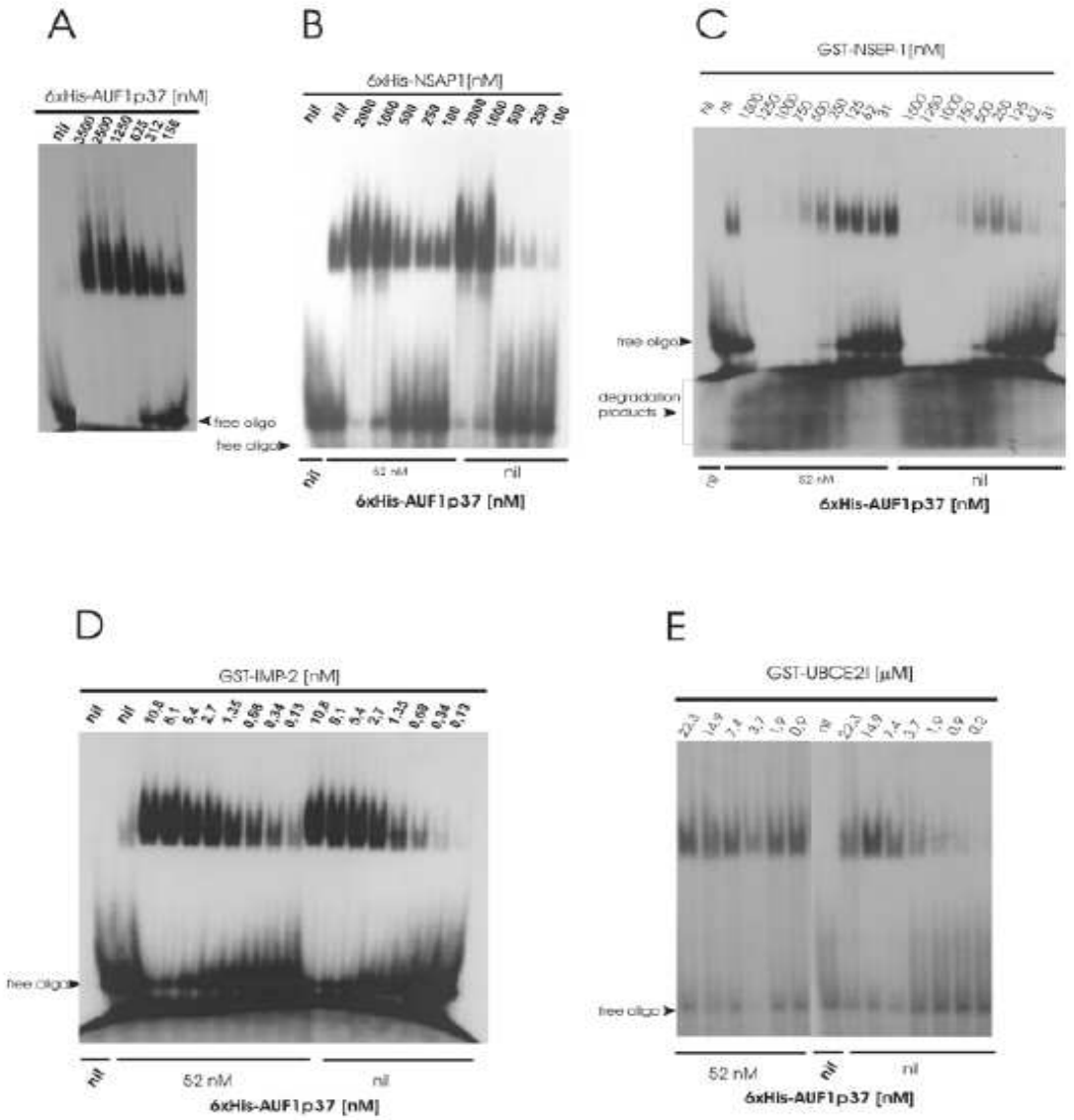

Fig. 4 Binding of the Proteins AUF1, NSAP-1, NSEP-1, IMP-2 and UBCE2I to an AU-Rich RNA Oligonucleotide (Electrophoretic Mobility Shift Assay, EMSA).
A 38-mer AU-rich oligonucleotide was radicactively labeled with [^{32}P]. EMSA of the different proteins and the labeled 38-mer RNA oligonucleotide was performed. Free oligonucleotide (nil, nil) served as a control. (A) Binding of 6xHis-AUF1 to the AU-rich RNA oligonucleotide. (B) Binding of 6xHis-NSAP-1 to the AU-rich RNA oligonucleotide. (C) Binding of GST-NSEP-1 to the AU-rich RNA oligonucleotide. (D) Binding of GST-IMP-2 to the AU-rich RNA oligonucleotide. (E) Binding of GST-UBCE2I to the AU-rich RNA oligonucleotide. (B-E) Binding of the interacting proteins both in the absence (nil) and presence of 52 nM 6xHis-AUF1p37.

shown). Although NSEP-1 bound to a 25-mer poly-G ribonucleotide (data not shown), it showed no ribonuclease activity toward it and neither bound nor degraded a single-stranded AT-rich DNA oligonucleotide (30-mer: ATTATTTATTATTTATTTATTATTTATTTA, not shown).

NSEP-1 Has an Endo-Ribonuclease Activity *in vitro*

When we incubated the AUF-1 interacting protein NSEP-1 with the AU-rich 38-mer test RNA oligonucleotide in the gel shift assay, we noted that the RNA was degraded and low molecular weight degradation products appeared in the lower part of the gel (Figure 4C). To examine this activity in more detail we incubated a 5′-end radioactively-labeled AU-rich 38-mer RNA oligonucleotide for different times with the GST-NSEP-1 protein (Figure 5A). The resulting RNA degradation fragments were resolved in a denaturing 20% polyacrylamide gel. We observed the appearance of intermediate degradation fragments of ~9 to 4 bases in length, whose length progressively short-

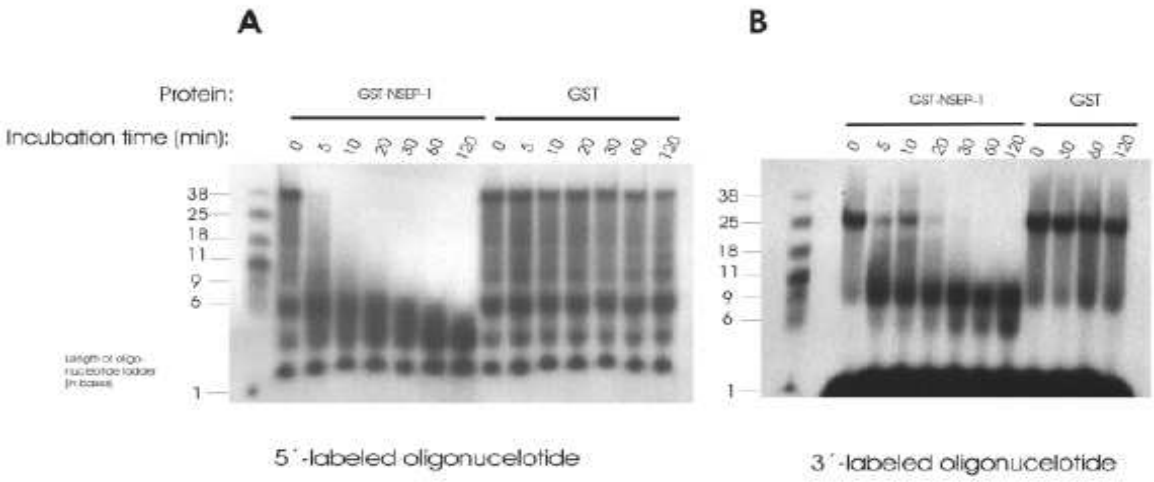

Fig. 5 *In vitro* RNA Oligonucleotide Degrading Assay.
(A) GST-NSEP-1 (0.25 μM) was incubated with the 5′-end [^{32}P]-labeled AU-rich RNA oligonucleotide (AU-rich 38-mer; 600 fmol) for the indicated times at 37 °C. GST (0.25 μM) served as a control and was incubated with the RNA oligonucleotide as indicated. After the incubation reactions the RNA degradation products were resolved on a denaturing (7 M urea) 20% polyacrylamide gel and the gel was exposed to an X-ray film. (B) Degradation of a 3′-end labeled AU-rich RNA oligonucleotide (5′-GGUUAUUAUUU-AUUUAUUAUU-UA[^{32}P]C[^{32}P]C-3′, 600 fmol). For details see the Materials and Methods section.

ened with longer incubation times. At the longest incubation times (120 min) we detected the accumulation of oligonucleotides of ~4-1 bases in length. The control protein GST did not degrade the oligonucleotide (Figure 5A). The additional lower molecular weight bands that can be seen at the zero time points and in all GST lanes probably represent co-labeled oligonucleotide synthesis products, since they were already present prior to incubation with either protein. These results suggested that NSEP-1 either operates in the 3′- to 5′-direction or that it has an endo-ribonuclease activity. If NSEP-1 operated in the direction 5′ to 3′, it would have readily generated only the free mononucleotide at the running front of the electrophoresis, but would not generate any products of intermediate molecular weight like those that can be seen in Figure 5A.

To distinguish between the possibilities that NSEP degrades the RNA in the 3′ to 5′ direction or that it is rather an endoribonuclease, we generated a 25-mer RNA oligonucleotide that is labeled radioactively at its 3′-end (see Materials and Methods), and performed another degradation assay with NSEP1 (Figure 5B). The incubation of such a 3′-labeled 25-mer ribo-oligonucleotide with NSEP-1 resulted in the appearance of intermediate degradation products of ~11–9 bases in length at incubation times from 5–20 min. At longer incubation times (20 min to 120 min) shorter degradation products of ~9–4 bases were generated. Such a result is incompatible with the interpretation that the ribonuclease activity of NSEP1 operates in the 3′ to 5′ direction, because this would have immediately generated degradation products of 1–2 bases in length after the shortest incubation times.

In summary, the degradation studies with the two differently-labeled ribonucleotides suggest that NSEP1 has an endoribonuclease activity, since the oligonucleotides that had been labeled at both ends yielded degradation products of intermediate size (~11 to ~6 bases) at short incubation times (up to 20 min). At longer incubation times (120 min), however, we observed the accumulation of very small degradation products: down to 1 base in the case of 5′-end labeled oligonucleotide and to ~4 bases in the case of the 3′-end labeled oligonucleotide.

Discussion

In mammalian cells AU-rich elements in the 3′-untranslated regions of certain mRNAs are potent *cis*-acting determinants of rapid mRNA turnover. AUF1 was the first RNA-binding protein identified to bind specifically to these AU-rich elements (Zhang *et al.*, 1993). Since AUF1 itself does not possess ribonuclease activity, it had been suggested that it rather targets other proteins to the AU-rich element. We speculated that AUF1 might be engaged in protein-protein interactions and performed a yeast two-hybrid screen with AUF1p37. Aside from AUF1 itself, we identified four other proteins that interact with AUF1: NSAP-1, NSEP-1, IMP-2 and UBCE2I.

NSAP-1 is made up of four RRM domains. The interaction between different proteins containing RRM domains has been described previously (Kim *et al.*, 2000). Like these authors had shown, the interactions between proteins of the RRM family do not occur randomly but in a very specific fashion. Depending on the pair of interacting hnRNP containing multiple RRMs, one, two or three of the RRMs are involved in the interaction (Kim *et al.*, 2000). In the interaction of AUF1 with NSAP-1 we found

that pairwise combinations of specific adjacent RRMs of NSAP-1 are both necessary and sufficient for the interaction with AUF1. Interaction can also occur when more than two RRMs are present but not with single RRMs of NSAP-1. This result indicates that the affinity of single domains might be too low for detection with the yeast two-hybrid method or that the inter-domain linker region is involved in the interaction. Kim *et al.* demonstrated that interactions among hnRNPs not only occur between RRM domains, but also among KH and between KH and RRM domains (Kim *et al.*, 2000).

The interaction of the two proteins AUF1 (=hnRNP D) and NSAP-1 has been described previously by methods other than the yeast two-hybrid method (Grosset *et al.*, 2000). Grosset and co-workers demonstrated that AUF1 and NSAP-1 are part of a multiprotein complex that is associated with the major coding region determinant (mCRD) of instability of the *c-fos* mRNA. Aside from AUF1 and NSAP1 these researchers were able to identify three other proteins in this multiprotein complex: Unr, PABP, and PAIP1. They proposed that this multiprotein assembly functions as a bridging factor between the mCDR and the poly-(A) tail (*via* PABP) and they further speculated that the transit of the ribosome might disrupt this complex and thereby cause the rapid deadenylation and decay of the *c-fos* mRNA. These findings and interpretations are in agreement with our own data that suggest that the interaction of AUF1 and NSAP1 might be of importance to the mechanism of accelerated mRNA decay.

We observed that the incubation of an AU-rich RNA oligonucleotide with the AUF1 binding protein NSEP-1 resulted in its degradation *in vitro*. A more detailed analysis of this RNA degradation process revealed that NSEP-1 possesses an endo-ribonuclease activity. NSEP-1 is a member of the Y-Box family of proteins, which are multifunctional proteins that have a highly conserved cold-shock domain (CSD) but rather variable C-terminal domains (Matsumoto and Wolffe, 1998; Shnyreva *et al.*, 2000). Due to their DNA and RNA binding activities, Y-Box proteins have been functionally implicated in the regulation of transcription (Coles *et al.*, 1996; Diamond *et al.*, 2001) and translation (Matsumoto *et al.*, 1996; Matsumoto and Wolffe, 1998). Our finding that the Y-Box protein NSEP-1 not only binds nucleic acids, but also interacts with the human mRNA destabilizing protein AUF1 and has an endoribonuclease activity, suggests an additional functional context for NSEP-1. The NSEP-1 ribonuclease activity has a base preference: a polyG ribonucleotide probe was not degraded by NSEP-1, whereas an AU-rich 38-mer ribonucleotide was efficiently degraded.

IMP-2 is another RNA binding protein that we found to interact with AUF1 p37 and p40 *in vivo* in the yeast two-hybrid system. The interaction of the KH domain-containing protein IMP-2 with AUF1 occurs only when KH domains 2 and 3 or 3 and 4 are present. These results are also in agreement with the findings of Kim *et al.* (2000), who had shown that a single KH domain, in contrast to a single RRM domain, is not able to interact with other KH or with RRM domains. The mapping studies of protein domains involved in protein-protein interaction among hnRNPs suggested that the multidomain structure of the hnRNPs might have evolved not only for its interaction with RNA but also to mediate protein-protein interactions that might be important for mRNA processing, export and degradation.

Finally, we identified the ubiquitin-conjugating enzyme E2I (UBCE2I) as an AUF1-interacting protein partner. This is a very interesting finding, since it had been previously demonstrated that AUF1 is involved in the control of mRNA decay, which has been linked to the heat shock-ubiquitin-proteasome pathway (Laroia *et al.*, 1999; 2002). These authors demonstrated that the degradation of AU-rich mRNAs is associated with the ubiquitination of AUF1 and its degradation in the proteasome. Heat shock, inhibition of the proteasome pathway or inactivation of the ubiquitin-activating enzyme E1 resulted in the accumulation of undegraded AUF1 and was accompanied by a block of the decay of AU-rich mRNAs. These results suggested that the decay of the AU-rich mRNAs is coupled with the ubiquitin-dependent degradation of AUF1. Our results give further support to this line of argumentation, since we identified the ubiquitin-conjugating enzyme E2I as an AUF1 interaction partner. UBCE2I interacts with AU-rich RNA (Figure 4E), however with relatively low affinity. It is tempting to speculate that the RNA binding activity might be important to ensure that only AUF1 bound to its RNA substrate becomes ubiquitinated.

We demonstrated here that all four AUF1-interacting proteins are able to bind to an AU-rich 38-mer RNA oligonucleotide (Figure 4B-E). When we added AUF1 protein to the AUF1-binding proteins and the RNA oligonucleotide we observed an intensified signal of the shifted bands in comparison to the conditions where AUF1 was absent. This result suggests that AUF1 and its interacting proteins bind simultaneously to the large 38-mer oligonucleotide. We did not, however, detect any qualitative differences such as supershifts under these experimental conditions. This might indicate that the gel shift method is not sensitive enough for the detection of interactions between AUF1 and its interacting proteins in the presence of the oligonucleotide. Therefore we also employed the UV cross-linking technique (Gao *et al.*, 2001) in order to analyze whether qualitative changes in the shift patterns would occur when AUF1, its interacting proteins and an AU-rich 11-mer oligoribonucleotide are incubated and crosslinked (data not shown). We could observe that three of the four AUF1-interacting proteins (NSAP1, IMP-2 and UBCE2I), when incubated together with AUF1, caused a supershift when compared to AUF1 incubated alone with the 11-mer oligoribonucleotide. The supershifts were most pronounced at the highest interacting protein concentrations tested. These results may suggest that the interacting proteins had been crosslinked and that their covalent coupling had caused a reduced electrophoretic mobility of the bound AU-rich

11-mer oligonucleotide. It is noteworthy that the 11-mer oligonucleotide is too short to accommodate more than one of the proteins, since an RNA binding protein that contains two RRM domains like AUF1 makes contact to ~8 bases (Moraes *et al.*, 2002). The fact that the supershift in the UV-crosslink assay occurred only in the presence of both AUF1 and its interacting protein partners suggests that these proteins can interact when bound to the 11-mer oligonucleotide. Future experiments will address the detailed mode of these interactions.

We found that the AUF1-interacting proteins, with the exception of UBCE2I, interact only with AUF1 p37 and p40. Neither of these isoforms contain the 49 amino acid insertion 'b' (Figure 1A) that is encoded by its own exon. This insertion might be located in a region of AUF1 that is involved in its interaction with IMP-2, NSEP-1 and NSAP-1, since its presence abrogates the interactions. One other group performed a yeast two-hybrid screen with the protein AUF1p45 as 'bait' (Arao *et al.*, 2000). These authors studied the isoforms p42 and p45 that both contain the region 'b' (Figure 1A) and found an interesting interacting protein partner: the nuclear matrix-associated factor SAF-B, which is involved in gene regulatory functions (Stief *et al.*, 1989). The observation of this interaction explained the long-known fact that the AUF1 p37/p40 isoforms are preferentially located in the cytoplasm, whereas p42/p45 are rather located in the nucleus (Arao *et al.*, 2000). AUF1 p42/p45 are probably retained in the nucleus, because they are engaged in interaction with the nuclear protein SAF-B. The specific distribution of the AUF1 isoforms between cytoplasm and nucleus suggests that they perform distinct functions in these two compartments. It is possible that the nuclear AUF1 isoforms p42 and 45 are involved in mRNA processing and/or export, and the cytoplasmic isoforms in the regulation of the mRNA degradation. The identification of AUF1 isoform-specific interacting proteins reported here might prove to be an insightful finding in the steadily ongoing analysis of AUF1´s manifold functions in gene regulatory events.

Materials and Methods

Plasmid Construction and Antibodies

Plasmids pcDNA3.1 (Invitrogen) containing insert DNAs encoding AUF1 p37, p40, p42 and p45, were kindly provided by Dr. R. Schneider (New York University, USA), and served as the template DNA in the PCR reactions. AUF1 protein p37 cDNA was amplified by PCR (Pet-s: 5´AGG AAT TCC ATA TGC ACC ATC ATC ACC ATC ACA AGC; Pet-as: 5´ATG GAT CCT TAT TAG TAT GGT TTG TAG CTA TTT TG), and subcloned into bacterial expression vector pET3c (Stratagene). For the yeast two-hybrid analysis, AUF1-p37 cDNA was PCR-amplified (pBTM-s: C GGG ATC CGT ATG TCG GAG GAG CAG TTC GGC; pBTM-as: TA TGC GCT GAC TTA GTA TGG TTT GTA GCT ATT TTG) and inserted into the yeast bait expression vector pBTM116 (Durfee *et al.*, 1993; Bartel and Fields, 1995; Vojtek and Hollenberg, 1995). Equivalent strategies were also employed to insert the cDNAs encoding the other three isoforms of AUF1 (p40, p42 and p45) into the yeast expression vector pBTM116 and the cDNA of AUF1p37 into the yeast expression vector pACT2 (Clontech, prey vector), which encodes the Gal4 activation domain (Gal4 AD). To express the AUF1-interacting proteins NSEP-1, IMP-2, and UBE2I as fusion proteins with glutathione S-transferase (GST), their DNAs were subcloned from the pACT2 library plasmids into the bacterial expression vector pGEX-2TK (Amersham Pharmacia). In a similar manner NSAP-1 was subcloned into the plasmid pET-28 and expressed as a 6xHis fusion protein. The following oligonucleotides were used to amplify the cDNAs encoding the proteins NSEP-1 (sense: 5´GC GGA TCC AAG AAG AAT AAC CCC AGG AAG TAC C 3´; antisense: 5´TCC CCC GGG TTA CTC AGC CCC GCC CTG CTC AGC 3´), NSAP-1 (sense: 5´ CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC C 3´); antisense: 5´ TCT CCC GGG TTA CCC TCG ACC TCT TGT TGG AGG GGG 3´), IMP-2 (sense: 5´ GC GGA TCC ACC ATA AAG AAC ATC ACT AAG CAG 3´; antisense: 5´CG GAA TTC TCA CTT GCT GCG CTG TGA GGC GAC 3´), and UBE2I (sense: 5´ GC GGA TCC CTC GCC CAG GAG AGG AAA GCA TGG 3´; antisense: 5´ CG GAA TTC TTA GGG CGC AAA CTT CTT GGC 3´). For the generation of the deletion mutants for the proteins NSAP-1, NSEP-1 and IMP-2 as utilized in the yeast two-hybrid system, the oligonucleotides shown in Table 2 were used. A specific rabbit anti-AUF1 immunoserum was obtained by eight successive immunizations of a rabbit with 1 mg of recombinant 6xHis-AUF1p37 protein in intervals of two weeks.

Yeast Two-Hybrid Screen and Interaction Analysis

AUF1-p37-pBTM116 (lexA DNA binding domain fusion) was co-transformed in the yeast reporter strain L40 (Bartel and Fields, 1995) together with a human fetal brain cDNA library (Clontech) cloned C-terminally to the activation domain of Gal4 in plasmid pACT2 (Durfee *et al.*, 1993). A total of ~2×10^6 transformants were plated on synthetic minimal medium (MM) lacking tryptophan, leucine and histidine but supplemented with adenine. After 5 days the 380 grown clones were restreaked on MM plates and tested by a ß-galactosidase filter assay. Yeast DNA was isolated from LacZ+ clones, and the library plasmids were rescued into *E. coli* strain HB101 (Promega). After re-transformation into L40 together with LexA-AUFp37, 35 clones were confirmed to be His+, LacZ+. The DNA inserts of the library plasmids were sequenced by automated sequencing (ABI PRISM™ 377).

Bacterial Expression and Purification

E. coli strain BL-21 was transformed with recombinant expression vectors: AUF1p37 in pET-3c, the AUF1-interacting proteins NSEP-1, UBE2I, or IMP-2 in frame with an N-terminal GST (in pGEX-2TK) and NSAP-1 with an N-terminal 6xHis fusion tag (in pET-28a). Recombinant bacteria were induced with isopropylthio-β-D-galactoside (IPTG) for 3 hours at 37°C. Cells were lysed by the addition of lysozyme (1 mg/ml), incubation for 30 min at RT and four subsequent freeze-thaw cycles. DNA was digested by addition of DNase (1 µg/ml). The bacterial lysate was centrifuged at 14 000 *g* for 20 min and the supernatant containing soluble 6xHis or GST fusion proteins was applied to the appropriate columns. The 6xHis fusions were applied to a Ni-NTA column (Qiagen). Column washing and elution were performed according to the manufacturer's instructions. The cell lysates containing the GST fusion proteins were applied to a glutathione Sepharose 4B column (Amersham Biosciences). The column was washed and eluted according to manufacturers instructions. Five µl aliquots of fractions were analyzed by SDS-PAGE.

In vitro Binding Assay and Western Blot Analysis

Equal amounts of free GST or GST fusion proteins were allowed to bind to glutathione Sepharose 4B resin (Amersham Pharmacia) in 1 ml of PBS for 2 hours at 4°C. After incubation, the resin-bound samples were washed three times with PBS. 0.2 µg recombinant 6xHis-AUF1p37 fusion protein were added to the resins and incubated in 1 ml PBS, 0.2% Triton X-100, pH 8.0, for two hours at 4°C to allow for protein-protein interaction. This incubation was performed in the presence of 10 µg of RNase A, to remove possible contaminating RNA and therefore rule out that the interactions could occur indirectly through the presence of minor amounts of contaminating RNA. The resin was then washed three times with 0.6 ml PBS and the resin-bound proteins were resolved in an SDS-10% polyacrylamide gel. After electrophoresis the proteins were transferred to a PVDF membrane by semi-dry electroblotting for two hours. After saturation with unspecific protein (Blotto: PBS with 10% nonfat dry milk) the membrane was incubated with a rabbit anti-AUF1 antiserum (1:20, in Blotto) for 1 h. After 3 washes with PBS/0.05% Tween-20 it was incubated with secondary HRP-conjugated goat anti-rabbit IgG antibody (1:5000; Santa Cruz Biotech) for 1 h and washed again 3 times. Finally, the membrane was developed by chemiluminescence (Luminol reagent, Santa Cruz Biotech). In the case of NSAP-1 the 6xHis-NSAP-1 fusion protein was coupled to a Ni-NTA-Sepharose resin (Qiagen) and 6xHis-RACK coupled to the same resin served as a control protein. To prevent unspecific interaction of 6xHis-AUF1 fusion protein with the Ni-NTA-Sepharose the recombinant protein was digested with thrombin to remove the 6xHis-tag fusion part of the protein. Repurified AUF1 was then incubated with the beads as above.

Electrophoretic Mobility Shift Assay (EMSA) and RNA Degradation Assays

EMSA was essentially performed as described (Rui *et al.*, 2001; Moraes *et al.*, 2002). Briefly, an AU-rich RNA oligonucleotide (38-mer: GUGAUUAUUUAUUAUUUAUUUAUUAUUU AUUUAUUUAG), representing a region of the 3′-portion of the human TNF-α mRNA, was 5′-labeled with [γ-^{32}P]-dATP using T4 polynucleotide kinase. The radiolabeled probe was column-purified and aliquots of 30 fmol (~10 000 cpm) were used in each binding reaction. The oligonucleotide probe and the proteins were incubated in a final reaction volume of 25 µl, containing 100 mM potassium acetate, 10 mM Tris (pH 7.5), 5 mM magnesium acetate, 20% glycerol, 0.1 ng/µl BSA, 80 ng/ml ssDNA, at 4°C for 20 min (Moraes *et al.*, 2002). The RNA-protein complexes were run out on a non-denaturing 6% polyacrylamide gel in 0.5× TBE buffer with constant water-cooling. Dried gels were exposed for 12 h to X-ray film at –80°C with an intensifying screen.

The NSEP-1 degradation assay (Figure 5) was essentially performed as follows: proteins and AU-rich RNA (38-mer, 5′-labeled with [^{32}P] as above, 600 fmol) were incubated at RT for the times indicated in Figure 5A. The degradation products were run out on a denaturing 20% polyacrylamide gel in the presence of 7 M urea. A 3′-labeled AU-rich RNA oligonucleotide was generated in the following fashion: two DNA oligonucleotides encoding a T7 RNA polymerase binding site (T7AU-S: CCCGCGTAATACGACTCACTATAGGGTTATTATTTATTTATTATTTACC, T7AU-AS: GGTAAATAATAAATAAATAATAACCCTATAGTGAGTCGTATTA-CGCGGG) were annealed and used for an *in vitro* transcription with T7 polymerase (SP6/T7 Transcription Kit, Roche) in the presence of [γ-^{32}P]-CTP (procedure as recommended by the manufacturer). This generated an RNA oligonucleotide of 25 nucleotides in length that was labeled only at its 3′-end with two terminal [^{32}P]-CTPs (GGUUAUUAUUUAUUUAUUAUUUA[^{32}P]C[^{32}P]C). This RNA oligonucleotide was subsequently incubated with NSEP-1 and GST in the degradation assay (Figure 5B) and the resulting degradation products were run out on a denaturing (7 M urea) 20% polyacrylamide gel and analyzed by autoradiography. For the determination of the approximate size of the degradation fragments a radioactively labeled 'base marker' was generated by the labeling procedure described above. Six oligonucleotides of 38, 25, 18, 11, 9 and 6 bases in length were used and free ATP was added to mark the one base running front.

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3. Resultados

Artigo II

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

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

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Abstract

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

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

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

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

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

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

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

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

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

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

Results and discussion

Yeast two-hybrid screen using PRMT1 as a bait

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

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

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

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

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

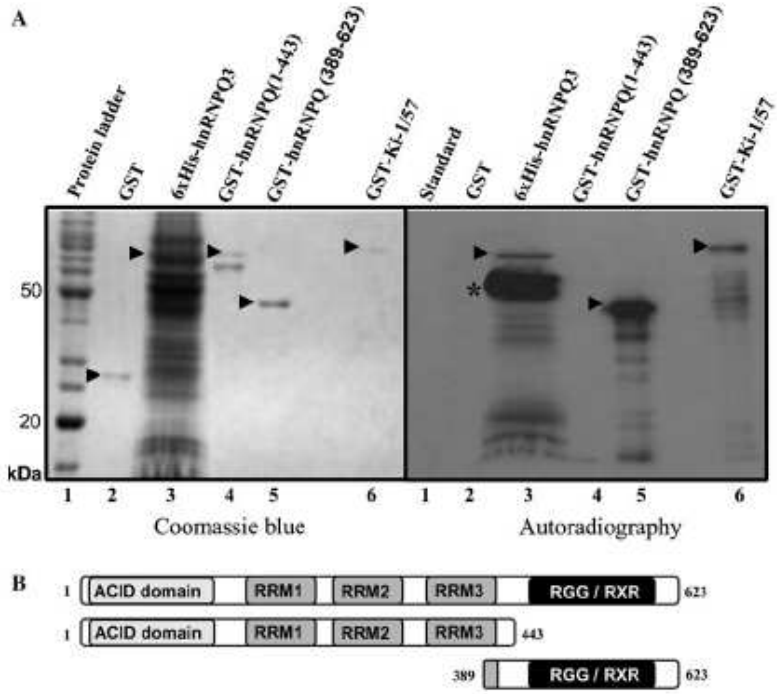

Fig. 1. hnRNPQ is a substrate for PRMT1 *in vitro*. (A) The indicated proteins were methylated by GST-PRMT1 *in vitro*. PRMT1 was expressed and purified as a GST fusion protein and incubated with the indicated recombinant proteins, all expressed in and purified from *E. coli*. Full-length hnRNPQ was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

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

hnRNPQ is methylated in vivo in HeLa cells

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

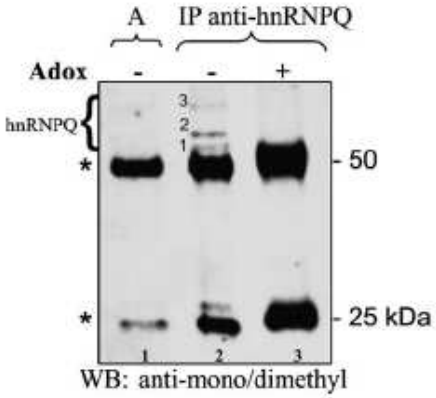

Fig. 2. hnRNPQ isolated from human cells shows methylation *in vivo*. hnRNPQ was immunoprecipitated (IP) from the whole cell lysate of HeLa cells treated (+) or non-treated (−) by methylation inhibitor Adox. Then the immunoprecipitated proteins were run out on by SDS–PAGE and transferred to a PVDF-membrane.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

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

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

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

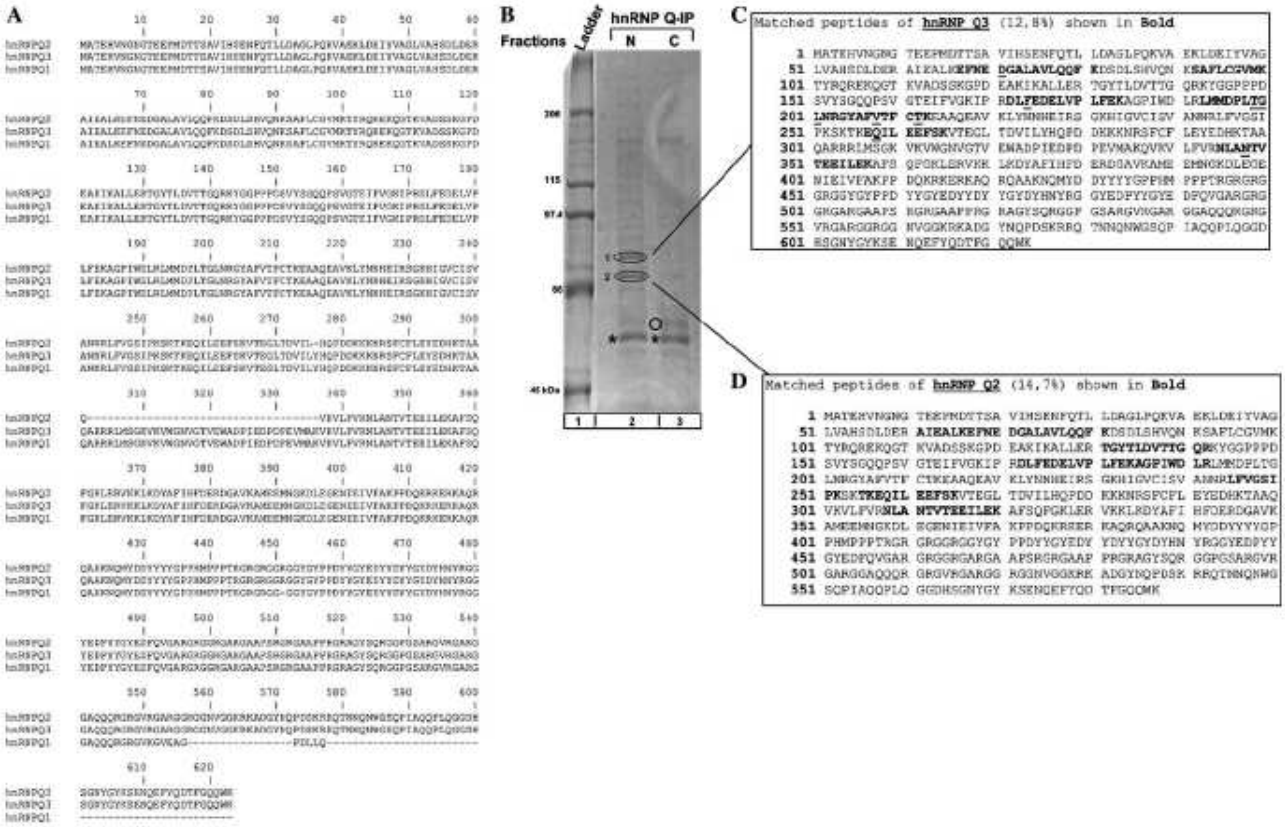

Fig. 3. Identification of the nuclear isoforms hnRNPQ2 and 3 by mass spectrometry. (A) Protein sequence alignment of the three hnRNPQ isoforms 1–3. (B) HeLa cells' nuclear and cytoplasmic fractions were separately immunoprecipitated with anti-hnRNPQ/R antibody and co-precipitated proteins were run out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

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

In vitro methylation of endogenous cellular hnRNPQ by PRMT1

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

We found two bands of ca. 67 and 70 kDa, corresponding to hnRNPQ2 and 3, in nucleus and cytoplasm of untreated HeLa cells (lanes 3 and 4). However, the nuclear hnRNPQ bands (lane 3) present significantly more methyl-H^3 incorporation than the cytoplasmic bands (lane 4). On the other hand, we only were able to observe the band corresponding to hnRNPQ3 in the Adox-treated cells. In this case the cytoplasmic protein fraction (lane 2) was methylated stronger *in vitro* by GST-PRMT1 than the nuclear fraction (lane 1). This could reflect that hnRNPQ3 in presence of inhibitor Adox is re-distributed to the cytoplasm. This

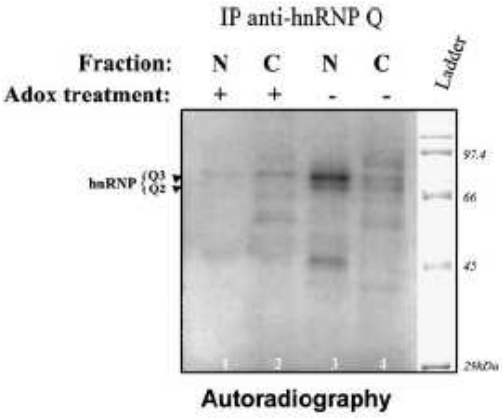

Fig. 4. *In vitro* methylation assay. HeLa cells were (+) or were not (−) incubated with the inhibitor of endogenous protein methylation, Adox. Next nuclear (N) and cytoplasmic (C) fractions of these cells were prepared and submitted to immunoprecipitation (IP) with anti-hnRNPQ antibody. Immunoprecipitated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

hypothesis obtains further support from the *in vivo* immunofluorescence experiment reported below in Fig. 5. At this point we do not yet understand why the hnRNPQ proteins from the nucleus of Adox-treated cells are less well methylated *in vitro* than those of untreated cells, since the contrary may be expected. We speculate that the inhibition of hnRNPQ methylation by Adox may lead to an increase in its association with other proteins, to conformational change or even to a decrease in protein stability. So the fraction corresponding to the nucleus of treated cells showed a significant reduction in its *in vitro* methylation by GST-PRMT1.

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

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

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

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

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

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

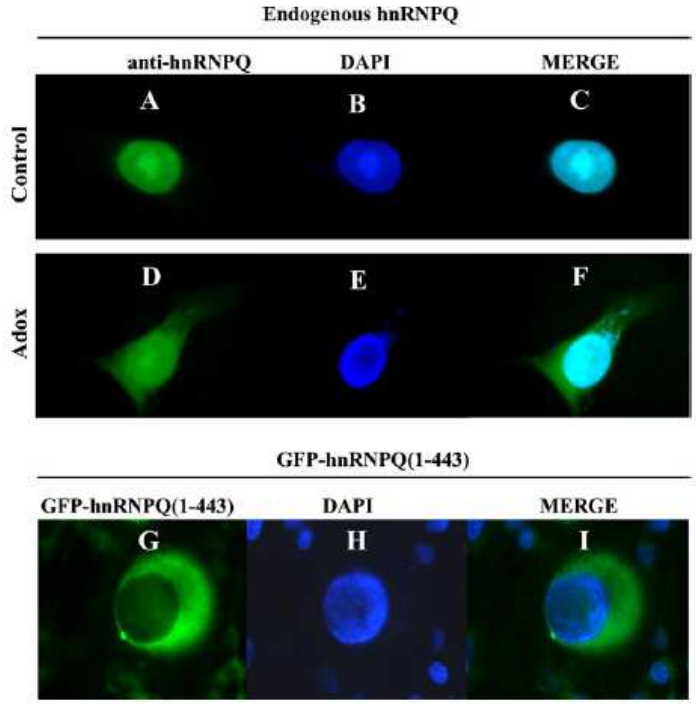

Fig. 5. Inhibition of the methylation of hnRNPQ leads to its re-distribution to the cytoplasm, and hnRNPQ requires its C-terminal region for nuclear localization. HeLa cells were grown on glass coverslips and incubated for 16 h with (D–F) or without Adox (A–C) at 37 °C. Cells were fixed with 100% methanol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

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

Materials and methods

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

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

Expression and purification of recombinant proteins. To generate the proteins GST, GST-hnRNPQ(1–443), GST-hnRNPQ(389–623), GST-PRMT1, and GST-Ki-1/57, the cDNAs were amplified by PCR and

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

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

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

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

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

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

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

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

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3. Resultados

Artigo III

The acidic domain of hnRNPQ (NSAP1) has structural similarity to Barstar and binds to Apobec1

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The acidic domain of hnRNPQ (NSAP1) has structural similarity to Barstar and binds to Apobec1 ☆,☆☆

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Abstract

Apobec1 edits the ApoB mRNA by deaminating nucleotide C^{6666}, which results in a codon change from Glutamate to stop, and subsequent expression of a truncated protein. Apobec1 is regulated by ACF (Apobec1 complementation factor) and hnRNPQ, which contains an N-terminal "acidic domain" (AcD) of unknown function, three RNA recognition motifs, and an Arg/Gly-rich region. Here, we modeled the structure of AcD using the bacterial protein Barstar as a template. Furthermore, we demonstrated by *in vitro* pull-down assays that 6×His-AcD alone is able to interact with GST–Apobec1. Finally, we performed *in silico* phosphorylation of AcD and molecular dynamics studies, which indicate conformational changes in the phosphorylated form. The results of the latter studies were confirmed by *in vitro* phosphorylation of 6×His-AcD by protein kinase C, mass spectrometry, and spectroscopic analyses. Our data suggest hnRNPQ interactions via its AcD with Apobec1 and that this interaction is regulated by the AcD phosphorylation.

Keywords: hnRNPQ/NSAP1; Apobec1; AcD domain; RNA editing; Molecular modeling; Molecular dynamics; Protein–protein interactions; Circular dichroism

We reported earlier on the domain-mediated interaction between two modular RNA-binding proteins called AUF1 and heterogeneous nuclear ribonucleoprotein Q (hnRNPQ), which is also known as NSAP1 [1]. AUF1 is a protein that exerts a destabilizing effect on many target AU-rich mRNAs [2], to which it binds, but it also increases the internal ribosomal entry site-mediated translation of hepatitis C virus mRNA [3].

The protein hnRNPQ on the other hand had been initially identified as a prey in a yeast two-hybrid screen of a HeLa cDNA library using NS1, a non-structural protein from minute virus of mice [4]. It has been reported to preferentially bind to poly(A) RNA [5] and it is a component of a multi-protein complex that regulates the translationally coupled c-*fos* mRNA degradation [6]. An additional proposed function for this protein is the specific cytoplasmic regulation of the degradation of mRNA that is related to the circadian rhythm [7]. It can further act on the hnRNPs complex which assembles on nascent RNA

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☆☆ *Abbreviations:* AcD, acidic domain of hnRNPQ; AcD-P, phosphorylated acidic domain; ACF, Apobec1 complementation factor; ApoB, apolipoprotein B; Apobec1, apoB editing catalytic subunit 1; CDAR, cytidine deaminases active on RNA; hnRNPQ, heterogeneous nuclear ribonucleoprotein Q; NSAP1, non-structural associated protein 1; RGG box, Arginine Glycine-rich Box; RMSF, root-mean-square fluctuation; RRM, RNA recognition motif; SMN, survival of motor neurons.
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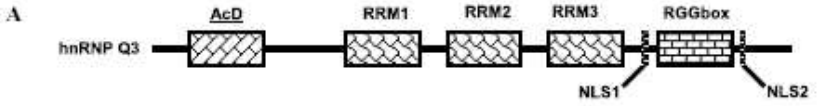
polymerase II transcripts, where it co-localizes with small nuclear (sn)RNPs [8] and is also found in the SMN (*survival of motorneurons*) complex [9].

Recently, Blanc and co-workers reported that hnRNPQ is an inhibitory component of the so-called apoB RNA editing complex, which inhibits the mRNA editing action of a protein called Apobec1 [10]. Mammalian mRNAs can be post-transcriptionally modified by site-specific adenosine or citidine deaminases in a process known as mRNA editing [11]. Editing by deamination is catalyzed by enzymes known as CDARs (cytidine deaminases active on RNA) and results in a change from citidine to uridine. The best-studied editing protein is Apobec1, which edits the ApoB mRNA by deaminating nucleotide C^{6666}. This editing results in a codon change from Glutamate to stop, provoking thereby the expression of a shorter protein variant of 48 instead of 100 kDa molecular mass [12]. An 11-nucleotide "mooring" motif downstream of the editing site, from nucleotide 6671 to 6681, is absolutely required for the editing reaction [13], which also requires the essential cytidine deaminase domain of the editing enzyme Apobec1 [14]. Lipoprotein particles containing the shorter ApoB-48 variant of the protein are cleared faster from the circulation and do not associate with other lipoproteins, resulting in a reduced atherogenic risk [15]. Apobec1 transgenic mice and rabbits, however, develop hepatocellular dysplasia and carcinoma, indicating that Apobec1 has to be tightly regulated by inhibitory protein factors that are also present in the apoB mRNA editing complex [16].

One of these negative regulator proteins is ACF (Apobec1 complementation factor), which has been simultaneously described by two groups. Their discoveries have greatly contributed to our understanding of the essential protein components involved in the apoB RNA-editing




```
B
          1                                                                   70
HnRNPQ3   MATEHVNGNG TEEPMDTTSA VIHSENFQTL LDAGLPQKVA EKLDEIYVAG LVAHSDLDER AIEALKEENF
  ACF     .......... .......... .......... .......... .......... .......... ..........
         71                                                                  140
HnRNPQ3   DGALAVLQQF KDSDLSHVQN KSAFLCGVMK TYRQREKQGT KVADSSKGPD EAKIKALLER TGYTLDVTTG
  ACF     .......... .......... .......... .MESNHKSGD GLSGTQK... EAALRALVQR TGYSLVQENG
                                              ....*.*   ::.:.*    ** ::**::* ***:*   .*
        141                                                                  210
HnRNPQ3   QRKYGGPPPD SVYSGQQPSV GTEIFVGKIP RDLFEDELVP LFEKAGPIWD LRLMMDPLTG LNRGYAFVTF
  ACF     QRKYGGPPPG ..WDAAPPER GCEIFIGKLP RDLFEDELIP LCEKIGKIYE MRMMMD.FNG NNRGYAFVTF
          *********.   :.. *.  * ***:**:* ********:* * ** * *:: :*:*** :.*  *********
        211                                                                  280
HnRNPQ3   CTKEAAQEAV KLYNNHEIRS GKHIGVCISV ANNRLFVGSI PKSKTKEQIL EEFSKVTEGL TDVILYHQPD
  ACF     SNKVEAKNAI KQLNNYEIRN GRLLGVCASV DNCRLFVGGI PKTKKREEIL SEMKKVTEGV VDVIVYPSAA
          ..*  *:.*: *  **:***. *: :*** **  * *****.* **:*.:*:** .*:.*****: .***:* ..
        281                                                                  350
HnRNPQ3   DKKKNRSFCF LEYEDHKTAA QARRRLMSGK VKVWGNVGTV EWADPIEDPD PEVMAKVKVL FVRNLANTVT
  ACF     DKTKNRGFAF VEYESHRAAA MARRKLLPGR IQLWGHGIAV DWAEPEVEVD EDTMSSVKIL YVRNLMLSTS
          **.***.*.* :***.*:.**  ***:*:.*: :::***: :* :**:*  : *  :.*:.**:* :****  :.:
        351                                                                  420
HnRNPQ3   EEILEKAFS. .QFGKLERVK KLKDYAFIHF DERDGAVKAM EEMNGKDLEG ENIEIVFAKP PDQKRKERKA
  ACF     EEMIEKEFNN IKPGAVERVK KIRDYAFVHF SNREDAVEAM KALNGKVLDG SPIEVTLAKP VDKDSYVRYT
          **::** *.   : * :**** *:.****:** .:*:.**:** : :*** *:*   **:.:***  *:.   *
        421                                                                  490
HnRNPQ3   QRQAAKNQMY DDYYYYGPPH MPPPTRGRGR GGRGGYGYPP DYYGYEDYYD YYGYDYHNYR GGYEDPYYGY
  ACF     RGTGGRGTML QGEYTYSLGQ VYDPTTT... .....YLGAP VFYAPQTYAA IPSLHFPATK GHLSNRAIIR
          :  ..:. *  :. * *.  : :   **          *   *  :*: : *       :     *   :
        491                                                                  560
HnRNPQ3   EDFQVGARGR GGRGARGAAP SRGRGAAPPR GRAGYSQRGG PGSARGVRGA RGGAQQQRGR GVRGA.....
  ACF     APSVRGAAGV RGLGGRGYLA YTGLGRGY.Q VKGDKREDKL YDILPGMELT PMNPVTLKPQ GIKLAPQILE
                ** *   * * **      * *              :        *                *:: *
        561                                                                  630
HnRNPQ3   ....RGGRGG NVGGKRKADG YNQPDSKRRQ TNNQNWGSQP IAQQPLQGGD HSGNYGY.KS ENQEFYQDTF
  ACF     EICQKNNWGQ PVYQLHSAIG QDQRQLFLYK ITIPALASQN PAIHPFTPPK LSAFVDEAKT YAAEYTLQTL
             :   *   *     * *  .* .             **   *  *        *      *     *:  .*
        631                                                                  700
HnRNPQ3   GQQWK..... .......... .......... .......... .......... .......... ..........
  ACF     GIPTDGGDGT MATAAAAATA FPGYAVPNAT APVSAAQLKQ AVTLGQDLAA YTTYEVYPTF AVTARGDGYG
          *
        701
HnRNPQ3   ..
  ACF     TF
```

Fig. 1. Comparison between amino acid sequences of the human proteins: hnRNPQ3 and ACF. (A) Schematic representation of the domain organization of the human protein hnRNPQ3: acidic domain (AcD), RNA recognition motifs (RRM), RGG box (Arg–Gly–Gly box), and nuclear localization sequences (NLS). (B) Alignment between amino acid sequences of hnRNPQ3 and ACF. The modeled acidic domain of hnRNPQ3 is underlined with *, showing identical residues; :, residues with high similarity; ., residues with low similarity.

complex. ACF is a 586-residue, ~65-kDa, RNA-binding protein, that contains a C-terminal dsRNA binding domain and three RNA recognition motifs (RRMs) [17]. Its functional importance was confirmed in studies showing that ACF binds both the protein Apobec1 and the mRNA of ApoB [10,18].

As mentioned above, another inhibitory component of the ApoB RNA editing complex is the hnRNPQ protein, which interestingly shares several of the domains presents in ACF, but is not directly involved in mediating C to U editing of apoB mRNA [10]. HnRNPQ has five protein domains or motifs (Fig. 1A): an N-terminal "acidic domain" (AcD) of yet unknown function, three RNA recognition motifs (RRMs) (also known as RNA binding domains RBD), and an unusually large ~120 amino acids long arginine–glycine rich domain (RGG box) [9].

By means of amino acid sequence alignments of the functionally associated proteins Apobec1 and hnRNPQ, we found that the main difference of the latter in comparison with Apobec1 is that it contains the mentioned acidic domain. This called our attention and motivated us to express this domain separately, to be able to study its function. Furthermore, we performed homology modeling and molecular dynamics studies on this domain and analyzed its secondary structure by CD spectroscopy. In summary, our biochemical and structural data on the acidic domain of hnRNPQ show that it interacts with Apobec1 and suggest that hnRNPQ, via binding through its AcD, could be a regulator of Apobec1.

Materials and methods

Modeling of the hnRNPQ acidic domain. Different regions of the amino acid sequence of the acidic domain (AcD) of human hnRNPQ protein (GenBank Accession No. AAK59703) were used as templates to search the Protein Data Bank (PDB) using the program Blastp [19]. When using the region Arg12–Cys86 of the acidic domain several high-resolution crystal structures were identified but after analysis with the program Threader [20] only one of them was selected as a possible template for modeling: Barstar from *Bacillus amyloliquefaciens* (1B2S) [21]. Several homology models for the AcD domain were generated using the program Modeller [22] according to the initially proposed alignment. The original alignment was generated using the Multialign program [23] but was further adjusted manually until an acceptable model was achieved. Model building and structural analyses were performed on a Compaq Alpha Server ES40 and molecular visualization was carried out using the WebLab Viewer (Accelrys). The programs: Procheck [24] and Verify_3D [25] were used to evaluate the stereochemical quality of the final model.

Plasmid constructions. The cDNA segment encoding the N-terminal domain (12–96) of human hnRNPQ was amplified by PCR using the plasmid construction pGEX4T2-hnRNPQ3 (1–623) as template and the oligonucleotides: AcD–S: (ccaggatccgaagacccatggatactacttc) and AcD–AS: (ctctcgagctataaaaaggcacttttgttctg). The PCR product was cloned into pGEM-T vector (Promega), confirmed by DNA sequencing, and subcloned into bacterial expression vector pET28a via *Bam*HI and *Xho*I restriction sites, to allow its expression as a 6×His-tagged fusion protein. The clone of Apobec1 protein from rat had been generously provided by Prof. Dr. Tasuku Honjo (Kyoto University, Japan) [26]. The rat Apobec1 amino acid sequence shows an identity of ~74% and a similarity of ~88% with the human Apobec1 amino acid sequence. Full length Apobec1 cDNA was PCR amplified similar as above and sub-cloned from that vector into the bacterial expression vector pGEX to allow its expression as a GST-tagged fusion protein.

Expression and purification of recombinant proteins. BL21 strain (Invitrogen) was transformed with recombinant pET28a-hnRNPQ-AcD vector and grown in LB medium with 30 μg/ml of kanamycin. When reaching the logarithmic growth phase the recombinant bacteria were induced for protein production with 1 mM of isopropyl-β-D-thiogalactoside (IPTG) at 37 °C for 4 h. After harvest and lysis the resulting suspension was cleared by centrifugation. GST and 6×His fusion proteins were purified using Glutathione–Sepharose 4B (GE Healthcare, Waukesha, WI) or Ni-chelating sepharose Fast Flow (GE Healthcare), respectively, as described before [1]. The concentration of the recombinant protein was determined spectroscopically using the calculated extinction coefficient for the denatured proteins [27].

In vitro phosphorylation. Purified recombinant protein 6×His-AcD was phosphorylated in complete kinase buffer in a final volume of 50 μl at 30 °C with purified PKC-Pan and radioactive [^{32}P]ATP for 20 min as described before for another protein substrate [28]. The commercial PKC-Pan had been purified from rat brain and consists predominantly of the PKC isoforms α, β, and γ (Promega). Radioactively labeled protein was then run out by SDS–PAGE. The gel was stained with Coomassie blue, dried, and exposed to X-ray film.

Mass spectrometry analysis. For identification of the phosphorylated amino acids in 6×His-construction, 100 μg/ml of the purified and phosphorylated 6×His-AcD were dialyzed in 10 mM ammonium bicarbonate buffer, pH 8.0. The proteolysis was performed with 0.1 μg/ml trypsin (Sigma, St. Louis, MO, USA) at 22 °C for 18 h. The reaction was stopped by addition of a CHCA matrix solution (10 mg/ml in the solvent: H_2O: acetonitrile: 3% trifluoroacetic acid; 4:5:1; v:v:v). Next, samples were prepared for MALDI-TOF-MS as previously reported [29], using immobilized Fe(III) affinity chromatography columns for the enrichment of the phosphopeptides. The mass spectra were recorded in linear and reflection modes using a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA) and observation of masses ranging from m/z 200 to 4000.

For the identification of protein bands through peptide fingerprint analysis, similar procedures were applied. In brief, 6×His-AcD or GST–Apobec1 bands were cut from Coomassie-stained SDS–PAGE gels and submitted to tryptic digestion in gel. Resulting peptides were submitted to analysis on a MALDI- TOF-MS, as described above.

Pull-down assay. GST–Apobec1 or control protein GST was coupled to glutathione sepharose beads. After washing, beads were incubated with 6×His-AcD or 6×His-AcD-P, washed again, and then eluted with Tris–Cl 20 mM, glutathione 20 mM, pH 8.0. The supernatants were boiled for 5 min in SDS-loading buffer. Bound proteins were separated by SDS–PAGE, stained with Coomassie blue or transferred to a PDVF membrane, analyzed by Western blot using immuno-chemiluminescence detection. Primary antibodies were: mouse anti-GST antibody 5.3.3 (for control of equal loading of beads) [30] or mouse anti-5×His monoclonal antibody (Qiagen). As secondary antibody we used anti-mouse IgG-HRP conjugate.

Molecular dynamics simulation. Energy minimization calculations, molecular dynamics simulations, and trajectory analysis were performed using the Gromacs program [31] running on a LNCC4 processor. Molecular visualization was performed in an Insight II graphical environment (Accelrys) operating on an Octane 2 workstation (Silicon Graphics). For the system setup the model with the lowest energy and best Procheck parameters among the 40 models generated was used as the starting coordinates. The in silico phosphorylation was performed using the Biopolymer module implemented in the InsightII package. Apo- and phosphorylated forms of the AcD model were immersed in a rhombic dodecahedron box filled with 3409 simple point charge (SPC) water molecules [32] and neutralized by addition of the necessary number of chloride counter ions. Simulation parameters: The calculations were performed using the Gromacs "all-hydrogen plus phospho protein" force field. The molecular dynamics simulation for both apo- (AcD) and phosphorylated (AcD-P) models was performed using periodic boundary conditions with a cut-off radius of 0.8 nm for short-range interactions and by updating the neighbor pair list every 10 steps. Long-range electrostatic interactions were treated by the "Particle Mesh Ewald method" [33]. The

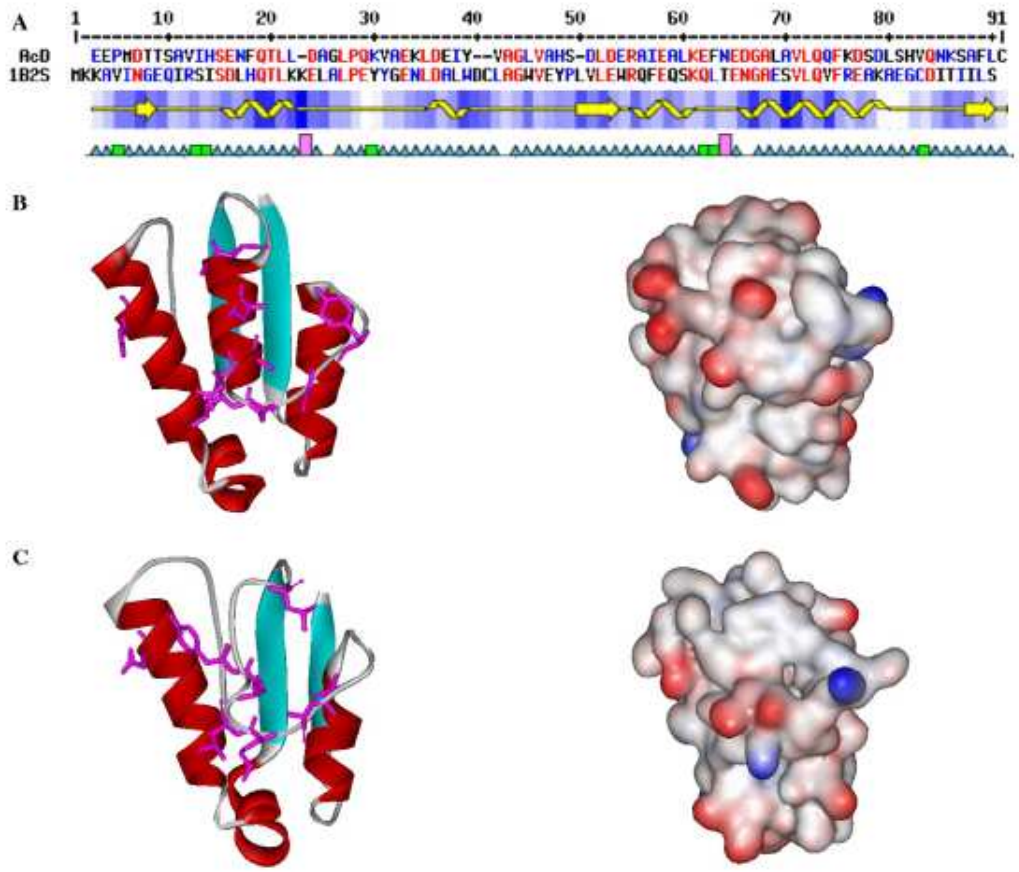


Fig. 2. Molecular modeling of the acidic domain of hnRNPQ. (A) Alignment of the amino acid sequences of the hnRNPQ acidic domain and the bacterial protein Barstar with a secondary structure analysis of the modeled domain created by the Procheck program shown below. In the background of the upper part the accessibility of each amino acid is given between shades of completely buried regions (dark blue) to accessible regions (white). The last line represents the region in the Ramachandran plot occupied by each residue: triangles are in favourable regions, small columns in allowed regions and large columns in generously allowed regions. No residues were present in disallowed regions. (B) Views of the structure of Barstar. Left: ribbon structure depicting amino acids responsible for the interaction with Barnase in purple. Right: accessible surface showing four negatively charged amino acids in red. (C) Model of hnRNPQ acidic domain. Left: ribbon representation with selected conserved amino acids (purple) that correspond to those of Barstar shown in (B), left side panel. Right panel: region with three negatively charged amino acids labeled red that correspond to those of Barstar indicated in (B), right side panel.

"LINCS" algorithm "SETTLE" [34] was employed to constrain the water geometries. A time step of 2 fs was chosen for integrating the equations of motion. Simulation protocol: Initially, relaxation of solvent and ions was performed through 500 steps of the steepest descendent energy minimization algorithm, while keeping the backbone of the models fixed. After removing any positional restraints, the system was submitted to another short minimization step that included the model-derived distance restraints, applied to a constant force of 10,000 kJ/mol/nm^2. Therefore, the system was equilibrated during a 200 ps step of molecular dynamics (MD) simulation with identical restraints, at 308 K, followed by another 800 ps step, using a reduced force constant of 2000 kJ/mol/nm^2. After equilibration, two consecutive MD simulations were carried out. In the first 1 ns MD simulation of the AcD models, the restraints were applied with a force constant of 2000 kJ/mol/nm^2. The second MD simulation was carried out without restraints for 5 ns. The structures were collected every 10 ps during the equilibration step and every 0.1 ps during the remainder of the simulation. Simulation analysis: The root mean square deviation (RMSD) was calculated for each structure collected during the simulations for both the apo- and phospho-forms of the model. A pairwise RMSD for the backbone atoms was also determined, where each structure in the trajectory was compared with respect to all other structures (data not shown). Finally, the root mean square fluctuation (RMSF) of the backbone atoms was computed on a per residue basis.

Far UV circular dichroism spectroscopy. CD spectra were acquired using a J-810 circular dichroism spectropolarimeter (Jasco) coupled to a Peltier Jasco PFD-425 S system for temperature control. Spectra were recorded in the range 205–260 nm and averaged over five scans at 20 °C, with a quartz cuvette of 1 mm path length. 0.5 nm step resolution, 50 nm/min speed, 4 s response time, and 1 nm bandwidth were used. The sample concentration was 2 mg/ml in 50 mM Tris–HCl, 100 mM NaCl, pH 8.0. Following baseline correction by subtracting the blank spectra, the observed ellipticity, θ (mdegree), was converted to the molar mean residue ellipticity [θ] (degree cm^2 dmol^{-1}). All buffers were filtered before use to avoid scattering from small particles.

Fluorescence spectroscopy. The experiments were performed with an Aminco Bowman^R Series 2 (SLM-Aminco) spectrofluorimeter equipped with a 450W lamp. Experiments were carried out at 25 °C in PBS buffer, pH 7,4. The recombinant proteins were analyzed at concentration of 0,5 mg/ml. The intrinsic Tyrosine fluorescence was investigated with an excitation wavelength of 283 nm and an emission wavelength of 306 nm using a spectral band pass of 4 nm for both excitation and emission.

Results and discussion

ACF has significant amino acid sequence similarity to hnRNPQ but lacks the acidic domain

When we searched sequence databanks for proteins with sequence similarity with human hnRNPQ (NSAP1/Gry-Rbp) we were able to identify the protein ACF, which shows ~50% amino acid sequence similarity (and ~34% amino acid sequence identity) and is functionally involved in mRNA editing processes (Fig. 1). The alignment of both amino acid sequences shows that the main difference between the two proteins is located in their N-terminal region, since only NSAP1/hnRNPQ contains the so-called acidic domain (AcD), which is lacking in ACF (Fig. 1B). Nonetheless, NSAP1/hnRNPQ can be grouped in the ACF gene family [10].

HnRNPQ is listed as a unique sequence in GenBank database (Accession No. AAK59703). However, database searches revealed the presence of expressed sequence tags (ESTs) coding for two additional, alternatively spliced forms of NSAP1/hnRNPQ. The isoforms of the protein have been consequently named hnRNPQ1-3 (Accession No. AY034483, AY034482, and AY034481, respectively). All hnRNPQ isoforms have five domains and mediate pre-mRNA splicing. The N-terminal presents a region with several acidic amino acids and therefore has been called "acidic domain" (AcD). This domain has no sequence differences between the three isoforms and its function has not been assigned. It is followed by three consecutive RNA recognition motifs (RRMs) (also known as RNA binding domains RBD), a putative nuclear localization signal (NLS), an unusually large ~120 amino acid long arginine–glycine rich domain (RGG box), a second putative NLS and an ~40 amino acid in the C-terminal region, rich in glutamine and asparagine residues [9].

Modeling of hnRNPQ acidic domain

The alignment shown in Fig. 1B called our attention to the possible importance of the acidic domain in hnRNPQ. In order to get first clues about the structure and function

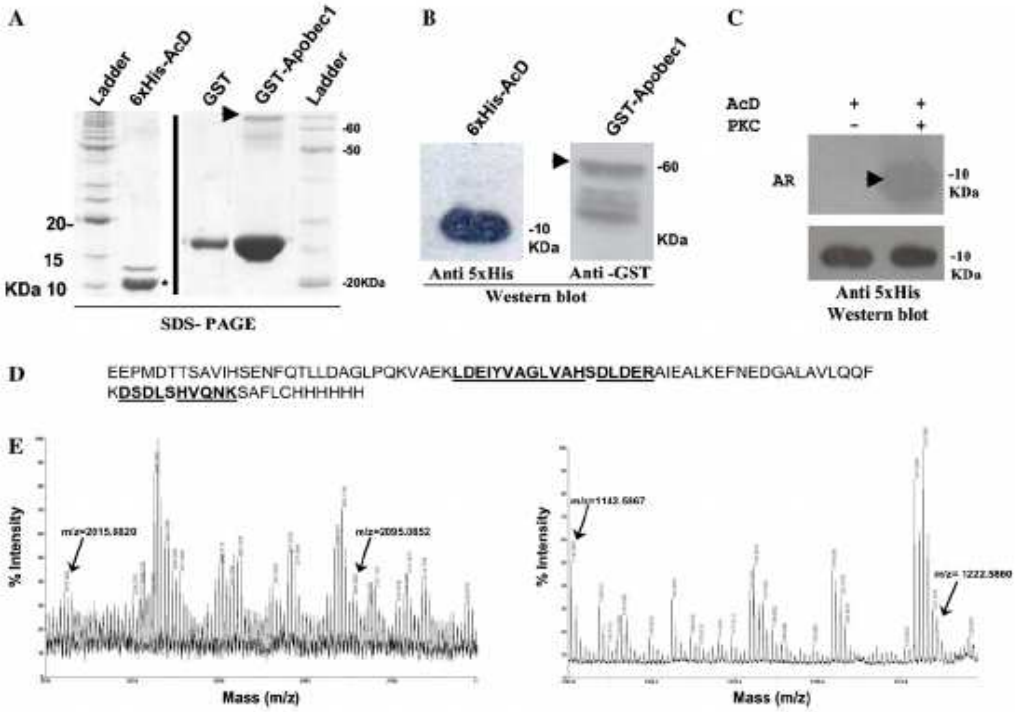


Fig. 3. Expression and purification of 6×His-AcD and GST–Apobec1 and *in vitro* phosphorylation of 6×His-AcD by PKC with subsequent analysis by mass spectrometry. (A) Purification of recombinant GST–Apobec1, GST, and 6×His-AcD. SDS–PAGE analysis of peak fractions after affinity purification. The asterisk (*) marks 6×His-AcD as identified by mass spectrometry analysis. The arrow head (►) indicates the full length GST–Apobec1 protein, also identified by mass spectrometry. (B) Western blot analysis of 6×His-hnRNPQ-AcD (left) and GST–Apobec1 (right) with indicated antibodies. The arrow-heads (►) mark the protein bands, which correspond to the bands with the same molecular masses of 6×His-AcD and GST–Apobec1, observed in the SDS–PAGE in (A). (C) *In vitro* phosphorylation of 6×His-AcD by PKC-Pan. Upper panel: the radiolabeled protein was detected by autoradiography (AR). Lower panel: equal loading of radioactive labeled and non-labeled protein was demonstrated by anti-6×His Western blot. (D, E) Mass spectrometry analysis of *in vitro* phosphorylated AcD. (D) Amino acid sequence of the recombinant hnRNPQ-AcD with underlined peptide fragments obtained by trypsin digestion. Two serine residues predicted for phosphorylation by PKC (S44 and S75) are embedded in these peptide regions. (E). Mass spectra of *in vitro* phosphorylated 6×His-hnRNPQ-AcD, digested for 16 h with trypsin at 37 °C. Right panel: identification of the predicted peptide masses that contains S44 in apo- and phospho-forms: ($m/z = 2015.08$ LDEIYVAGLVAHSDLDER, and $m/z = 2095.08$). Left panel: Identification of the predicted peptide mass containing S75 in both apo- and phospho-forms ($m/z = 1142.58$ DSDLSHVQNK and $m/z = 1222.58$).

of the AcD domain, we decided to analyze its primary amino acid sequence with the intuition to perform homology modeling. In previous studies Mourelatos and co-workers had identified the acidic domain [9].They defined the domain as consisting of 119 amino acids, spanning from Q12 to R130 of the hnRNPQ amino acid sequence. We found a good match of the alignment in a region of 85 amino acids length, ranging from Q12 to C96, with the protein Barstar from *B. amyloliquefaciens*. Barstar is a protein of 90 amino acids in length, which strongly interacts with and inhibits the *B. amyloliquefaciens* protein Barnase, an extracellular ribonuclease of 110 amino acids length. The binding of Barstar to Barnase is pH-dependent, mediated through hydrogen bonds and salt bridges, and results in the complete inhibition of the ribonuclease activity of Barnase [21]. The sterical inhibition of Barnase is mediated by a flexible loop of Barstar, which contains a short α-helical segment. Although Barstar has not been shown yet to be able to edit mRNA, its use as a template can be justified through its functional relatedness, since the regulatory protein pair Barstar/Barnase is involved in RNA binding and degradation. In this context it is noteworthy that the binding of Barstar to Barnase, and not to the target RNA, inhibits the RNA binding and degradation by Barnase [21].

After these initial analyses, we decided to model the acidic domain of hnRNPQ using the atomic coordinates of Barstar (1B2S) as a template [21]. The amino acid sequences of Barstar (1–90) and hnRNPQ (12–96) share an identity of 27% and a similarity of 37% (Fig. 2A and B). Over 40 models of the AcD were generated, using the program Modeller, and subsequently analyzed for stereochemical quality by using the programs Verify_3D, What_If, and Procheck (Fig. 2B). The generated model of the AcD of hnRNPQ showed the same topology as the template used: β-α-α-β-α-α-β. With the intention of clarity we number the amino acids from here on forward according to the alignment from Fig. 2A.

The principal amino acid residues involved in the binding of Barstar with Barnase are Y31, G32, N34, L35, D36, D40, A43, and G44, which are located in a flexible loop that contains an α-helix, and E77 (Fig. 2C). Our generated model of AcD also contains this loop region, however only with a short α-helical segment. The alignment in the flexible loop region between the two amino acid sequences (Fig. 2A) shows an identity of 44% and involves residues K27 to G39 and the amino acid D71 in the AcD (Fig. 2D). This region in the AcD is similar to that in Barstar with the following conserved residues: K27, A29, K31, L32, D33, A38, G39 and D71. When we analyzed the surface of the two proteins, we could observe that both have an extended area with negatively charged amino acids: four in Barstar; three on the surface of the AcD model (Fig. 2B and C). This region L27–P49 of Barstar is localized in a chemical environment comparable to the region D21–S44 of the acidic domain of hnRNPQ and both regions are rich in negatively charged amino acids. The amino acids D36, D40, E77, and E81 of Barstar correspond to the following ones in the acidic domain of hnRNPQ: D21, E30, D33 and D71, where the underlined amino acids are actually part of the "flexible loop".

Another interesting feature of the modeled structure of the hnRNPQ-AcD is the surface exposition of several hydrophobic amino acids, which most interestingly can also be found on the modeled structure of Apobec1, which had been kindly provided to us by Dr. Joseph Wedekind (University of Rochester; Department of Biochemistry and Biophysics) [35]. Furthermore, the Apobec1 model showed a region rich in basic amino acids. It may be speculated that the negatively charged surface of hnRNPQ-AcD contacts the positively charged region of Apobec1 in the complex of both proteins. The hydrophobic patches found in both AcD and Apobec1 on the other hand are very likely to serve as docking sites for the interaction of both proteins or in their interaction with other protein components of the multi-protein RNA editing complex.

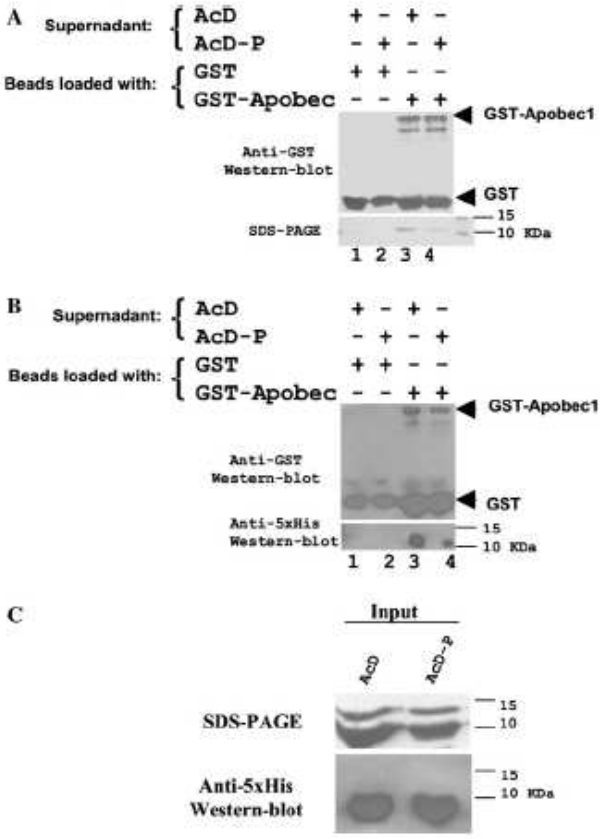


Fig. 4. *In vitro* pull-down assay between 6×His-AcD (AcD) or 6×His-AcD-Phospho (AcD-P) and GST–Apobec1. Gluthathione–Sepharose 4B beads were loaded with GST (control protein) or GST–Apobec1, washed, and then incubated with AcD or AcD-P. (A) Upper panel: Anti-GST Western blot for demonstration of equal loading of gluthathione–Sepharose 4B beads with GST (lanes 1 and 2) or GST–Apobec1 (lanes 3 and 4). Lower panel: SDS–PAGE gel stained with Coomassie Blue. (B) Repetition of the experiment in (A) but this time 6×His-AcD was detected by Western blot using anti-5×His antibody (lower panel). (C) Lanes 1 and 2: Input controls of AcD and AcD-P. Positions of molecular masses of selected marker proteins are indicated in all panels.

Future experiments must address whether the interaction of AcD with Apobec1 has inhibitory or stimulatory effects *in vivo* on the editing process of the ApoB RNA, and how the concerted action of the different proteins found in the editing complex is fine-tuned by protein phosphorylation and possibly other post-translational modifications.

Two predicted serines in the AcD are targets of phosphorylation by PKC in vitro

Early experiments with hnRNPQ had demonstrated that this protein is phosphorylated on serine residues in vivo and also represents a Phospho-CTD associated protein (PCAP) [36]. As studies on other human regulatory proteins have previously shown, post-translational modifications can interfere in protein function by blocking out specific interactions among proteins [28]. Our analysis of the AcD amino acid sequence with the program NetPhos [37] resulted in the prediction that only S44 and S75, among a total of six serine residues, have a high probability of phosphorylation (data not shown).

Analyzing the AcD tertiary structure (Fig. 2) we saw that both of these residues are indeed exposed on the surface of the model and therefore accessible to phosphorylation. In order to study the phosphorylation of the 6×His-AcD by PKC *in vitro*, we expressed and purified the fusion protein 6×His-AcD (Fig. 3A). The identification of the fusion protein was carried out by Western blot with an anti-5×His mAb (Fig. 3B, left panel).

Next we performed the *in vitro* phosphorylation by adding PKC-Pan to the 6×His-AcD (Fig. 3C) and subsequently were able to identify tryptic peptide fragments of the AcD, which contain S44 and S75 as phosphorylated amino acids, by mass spectrometry experiments. The peptides represent AcD domain fragments LDEIYVAGLVAH-$S^{44}(PO_4)$-DLDER (aa 32–49, identified $m/z = 2095.08$) and DSDL-$S^{75}(PO_4)$-HVQNK (aa 71–80, identified $m/z = 1222.58$), with the identified mass values being identical or very close to those predicted (2095.08 or 1222.54, respectively) [38].

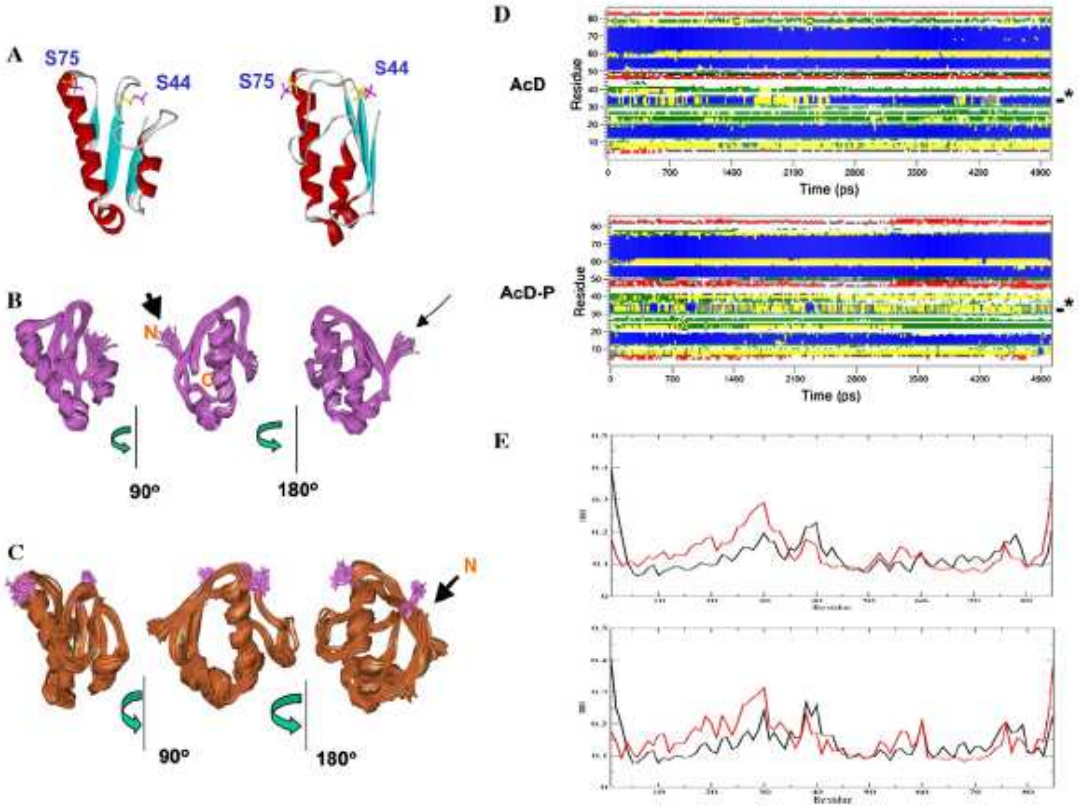


Fig. 5. Molecular dynamics study of hnRNPQ-AcD-6×His after *in silico* phosphorylation. (A) Ribbon representation of the modeled hnRNPQ-AcD depicting two phosphorylated serine residues (yellow) and the attached phosphate groups (purple). (B) Superposition of the 20 structural models of hnRNPQ-AcD taken randomly from the last 1500 ps of the 5000 ps molecular dynamics (MD) analysis. N-terminal regions indicated by black arrows. (C) *In silico* phosphorylation of the S44 and S75. Superposition of the 20 structural models of the acidic domain (phosphorylated form) simulated by (MD) chosen from the last 1500 ps, based on the minimum pairwise rmsd of the peptide backbone residues. (D) Dynamic of the secondary structure elements of the acidic domain in apo (AcD) and phosphor forms (AcD-P) during the 5000 ps time period. The region of hnRNPQ-AcD marked by an asterisks (*) corresponds to a flexible loop region in Barstar. (E) Root-mean-square fluctuation (Rmsf) of the backbone atoms (top) and all atoms (bottom) of the apo (black) and phospho (red) forms of hnRNPQ-AcD.

Furthermore, as a control, we also identified the same peptide fragments in their non-phosphorylated forms with the molecular masses of 2015.08 and 1142.58, respectively (Fig. 3D and E).

Interaction of GST-Apobec-1 with 6×His-AcD is inhibited by phosphorylation of the latter

Next, we carried out pull-down assays with purified recombinant proteins that had been expressed in *Escherichia coli* (GST, GST–Apobec1, and 6×His-AcD) (Fig. 3A and B). Furthermore, in order to understand the possible relevance of phosphorylation of the AcD for the interaction with Apobec1 we performed *in vitro* phosphorylation of 6×His-AcD to generate 6×His-AcD-P (Fig. 3C). As shown in Fig. 4 both 6×His-AcD and 6×His-AcD-P bound to GST–Apobec1, but not to GST. However, the interaction of 6×His-AcD-P with GST–Apobec1 was clearly and reproducibly inhibited in comparison to apo-6×His-AcD. We controlled the equal loading of the Glutathione–Sepharose 4B beads with GST and GST–Apobec1 proteins by performing a Western blot with anti-GST antibody.

In summary, these data seem to suggest that the phosphorylation of the AcD of hnRNPQ may represent an important post-transcriptional modification of hnRNPQ. It may further be speculated that phosphorylation could be a regulatory event for hnRNPQ interaction with other cellular proteins, including Apobec1. Our data seem to suggest that in the latter case, the phosphorylation of hnRNPQ-AcD has a negative effect on the interaction with Apobec1 (Fig. 4). Another group has previously reported that hnRNPQ actually suffers phosphorylation *in vivo* [36] and we demonstrated here by mass spectrometry experiments that the AcD of hnRNPQ can be phosphorylated by PKC *in vitro* on S44 and S75. These data clearly emphasize the likely relevance of hnRNPQ phosphorylation for the regulation of its functions.

In silico phosphorylation and molecular dynamics simulation of apo- and phospho-AcD

An in silico phosphorylation of residues S44 and S75 was performed in order to see if our AcD model would undergo structural changes (Fig. 5). The phosphorylated AcD (AcD-P) showed a increased flexibility of its N-terminal regions as well as of the flexible loop which moves towards the protein core upon phosphorylation (K27 to G39). Most interestingly, this region corresponds to the

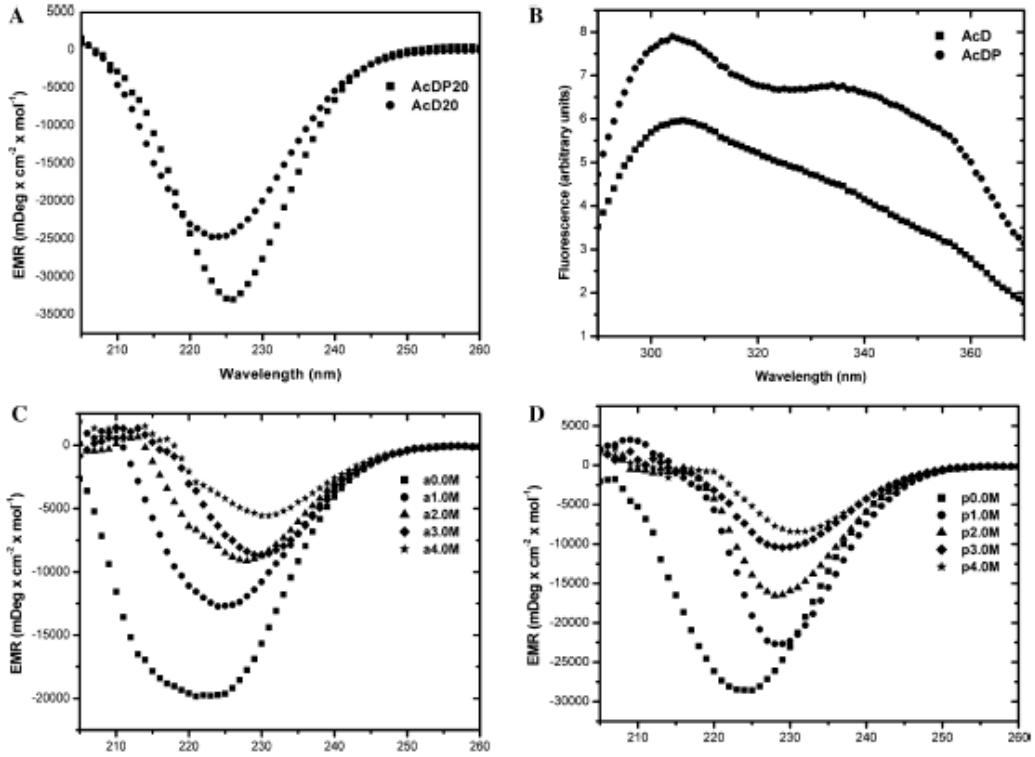


Fig. 6. Analysis of the stability of the apo- and phospho-forms of 6×His-AcD by circular dichroism and fluorescence spectroscopy. (A) Far-UV CD spectra of 6×His-AcD in its apo- (circles) and phospho- (squares) forms in PBS, pH 7.4 at 20 °C. (B) Fluorescence emission spectra of 6×His-AcD (circles) and 6×His-AcD-P (squares) (both at 0.5 mg/ml). The samples were excited at 283 nm and the emission was measured at 303 nm. (C,D) Chemical denaturation circular dichroism spectroscopy experiment using 6×His-AcD (C) and 6×His-AcD-P (D) with the indicated concentrations of guanidine hydrochloride in PBS, pH 7.4, 5% TFE at 20 °C.

flexible loop in Barstar, which is involved in the binding to Barnase from *B. amyloliquefaciens* (Fig. 5C). Inspection of the two proteins secondary structure elements, during the 5 ns of the MD simulation, showed that the largest conformational modifications after phosphorylation are found in a region located between amino acids 30 and 40 (Fig. 5D). This is also shown by an RMSF analysis (Fig. 5E). This is the region corresponding to the flexible loop region in Barstar, suggesting the possible disruption of its α-helix and a subtle overall change in the structure of the loop region after phosphorylation, as evidenced by spectroscopic analysis (Fig. 6A and B).

Conformational analysis of 6×His-AcD and 6×His-AcD-P by circular dichroism and fluorescence emission spectroscopy

To understand in more detail the structural changes in the AcD upon phosphorylation, we performed circular dichroism and fluorescence emission spectroscopy experiments. An analysis of the shape of the far UV CD spectrum, shown in Fig. 6A, suggests that the 6×His-AcD contains predominantly α-helices and some β-sheets. However, the CD spectrum of 6×His-AcD-P showed a more pronounced β-sheet characteristic (Fig. 6A).

The AcD domain contains a unique tyrosine residue (Y29), located in the flexible loop region, which can be used as probe in fluorescence emission experiments. Thus, we compared 6×His-AcD and 6×His-AcD-P by fluorescence emission spectroscopy (Fig. 6B). The phosphorylation induced a blue-shift, an increased overall fluorescence emission, and an increase in the fine structure of the emission spectrum. All of this suggests that the phosphorylation of the AcD results in an increased burial of the tyrosine residue (Fig. 6B). This may contribute to the observed inhibition of the interaction with Apobec1 upon phosphorylation of AcD (Fig. 4).

Temperature induced unfolding studies, using circular dichroism spectroscopy, showed that the AcD seems to represent a rather stable structure, since there were only small changes in the form of the CD spectrum upon heating the sample from 20 to 80 °C (data not shown). The molar ellipticity value of the minima at 224 nm decreased from ca. −24,000 (at 20 °C) to ca. −22,000 (at 80 °C), suggesting only small alterations in the global structure of the AcD upon heating. The chemical unfolding assay with guanidine hydrochloride showed an equivalent loss of the secondary structure for both AcD and AcD-P, suggesting that there is a general loss of α-helices, and maybe also of β-sheets structure (Fig. 6C and D).

Acknowledgments

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3. Resultados

Artigo IV

HnRNP Q interacts with HSP70/BiP and co-localizates with it upon PMA, Tapsigargin and heat treatment in the Endoplasmaic reticulum

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Manuscrito em preparação

HNRNPQ INTERACTS WITH HSP70/BIP AND COLOCALIZES WITH IT UPON PMA, TAPSIGARGIN AND HEAT TREATMENT IN THE ENDOPLASMIC RETICULUM

Short title: Induced HnRNPQ translocation to stress granules

Keywords: protein-protein interactions, stress granules, protein bodies, sub cellular localization

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Abstract

Introduction

The eukaryotic gene expression is regulated on different levels ranging from pre-mRNA processing, mature mRNA export, mRNA turnover, localization, to translation (1). Each regulation step involves various combinations of several RNA binding proteins, which form dynamic multiproteins complexes with the transcript, that differ from the nascent to the mature processes transcript and perform specific funcions (2). In the cytoplasm most of the proteins involved in these processes are frequently found in two specialized structures, known as processing bodies (PBs) and stress granules (SGs), which may be formed in response to specific stimuli (3,4).

PBs have been early identified as cytoplasmic *foci* containing enzymes involved in mRNA decay (5). These cytoplasmic *foci*, previously identified in yeast cells as "processing bodies" (P-bodies) (PBs), were originally thought to function as a small site of mRNA degradation, since related foci (also termed GW, XRN1 or DCP-bodies) (6-10) were identified in mammalian cells. Besides other studies could link these foci to the decay of mRNAs targeted by the RISC complex (11, 12). However, other work showed, that in yeast, the PBs may also serve to store mRNAs under stress conditions. Upon stress release, the stored mRNAs may return to active, translating polysomes (13, 14).

In a similar fashion, stress granules (SGs) have been describes as cytoplasmic aggregates, which contain mRNAs that accumulate in the cells under stress conditions and which present reduced or inhibited translation (4, 15). Under stress conditions, the subunit of the eukaryontic initiation factor 2 (eIF-2) is phosphorylated. This leads to the formation of a ternary complex of eIF-2, GTP, and methionyl-tRNA, which promotes the disassembly of the polysome (16). In response to these events, the SGs are assembled and there is a global repression of the protein synthesis (4). However, the inhibition of the global translation goes together with a selective translation of specific mRNAs that possess internal ribosome entry sites (IRES) (17, 18). In particular, translation mediated by IRES appears to promote the expression of several stress-response genes, which either promotes cell survival or cell death depending on the kind, severity and duration of the stress (19, 20). The key protein for the formation of the SG´s is TIA1 (cytotoxic granule-associated RNA binding protein), which is also one of the marker proteins for SG. TIA promotes SG formation through aggregation of its C-termini, which functions as a prion-like domain (PRD) and is able to bind to HSP70 (21). SGs and PBs interact in stressed cells (22) and at least some unstable mRNAs may be stabilized in stressed cells (23).

The hnRNP proteins are related to a wide array of RNA related functions, including: transcription, pre-mRNA processing, mRNA transport and translation, and contain at least 20 proteins. HnRNPs form large heterogenic complexes, which precise roles in RNA metabolism need still to be fully defined (24). HnRNPQ (also called SYNCRIP and NSAP1) has been earlier described as a protein which interacts with a non-structural protein from the minute virus of mice [25, 26, 27]. hnRNPQ appears to be a sub-family of hnRNPs, with three protein isoforms called hnRNP Q1–Q3, which are derived from alternative splicing of a single gene. Its smallest isoform Q1 has a molecular mass of 62 kDa, whereas Q2 has 65 kDa, and Q3 about 70 kDa. The

exact molecular functions of each of these three isoforms are still not well understood [24]. In general hnRNPQ has been functionally implicated in several different steps of mRNA maturation. One of the attributed functions for this protein was its association with the editosome complex, through of its interaction with the mRNA editing cytidine deaminase Apobec1 [26-28]. Furthermore, hnRNPQ has been identified to be a component of the SMN-complex (survival of motor neurons), where it interacts with the wild-type form of the protein SMN. It however, ceases to interact with the truncated form of SMN, which is found in spinal muscular atrophy [24, 29]. hnRNP Q proteins also are associated with polysomes and prevent the deadenylation of unstable mRNAs (30-33). Furthermore it has ~80% similarity with hnRNP R and both proteins are components of mRNA granules transported in neuronal dendrites (34, 35). Both proteins are further required for efficient pre-mRNA splicing in an *in vitro* assay (24). Finally, hnRNPQ has been also functionally associated to a complex that regulates the translation of some mRNAs and the degradation of specific mRNAs such as that of c-*fos* and related to the circadian rhythm [37, 38]. The association to the latter context is emphasized by the finding that hnRNPQ has been described to interact with the protein AUF1 [39], which is an important element for the destabilization of AU-rich mRNAs [40]. Here, we report that the sub-cellular localization of the human protein hnRNP Q can be modified under specific stress conditions, including PMA and tapsigargin treatments as well as heat shock. Furthermore, our results suggest that hnRNP Q interact to HSP70, GW182 and TIA-1 and colocalizes with the latter in PBs and SGs.

Materials and Methods

Plasmids construction

For the generation of the construction pGEX4T1-hnRNPQ(1-443), a DNA fragment encoding amino acids 1–443 of hnRNPQ was amplified via polymerase chain reaction (PCR) from the library of fetal human brain (Clontech) by using of the oligonucleotides hnRNPQDRGGbox-S-XhoI (CGATCTCGAGATGGCTACAGAACATGTTAAT GGAAATGG) and hnRNPQDRGGbox-AS-NotI (CTTTGCGGCCGCCTACTTCCACTG TTGCCCAAAAGTATC), purified and cloned into the SalI/XhoI sites of the pGEX4T1 vector. For the generation of construction pET28a-HSP70-1A see reference [41].

Protein expression

Expression and purification of recombinant proteins. To generate the construction GST-hnRNPQ(1–443), the cDNAs were amplified by PCR and inserted into pGEX4T1 bacterial expression vector as described above. The recombinant plasmid pGEX4T1-hnRNPQ (1-443) was co-transformed with pRARE vector into the *E. coli* strain BL21 (Stratagene). The protein expression was induced by 0,5 mM IPTG for 6 h at 37 °C. Protein affinity purification was performed using glutathione–Sepharose 4B (GE Healthcare). To generate the protein fused by 6xHis tail, the cDNA encoding HSP70 was cloned into the pET28a vector (Novagen). The

recombinant plasmid pET28a-HSP70 was transformed into the BL21 strain, without pRARE vector. The protein expression was induced by 0,5 mM IPTG for 6 h at 37 °C. Protein affinity purification was performed using Ni-NTA-Sepharose (Amersham).

***In Vitro* Phosphorylation**

Purified recombinant protein GST-hnRNPQ(1-443) (1 mg/ml) was phosphorylated in kinase buffer (20 mM Tris; 10 mM $MgCl_2$; 20 mM ATP; 2,5 mCi γ^{32}P-ATP at pH 7,5), using 50 ng of commercial PKC (alfa, delta and zeta) fused to 6xHis tail and radioactive ^{32}P-ATP in a final volume of 50 µl at 30 °C, for 30 min, as described before for another protein substrate [42]. In the reaction containing PKCα we added 100 nM of PMA plus 1,7 µM of $CaCl_2$ and 100 nM of PMA were added to the reaction with PKCδ. Radioactively labeled proteins were then run out by SDS–PAGE. The gels were stained with Coomassie blue, dried, and exposed to X-ray film.

Pull-down assay

The constructions 6xHis–HSP70(SBD) and 6xHSP70 and the control protein 6xHis-DRAP1 were coupled to Ni-NTA sepharose beads. After washing, beads were incubated with free GST or GST-hnRNPQ in the presence of ATP and ADP. The beads were washed again, and then eluted with PBS + Imidazol 500 mM. The supernatants were boiled for 5 min in SDS-loading buffer. Bound proteins were separated by SDS–PAGE, stained with Coomassie blue or transferred to a PDVF membrane, analyzed by Western blot using immuno-chemiluminescence detection. Primary antibodies were: mouse anti-GST antibody 5.3.3 (for control of equal loading of beads) [43] or mouse anti-5xHis monoclonal antibody (Qiagen). As secondary antibody we used an anti-mouse IgG-peroxidase conjugate.

Immunoprecipitation and Immunoblotting

In vivo binding assay. Hela cells (1.10^7 x cells) were collected and lysed in 1 ml 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). Lysate was treated with DNAse (Promega) and cleared at 14.000 xg at 4oC for 15 min. Next, 20 µl of protein A–Sepharose (for polyclonal antibody) or G–Sepharose (for monoclonal antibody) beads (Pharmacia) were loaded with the antibodies, washed with lysis buffer, and incubated or not with the indicated lysates for 2 h at 4 °C. After further three washes with lysis buffer, the beads were re-suspended in SDS–PAGE loading buffer and analyzed as described above.

Cell Cultivation and Stress-induced assay

HeLa cells were cultivated in Dulbecco's modified Eagle's medium - DME (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin/G-streptomycin (Biochrom, Berlin, Germany) and 10% heat-inactivated fetal bovine serum (Biochrom) at 37 °C and 5 % CO_2. For stress-induced assays, cells were grown until a confluency of 50 - 70 % on glass cover slips and treated with chloramphenicol (40 µg/ml) and cylohexamide (100 ug/ml) for 4h, in order to inhibit translation; PMA (phorbol 12-myristate 13-acetate) (400 nM) for 3h, for PKC pathway activation; actinomycin D (5 µg/ml) for 1 h for transcription stop; tapsigargin (2 µM) for 4h, to induce ER stress or Arsenite (0.5 mM) for 30min, with additional 30 min for the recovery from Arsenite treatment.

Preparation of Cell extract

Hela cells were washed twice with ice-cold PBS and then suspended in "low salt buffer" (LSB) (10 mM Tris-HCl, pH 7.4, 320 mM sacharose, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 0.5 mM DTT, 0.4 % NP-40) for 12 min on ice and centrifuged for 5 min at 800 xg at 4°C. . The supernatant was called cytoplasmic fraction. In order to obtain the nuclear fraction, the pellets (nuclei) were then resuspended in 300 ul of "high salt buffer" (HSB) (25 mM Tris-HCl, 250 mM NaCl, 1 mM EDTA, 1% NP-40, pH 8.0) and incubated for 12 min on ice. After incubation, they were centrifuged at 20.000 x g for 10 min at 4 °C. Then the cytoplasmic and nuclear fractions were run out on a SDS-PAGE gel.

Immunofluorescence microscopy

After treatments, cells grown on cover slips were washed with phosphate-buffered saline (PBS), fixed in 2% paraformaldehyde in PBS for 30 min at 25 °C, and then permeabilized for 10 min in PBS + Triton 0,3 % at 25 °C. After three washes in PBS of 5 min each, the cells were treated with PBS + 100 mM of Glicine for 5 min at 25 °C. After three washes the cells were incubated in blocking buffer (BSA 1 % in PBS) for 1 h at 25 °C. Cells were then exposed, after five more washes, to primary antibodies (e.g. mouse anti-hnRNPQ monoclonal, rabbit anti-BiP polyclonal, goat anti-GW182 polyclonal and goat anti-TIA1 polyclonal all diluted in blocking buffer) for 2 h at 25 °C, followed by five washes in PBS. Cells were then incubated in secondary antibody (e.g., donkey anti-goat rhodamine-conjugated, bovine anti-mouse FITC-conjugated, and mouse anti-rabbit rhodamine-conjugated) containing DAPI diluted in blocking buffer and incubated for 1h. Finally cells were then washed five times in PBS for 5 min, mounted with Fluoromouting reagent, and analyzed with a fluorescence microscope (Eclipse E600, Nikon).

Results

hnRNPQ was phosphorylated by PKC

Previous works were able to identify hnRNPQ as a protein that specifically interacts with polysomes in 3T3-L1 adipocytes cells. Furthermore, hnRNPQ was identified as a phosphorylation target of "Insulin receptor tyrosine kinase" (IRK). This receptor may phosphorylate some tyrosines that flank the RGG-box, presented in its C-terminal region. After the receptor activation and phosphorylation, there was a considerable reduction in the amount of hnRNP Q linked to polysomes (35). Furthermore, we showed that the acidic domain (AcD) present at its N-terminus could be *in vitro* phosphorylated by PKC-Pan promoting a considerable reduction in the interaction between AcD and Apobec1 (28). It is worth noting that the protein kinase C (PKC) family consists of several isoforms, which contain at least two functional domains: the N-terminal regulatory domain and the C-terminal catalytic domain, which may or may not be regulated by Ca^{+2} or phospholipids.

We set out to investigate which PKC isoform could specifically phosphorylate hnRNPQ *in vitro*. Thus, we performed an *in vitro* phosphorylation essay using GST-hnRNPQ(1-443) and three different PKCs (α, δ and ζ) (Fig. 1A). The results showed that PKCδ most efficiently phosphorylated GST-hnRNPQ(1-443), whereas PKCα and PKCζ showed only moderate activity. We also analyzed the recombinant protein 6xHis-FEZ1 (49) as a positive control (Fig. 1B) and free GST, as a negative control (data not shown). Furthermore, we performed the phosphorylation reaction without recombinant protein to show that there was no significant auto-phosphorylation by PKC under these conditions (Fig. 1B). To evaluate if the interaction between PKCδ and hnRNPQ occurred also *in vivo*, we performed a co-immunoprecipitation of hnRNPQ and PKCδ. PKCδ was immuno-precipitated from extracts of Hela cells treated or not with PMA, resolved by SDS-PAGE and probed with an anti-hnRNPQ antibody. We observed that PKCδ interacted with hnRNPQ in both the presence and absence of PMA (not shown). These data suggest that function and localization of hnRNPQ may be regulated by phosphorylation through PKC. It is already known that several proteins that interact with the regulatory domain of PKC isoforms have emerged to be the determinants for the subcellular localization of these PKC isoforms (46, 47, 48). On the other hand the phosphorylation through PKC may regulate the sub-localization of the protein-targets (43).

The activation of PKCs pathways can modify the sub-cellular localization of hnRNPQ

HnRNPQ is predominantly a nuclear protein (24). However, it was also identified in cytoplasmic granules that consist of large complexes of mRNA-binding proteins, which may be transported by cellular motors, such as proteins of the kinesin superfamily (KIFs) (50). Furthermore, both lack of Arginine methylation by PRMT and its tyrosine specific phosphorylation by insulin receptor can re-distribute hnRNPQ from the nucleus to the cytoplasm (30, 45).

To investigate, whether the cellular localization of hnRNPQ could be modified by the activation of PKCs, Hela cells were treated with PMA (Fig. 2). There was a clear shift in its localization from the nucleus to the cytoplasm when the HeLa cells were treated with PMA. Curiously, there was also the formation of small granules spread throughout the cytoplasm. In order to avoid the translation of new proteins, the cells were also treated with chloramphenicole and cyclohexamide. The results of the control experiments showed that the inhibition of translation alone or the treatment with the PKC inhibitor $Ro_{(32-0432)}$ did not make a considerable difference in the localization of hnRNPQ.

We further performed a fractionation of cytoplasmic and nuclear fractions of both untreated and PMA treated cells (not shown). We were not able to identify a visible difference in the Western blot of the PMA treated versus non-treated cells. This could suggest that the cytoplasmic spots detected may represent endoplasmic reticulum, which is a continuation of the nuclear envelope membranes.

hnRNPQ co-localized with BIP, a specific ER protein

We reasoned that hnRNPQ could be localized in the endoplasmatic reticulum (ER) or close to it, since we could not identify it in the supernatant, cytoplasm fraction, despite the fact that the immuno-fluorescence had clearly shown a granular cytoplasmic staining. Therefore, we addressed its cellular co-localization with an ER specific "marker" or BiP (Binding Protein, which belongs to the Hsp70 family) to further characterize the nature of the observed hnRNPQ granules, which were formed after PMA treatment. Moreover, recent work demonstrated that hnRNPQ has an essential role in the translation of BiP mRNA, by its strong binding to an IRES (*Internal Ribosomal Entry Site*), which is found in its un-translated region (5´-UTR) (51). To evaluate this, a co-immunofluorescence was performed between hnRNP Q and BIP, a protein that has been identified as an immunoglobulin heavy-chain-binding protein that also binds transiently to several nascent, wild-type secretory and transmembrane proteins and permanently to misfolded proteins that accumulate in the endoplasmic reticulum (ER) (52, 53). Many roles for the BiP have been proposed, such as: the assembly of nascent proteins and the mediation of proper folding (51, 54). The BiP protein, also known as glucose-regulated protein 78 (GRP78), is a member of the heat shock protein 70 (HSP70) family (55).

The data show that in untreated Hela cells as much as in cells with the translation inhibited, both proteins did not co-localize in the cytoplasmic granules (Fig. 3). After PKC activation, however, both proteins co-localizated in evidently the same perinuclear spots, which we saw previously (Fig. 2). In summary, this may suggest that hnRNPQ could interact, under specific conditions, not only with the mRNA of BiP, but also with the protein BiP, in the ER.

Human Hsp70-1A, protein which was cloned, purified and characterized[41], is 64% identical to its homologue BiP[56]. Proteins from Hsp70 family have structural and functional conservation: . the N-terminal region presents an ATPase domain, which binds to and hydrolyzes ATP, and the C-terminal region presents a substrate binding-domain (SBD) (Fig. 4A), which contains a conserved signature EEVD motif, that interacts

with other chaperones, including HSP40 and HSP90 [57]. This motif also regulates the repair of other proteins (58) (Fig. 4A). Hsp70-1A and BiP are more than 80% identical in the C-terminus.

In order to test if the interaction between hnRNPQ and HSP70 occurs *in vitro*, we performed a semi in vivo pull-down assay with purified recombinant proteins. For this, 6xHis-HSP70(SBD) was immobilized on Ni-NTA sepharose beads and Hela cell extracts were incubated with loaded or non loaded beads. We observed that hnRNPQ and HSP70 interacted (data not shown). The C-terminal of hnRNP Q presents a RGGbox, rich in charged amino acids and believed to be an unfolded region (Fig. 5A).

To further map the interaction between HSP70 and hnRNPQ, we decided to use the recombinant constructions GST-hnRNPQ (1-443) and 6xHis-SBD-HSP70 in an *in vitro* pull down assay (Fig. 4), using free GST as a control protein. The data clearly showed an interaction between GST-hnRNPQ(1-443) and the 6xHis-HSP70(SBD) (Fig. 4B,C). Both phosphorylated and un-phosphorylated forms of GST-hnRNPQ(1-443) interacted with 6xHis-HSP70(SBD). This suggests that this interaction was independent of phosphorylation (Fig. 4C). Moreover, GST-hnRNPQ(1-443) was sufficient to bind to the SBD domain, even without the supposed unfolded C-terminal region containing the RGG-box. Furthermore, we also performed the pull down assay in the presence of ATP or ADP as an additional control. Since our Hsp70 construct did not contain the ATPase domain there were no significant differences in the strength of interactions observed (Fig. 4B).

The hnRNPQ localization is altered by stress

It is already known that the expression of BiP protein is mainly regulated at the level of translation (59, 60) and that the translation of the BiP mRNA increases in poliovirus-infected cells, where translation of most host cellular mRNAs is inhibited (60). Moreover, heat-shock treatment was shown to increase the IRES-dependent expression of the BiP mRNA (61). This behavior suggests that increased BiP expression is stress-dependent. We therefore reasoned that the localization of hnRNPQ could be also modulated by stress conditions.

We therefore set out to induce ER stress by Tapsigargin, known as a specific ER stress inductor. To better control the experiment the Hela cells were also treated them with chloramphenicole and cyclohaxamide to inhibit the on-going *de novo* translation. After 4h of treatment with tapsigargin, our results showed that the hnRNPQ localization is very similar to that previously found upon PKC activation (Fig. 5, 2 and 3). There was however again no difference between the cells only treated with tapsigargin and the cells treated with tapsigargin plus translation inhibitors. These data may suggest that localization of hnRNPQ could be directly altered by ER stress. We may speculate that there may be a dynamic role for hnRNPQ in the light of the stabilization of some mRNA under stressed conditions.

Co-localization of hnRNPQ and BiP after both ER and heat shock stresses

Since hnRNPQ occurred at specific granules after PKC activation and ER stress, we decided to investigate the co-localization of hnRNPQ and BiP under different stress conditions. For this, we induced ER stress with both tapsigargin (Fig. 6A) and heat shock at 42 °C for 1h (Fig. 6B). Both stress conditions were again performed in the presence of translation inhibitors.

We observed a clear co-localization of hnRNPQ and BiP in the cytoplasmatic granules after both types of stresses. Interestingly, both conditions may activate different kinase pathways. It is already known, that under thermal stress a specific kinase is activated, PKR, which works like a temperature sensor and promotes the phosphorylation of eIF2α. PKR can also be activated if there is DNA damage by UV, or by viral infections or oxidative stress (61). On the other hand the ER stress activates a specific kinase known as PERK. This kinase is presented on the ER´s membrane. PERK could be also characterized as an ER stress sensor and is able to phosphorylate eIF2α (61). Curiously, the formation of stress granules (SGs) depends on eIF2α phosphorylation (64) and the pattern of localization of the SGs is similar to that of the hnRNPQ granules observed a after ER stress conditions, heat shock or PKC activation. This may suggest that, under stress conditions hnRNPQ could be translocated to SGs.

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The abbreviations used are: PKC, protein kinase C;

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Figure legends

Figure 1. *In* vitro phosphorylation of the recombinant protein GST-hnRNPQ(1-443). A. The recombinant protein was phosphorylated with different PKCs, which represented the three big family: classical (PKCα), novel (PKDδ) and atypical (PKCζ). Upper panel: Purified recombinant protein was run out on by SDS-PAGE. Lower panel: The radiolabeled hnRNPQ(1-443) was detected by autoradiography (AR). All phosphorylation assays were performed with the indicated kinases and [^{32}P]-ATP. B. Phosphorylation control. The recombinant protein 6xHis-FEZ was used as positive control. Top panel: Purified recombinant 6xHis-FEZ1 and PKC were run out by SDS-PAGE. Lower panel: Radiolabeled 6xHis-FEZ1 was detected by autoradiography. The purified PKCs were also incubated with [^{32}P]-ATP and run out alone (negative control).

Figure 2. Subcellular localization of endogenous hnRNPQ, after cellular activation with PMA or inhibition of PKC pathways by Ro. Hela cells were grown on glass cover slips and stimulated with PMA (400 nM) or Ro (200 nM) for 4h at 37 °C. To inhibit the translation of new proteins, cells were also treated with cyclohexamide (100ug/ml) and chloramphenicol (40 ug/ml). After the indicated treatment the cells were fixed with 2 % of paraformaldehyde in PBS. hnRNPQ was immuno-detected with a primary monoclonal antibody anti-hnRNPQ and a secondary anti-mouse antibody coupledwith Fluorescein protein (FITC) (green). The nuclei were counter-stained with DAPI (blue). Arrow: cytoplasmatic "spot", appearing after PKC activation.

Figure 3. Subcelluar co-localization of hnRNPQ and BiP. A. Co-localization of hnRNPQ and BiP in Hela cells treated or not with cyclohaxamide (100 μg/ml) and chloramphenicole (40 μg/ml). B. Co-localization after PKC pathway activation with PMA (200 nM) for 3h at 37 °C. After treatment, cells were fixed with 2 % of paraformaldheyde in PBS. The proteins were immuno-stained with an primary antibody to anti-hnRNPQ (monoclonal) and a serum against anti-BiP (polyclonal).

Figure 4. *In vitro* interaction between hnRNPQ and HSP70. A. Schematic representation of the domain organization of hnRNPQ and Hsp70. Top: HSP70 organization: ATPase domain (grey) at the N-terminus and the substrate binding-domain (SBD) (yellow, ~40kDa) at the C-terminus. Our construction contains only the SBD domain fused to a 6xHis tag. Full length hnRNPQ (~70kDa) organization: Acidic domain (AcD, blue) at the N-terminus, followed by 3 RNA binding domains (RRM, red) and the RGG-box (yellow) at C-terminus. Our construction, fused to GST (white) and lacking the RGGbox. B. *In vitro* pull-down assay between 6xHis-HSP70(SBD) and GST-hnRNPQ(1-443). Ni-NTA beads were loaded with equal amounts of 6xHis

HSP70(SBD), washed and incubated with GST (negative control) or GST–hnRNPQ(1-443) in the absence or presence of ATP or ADP. Upper panel: Anti-GST Western blot (WB) . Lower panel: Anti-6xHis WB for demonstration of equal loading of the Ni-NTA-Sepharose beads. C. *In vitro* pull-down assay between 6xHis-HSP70(SBD) and phosphorylated or unphosphorylated forms of GST-hnRNPQ(1-443). The construction GST-hnRNPQ(1-443) was previously phosphorylated byPKCpan or PKC□. Ni-NTA beads were loaded with equal concentration of 6xHisHSP70(SBD), washed and incubated with GST (negative control) or apo- or phosphor GST–hnRNPQ(1-443). Upper panel: Anti-GST Western blot (WB) of GST–hnRNPQ(1-443). Lower panel: Anti-6xHis WB for demonstration of equal loading.

Figure 5. Subcellular localization of hnRNPQ under stress induced by tapsigargin. Hela cells were grown on glass cover slips and treated with Tapsigargin (1μM) for 2h. After treatment cells were fixed with 2 % parafhormaldehyde in PBS for 30 min. The hnRNPQ immuno-detection was performed with primary antibody anti-hnRNPQ and secondary antibody, coupled by FITC (green). The nuclei were stained with DAPI (blue). The cells were analyzed with a fluorescence microscope (Nikon). Arrows indicate the cytoplasmic hnRNPQ spots, which appear after ER stress.

Figure 6. Subcelular co-localization of hnRNPQ and BiP after tapsigargin treatment and and heat shock. A. Co-immuno-detection of endogenous proteins under ER stress with tapsgargin (1 μM). Cells were also treated with translation inhibitors. B. Co-immuno-staining of endogenous proteins after heat shock (incubation for 1h at 42 °C). After treatments, cells were fixed and immuno-stained as above. Arrows indicate cytoplasmic hnRNP/BiP spots, which appear after stress conditions.

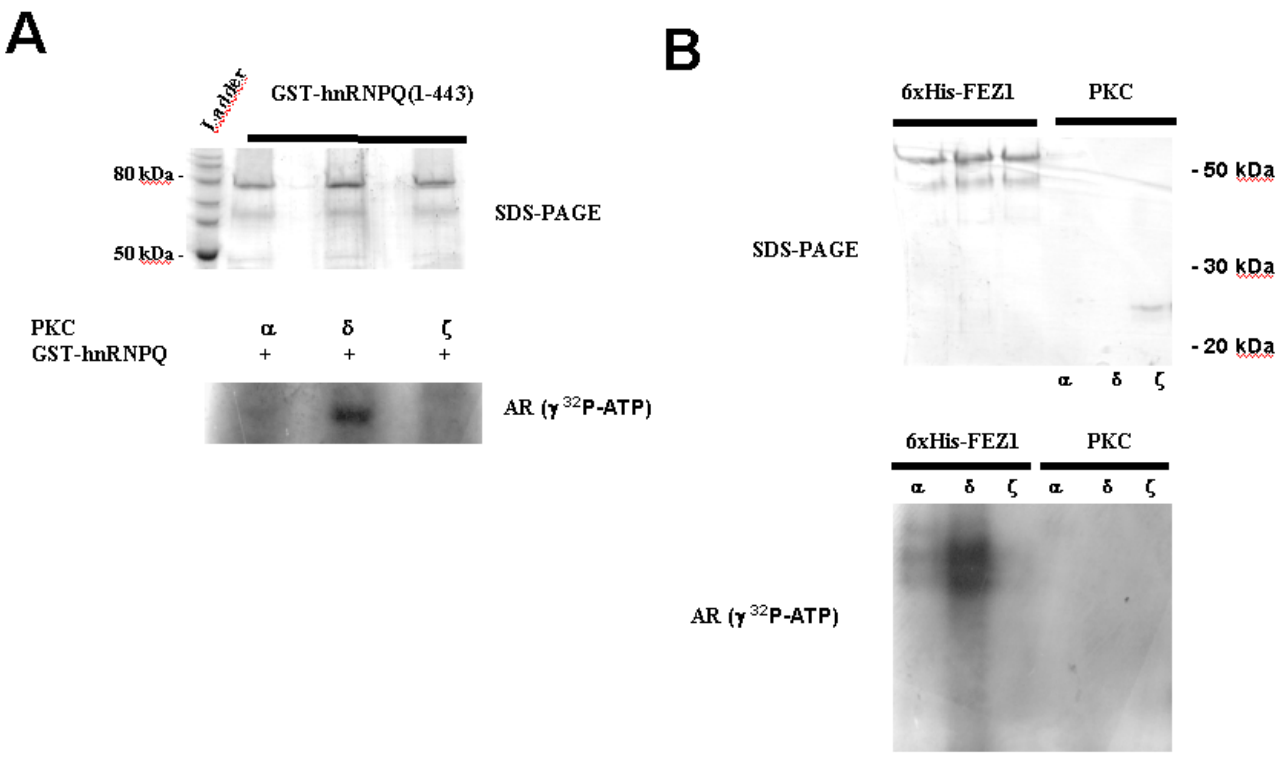
A
Ladder
GST-hnRNPQ(1-443)
80 kDa -
50 kDa -
SDS-PAGE
PKC
α
δ
ζ
GST-hnRNPQ
+
+
+
AR (γ 32P-ATP)
B
6xHis-FEZ1
PKC
SDS-PAGE
- 50 kDa
- 30 kDa
- 20 kDa
α
δ
ζ
6xHis-FEZ1
PKC
α
δ
ζ
α
δ
ζ
AR (γ 32P-ATP)

Figura 1

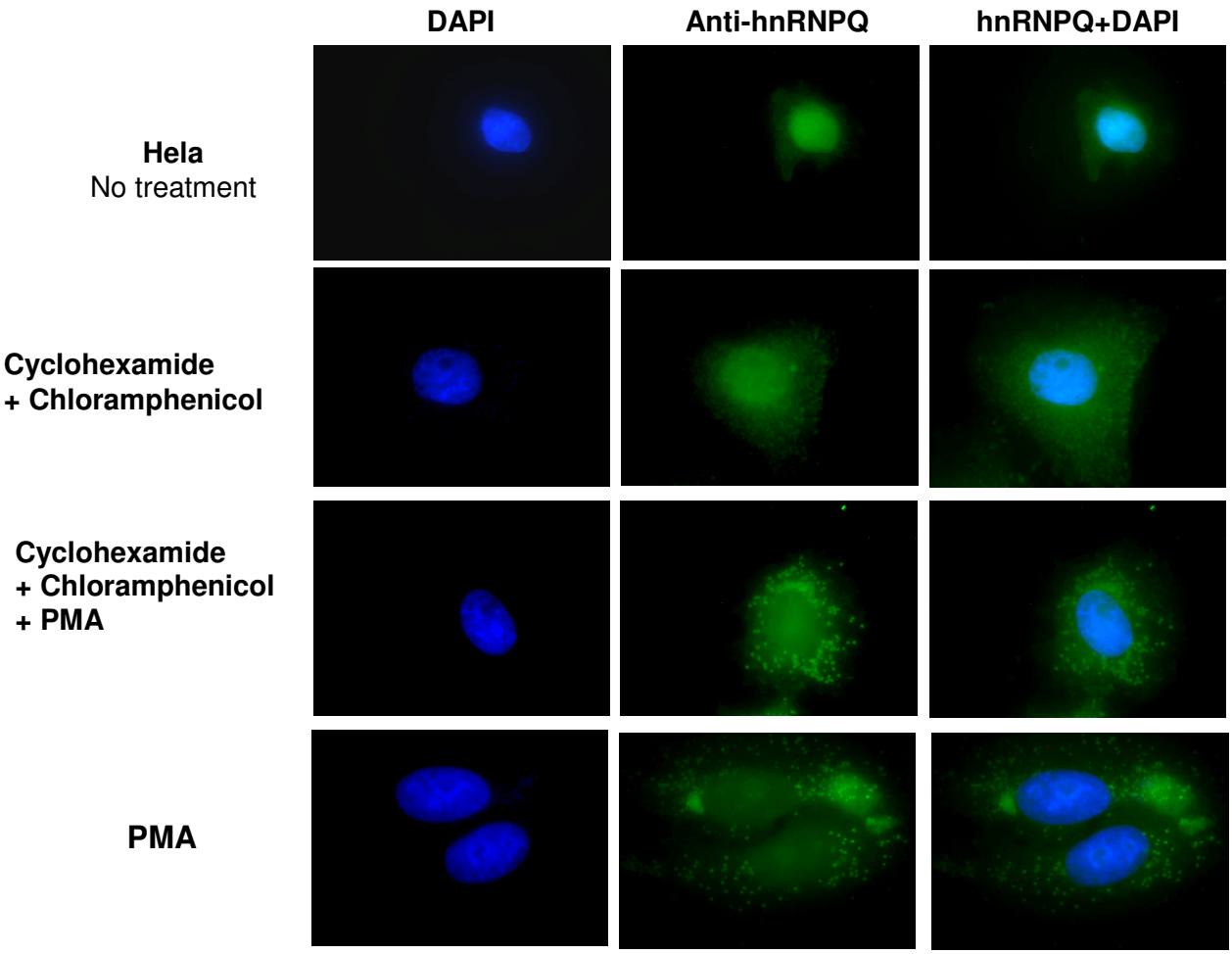
DAPI
Anti-hnRNPQ
hnRNPQ+DAPI
Hela
No treatment
Cyclohexamide
+ Chloramphenicol
Cyclohexamide
+ Chloramphenicol
+ PMA
PMA

Figura 2

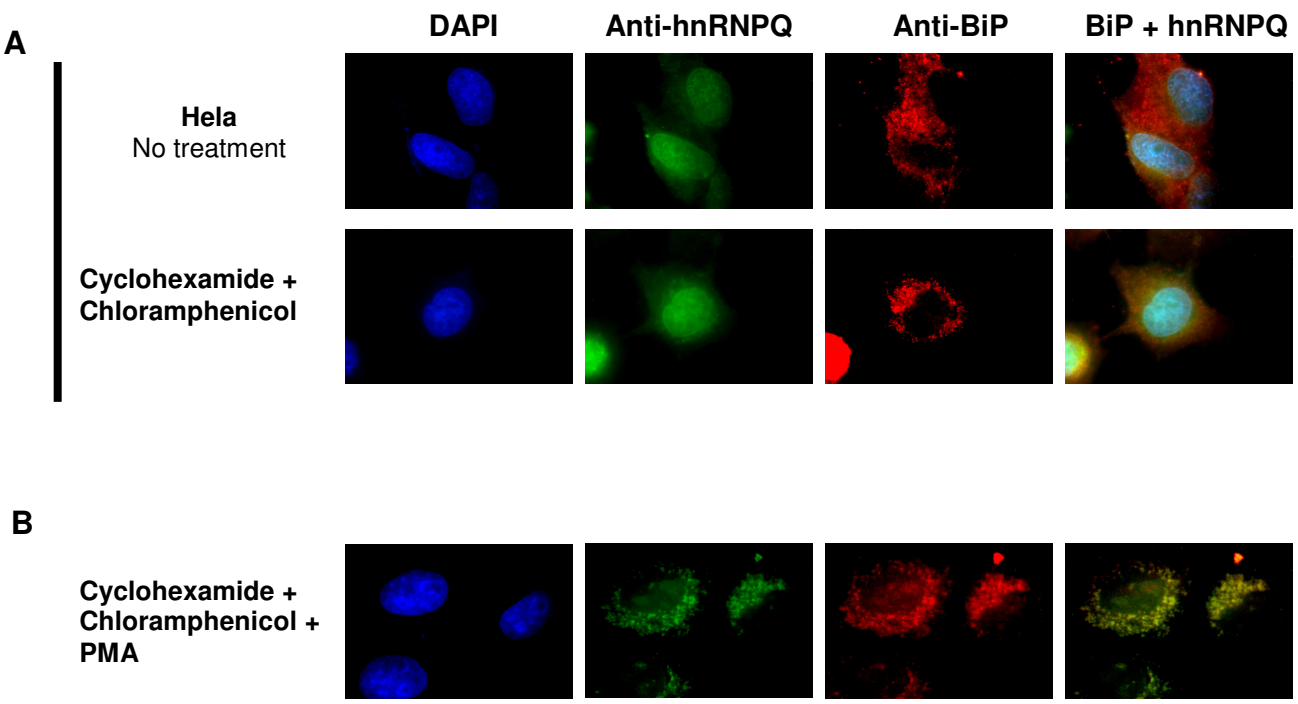
A
DAPI
Anti-hnRNPQ
Anti-BiP
BiP + hnRNPQ
Hela
No treatment
Cyclohexamide +
Chloramphenicol
B
Cyclohexamide +
Chloramphenicol +
PMA

Figura 3

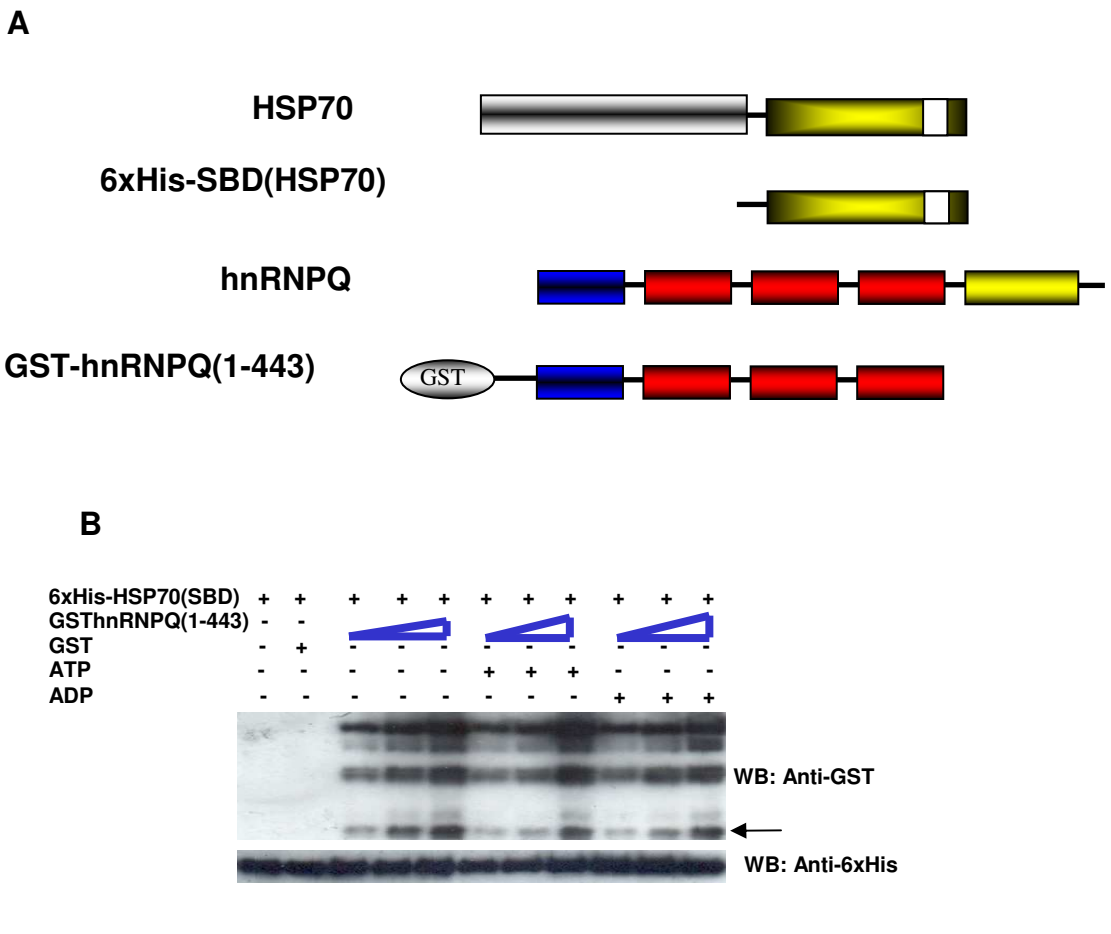
A
HSP70
6xHis-SBD(HSP70)
hnRNPQ
GST-hnRNPQ(1-443)
GST
B
6xHis-HSP70(SBD)
GSThnRNPQ(1-443)
GST
ATP
ADP
WB: Anti-GST
WB: Anti-6xHis
C
PKCPan
PKCδ
6xHis-HSP70(SBD)
GST-hnRNPQ(1-443)
GST
WB: Anti-GST
WB: Anti-6xHis

Figura 4

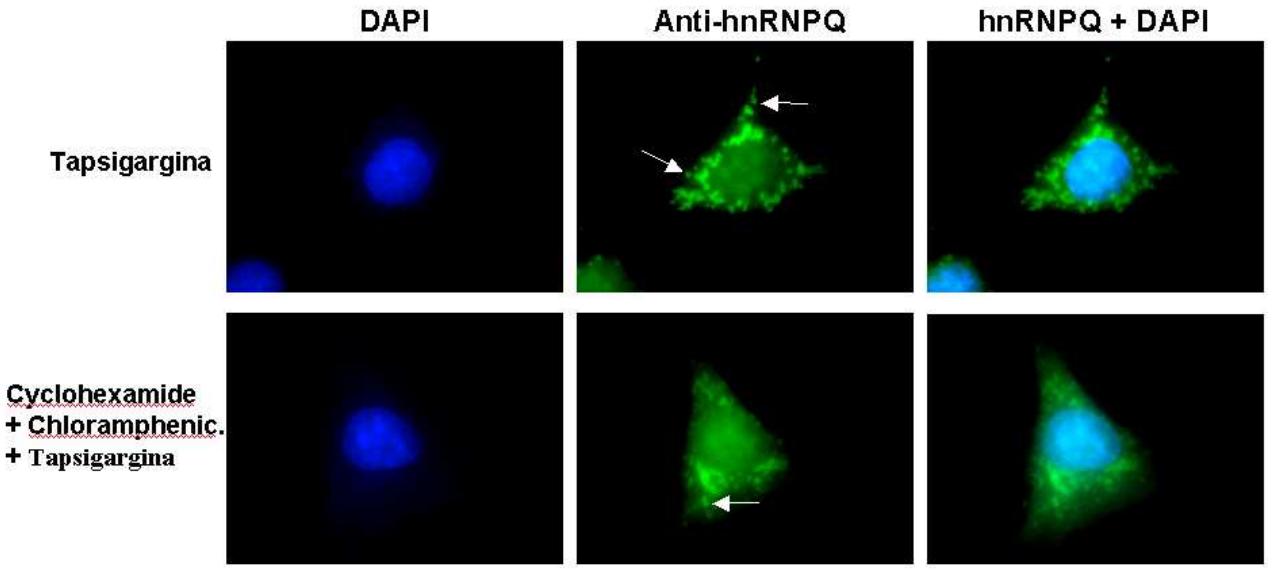
DAPI
Anti-hnRNPQ
hnRNPQ + DAPI
Tapsigargina
Cyclohexamide
+ Chloramphenic.
+ Tapsigargina

Figura 5

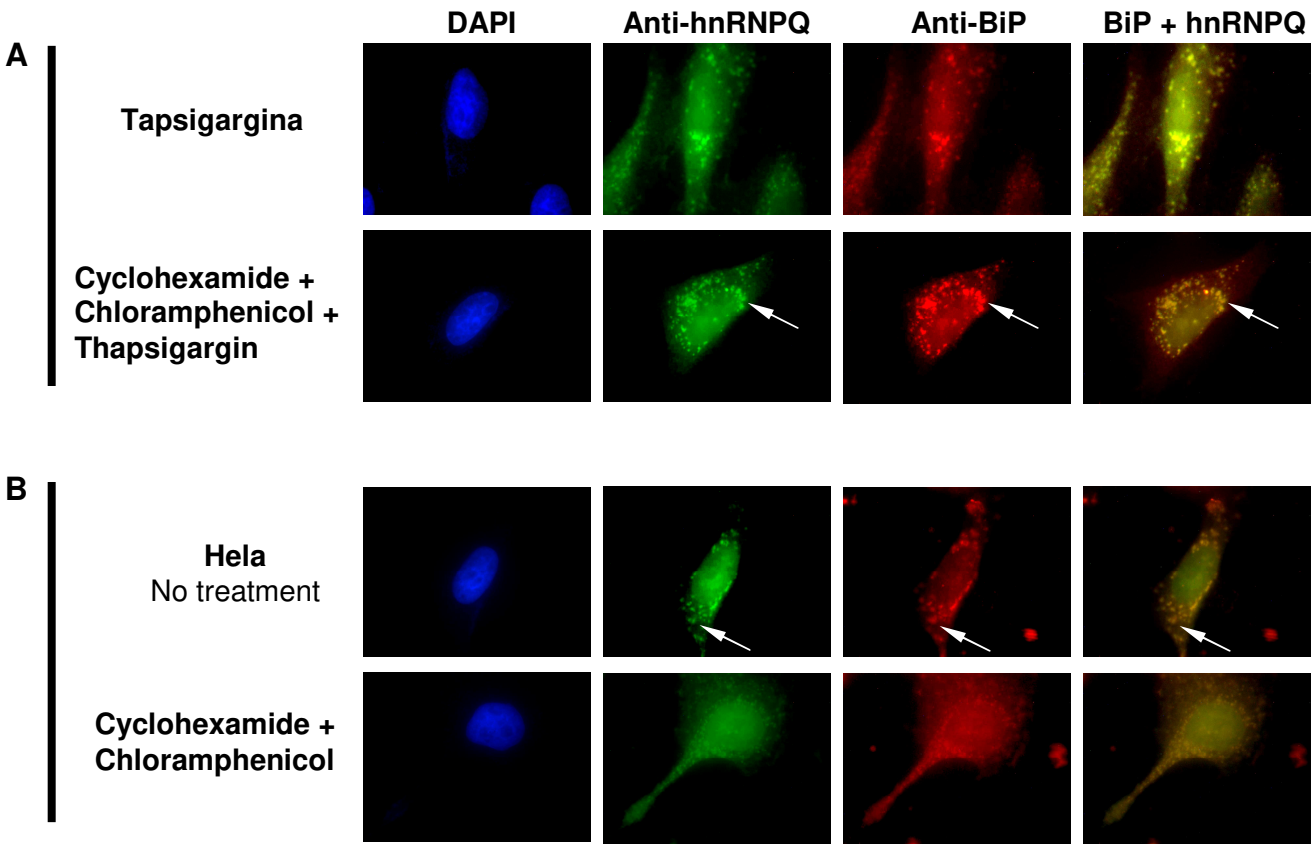
DAPI
Anti-hnRNPQ
Anti-BiP
BiP + hnRNPQ
A
Tapsigargina
Cyclohexamide +
Chloramphenicol +
Thapsigargin
B
Hela
No treatment
Cyclohexamide +
Chloramphenicol

Figura 6

6. Resultados complementares

Identificação das proteínas que interagem com a proteína humana IMP2 (*IGF- II mRNA-binding protein*).

Primeiramente realizamos um ensaio de duplo híbrido em levedura (Y2HS) utilizando para isso a proteína humana IMP2, como isca. Resolvemos fazer esse ensaio porque ela foi uma das proteínas pescadas por AUF1 (Moraes, KCM et al 2003. Artigo I, p18).

IMP2 faz parte de uma subfamília de proteínas (IMPs: IMP1, IMP2 e IMP3). Essa subfamília de proteínas faz parte de uma super-família de proteínas chamada VICKZ, nome baseado na primeira letra dos membros dessa família. (Vg1 RBP/Vera; IMP-1, IMP-2, IMP-3; CRD-RBP; KOC e ZBP-1) (Ross *et al*.1997, Havin *et al* 1998 e Deshler *et al*. 1998). Altos níveis das proteínas VICKZ são encontrados em muitos tipos de câncer, incluindo tumores de rim, mama, pâncreas, pele e cólon do útero. Em contraste, baixos ou indetectáveis níveis dessas proteínas são encontrados em tecidos normais (Yaniv *et al.,* 2002). A subfamília de IMPs é caracterizada por dois domínios RRMs ("*RNA recognition motif"*) na região N-terminal e quatro domínios KH (*"hnRNP K homology"*) na região C-terminal (Nielsen et al., 1999). Esta proteína está envolvida na localização sub-citoplasmática de determinados mRNAs durante a embriogênese (Nielsen *et al.* 1999).

Após os devidos testes, obtivemos em torno de 15 seqüências. As 15 seqüências obtidas foram analisadas e apenas as interações que julgamos funcionalmente mais próximas à IMP2 foram sumarizadas na tabela 3.

Tabela 3 – Identificação das proteínas, provenientes da biblioteca de células HeLa, que interagem com a proteína humana IMP2 por duplo-híbrido.

Nome do clone	Número de clones pescados	Tamanho do inserto identificado	Tamanho original da proteína	Função	Referências
PIG-U	3	~1000pb	1306nt-436aa	Subunidade do complexo de GPI transamidases.	Hong Y, et al. (2002)
p80/nucleophosmin anaplastic lymphoma kinase fusion protein	2	~750pb	2040nt-680aa	Proteína híbrida (NPM-ALK) formada em limfomas. Hiperfosforilada em limfomas Ki-1	Fujimoto, J. et al. (1996) Bischof, D. Et al. (1997)

As outras proteínas pescadas e não mostradas aqui, não apresentam significativa relação funcional com IMP2, como é o caso das proteínas ribossomais L7a e L10.

A principal interação vista foi entre a proteína IMP2 e a proteína híbrida, NPM-ALK (*"p80/nucleophosmin anaplastic lymphoma kinase fusion protein").* Essa proteína é resultado de uma translocação - t(2;5) (p23;q35) – entre o gene que codifica a kinase ALK, fusionado *in frame* com o gene que codifica a proteína nucleolar NPM. Essa translocação ocasiona o surgimento de uma proteína híbrida NPM-ALK, que é uma quinase constitutivamente ativa e que possui um papel central na formação de determinados linfomas. A proteína é encontrada em linfomas de Hodgkin e de não Hodgkin e é fosforilada pela proteína humana Ki-1 (Pulford, K. *et al* 1997 e Fallini, B. *et al* 2007).

Outra proteína de importância funcional é a proteína humana PGI-U, membro de um complexo humano, denominado GPIT ("*Glycosylphosphatidylinositol trasamidase"*). GPIT é um multi-complexo protéico que ancora o lipídio fosfatidilinositol a determinadas proteínas de membrana. GPIT é formado pelas proteínas Gaa1, Gpi8, PIG-S, PIG-T, and PIG-U (AAH30512). A natureza das interações funcionais entre as sub-unidades de GPIT, bem como as proteínas que interagem com os membros de complexo, ainda é desconhecido.

Num segundo momento, utilizamos novamente a proteína humana IMP2 como isca num ensaio de duplo híbrido. Neste caso, utilizando uma biblioteca de cérebro fetal humano. A mesma biblioteca utilizada anteriormente para a proteína AUF1 (Moraes *et al* 2003 – Artigo I, p. 18). Dentre as interações obtidas pelo ensaio, sumarizamos somente a que julgamos relevante, demonstrada na tabela 4.

Tabela 4 – Identificação das proteínas, provenientes da biblioteca de cérebro fetal humano, que interagem com IMP2.

Nome do clone	Número de vezes que interagiu	Tamanho do inserto identificado	Tamanho original da proteína	Funcão	Referências
Heat shock 90kDa protein 1	1	~500nt	732 aa	Auxilia no correto enovelamento de diversas proteínas. Sua função é dependente da capacidade de hidrolisam ATP	Piper, P.W. et al. (2003) Richter, K. et al. (2002)

Ao utilizarmos a biblioteca de cérebro fetal humano identificamos a proteína Hsp90. Hsp90 apresenta três domínios: na região N-terminal há um domínio de ligação a ATP, um domínio no meio da proteína e um domínio na região C-terminal, responsável pela sua dimerização. A dimerização da proteína HSP90 é um processo essencial para o seu correto funcionamento (Richter, K *et al* 2001 e Louvion, J. F *et al* 1996).

Em eucariotos superiores essa proteína já foi encontrada interagindo com receptores de hormônios esteróides, com algumas tirosina quinases de retrovírus e com proteínas do citoesqueleto – actina, dineína e miosina (Zhao, R et al 2005, Pratt, W.B. et al 1997 e Whitesell, L *et al* 1994). Em neoplasias a proteína Hsp90 auxilia fortemente na maturação de oncoproteínas (Calderwood, S.K. *et*

al 2006 e Chiosis, G. *et al* 2006). Estudos recentes utilizam Hsp90 como alvo de novos fármacos como 17-AAG ("*ansamycin 17-allylaminogeldanamycin*"), na tentativa de evitar que essa proteína consiga auxiliar no enovelamento de determinadas oncoproteínas (Chiosis, G. *et al* 2006).

Além disso, outros trabalhos mostraram que há uma forte ligação entre a proteína NPM-ALK e Hsp90 (Bonvini P, *et al.,* 2002). Curiosamente, foi demonstrado que AUF1 interage com HSP70 (Laroia *et al.,*1999) e sabe-se ainda que Hsp90 forma um dímero e que este dímero interage com Hsp70, além de outras co-chaperons, quando estão atuando. Trabalhos *in vivo* demonstraram que IMP2 também interage com proteínas do citoesqueleto, o que corrobora com o nosso achado (Nielsen *et al.* 2002).

Identificação das proteínas que interagem com a proteína humana hnRNP Q

Nós fizemos também um ensaio de duplo híbrido em levedura, utilizando como isca a proteína humana hnRNPQ/NSAP1. Nesse ensaio, 29 seqüências foram obtidas e analisadas. Dentre as interações vistas, sumarizamos na tabela 4 as mais relevantes.

Tabela 5 – Identificação das proteínas, provenientes da biblioteca de células HeLa, que interagem com a proteína humana hnRNPQ

Nome do clone	Número de clones pescados	Tamanho do inserto identificado	Tamanho original da proteína	Função	Referências
DJ-1/PARK7	5	~300pb	189aa	Regulação da transcrição sobre estresse oxidativo	Nagakubo, D., et al (1997)
Praja-2/Neurodap1	3	~650pb	708aa	Proteína híbrida (NPM-ALK) formada em linfomas. Hiperfosforilada em linfomas Ki-1	Takahashi, K. *et al* (2001)

A principal interação vista foi entre a proteína NSAP1 e a proteína DJ-1/PARK7 (NCBI: Q99497), interação verificada por 5 vezes. Esta proteína interage com PIAS2 e está intimamente ligada à doença de Parkinson. Essa doença é caracterizada por ser uma desordem multi-fatorial de base genética, que degrada os neurônios da região central do cérebro e termina por desorganizar os movimentos das pessoas afetadas (Takahashi, K. *et al* 2001). A proteína DJ-1/PARK7 funciona como uma chaperone sensível ao estresse oxidativo, tendo como principal função proteger a integridade das células neuronais diante de tal estresse (Canet-Aviles, R.M. *et al* 2004). Foram encontradas mutações autossômicas recessivas no gene DJ-1, que provavelmente são

responsáveis pela perda da função da proteína, cujas principais são: regular a transcrição e responder adequadamente ao estresse oxidativo (Miller, D.W. *et al* 2003).

Outra importante interação, identificada por três vezes com NSAP1, é com a proteína humana Praja-2/Neurodap1(NCBI: NP055634), uma proteína da família das *zinc-finger*, que possui um domínio RING-H2 e que está intimamente relacionada com o retículo endoplasmático (Nakayama M, *et al* 1995). Essa proteína chama a atenção devido a sua capacidade de interação com proteínas da família das ubiquitinas (Yu, P. *et al* 2002). Curiosamente, já havíamos demonstrado que a proteína AUF1 também interage com um membro dessa família UBC9. Outra importante proteína que também foi "pescada" através desse ensaio de Y2HS foi β-tubulina.

7. Considerações Finais

Uma rede regulatória envolvendo as proteínas hnRNP D, Q e IMP2

Interessantemente, algumas interações, identificadas por nós, têm sido confirmadas pela literatura. É o caso da proteína híbrida NPM-ALK que foi pescada, no ensaio Y2HS, por IMP2. Trabalhos recentes demonstraram que essa proteína interage com AUF1 e que ambas co-localizam em específicos focos citoplasmáticos (Fawal, M *et al* 2006). Portanto, não somente AUF1 é capaz de interagir com NPM-ALK, mas também HSP90 e ambas são capazes de interagir com IMP2 (Figura 4).

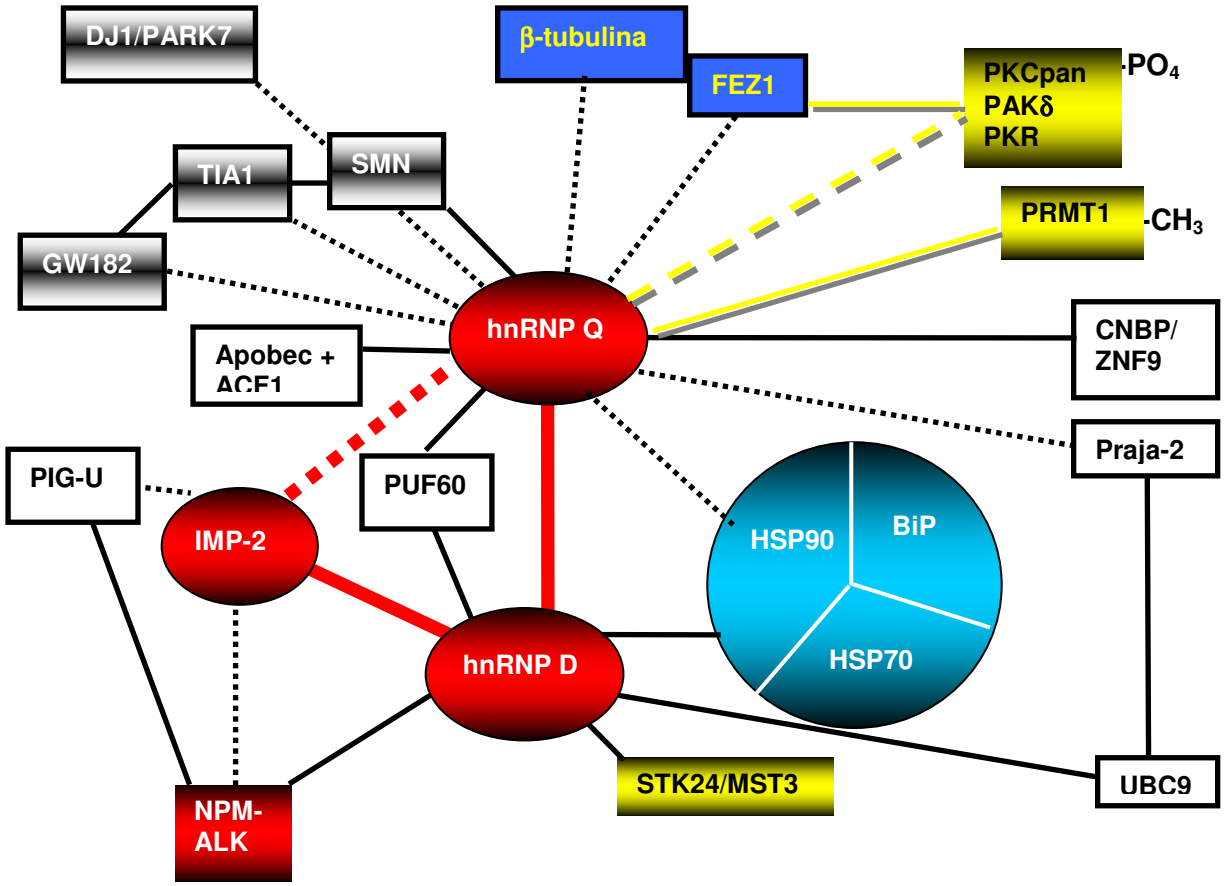

Figura 4. Representação parcial da rede de interações em que as proteína humanas hnRNPQ, hnRNPD e IMP2 participam (círculos vermelhos). As linhas cheias significam interações já comprovadas e publicadas, já as linhas tracejadas identificam interações vistas por nós, porém ainda não publicadas. Os retângulos amarelos representam enzimas responsáveis por modificações pós-traducionais, seja por fosforilação (-PO4), seja por metilação (-CH3). O círculo tripartido representa os membros da família das chaperones que participam dessa rede. Os retângulos em azul, no alto, representam as proteínas que fazem parte do citoesqueleto (b-tubulina) ou que se associam a elas (FEZ1).

Outra interação, que julgamos relevante, foi entre PIG-U e IMP2. Essa interação nos trouxe uma importante informação, pois alguns trabalhos realizados com a proteína IMP2 já haviam demonstrado que ela tinha uma interessante dinâmica de localização celular. IMP2 é uma proteína predominantemente citoplasmática, porém acumula-se, principalemente na região perinuclear. Após determinado estímulo, essa proteína dirige-se à membrana celular, onde se acumula logo abaixo da membrana. Durante esse percurso ela interage com diversas proteínas do citoesqueleto. Dentre essas proteínas do citoesqueleto, podemos citar a β-tubulina (Nielsen, F.C *et al* 2002).

Através do ensaio de Y2HS com a proteína humana hnRNPQ conseguimos identificar β-tubulina como uma proteína que interage com hnRNPQ. Essa hipótese já havia sido discutida em um trabalho que demonstrou hnRNPQ está organizada em específicos grânulos citoplasmáticos, presentes em neurônios (Kanai, Y *et al* 2004). Ainda, esses grânulos estão intimamente associados com algumas kinesinas, membros da família das KIFs. Nesse caso, hnRNPQ transportaria algumas moléculas de mRNA ao longo dos dendritos desses neurônios. Provavelmente ela consegue fazer isso se associando a proteínas do citoesqueleto ou a proteínas adaptadoras (Kanai, Y *et al* 2004).

Em trabalhos realizados no grupo, vimos que hnRNP Q interage *in vitro* (dados não mostrados) com FEZ1 e ambas estão na mesma fração celular (Lanza, D.F. *et al* 2008, submetido). FEZ1 é uma proteína humana que interage diretamente com α- e β-tubulina, constituintes do microtúbulo, assim como as kinesinas. Além disso, FEZ1 também interage com Clasp, que é uma proteína presente na ponta dos microtúbulos (Assman, E. Et al 2006, Lanza, D.F. et al 2008, submetido). Assim, acreditamos que tanto IMP2, quanto hnRNPQ transitam pelo citoplasma, associando-se diretamente ou indiretamente à proteínas do citoesqueleto.

Um outro importante aspecto que podemos especular diante dos nossos resultados e das informações contidas na literatura é a influência de modificações pós-traducionais nas proteínas AUF1, IMP2 e hnRNPQ. Podemos citar, como exemplos, a metilação e a fosforilação de hnRNPQ. Nossos resultados mostram que a metilação é uma modificação chave para a localização celular de hnRNPQ (Passos, D.O. *et al* 2006. Artigo II, p.19). Interessante notar que essa modificação assume uma função quase que de “edição” ou marcação na proteína em questão uma vez que ela não é reversível, como acontece com a fosforilaçõa em geral. Nossos resultados também mostram que o domínio N-terminal (AcD) de hnRNPQ é passível de fosforilação (*in vitro*) por PKC e que essa fosforilação perturba fortemente a interação entre o domínio AcD de hnRNPQ e a proteína Apobec1 (Quaresma,A.J.C. e*t al* 2006. Artigo III, p.20). Além disso conseguimos mostrar que hnRNPQ não somente interage, como também é fosforilada *in vitro* pela proteína PKCδ. Ensaios *in vivo* demonstraram que a ativação da via das PKCs, via tratamento com PMA, mostrou-se relevante para a localização celular de hnRNPQ (Quaresma,AJC. e*t al* 2008, manuscrito, p.21).

Há ainda a relação entre hnRNPQ e algumas estruturas citoplasmáticas, organizadas em específicos focos, como é o caso dos grânulos de estresse (SG) e dos corpos de processamento (PBs). Por exemplo, com a proteína SMN, envolvida na atrofia espinho-muscular (do inglês: *Spinal muscular atrophy* – SMA) (Mourelatos, Z *et al* 2001), que co-localiza com os SGs (Hua, J. and Zhou, J. 2004).

Para isso, investigamos a interação entre hnRNPQ e BiP, que é membro da família das HSP70, específica de retículo endoplasmático (ER)(Kida *et al* 2005). Sabe-se ainda que HSP70 apresenta papel fundamental na formação dos SGs e co-localiza com TIA1 (Gilks, N. *et al* 2004). Após diversos tratamentos, vimos que hnRNPQ, não somente co-localiza com HSP70, mas também com as outras proteínas específicas desses focos citoplasmáticos. Nesse caso, vimos que hnRNPQ pode ser co-imunoprecipitada tanto com TIA1 (proteína específica de SGs) quanto com GW182 (proteína específica dos PBs). Após alguns ensaios de co-imunolocalização, feito em células Hela, vimos que hnRNPQ co-localiza com HSP70, assim como com TIA1 e GW182. Entretanto essas co-localizações dependem do estímulo dado às células.

Estudos recentes de interactoma conseguiram identificar outras proteínas como possíveis parceiras de hnRNPQ (Ewing RM *et al* 2007). Dentre elas temos a proteína ZNF9, que apresenta 9 domínios do tipo *zinc-finger* e está relacionada a uma distrofia miotônica do tipo 2 (MIM2)(Botta,A. *et al* 2006), a kinase STK24/MST3, que é membro da subfamília das GCK quinases e participa da via das MAP quinases (MAPK) (Zhou,T.H *et al* 2005), bem como a proteína PUF60 que apresenta importante papel no *splicing* de determinados mRNAs e participa do complexo de spliceossomo (Page-McCaw, P.S *et al* 1999) (Figura 4).

8. Conclusões

Artigo I

O nosso ensaio de duplo híbrido em levedura, utilizando a isoforma AUF1p37, mostrou que AUFp37 interage não somente com ela mesma mas com a outra isoforma AUF1p40. Além disso, proteínas que apresentam domínios de ligação a RNA também foram "pescadas", como é o caso das proteínas IMP2, NSEP1 e hnRNPQ/NSAP1.

Todas as proteínas identificadas no ensaio de duplo híbrido em levedura (Y2HS), como potenciais parceiras de AUF1, também foram capazes de interagir *in vitro*.

O mapeamento das interações entre as proteínas e AUF1p37 mostrou que há especificidade nas interações. Além disso, ficou claro que os domínios das proteínas cooperam entre si a fim de promover a interação do tipo proteína-proteína.

Todas as proteínas pescadas no ensaio de 2THS, bem como AUF1p37, foram capazes de interagir com uma sonda de RNA (com 38-mers) rica em AU.

Artigo II

Outro ensaio de duplo híbrido em levedura, utilizando a proteína humana arginina metiltransferase (PRMT1), como isca, mostrou que PRMT1 também interage com hnRNPQ/NSAP1.

Ensaios de metilação *in vitro* demonstraram que NSAP1 é metilada por PRMT1 na região rica em aminoácidos arginina e glicina (RGGbox), situado na região C-terminal.

Imunoprecipitações, realizadas com extrato de célula Hela, sugerem fortemente que as isoformas hnRNPQ2 e Q3 são substratos de arginina metiltransferases.

Ensaios de localização celular, realizados com célula Hela, demonstram que a inibição da metilação pode regular a localização celular da proteína humana hnRNPQ/NSAP1.

Artigo III

hnRNPQ e ACF apresentam grande similaridade seqüencial e provavelmente estrutural, entretanto a região que compreende o domínio ácido (AcD) está presente apenas em hnRNPQ.

Apesar de apresentar cerca de 30% de identidade, o domínio AcD pode ser modelado, utilizando a proteína Barstar como molde. Além disso os modelos gerados apresentaram considerável qualidade de estrutura quando validados pelos programas: PROCHECK, Verify_3D, dentre outros.

Ensaios de espectrometria de massa confirmaram que o domínio AcD de hnRNPQ apresenta duas serinas (S44 e S75), que podem ser fosforiladas *in vitro* por PKC.

Ensaios de interação *in* vitro demonstraram que o domínio AcD é suficiente para interagir com Apobec1.

A fosforilação do domínio AcD inibe sua interação com proteína Apobec1.

Através de técnicas experimentais, como: dicroísmo circular e fluorescência e teóricas, como: dinâmica molecular, podemos sugerir que a fosforilação causa considerável alteração na estrutura secundária e terciária do domínio AcD.

Artigo IV

Ensaios de fosforilação *in* vitro mostraram que hnRNPQ/NSAP1 é especificamente fosforilada por PKCδ.

O *Western Blot* anti-hnRNPQ do imunoprecipitado de PKCδ mostra que essas proteínas interagem entre si *in vivo.*

Ensaios imunofluorescência em células Hela mostraram que a localização da proteína hnRNPQ/NSAP1 endógena é predominantemente nuclear e que essa localização não é dependente da tradução. Entretanto, essa localização sofre grande alteração, quando ocorre ativação da via das PKCs, via tratamento com PMA.

Ensaios de co-imunofluorescência, em células Hela, sugerem que hnRNPQ/NSAP1 co-localiza com a HSP70 de retículo endoplasmático (ER) – BiP - em específicos grânulos citoplasmáticos, após o tratamento com PMA.

Ensaios de interação *in vitro* demonstraram que o domínio hnRNPQ/NSAP1 interage com o domínio de ligação ao substrato de HSP70, bem como com a proteína inteira.

Ensaios imunofluorescência com células Hela demonstram que a localização celular de hnRNPQ/NSAP1 sofre significativa alteração após indução de estresse de ER, via tratamento com tapsgargina.

Ensaios de co-imunofluorescência com células Hela demonstram que hnRNPQ/NSAP1 co-localiza com HSP70 após indução de estresse de ER e térmico.

O *Western Blot* anti-hnRNPQ do imunoprecipitado de GW182 (proteína específica de P-bodies) e TIA1 (proteína específica de grânulos de estresse) mostra que essas proteínas interagem entre si *in vivo.*

9. Referências Bibliográficas

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