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N-teminal interaction domain implicates PAK4 in translational regulation and reveals novel cellular localization signals

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List of abbreviations: AID: auto-inhibitory domain; CHX: cycloheximide; CRIB: CDC42/Rac interactive binding domain; EMCV: encephalomyocarditis virus; FMRP: fragile X mental retardation protein; egfp: enhanced green fluorescent protein; GSH: glutathione; GST: glutathione *S*-transferase; HRV: human rhinovirus; IRES: internal ribosome entry site; NES: nuclear export signal; PAK: p21-activated kinase; PBD: p21 binding domain; RNP: ribonucleoprotein; rp: ribosomal protein.

ABSTRACT

The serine/threonine kinase PAK4 is a Rho GTPases effector protein implicated in many critical biological processes, regulating cell morphology and motility, embryonic development, cell survival, response to infection and oncogenic transformation. Consistently with its pro-oncogenic features, PAK4 was found overexpressed in many cancer cell lines and tissues, and necessary to promote survival pathways activation. Together with other Paks, it is now considered as a promising target for specific cancer therapy, however, little is still known on its modes of regulation, molecular partners and substrates. Since the N-terminal regulatory moiety plays important roles in PAK4 activity and functions, also independently of GTPase interactions, in this study we looked for N-terminal domain binding partners with an affinity chromatography approach. We identified a novel interaction domain involved in association with ribonucleoprotein (RNP) complexes, suggesting PAK4 implications in translational regulation. Indeed we found that active PAK4 can affect (cap independent) translation from specific IRES sequences *in vivo*, and that the N-terminal domain is critical for such regulation. Further, we could establish that within the RNP interacting sequence, PAK4 regulatory domain contains targeting elements which drive cytoplasmic localization and function as a nuclear export signal. Analysis of endogenous PAK4, which was found in both cytoplasmic and nuclear fractions, and implicated in IRES mediated translation, further supports the significance of our findings. Our data indicate novel means for PAK4 regulation of gene expression, and provide new elements to understand the molecular mechanisms that determine PAK4 cellular localization and functions.

INTRODUCTION

The PAK4 protein belongs to the Pak (p21-activated kinase) family of serine/threonine kinases that were firstly identified as Rho GTPases effector proteins (Manser et al., 1994; Zhao and Manser, 2005). Paks have been implicated in diverse cellular processeses that include cytoskeletal rearrangements, motility, regulation of gene expression, survival, and response to infection, with GTPase dependent and independent mechanisms (Arias-Romero and Chernoff, 2008).

Paks have also been implicated in oncogenic transformation and were found overexpressed in many kinds of tumors, and are considered an attractive target for specific therapy design (Molli et al., 2009; Eswaran et al., 2009).

The 6 known mammalian Pak isoforms are differentially expressed in adult tissues and during development, and are divided in subgroups A (PAK1-3) and B (PAK4-6), depending on their sequence similarity, regulatory mechanisms and functions (Arias-Romero and Chernoff, 2008; Zhao and Manser, 2005). Their common structural features consist in a C-terminal protein kinase domain and an N-terminal regulatory region that includes the p21-Binding-Domain (PBD) (also designated as CRIB) that binds the active, GTP-bound form of CDC42 or Rac.

Besides to higher relative sequence similarity within the kinase and PBD domains, group A and group B Paks can be further distinguished for the presence, in group A, of additional conserved domains in their regulatory moiety, implicated in their regulation and interaction with signaling proteins and substrates. For example, an auto-inhibitory domain (AID) partially overlaps the PBD of group A Paks and maintains the kinase in a dimeric inactive state. Moreover, recent structural data of B Paks kinase domain further indicate that the two groups show unique features for transition to the active state (Eswaran et al., 2009).

As compared to group A, group B regulatory portion is much less conserved outside the region comprising the PBD, and only poly-proline sequences, differentially located within each isoform, and an N-terminal basic region, can be recognised as conserved motifs. Importantly, PAK4 and PAK6 kinase activity does not seem to be increased by interaction with the active GTPase and they do not contain a recognisable AID. Nontheless, for all group B Paks, the N-terminal moiety seems to play important regulatory roles, from cellular localization to inhibition of full length protein kinase activity.

To date PAK4 is the best characterized group B Pak and was firstly identified as a CDC42 target, regulating filopodia formation by active CDC42 (Abo et al., 1998). It is now known that it plays critical roles during development, regulating migratory and differentiation properties of neuronal and non neuronal cells, probably by its action on cellular cytoskeleton and adhesion (Qu et al., 2003). PAK4 overexpression has been observed in many human tumor cell lines, and its gene locus amplification is recurrent in many cancers, including pancreatic tumors (Callow et al., 2002; Liu et al., 2008; Molli et al., 2009). Indeed its overexpression is sufficient to drive cellular transformation in cell lines, to promote survival and protect cells from apoptosis induced by different stimuli. Its function has been shown to be necessary for transformation by Dbl and Ras oncogenes, and for survival signals induced by TNFR signaling (Qu et al., 2001; Gnesutta et al., 2001; Gnesutta and Minden, 2003; Callow et al., 2002; Li and Minden, 2005; Liu et al., 2008). Reported interaction and activation by KGF, HGF receptors and integrin subunits also underlined its relevance in cell adhesion, motility and survival processes (Lu et al., 2003; Wells et al., 2002; Zhang et al., 2002; Lotti et al., 2007).

However, PAK4 interactions with cellular pathways are still not completely elucidated and novel information on its partners and regulatory mechanisms could give an important contribution in understanding its biological roles and for proper design of specific therapies.

With our study we have found that PAK4 N-terminal domain can associate with components of the translational machinery, suggesting novel means for PAK4 regulation of gene expression. We have also found that active PAK4, through interactions of the regulatory domain, can affect cap independent translation of specific IRES sequences in vivo, and identified a specific region that could regulate nucleocytoplasmic shuttling of the protein. Our data give new insights on the role of the regulatory domain in PAK4 biological functions, suggesting distinct mechanisms for nuclear and cytoplasmic localized PAK4 protein in gene expression regulation.

MATERIALS AND METHODS

Plasmids: HA-tagged full length PAK4 wt, PAK4 Kinase domain (KDom) or inactive Kinase domain (KDomKM), PAK4 DeltaCRIB in Srα3 (described as PAK4Δ,

PAK4ΔK350M and PAK4ΔGBD); Myc-tagged PAK4wt and PAK4NE (containing the activating mutations S445N, S474E) in pCANmyc2, were described in (Abo et al., 1998; Qu et al., 2001; Gnesutta and Minden, 2003). The egfp-KDom construct was generated by subcloning in frame with egfp a Flag-tagged version of PAK4Δ in pEGFP-C3 (Clontech), resulting in a egfp-Flag-PAK4 kinase domain expression vector. The correct protein size was verified by WB with anti-GFP and M2-Flag Abs. Myc tagged active PAK2 (T402E) (Ling et al., 2005) was generated by site directed mutagenesis and confirmed by sequencing. PAK4 N-terminal domain constructs were generated as follows: PAK4 sequences coding for aa 1-297 (denominated N-ter in this work), 1-113 (CRIB), 65-175 (RDP) and 65-116 (RDC), were obtained by PCR using human PAK4 cDNA (Abo et al., 1998) as a template including BamHI and HindIII sites in amplification primers for subcloning purposes. Amplicons were cloned in pGEM-T (Promega) and sequenced. BamHI-HindIII PAK4 fragments were then inserted in pGEX-KG to generate GST fusion proteins expression plasmids. The GST-N-ter construct was used to generate C-ter deletion mutants RD (aa 1-174) and PBD (1-64): after linearization by partial digestion with SmaI, 3' sequences were excised with HindIII. After fill-in DNAs were ligated and transformed to obtain the recombinant plasmids. To obtain myc-RDP, egfp-RDP and egfp-RDC mammalian expression plasmids, RDP and RDC sequences were inserted in-frame in pCANmyc1 or pEGFP-C1 vectors.

pR-F, pR-Myc-F and pR-HRV-F (pGL3R, pGL3Rutr, pGL3RHRV (Stoneley et al., 2000)) dicistronic vectors were kindly provided by A.E. Willis (Nottingham, UK) and B. Morrish (Parkville, Australia). pR-EMCV-F was obtained by subcloning the EMCV IRES sequence from pIRES2-EGFP (Clontech) as a 622bp EcoRI-NcoI fragment in pR-F. egfp-rpP0 expression plasmid (pEGFP-C1-P0 (Tchórzewski et al., 2003)) was kindly provided by M. Tchórzewski (Lublin, Poland).

Protein purification and affinity chromatography: GST fusion proteins were expressed in BL-21 *E. coli* cells and purified using GSH-sepharose (GE Healthcare) according to manufacturer's instructions. Cultures were grown in LB medium with 100µg/ml ampicillin, and induced with 0.1µM IPTG (1.5 to 3hs at 24 ˚C). Cell pellets were lysed in 20mM NaPhosphate buffer pH 7.8, containing 0.5M NaCl, 0.5mM DTT, 0.5% Triton X-100 and protease inhibitors. For GSH-purification 10% glycerol was

added to clarified soluble fractions, and to lysis buffer for column washes. Affinity matrices were prepared by covalently coupling the purified proteins (2.5 mg/ml gel) to CNBr activated sepharose (SIGMA) according to manufacturer's instructions.
HeLa extracts were prepared from confluent plates in Triton Lysis Buffer (TLB: 10mM Tris pH 7.5, 100mM NaCl, 1% Triton X-100) containing 10µg/ml aprotinin and leupeptin, 1mM PMSF, 4mM EDTA and 10mM Na_3VO_4, and clarified by centrifugation (18000xg, 20'). For affinity chromatography, cell lysates (2mg/ml) were incubated with matrices for 4h at 4 ˚C (typically 20µl of affinity matrix with 2mg of cell extract). Beads were pelleted by low speed centrifugation and washed 5 times with TLB. Interacting proteins were eluted in 2V of 2X SDS Sample Buffer and heating 5' at 95 ˚C for subsequent analyses.
For ribosomal subunits dissociation/stabilization, lysates were treated essentially as described in (Jao and Chen, 2006; Corbin et al., 1997). For RNase treatments, extracts were incubated with 100µg/ml RNaseA (SIGMA) for 15' on ice, prior to interaction.
For CHX extracts, CHX was added to cell culture (1µg/ml) 15' before lysate preparation and TLB was supplemented with CHX (100µg/ml) for cell lysis and affinity beads washes. EDTA lysates were prepared by adding 40mM EDTA to cell extracts, prior to interaction. Where indicated, 40U/ml RNaseOUT RNase inhibitor (Invitrogen) was added to TLB for cell lysis and column washes.
To detect egfp-rpP0 association in GST pull-down experiments, 15µg of purified GST fusion proteins were loaded on GSH-sepharose and added to transfected Hek293 extracts (1mg) prepared in TLB as described below. Proteins were allowed to interact for 4hs at 4 ˚C and resins were washed 4 fold in TLB. Recombinant and interacting proteins were eluted by addition of 2X SDS-SB and heating.
Protein eluates from affinity chromatography or GST pull-down interaction assays were resolved by SDS-PAGE. Proteins were either visualised in gels by Silver Staining procedure, or transferred to nitrocellulose membranes and processed for Western Blotting.
MALDI-MS analyses and id of interacting proteins was performed by the Columbia University Protein Core Facility (NY, USA).

Western Blotting: After SDS-PAGE, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell) and decorated with indicated primary Abs and

HRP conjugated secondary antibodies (SIGMA). Protein bands were then visualized with the ECL method (GE-Healthcare).

Antibodies: For Western Blotting and immunofluorescence analyses we used the following antibodies: mouse monoclonal anti-FMRP (Chemicon), anti-PARP and anti-PAK4 (BD-Pharmingen), anti-αtubulin (SIGMA), anti-GFP (Roche), anti-GST (sc-138, Santa Cruz Biotechnology), anti-HA (HA.11) and anti-Myc (9E10) (Covance); rabbit policlonal anti-QM (sc-798, Santa Cruz Biotechnology), anti-rpS6 and anti-PAK4 (Cell Signaling Technology). Policlonal human Abs to Ribosomal P antigen (Biodesign) were used for detecting ribosomal P proteins.

Cell culture and transfections: HeLa and Hek293 cells were cultured in DMEM-glutamax (GIBCO) containing 10% FBS and antibiotics (Euroclone), in a humified 5% CO_2 atmosphere at 37 ˚C. For PAK4 knock-down (PAK4i) and pSUPER control HeLa stable cell lines (Li and Minden, 2005), kindly provided by A. Minden (Piscataway, NJ, USA), growth medium was supplemented with 1.5µg/ml puromycin (SIGMA). Before experiments, cells were passaged in medium without puromycin. For dual luciferase assays HeLa cells were transfected in 12 wells plates with pR-F dicistronic reporters and PAK4 expression constructs (1:2 ratio) using Lipofectamine2000 (Invitrogen) according to manufacturer's instructions. After 48 hours, cell lysates were prepared and assayed for Firefly and Renilla luciferase activities using the Dual-luciferase Reporter Assay System (Promega) and a Berthold Lumat luminometer. IRES driven translation was calculated, as described in (Stoneley et al., 2000a), by dividing Firefly by Renilla luciferase light units. F/R values were then normalized to the average pR-F control values of each independent experiment (Relative IRES translation).
For transfection controls and for exogenous expression of gfp-rpP0 in HeLa or Hek293 cells, after Lipofectamine2000 transfection, extracts were prepared in TLB buffer containing DTT and protease inhibitors.

Fluorescence analysis: Cells were plated on coverslips and transfected with indicated expression plasmids using Lipofectamine2000. 48hs post transfection, cells were fixed in 4% PFA in PBS and permeabilized in 0.1% Triton X-100. To detect PAK4

proteins, cells were decorated with Abs to the relevant epitope tag in 0.1% Triton X-100 as described (Abo et al., 1998), and with Alexa594 goat anti-mouse secondary Abs (Molecular Probes, Invitrogen). DAPI was also included in the last incubation step to visualise cell nuclei. Secondary Abs or egfp epifluorescence images were collected with an Olympus BX60 Fluorescence microscope, with a 60X UplanFl oil lens, using a Kodak digital camera and dedicated acquisition software (KodakMDS).

Cellular fractionation

To obtain cytosolic and nuclear fractions of HeLa and PAK4i cell lines, cells were collected by scraping in PBS and pelleted by low speed centrifugation. After 1 PBS wash, cell pellets were resuspended by pipetting in 5V of hypotonic CE buffer (10mM Hepes-KOH pH 7.9, 10mM KCl, 1 mM EDTA, 1mM EGTA, 1mM DTT), supplemented with 0.5% NP-40. After 15' gentle agitation at 4 ˚C, lysates were pelleted 5' at 380xg and the supernatant was saved as cytosolic extract. The nuclear pellet was washed once in 2V of CE, pelleted 1' at 1200xg and the supernatant combined with the cytosolic extract (Cytosolic fraction). Nuclei were resuspended by pipetting and briefly vortexing in 1V of high salt NE buffer (20mM Tris pH 8, 0.6M NaCl, 1.5mM MgCl2, 0.2mM EDTA, 25% glycerol). After 20' gentle agitation at 4 ˚C, insoluble material was pelleted 30' at 80000xg and the supernatant collected to obtain the Nuclear fraction.

During the procedure, buffers were all supplemented with protease and phosphatase inhibitors (10⌠g/ml aprotinin, 1⌠g/ml leupeptin, 1mM PMSF, 1mM Na_3VO_4).

To detect endogenous protein localization by WB, equal protein micrograms of the cytosolic and nuclear fractions were loaded on SDS-PAGE gels.

RESULTS

Affinity chromatography isolation of proteins binding to PAK4 N-terminal domain reveals RNA dependent interaction with ribosomal and RNA binding proteins.

In order to screen for proteins and/or protein complexes that can associate with the N-terminal portion of PAK4, we performed affinity chromatography on HeLa cells extracts using a GST fusion protein containing the N-terminal regulatory domain of

human PAK4 (aa 1-297; N-ter) as a bait. (See also fig. 1b for the schematic representation of PAK4 and protein domains used in this work for affinity chromatography experiments). The PAK4 domain or GST tag control were covalently coupled to sepharose and cell lysates were allowed to interact with affinity matrices (see Materials and Methods). After washing, eluted samples were analysed by SDS-PAGE and silver staining. While we found some proteins were bound by the GST tag, several other proteins were specifically associated to PAK4 N-ter (not shown, and fig. 1a top panel, lanes 5 and 2). Some of the specific and most prominent protein bands were excised from gels after preparative experiments, and were identified by MALDI-Mass Spectrometry (MS). Such analysis indicated that most of the isolated bands (10 out of 13 analysed), were proteins involved in RNA metabolism and translation, such as ribosomal proteins (rp*s*) of the large or small subunits, including rpP0, rpL10 (QM), rpS13, and RNA binding proteins, such as the Dead-Box RNA helicase DDX3 (Lai et al., 2008)(not shown).

These data suggested that many of the proteins we found associated to the N-ter domain might in fact be part of RNP (ribonucleoprotein) complexes, and could bind PAK4 by both direct or indirect interactions. We then decided to further test and characterize PAK4 association with RNPs.

We first asked if protein binding may be dependent on the presence of RNA, and performed affinity chromatography after RNase treatment of cell lysates, comparing the bound protein pattern with that obtained with untreated lysates, with or without RNase inhibitors (fig. 1a). As shown in the silver stained gel (top panel), while RNase inhibitors did not change the pattern of proteins associated to N-ter, RNase treatment decreased significantly the number of PAK4 interacting proteins (compare lanes 6 and 5), and the band pattern (taking into account bands due to partial elution of the bait, lanes 7 and 8) resulted very similar to that obtained with the GST tag control, indicating that most of N-ter specific interactions were dependent on the presence of RNA. No change was observed in the samples obtained with the GST control.

In order to confirm the identity of some protein bands identified by MS, we performed western blotting (WB) on parallel gels using antibodies recognising ribosomal P-protein P0, or rpQM. As shown in the bottom panels of figure 1a, specific bands were present only when the N-ter PAK4 protein was used as a bait (lanes 4 and 5), and interaction was markedly reduced in RNase treated samples (lane

6). Further, presence of rps from both the small and large subunits of the ribosome in the isolated complexes, not yet identified by MS, was confirmed with antibodies to rpS6 (fig. 1a, bottom panels), and by detection of rpP1 and rpP2 with P-proteins antisera (not shown).

These data strongly indicate that PAK4 N-terminal domain can interact with ribonuclear protein complexes also containing ribosomal subunits, and this association is dependent on RNA.

To identify the PAK4 region involved in RNP association, we generated and purified different fusion proteins with C-terminal and N-terminal deletions of the regulatory domain (fig. 1b), coupled them to sepharose, and used the matrices for affinity chromatography of HeLa extracts. Eluted proteins were analysed by WB for interaction with rpP0, rpS6 and rpQM (fig. 1b bottom panels). While the first 174aa (RD) efficiently bound the rps, aa 1-113 (CRIB), containing the Pak family p21-Binding-Domain (PBD) and a downstream sequence conserved in PakB proteins, lost part of this ability. Importantly, the PBD construct (aa 1-64) did not interact with rps. N-terminal deletions of the RD protein (RDP aa 65-175; RDC aa 110-175) allowed us to define that the protein region spanning aa 65-175 of human PAK4 (the RDP domain) can interact in vitro with rps as efficiently as the N-terminal 174aa (RD) (or 297aa, N-ter) portion. These data also show that the PBD portion is not necessary for rp binding by PAK4 N-ter.

We then verifyed if RNP association to the RDP protein is also RNA dependent. Affinity chromatography was performed as for the N-ter bait, with or without RNase pre-treatment, and protein association was assayed by silver staining or WB (fig. 2a). The silver stained gel (top panel) shows that, compared with the GST control, the RDP protein specifically interacts with several proteins (see lanes 2 and 6), as we already observed for the N-ter construct. Also in this case, specific associations are markedly decreased, if not abolished, upon RNase pretreatment of the lysates (lanes 2 and 3), and RNA dependent binding was also confirmed by WB to rps (bottom panels). Total RNA analysis of cell lysates, before and after interaction, confirmed RNA integrity in all RNase untreated samples (not shown).

Considering that several ribosomal proteins were isolated by our approach, we asked if binding could be perturbed by treatments that affect ribosome subunits association. For these experiments, HeLa cells were lysed in the presence, or not, of Cycloheximide (CHX). CHX blocks the elongation step of translation and is used to

stabilyse polisomes during cell lysis. EDTA was used to chelate Mg^{2+} ions that are required for ribosomal subunits association. After interaction of the RDP affinity matrices with treated or untreated lysates, protein eluates were analysed by WB to ribosomal proteins. Binding of FMRP, a RNA binding protein found in RNPs and involved in traslational control of mRNAs (Corbin et al., 1997; Zalfa et al., 2006), was also tested. Results in figure 2b, show that while CHX slightly increases association to RDP, EDTA treatment markedly reduces binding of both ribosomal proteins and FMRP. (rpP0 association seems to be less affected by this treatment, however we think that P0 WB signal is in part aspecific due to comigration of the RDP bait with P0 - see also RDP no lysate lane in fig. 1b).
Taken together these data indicate that PAK4 N-terminal domain binding to ribonuclear protein complexes is dependent on RNA and ribosomal subunit association and/or presence of divalent ions.

To further validate and assay the interaction of the RDP portion with ribosomal proteins as compared to other PAK4 N-terminal fragments, we performed GST pull-down experiments and tested association with rpP0, exogenously expressed as a egfp fusion protein in Hek293 cells.
GSH beads preloaded with equivalent GST fusion protein amounts were allowed to interact with egfp-P0 or egfp extracts obtained from transfected Hek293 cells, and binding was assayed by WB to the egfp tag (Fig. 3, top panels) following the pull-down experiment. As expected, when the RD was used as a bait, egfp-P0 was specifically pulled down, and no interaction was observed with the egfp control, or using the GST tag as a bait. Moreover, similar amounts of egfp-P0 were bound by the RDP bait. The smaller RDC construct showed lower egfp-P0 binding, while no association could be observed using the N-terminus PBD construct. Equal loading of the GSH beads was confirmed by reprobing membranes with antibodies to GST to detect eluted bait proteins in the pull-down samples (fig. 3 bottom panel).
These data further support that the region(s) (aminoacids) involved in binding with ribosomal proteins lye within the RDP portion (aa 65-175) of PAK4 N-terminal domain, and that the PBD domain is dispensable for such association.

Activated PAK4 can regulate IRES driven translation in vivo.

We then looked for a functional role of PAK4 interactions with ribosomal proteins in vivo. Since PAK4 interacts with RNPs in vitro, and considering that PAK2 can

regulate translation (Ling et al., 2005), we wanted to test if PAK4 could be involved in translational control processes. Dicistronic constructs that give rise to transcripts containing two orfs coding for Renilla and Firefly luciferase enzymes separated by Internal Ribosome Entry Site (IRES) sequences are a widely used system to test for cap independent IRES driven translation. Because PAK4 can suppress apoptosis by different means (Gnesutta et al., 2001; Gnesutta and Minden, 2003; Lu et al., 2003; Li and Minden, 2005) and c-Myc 5' UTR is one of the best characterized cellular IRESs that is active during apoptosis (Stoneley et al., 2000; Spriggs et al., 2005), we started to test if PAK4 could regulate c-Myc IRES driven translation in this system (see in fig. 4 the schematic depiction of the pR-F derived constructs used in this work). HeLa cells were transfected with the pR-Myc-F dicistronic reporter or with the pR-F plasmid that does not contain the IRES sequence, with or without cotransfecting PAK4 expression constructs, and cell lysates were assayed for luciferase activities. The ratio of Firefly (coded by the downstream orf) versus Renilla (coded by the upstream orf) luciferase activity, normalised to the pR-F ratio (no IRES control), represents the IRES driven translational rate as compared to cap dependent translation (Stoneley et al., 2000a). As expected, when the c-Myc IRES reporter was transfected with empty vector alone, F/R values were about 50 fold higher than those given by the pR-F plasmid (see fig. 4 left panel, F luc vs R luc graph), indicating the IRES driven translational rate of the c-Myc sequence. Cotransfection of full length wild type PAK4 did not affect the Myc IRES driven activity, showing F/R values similar to the vector control sample. However, when we transfected an activated mutant of full length PAK4 (PAK4 NE), F/R values of pR-Myc-F were substantially reduced to 20 fold the pR-F control, indicating a repression of cap independent translation. Interestingly, when we used an activated PAK2 mutant, consistently with what already reported (Ling et al., 2005), we did not observe substantial changes in Myc-IRES driven translation, indicating that repression of F/R ratios is a specific effect of the PAK4 kinase.

In order to understand if active PAK4 could generally repress IRES driven translation, or F/R ratios, given by IRES dicistronic constructs, we tested different and well characterized sequences such as the EMCV (Encephalomyocarditis Virus) and HRV (Human Rhinovirus) IRESs (Jang et al., 1988; Borman et al., 1993) (fig. 4 right panels). When co-transfected with an empty vector alone, as already observed for the Myc IRES, EMCV and HRV IRESs substantially increase Firefly expression,

resulting in F/R values more than 20 and 10 fold, respectively, the pR-F control. Unexpectedly, opposite of what we observed for the Myc IRES, when PAK4 NE was cotransfected with the viral IRES reporters, F/R ratios were significantly increased. In fact, as evidenced by the Fold Change graph where IRES translational rate is normalised to the relative empty vector control (PAK4 NE *minus*), EMCV and HRV IRESs display about 2 fold their activity in the presence of PAK4 NE. This result indicates that the tested viral IRES sequences function can be upregulated by active PAK4 kinase mutant expression.

These data strongly suggest that in vivo the active PAK4 NE mutant can modulate IRES translational rates, as observed in the dicistronic assay system.

The N-terminal domain is required for active PAK4 regulation of EMCV IRES driven translation.

To understand if PAK4 interactions of the N-terminal region could be actually involved in modulation of IRES activity, we tested PAK4 N-terminal deletion mutants in the dicistronic assay. It has already been observed that removal of the N-terminal domain or the first 119 aa from the full length protein generates a constitutively active kinase (Abo et al., 1998; Callow et al., 2002; Wells et al., 2002). We therefore asked if active PAK4 can mimic the full length protein effects independently of the N-terminal domains.

The PAK4 kinase domain (KDom, aa 297-591) or the PAK4 DeltaCRIB (aa 119-591) deletion mutants were tested for their activity towards the pR-EMCV-F reporter vector, in parallel with the full length wild-type PAK4 (PAK4 wt), the active PAK4 NE mutant, or with a catalytically inactive kinase domain mutant (KDomKM) (figure 5). As shown in the F/R fold change graph, while PAK4 NE increases F/R ratios, activating the EMCV IRES driven translation, the kinase active mutant lacking the full N-terminal domain (KDom) cannot obtain this effect. Further, the DeltaCRIB mutant, which lacks the PBD domain and part of the RDP domain we identified to interact with RNPs, also fails to increase EMCV IRES function. Rather, PAK4 N-terminal deletants seem to decrease F/R ratios given by the EMCV IRES reporter. Full length wt PAK4 or the catalytically inactive Kinase Domain (KDomKM) did not significantly change EMCV IRES driven translation.

These data indicate that both kinase activity and the N-terminal domain are required for positive regulation of EMCV IRES driven translation by active PAK4.

PAK4 cellular localization is controlled by sequences within the RDP portion of the N-terminal domain.

Previous reports indicated that beside to relieving an inhibitory effect on kinase activity, deletion of PAK4 N-terminal portions can also cause nuclear localization of the kinase (Abo et al., 1998; Wells et al., 2002), which is otherwise reported to be cytoplasmic when expressed as a full length protein. Considering the different effects of the active PAK4 mutants in the dicistronic assay, and that RDP interactions could be relevant for PAK4 translational regulation, we wanted to better characterize cellular localization determinants within the N-terminal domain.

We then analysed cellular distribution of the full length protein or isolated domains by immunofluorescence. As shown in figure 6a, HeLa cells transfected with expression vectors for PAK4 full length protein or deletion mutants were decorated with antibodies to the relevant epitope tag. As already reported (Abo et al., 1998; Wells et al., 2002), full length PAK4 was prevalently found cytoplasmic and perinuclear, showing little or no staining in the nuclei, while the kinase domain alone and the DeltaCRIB were both cytoplasmic and nuclear, showing a strong nuclear staining. Kinase activity was not required for nuclear localization of the kinase domain, since the inactive mutant KDomKM was similarly distributed within the cell and nucleus (not shown). When we expressed the N-terminal regulatory domain, however, it showed a cytoplasmic and perinuclear staining similar to the full length protein, indicating that this region is mainly excluded from the nuclear compartment. Interestingly, also the myc tagged RDP domain showed a prevalently cytoplasmic staining, suggesting that beside to the well characterized p21 Binding Domain (which is not present in the RDP portion), other sequences within the N-terminal domain could also determine PAK4 subcellular localization, and could in fact direct nuclear exclusion and cytosolic localization.

To determine whether the RDP contains specific targeting sequences that could also drive redistribution of the full length protein, we constructed egfp fusions and analysed their cellular localization by fluorescence microscopy (fig. 6b). When the egfp protein is expressed in HeLa cells it is found distributed throughout the cell, including the nucleus. When the RDP domain was fused to egfp (egfp-RDP), the fusion protein was instead found to be cytoplasmic, showing that in this case egfp is preferentially excluded from the nuclear compartment. However, when we used the

smaller RDC PAK4 portion, the egfp fusion was no longer excluded from nuclei, showing a diffuse signal within the cytosol and nuclear accumulation.
Considering the difference in size of the two fusion proteins and that extra aminoacids might prevent egfp diffusion into the nucleus, we also evaluated localization of egfp-fused kinase domain (egfp-KDom), which contains a larger (unrelated) portion of PAK4. As shown in figure 6b, egfp-KDom is evenly distributed in the cells and clearly shows nuclear localization, indicating that a larger size of the fusion protein does not impede egfp, or PAK4 kinase domain, nuclear localization *per se*. Further dissection of the RDP portion allowed us to determine that aa 65-109, not included in RDC, are sufficient to drive egfp cytoplasmic localization (not shown).
Taken together these data indicate that specific sequences in the RDP protein, within PAK4 aa 65-109, can promote active export of egfp from the nucleus.
These data also suggest that PAK4 sequences we found implicated in ribonuclear protein complexes interactions could also be relevant for nucleocytoplasmic shuttling of the full length protein. In this view, cellular localization and/or transport may also have relevant implications in PAK4 mediated regulation of EMCV IRES driven translation.

Endogenous PAK4 is found in both cytoplasmic and nuclear fractions and is involved in translational regulation.

In order to support the relevance of our findings, we performed cellular fractionation experiments and evaluated endogenous PAK4 cellular distribution. While cytoplasmic fractions revealed, as expected, a prevalent band of the predicted molecular size of 64KDa, we observed that several protein bands were detected in the nuclear extracts by available antibodies (not shown). In order to discriminate for the specific PAK4 signal we compared HeLa cells with PAK4 knock down stable cell lines (PAK4i) (Li and Minden, 2005). As shown in figure 7a, HeLa extracts clearly show the presence of a specific PAK4 band in both cytosolic and nuclear fractions, which is markedly reduced in PAK4i control cells. These data indicate that in Hela cells endogenous full length PAK4 has both cytoplasmic and nuclear localization. Transfected full length PAK4 showed a similar distribution in cellular fractions (not shown).
Finally we tested IRES activity in PAK4 knock down cells. The dicistronic reporter vector containing the EMCV IRES was transfected in HeLa PAK4i or control cell lines and F/R values were normalised to those obtained with pR-F control. As shown

in fig. 7b, we found that in PAK4i cells IRES driven translation was consistently increased, when compared to control cell lines. These data indicate that endogenous PAK4 has a relevant function in translational control mechanisms of IRES sequences.

DISCUSSION

Protein kinase PAK4 is a pivotal regulator of cellular pathways that are involved in oncogenic transformation. Containing a conserved N-terminal PBD and C-terminal serine-threonine kinase domain, it has been identified as a Pak family Rho GTPases effector that mediates cytoskeletal rearrangements acting preferentially as a CDC42 target (Abo et al., 1998). Several studies reported its pro-oncogenic potential as an active or overexpressed kinase, and indeed its overexpression and gene locus amplification has been reported in cancer cell lines and tumors of different tissue origins, including pancreatic, colon, mammary tumors and squamous cells carcinoma. Further, its function was reported to be necessary for cancer cells features of oncogenic and metastatic transformation. (Qu et al., 2001; Callow et al., 2002; Wells et al., 2002; Li and Minden, 2005; Parsons et al., 2005; Liu et al., 2008; Kimmelman et al., 2008; Chen et al., 2008; Ahmed et al., 2008; Begum et al., 2009).

It is increasingly evident that PAK4 cellular functions are not limited to regulation of actin dinamics and cytoskeletal remodeling, and, importantly, not all of them require CDC42 interaction or the catalytic activity (Wells et al., 2002; Gnesutta and Minden, 2003; Li and Minden, 2005). In fact, the growth advantage of PAK4 overexpressing cells is also in part explained by conferred resistance to apoptotic pathways activation and by its role in promoting survival responses (Wells et al., 2002; Gnesutta et al., 2001; Gnesutta and Minden, 2003; Li and Minden, 2005; Lu et al., 2003).

In understanding PAK4 biological and pathological functions it is thus of great importance to clarify its interactions with cellular pathways and its modes of regulation. As for other group B Paks, PAK4 kinase activity is not increased by interaction with active GTPases, and lack of a conserved AID suggests that other regulatory modes should exist. Nontheless, it is also clear that in PakB kinases the N-terminal (regulatory) domain plays an important role in inhibiting their catalytic activity (Abo et al., 1998; Yang et al., 2001; Callow et al., 2002; Ching et al., 2003).

Current evidence rather supports the idea that PAK4, and PakB proteins, functional regulation mainly involves cellular relocalization and local interaction with specific targets. The PBD domain is required for PAK4 relocalization to Golgi structures and for sustained filopodia formation by active CDC42, while HGF migratory signals relocalize PAK4 to cell edges independently of CDC42 interactions. Active PAK4 also localizes to, and modifies, podosomal structures in migratory macrophages. (Abo et al., 1998; Callow et al., 2002; Wells et al., 2002; Gringel et al., 2006). PAK5 can shuttle to mitochondria and in nuclei, and PAK6 is localised to nuclei following AR interaction (Cotteret et al., 2006; Wu and Frost, 2006; Yang et al., 2001).

The high sequence divergence of the central portions of PakB proteins also suggests that specific signaling pathways regulation and/or target interactions could occur in this region of the proteins. In fact, PAK5 AID and cellular localization signals were mapped in this domain (Ching et al., 2003; Cotteret et al., 2006), and PI3K dependent PAK4 regulation in HGF signaling occurs in this protein portion (Wells et al., 2002). It's also interesting to note that PAK4 regulatory domain lacking the PBD efficiently acts as a dominant negative mutant for Dbl transformation (Qu et al., 2003), indicating relevant functional roles for this domain.

With the intent of isolating proteins interacting with the PAK4 N-terminal domain, in our study we decided to screen for potential targets/partners with an affinity chromatography approach, and found that several proteins were specifically isolated from Triton extracts of HeLa cells using the N-terminal regulatory domain as a bait. In these assay conditions we found that activated CDC42 is also bound by the N-terminal baits containing the PBD, further supporting the assay specificity (not shown).

Mass spectrometric analysis and identification of prominent interacting bands showed that most of the isolated proteins were ribosomal proteins, and presence of other RNA binding proteins suggested they could be part of a RNP complex. In fact, by WB analysis we confirmed that both small and large subunits components were specifically present in PAK4 N-ter precipitates, together with other RNA binding proteins such as FMRP. Association of all the proteins we analysed by WB was dependent on RNA integrity, indicating that ribosomal binding could occur only in the presence of RNA. While we cannot exclude that PAK4 directly interacts with a specific ribosomal protein(s) on assembled ribosomes, since RNaseA treatment also caused rRNA degradation (not shown), other treatments that affect ribosomal binding

to mRNAs and polysome stability such as EDTA, CHX (fig. 2), and high salt concentration (not shown), also affected rp binding to PAK4. These data strongly indicate that proteins isolated by affinity chromatography are part of a ribosome containing RNP complex. We also found that FMRP, a RNA binding protein involved in translational regulation, is present in PAK4 isolated RNPs and its binding is also disrupted by treatements that affect RNA and ribosome integrity. In brain cells FMRP is found associated to RNAs in heavy RNP particles, and could act on translating polysomes or be part of translationally inhibited RNP complexes (Corbin et al., 1997; Zalfa et al., 2006). It is interesting to note that PakA proteins were found implicated in mental retardation syndromes and PAK1 was found to interact with FMRP in brain extracts (Eswaran et al., 2008; Hayashi et al., 2007). Also considering neuronal cells defects in PAK4 knock-out embryos (Qu et al., 2003), our findings suggest that PAK4 might also play a part in FMRP mediated cellular events.

Among the ribosomal components we identified by MS in PAK4 precipitates, one of the most abundant was rpP0. Together with rpP1 and rpP2, rpP0 is a constituent of the ribosomal stalk of the large subunit, regulated by phosphorylation. This structure is connected to the GTPase center and is involved in binding of Elongation Factors, with pivotal functions in several steps of the translation process. Assembly of the stalk is a late step in ribosomal biogenesis and P proteins are also found in cytoplasmic pools, suggesting that loading could be regulated in the cytoplasm (Ballesta and Remacha, 1996; Tchórzewski et al., 2003). Interestingly, recent evidence also involved rpP0 in regulation of the FMDV IRES translational activity (Martínez-Azorín et al., 2008). rpQM/rpL10 is also a late biogenesis ribosomal component, required for subunit joining, with a role in nuclear export of the large subunit. Reports also indicated possible extraribosomal and tumor suppressor functions for QM (Zemp and Kutay, 2007; Monteclaro and Vogt, 1993).

Due to the diverse function and composition of RNPs in the cell (Moore, 2005), more studies will be needed to understand the nature of the complex(es) we found to interact with PAK4. However, presence of components from both ribosomal subunits together with RNA binding proteins like FMRP and DDX3 (Corbin et al., 1997; Zalfa et al., 2006; Lai et al., 2008) seems to suggest that PAK4 could be part of translating or stalled polysomes, or be involved in RNP transport.

Another interesting hypothesis supported by our in vivo data, is that PAK4 could regulate translational events of specific mRNAs, possibly by regulating association of specific factors.

Internal Ribosome Entry Site (IRES)- mediated translation initiation has been shown to be exploited in specific cellular conditions, in particular when cap dependent translation is inhibited, such as during specific cell cycle phases or in stress conditions, like apoptosis and viral infection (Hellen and Sarnow, 2001; Holcik and Sonenberg, 2005). This alternative allows expression of specific mRNAs, for the execution of the cellular response, or to exploit the translational machinery during global protein synthesis shutoff. Different canonical and non canonical factors are known to bind specific IRESs, regulating translation initiation from these sequences. Many of the known ITAFs (IRES Transacting Factors) also have other roles in mRNA metabolism and their cellular localization and/or (regulated) nucleo-cytoplasmic shuttling also plays an important part for IRES functionality (Spriggs et al., 2005; Lewis and Holcik, 2008; Sawicka et al., 2008; Cammas et al., 2007).

We found that translational activity of different IRES sequences is differentialy regulated by active full length PAK4 (PAK4 NE), with a decrease of the apoptotic c-Myc IRES, and an increase of viral IRESs mediated translation (fig. 4).

While we cannot exclude an indirect effect that results in repression of Myc IRES activity due to higher expression of the dicistronic mRNA (Stoneley et al., 2000a), these data are in good agreement with the pro-survival PAK4 role during apoptotic insults (Gnesutta et al., 2001; Li and Minden, 2005). In fact we could observe that F/R values are also partially repressed in the pR-F control, nontheless PAK4 NE induced a specific and statistically significant reduction in c-Myc IRES reporter activity (see fig. 4). On the other hand two different IRESs of viral origin (EMCV and HRV) share similar regulation by PAK4 NE, opposite of what we observed for c-Myc IRES, as their activity is substantially increased. These data further support a specific effect on translation by PAK4 NE.

Moreover, we also found that stably silenced PAK4 cell lines show increased IRES driven activity (fig. 7b), further indicating a relevant new role for PAK4 in translational control.

One of the possible explanations of the observed upregulation of EMCV IRES activity in knock-down cells is that endogenous PAK4 could for example repress

general IRES dependent translation in basal conditions, resulting in increased activity after PAK4 silencing, while activated PAK4 would mimick specific stress conditions. While some studies already reported a role for PAK4 in cellular responses to bacterial infection (Huang et al., 2007; Eswaran et al., 2008), to our knowledge no information is yet available on PAK4 implications during viral infection or life cycle. In this case, our data suggest a novel PAK4 pathway that could be involved, and possibly exploited, in viral infection, at least for regulating translation of viral RNA.

Implication of PAK4 N-terminal domain in translational regulation was assesed on the EMCV IRES activity. Our experiments (fig. 5) show that PAK4 deletion mutants, known to be activated by removal of N-terminal domain portions, cannot obtain similar activatory effects on EMCV IRES driven translation as observed with full length active PAK4. These data show that interactions occurring within aa 1-119 (deleted in PAK4 DeltaCRIB), have a relevant role in regulation of EMCV IRES function by active PAK4.

Interestingly, these interactions seem to specifically affect the viral IRES(s), since the same deletion mutants, when tested on the Myc IRES construct, had similar inhibitory effects as the full length active protein (not shown).

While coprecipitation experiments of endogenous or exogenously expressed proteins did not allow us to detect stable association of full length or N-terminal PAK4 domains with rps in cells (not shown), and to determine whether an association with RNPs occurs in vivo, by our analyses (figs. 1 and 3) we know that aa 65-110 significantly affect PAK4 N-terminal domain binding with ribosomal proteins in vitro. This region might thus be required for regulation of the EMCV IRES by active PAK4.

On the other side, we cannot exclude that the p21 Binding Domain (with the conserved motif spanning aa 10-48) or other sequences within aa 1-119, could also determine specific associations that affect PAK4 ability to regulate EMCV IRES driven translation. To address this point further analysis with internal deletants and/or point mutants will be needed.

Another explanation for such functional difference may reside in cytosolic/nuclear distribution of PAK4 protein domains. PAK4 nuclear accumulation can be observed, as already described (Abo et al., 1998; Wells et al., 2002), after deletion of the N-terminal domain or aa 1-119 (see fig. 6a). Again, cytosolic localization determinants seem to reside within this protein region, since the N-

terminal domain also shows cytosolic localization. However, considering that the RDP portion (aa 65-175), but not the RDC (aa 110-175) can drive cytoplasmic localization (fig. 6b), we can conclude that aa 65-110 contain a targeting domain that acts as a nuclear export signal. Considering that endogenous (full length) PAK4 is also found in nuclear fractions (fig. 7), we suggest that such region could act as a NES for the full length protein. In this view, association with RNPs, translational control and cellular distribution, or even protein activity, could be affected by interactions mediated by the RDP region of PAK4 N-terminal domain.

Sequence analysis of the RDP domain allowed us to identify a hydrophobic aminoacid stretch that matches a NES consensus (la Cour et al., 2004) (not shown) and we are currently investigating if such sequence is involved in one or more of these processes.

In conclusion our study allowed us to define a novel region that could play relevant functional roles in PAK4 cellular biology, and suggests new perspectives for understanding its modes of regulation in normal cells and in malignant transformation.

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FIGURE LEGENDS

Figure 1: Affinity chromatography of HeLa cell lysates with PAK4 N-terminal domains.

A) The N-ter domain of PAK4 interacts with ribosomal proteins in a RNA dependent manner. HeLa cells lysates prepared in the presence of RNase inhibitor, or treated with RNase A, as indicated, were used in affinity chromatography pull-down experiments, using PAK4 N-terminal region (aa 1-297) GST fusion protein (N-ter, lanes 4-6), or control GST tag (lanes 1-3), covalently linked affinity matrices. Control samples incubated with buffer alone (no lys, lanes 7, 8) were used for detecting bait proteins background. Eluted proteins were separated by SDS-PAGE and visualised by Silver staining (top panel), or Western Blotting (WB) (bottom panels) with indicated antibodies (Abs) to specific ribosomal proteins (rp). Protein standards migration positions (molecular weight -MW- in KDa) are indicated on the left side of the top gel. Arrows on the right indicate the migration positions of full length bait proteins (top arrow: GST-N-ter, bottom arrow: GST). WCL: whole cell lysate control.

B) The RDP region (aa 65-175) of PAK4 N-terminal domain is sufficient for efficient rp binding. Top panel: Schematic depiction of human PAK4 with the relative positions of the conserved Pak proteins PBD and Kinase Domains (in shaded boxes), and diagram of PAK4 N-terminal domain fragments, with corresponding aminoacid residues, that were used as purified GST fusion proteins in affinity chromatography experiments described in this work. Bottom panels: PAK4 fragments affinity matrices (indicated above the panels) were used in pull-down experiments using HeLa extracts, or buffer alone (lysate + or -). Eluted proteins were separated by SDS-PAGE and analysed by WB to detect interaction with specific rps, as indicated. WCL: whole cell lysate input control.

Figure 2: The RDP interaction with RNPs retains sensitivity to RNase and is disruped by EDTA treatment.

A) HeLa cells lysates treated, or not, with RNAse A or prepared in the presence of RNase inhibitor, as indicated, were used in pull-down experiments using GST-PAK4 aa 65-175 (RDP, lanes 1-3) or control GST tag (lanes 5-7) covalently linked affinity matrices. Control samples incubated with buffer alone (no lys, lanes 4, 8) were used for detecting bait proteins background. Eluted proteins were resolved by SDS-PAGE

and visualised by Silver Staining (top panel), or WB (bottom panels) with Abs directed to specific rps, as indicated. On the left side of the top panel migration positions of MW protein standards (in KDa) are shown. Arrows on the right indicate migration positions of full length bait proteins (top arrow: GST-RDP, bottom arrow: GST).

B) Affinity chromatography of HeLa cell lysates prepared in the presence, or not, of CHX and treated with EDTA as indicated (lysate treatment), was performed with RDP or GST tag matrices. Eluted samples were analysed by WB to detect interaction with specific rps, or FMRP, as indicated on the left side of the panels. WCL: whole cell lysate input control lane.

Figure 3: RDP associates to exogenously expressed rpP0.

Hek293 cells were transfected with egfp (egfp) or egfp-rpP0 (egfp-rpP0) expression vectors and cell lysates were used in GST pull-down assays with GSH beads preloaded with equivalent amounts of the indicated GST fusion protein (bait). Eluted proteins were separated by SDS-PAGE and analysed by WB with anti-GFP Abs to detect interaction with the prey protein (top panels). Stripped membranes were re-probed with anti-GST Abs to confirm equal loading of bait proteins (bottom panel). Arrows on the right side of the top panels indicate migration positions of egfp-rpP0 and egfp prey proteins (top and bottom arrows, respectively). WCL input: whole cell lysate input control.

Figure 4: Active PAK4 modulates IRES driven protein expression from dicistronic reporters.

Top: Schematic diagram of the pR-F dicistronic plasmid with the indicated position of the IRES sequences contained in the pR-F derived constructs used in this work. Left panel: HeLa cells were transfected with the pR-F plasmid or the c-Myc IRES containing reporter (pR-Myc-F), together with wt PAK4, active PAK4 (PAK4 NE) or active PAK2 (PAK2 K+) expression vectors, or empty vector control (vec). After transfection, Renilla and Firefly luciferase activities were measured in cell lysates and expressed as Relative IRES driven translation (F luc vs R luc, IRES dependent versus cap-dependent translation) normalised to the pR-F control sample. Right panels: HeLa cells were transfected with pR-F (none) or with c-Myc, EMCV or HRV IRES containing reporters, as indicated (pR-F IRES), together with full length active PAK4

(PAK4 NE) or control vector (+ or –, respectively). Relative IRES translation, calculated and expressed as described above, is shown in the top graph (F luc vs R luc). To compare PAK4 NE effects on different reporters (bottom panel, Fold Change graph), Relative IRES translation was also expressed as normalised to the correspondent vector control sample (PAK4 NE *minus*). For the Myc IRES reporter, an asterisk denotes statistically significant difference as compared to pR-F PAK4 NE (+) control (Student's t-Test $p<0.01$).

Values shown in graphs represent the average of data (± s.e.m.) from at least 2 experiments. Each experiment was performed in duplicate samples. Bar filling shades indicate samples transfected with the same IRES reporter vector.

Figure 5: PAK4 N-terminal domain is implicated in regulation of EMCV IRES translation by active PAK4.

Top panel: Comparison of PAK4 N-terminal deletion mutants effects on EMCV IRES. HeLa cells were transfected with pR-F, or with the pR-EMCV-F reporter together with full length wt or active PAK4 (PAK4 wt or NE), or with the following PAK4 deletion mutants: active Kinase Domain (KDom), inactive Kinase Domain (KDomKM), or active DeltaCRIB (DeltaCRIB). Luciferases' activities were measured and expressed as Relative IRES translation normalised to the empty vector control sample, as described in fig.4 (Fold Change graph). Values shown in graph represent the average of data (± s.e.m.) from 3 experiments. Each experiment was performed in duplicate samples. Bottom panel: Lysates from parallel samples were separated by SDS-PAGE and analysed by WB with the indicated Abs to detect PAK4 proteins relative expression levels. PAK4 deletion mutants do not contain the anti-PAK4 Abs epitope and were detected with HAtag Abs. For comparison, HAtag PAK4wt sample was loaded on both sides. On the left side of the panels an arrow indicates the endogenous PAK4 protein detected by anti-PAK4 Abs. Migration position of protein MW standards (in KDa) are indicated on the right side of the panels.

Figure 6: Cellular localization analysis of PAK4 protein domains by fluorescence microscopy.

A) PAK4 N-terminal domain and the RDP region show cytoplasmic distribution similar to full length PAK4. Top panels: HeLa cells were transfected with expression

vectors for epitope tagged full length PAK4 (PAK4wt) or PAK4 deletion mutants, as indicated on the left side of the pictures. Protein localization was visualised by immunofluorescence analysis of fixed cells with Abs to the relevant epitope tag, as indicated above the panels. Bottom panel: schematic representation of the relative PAK4 proteins with corresponding aminoacid residues. The PBD and Kinase Domains are indicated by shaded boxes.

B) The RDP domain is sufficient to drive egfp nuclear exclusion and cytoplasmic localization. Top panels: HeLa cells were transfected with plasmids encoding for egfp fusion proteins of different PAK4 domains, as indicated on the left side of the pictures. egfp proteins localization was visualised in fixed cells by fluorecence microscopy. Bottom panel: Schematic representation of the egfp and egfp-PAK4 fusion proteins and corresponding predicted MW. The relative PBD, Kinase Domain, and egfp portion positions are indicated by shaded boxes.

Figure 7: Endogenous PAK4 localization and functional analysis.

A) Cellular fractionation shows endogenous full length PAK4 is found in both cytoplasmic and nuclear fractions. HeLa cells or PAK4 knock-down stable cell lines control (PAK4i) were fractionated to obtain cytoplasmic (C) and nuclear (N) extracts. Equal protein amounts were loaded on SDS-PAGE and analysed by WB with Abs to PAK4. An arrow indicates the specific PAK4 band which is downregulated in PAK4i cells. An asterisk indicates an unspecific band detected in nuclear extracts. Anti PARP and tubulin Abs were used as fractionation control, to detect nuclear or cytoplasmic (respectively) localised proteins.

B) EMCV IRES translation is upregulated in PAK4 knock-down cells. HeLa PAK4i cells or vector control stable cell lines were transfected with pR-EMCV-F or pR-F. Bars indicate EMCV driven Relative IRES translation (calculated as in fig. 4) expressed as fold change of the control cells values. Values shown in graph represent the average of data (± s.e.m.) from 2 experiments. Each experiment was performed in triplicate samples. An asterisk denotes statistically significant difference from control cells (Student's t-Test $p<0.02$).

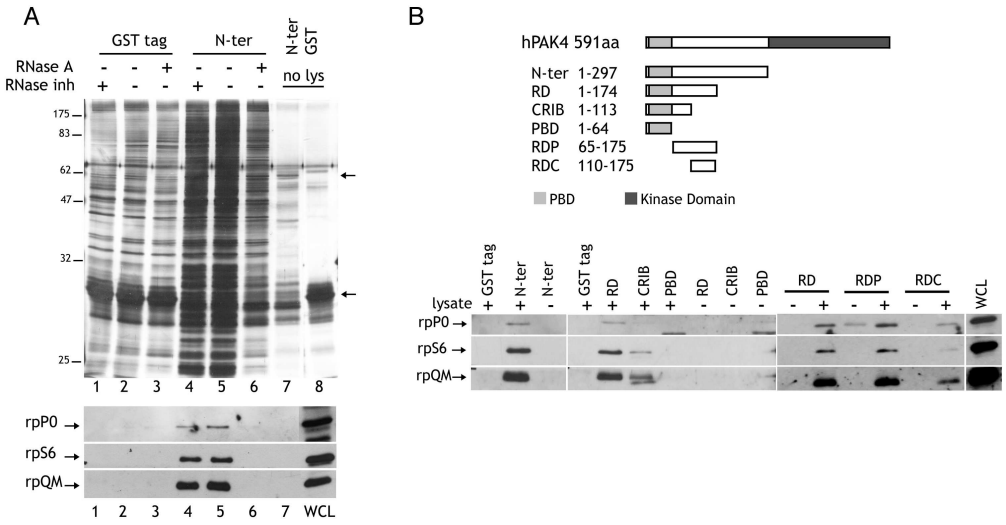


Fig.1

Affinity chromatography of HeLa cell lysates with PAK4 N-terminal domains
A) The N-ter domain of PAK4 interacts with ribosomal proteins in a RNA dependent manner
B) The RDP region (aa 65-175) of PAK4 N-terminal domain is sufficient for efficient rp binding

170x94mm (450 x 450 DPI)

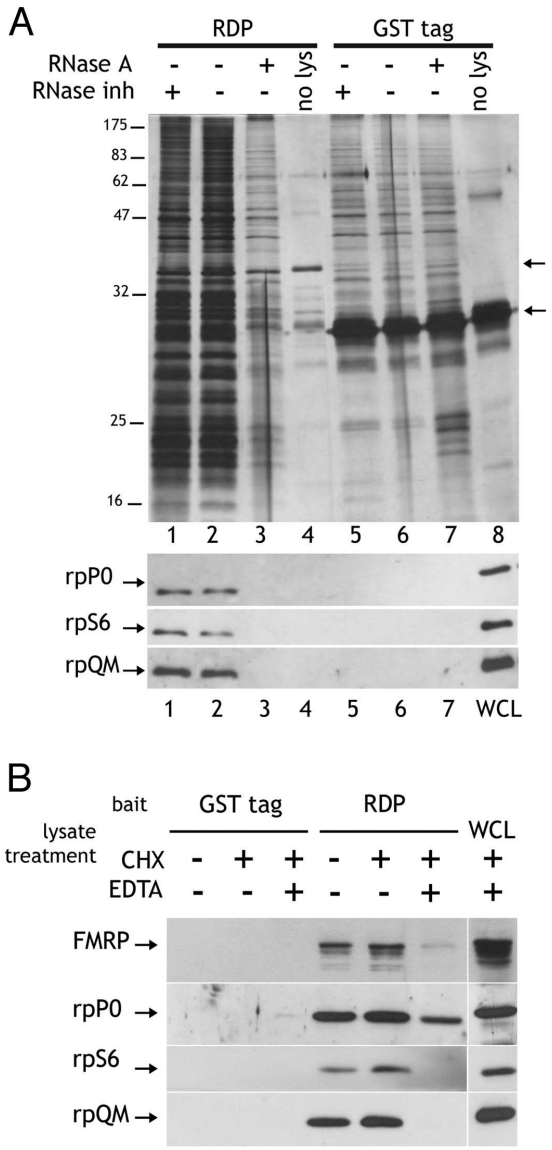


Fig. 2

The RDP interaction with RNPs retains sensitivity to RNase and is disruped by EDTA treatment
66x146mm (400 x 400 DPI)

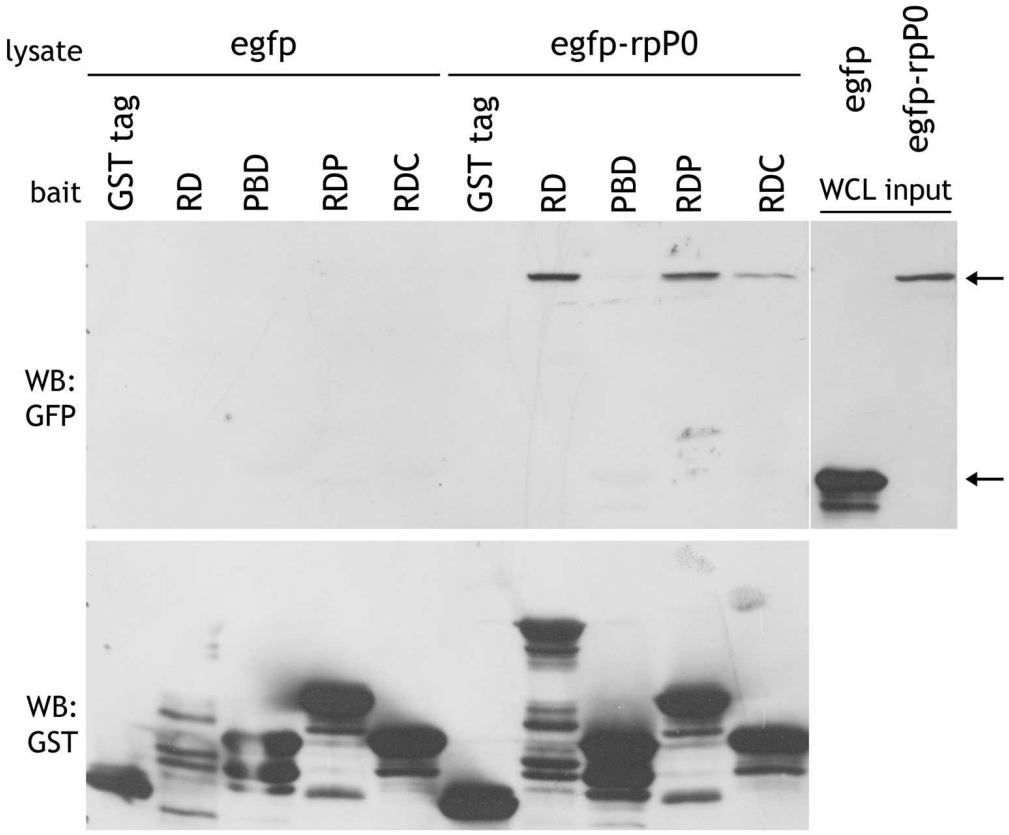


Fig. 3

RDP associates to exogenously expressed rpP0
64x58mm (600 x 600 DPI)

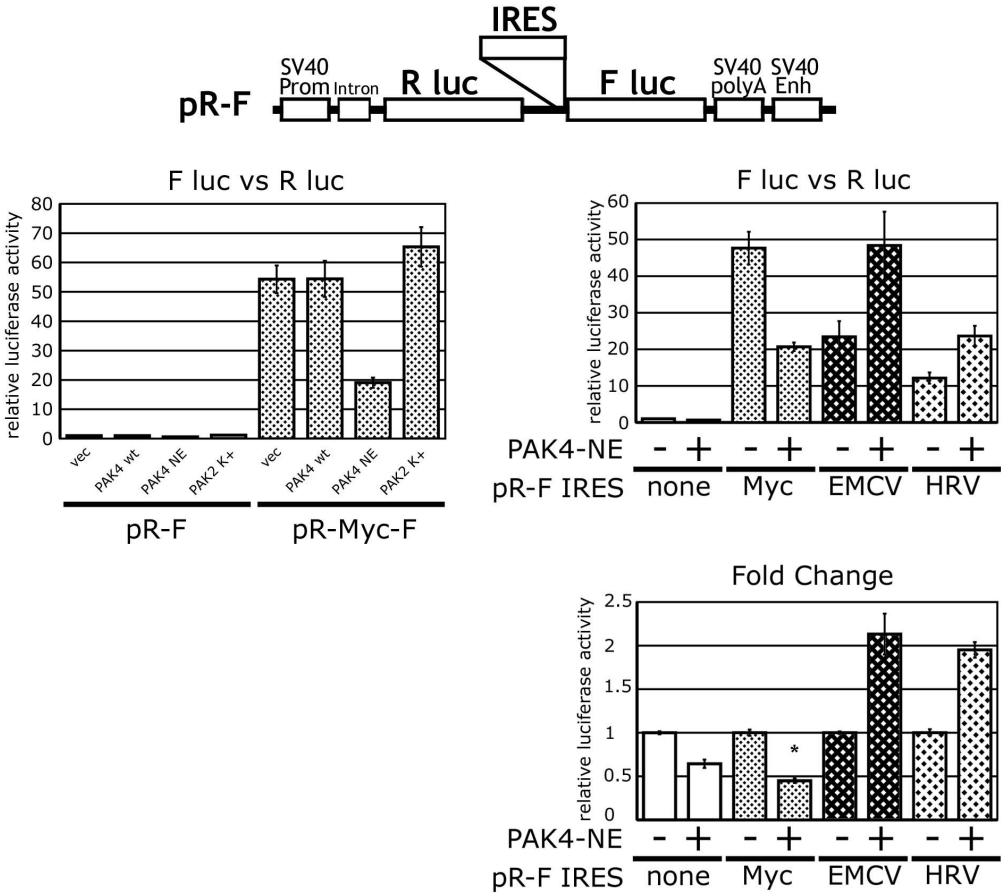


Fig. 4

Active PAK4 modulates IRES driven protein expression from dicistronic reporters
106x101mm (450 x 450 DPI)

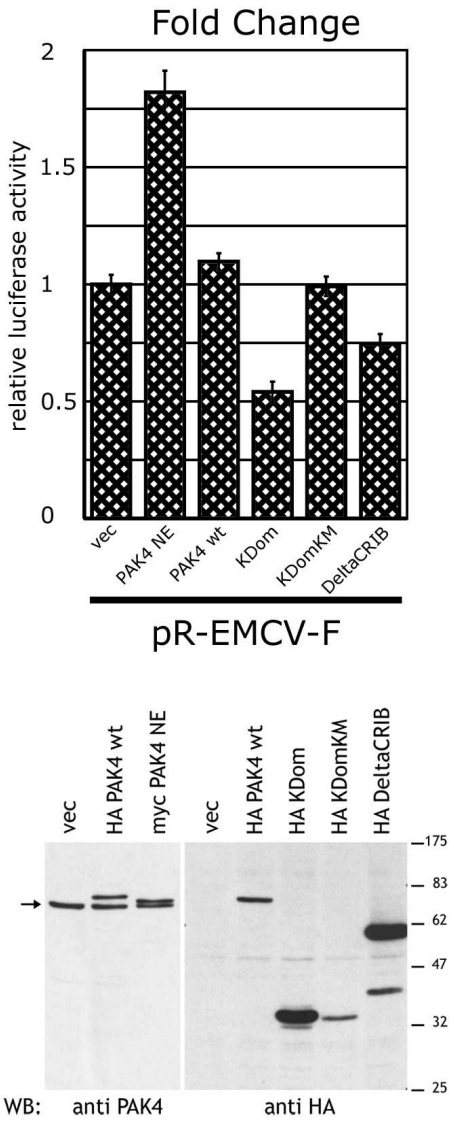


Fig. 5

PAK4 N-terminal domain is implicated in regulation of EMCV IRES translation by active PAK4
42x112mm (400 x 400 DPI)

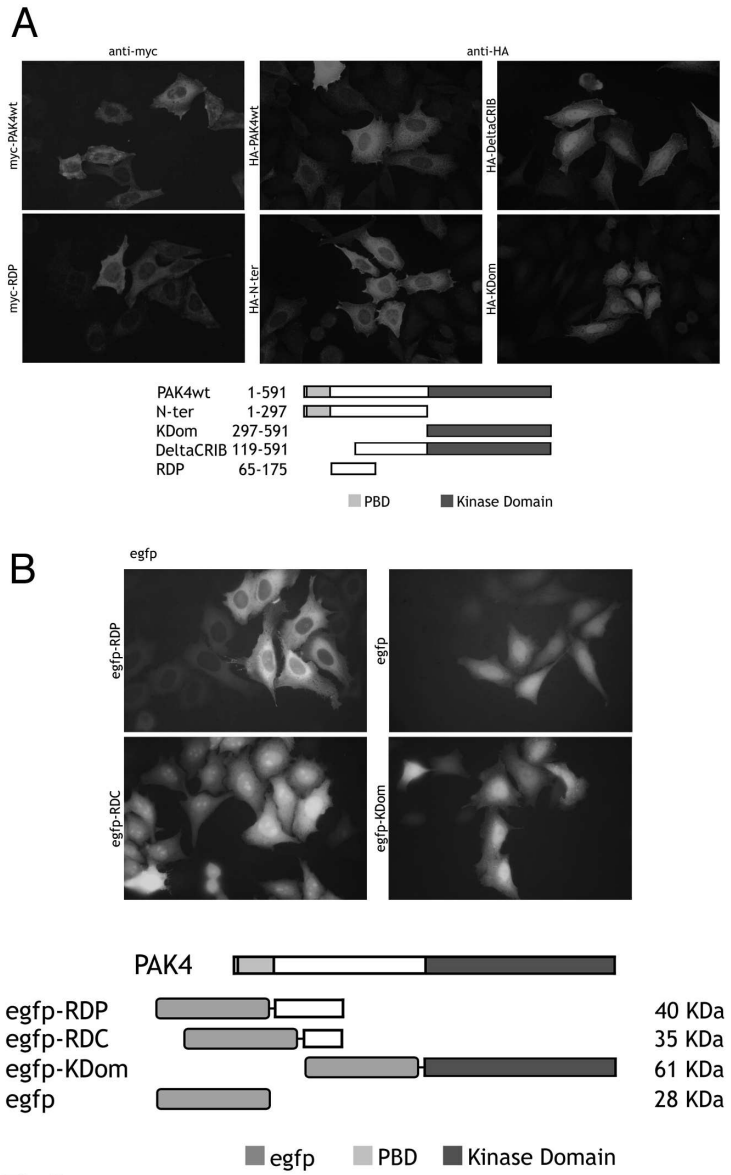
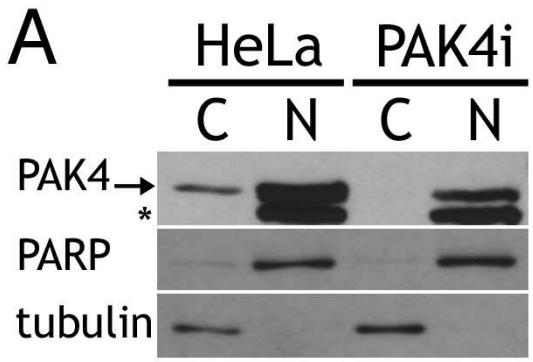


Fig.6

Cellular localization analysis of PAK4 protein domains by fluorescence microscopy.
A) PAK4 N-terminal domain and the RDP region show cytoplasmic distribution similar to full length PAK4
B) The RDP domain is sufficient to drive egfp nuclear exclusion and cytoplasmic localization

112x184mm (300 x 300 DPI)



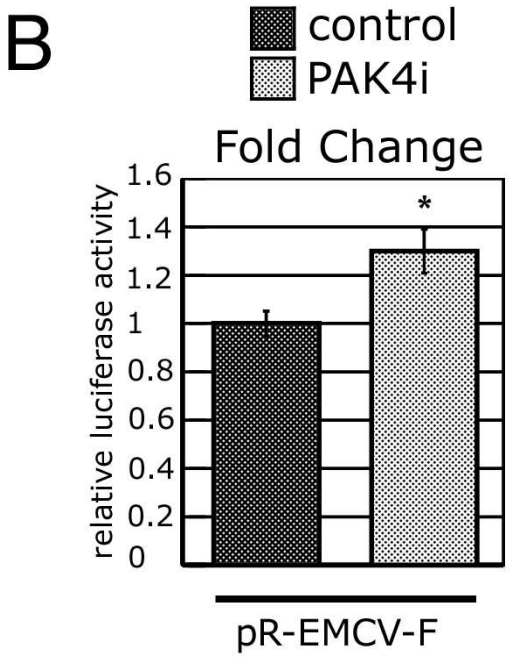


Fig. 7

Endogenous PAK4 localization and functional analysis
35x77mm (450 x 450 DPI)