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p120-Catenin Is a Binding Partner and Substrate for Group B Pak Kinases

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ABSTRACT

Pak5 is a member of the Group B p21-activated kinases, which are effectors of the Rho family GTPases Cdc42 and Rac. Pak5 has been shown to promote cytoskeletal reorganization, inducing filopodia formation and neurite outgrowth in neuroblastoma cells. In this study, we used affinity chromatography followed by SDS–PAGE and mass spectrometry to identify potential downstream effectors of Pak5. Using this approach, we isolated p120-catenin (p120), a known regulator of cytoskeletal reorganization and Rho GTPases. Using co-immunoprecipitation assays we found that p120 preferentially interacts with Pak5 among the Group B Paks. Results from immunofluorescence studies revealed that Pak5 and p120 co-localize in cells. Both Pak5 and constitutively active Pak4, the founding member of the Group B Paks, directly phosphorylate p120 in vitro. The phosphorylation was shown by Western blot and immunofluorescence to take place specifically on serine 288. This study is the first report of an upstream serine/threonine kinase that phosphorylates p120. J. Cell. Biochem. 110: 1244–1254, 2010.

KEY WORDS: Pak5; p120-CATENIN; KINASE; PHOSPHORYLATION

The p21-activated kinases (Paks) are a family of serine/threonine protein kinases that were first identified by their ability to bind to the Rho GTPases, Cdc42, and Rac. In mammals, Paks fall into two classes: Group A and Group B based on their amino acid sequences and architecture [reviewed in Jaffer and Chernoff, 2002]. The Group A Paks consist of Pak1, Pak2, and Pak3 [Parrini et al., 2002]. The Group B Paks, consisting of Pak4, Pak5, and Pak6 [reviewed in Jaffer and Chernoff, 2002], differ significantly from the Group A Paks. They lack the Nck-binding, PIX/COOL-binding, and autoinhibitory domains of the Group A Paks, and their GBD and kinase domains exhibit only 50% sequence homology with Group A Paks. Within the GBD and kinase domains the sequence identity among the Group B Paks is quite high, although outside these domains, they are almost completely different. Functionally, Group B Paks differ from Group A Paks in that Cdc42 or Rac binding to the GBD is not required for kinase activity. Furthermore, Group A Paks can complement defects in *Saccharomyces cerevesiae*, Ste20, the founding member of the Pak family of kinases while in contrast, Group B Paks cannot.

Pak4 was originally identified as an effector of Cdc42, linking it to filopodia and actin cytoskeletal reorganization [Abo et al., 1998]. Expressed ubiquitously, it has since been shown to play roles in anchorage-independent growth [Qu et al., 2001], embryonic development [Qu et al., 2003], cell survival [Gnesutta et al., 2001; Gnesutta and Minden, 2003; Li and Minden, 2005], cell senescence [Cammarano et al., 2005], and tumor formation [Liu et al., 2008]. Pak6 was identified as a protein that interacts with the androgen receptor [Yang et al., 2001]. Expressed largely in the testis and prostate as well as the brain, Pak6 has been shown to play a role in androgen receptor signaling [Yang et al., 2001; Lee et al., 2002] *Pak5/Pak6* double-knockout mice show defects in behavior, memory and learning [Nekrasova et al., 2008], suggesting that these two kinases may play roles in neural development.

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Pak5, which is highly expressed in the brain [Dan et al., 2002; Pandey et al., 2002], was originally shown to induce filopodia formation and neurite outgrowth in neuroblastoma cells [Dan et al., 2002]. Little is known about the mechanism by which Pak5 induces neurite outgrowth. One possibility is that Pak5 inhibition of RhoA may contribute to this process [Tigyi et al., 1996; Gebbink et al., 1997; Kozma et al., 1997; Dan et al., 2002]. Pak5 is also an inhibitor of the MARK2 kinase, which leads to stabilization of microtubules [Matenia et al., 2005]. This may also play a role in Pak5 regulation of cell shape changes that are necessary for neurite outgrowth. In addition to cytoskeletal organization, Pak5 has been shown to have other functions. These include inhibition of apoptosis, which involves targeting Pak5 to the mitochondria [Cotteret et al., 2003; Cotteret and Chernoff, 2006]. Pak5 has been shown to phosphorylate and activate Raf-1, which in turn controls signaling pathways such as the MAP Kinase pathway [Wu et al., 2008]. Finally, Pak5 has recently been found to be overexpressed in colorectal carcinoma cell lines and may regulate their adhesion and migration [Gong et al., 2009].

In this study, we describe the identification of a new candidate effector protein for Pak5. Using affinity chromatography we have found that Pak5 binds to p120-catenin (p120). p120, the founding member of the δ-catenin family, is an important regulator of cytoskeletal reorganization. It inactivates RhoA through a guanine dissociation inhibitor (GDI)-like mechanism and also activates Rac [reviewed in Anastasiadis and Reynolds, 2001]. It is implicated in a number of processes, including cadherin stabilization, metastasis, inflammation, and possibly transcriptional regulation [reviewed in Reynolds and Carnahan, 2004; Anastasiadis, 2007; Daniel, 2007]. We show here that Pak5 directly phosphorylates p120, and propose that it is involved in cytoskeletal reorganization mediated by Group B Paks. Interestingly, we found that Pak4 also strongly phosphorylates p120, although it is not a strong p120 binding protein. It will be interesting to determine whether p120 has an important role in Pak4 signaling pathways leading to changes to cell growth and survival, as well as the regulation of cell shape.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Mouse monoclonal anti-myc-tag, rabbit polyclonal anti-myc-tag, and HA-tag antibodies were purchased from Cell Signaling Technologies. Rabbit polyclonal anti-Pak5 antibody was described in [Nekrasova et al., 2008]. Mouse monoclonal anti-p120 antibody and antibodies specific for p120 phosphorylated on serine 268, serine 288, threonine 310, and threonine 916 were all purchased from BD Biosciences. Cy3-conjugated donkey anti-mouse and Cy2-conjugated donkey anti-rabbit IgG antibodies were purchased from Jackson Immunoresearch. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG antibodies were purchased from Sigma Life Sciences.

PLASMIDS

pGEX-Pak5 was obtained by subcloning Pak5 in frame with the GST protein into the pGEX-KG vector. pGEX-p120Δ was obtained by subcloning a fragment corresponding to the N-terminal 330 amino acids of the 1A isoform of murine p120 from RcCMV-Kpn1/mp120-1A into pGEX-KG. pLPC-mycPak4wt was described by Gnesutta and Minden [2003] and pLPC-mycPak4(S445N) was described by Qu et al. [2001]. pLPC-mycPak5WT, pLPC-mycPak5(S573N), and EGFP-Pak5 were described by Dan et al. [2002]. RcCMV-Kpn1/mp120-1A was described by Xia et al. [2003]. A vector expressing myc-tagged Pak6 was created by cloning wild-type murine Pak6 into pCANmyc1.

CELL CULTURE AND TRANSFECTION

All cell lines were grown at 37°C in 5% CO_2 cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 4 mM glutamine. Transfection of plasmid DNA into HEK293 cells and NIH-3T3 cells was performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturers protocol.

WESTERN BLOTS

Cell lysates were prepared using TL buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1% TritonX-100, 1 mM EDTA, 20 mM β-glycerol phosphate, 1 mM Na_3VO_4, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 10 μg/ml aprotinin). Protein concentrations were determined by microplate assay using the Bio-Rad Protein Assay reagent according to the manufacturer's protocol. Equal amounts of proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidenedifluoride membranes (Immobilon P, Millipore Corp.), blocked with 5% non-fat dry milk in Tris-buffered saline/0.1% Tween (TBS/T), and incubated with primary antibody diluted into TBS/T containing 5% BSA at the following dilutions: anti-myc tag, anti-HA tag, and anti-pS288 – 1:1000, anti-p120, anti-pS268, anti-pT310, anti-pT916, and anti-Pak5 – 1:500. Primary antibody incubations took place overnight at 4°C. After washing in TBS/T, the blots were incubated with secondary antibody diluted into TBS/T–5% non-fat dry milk for 1 h at room temperature. After washing in TBS/T, blots were incubated with ECL chemiluminescent reagent (GE Healthcare) and exposed to X-ray film (Kodak).

QUANTITATION OF WESTERN BLOT DATA AND STATISTICAL ANALYSIS

The amounts of total and phosphorylated HA-tagged p120 in cell extracts were quantitated by measuring the band intensities using ImageJ software (NIH). Low exposure films were scanned as JPEG files. ImageJ was then used to subtract the background and measure the integrated density of each band. The amount of p120 in samples transfected with Pak4 or Pak5 relative to those transfected with empty vector was determined according to the following formula: relative intensity = f/g, where f and g represent the integrated densities of the Pak and the empty vector samples, respectively. The results from triplicate experiments were averaged and plotted using Microsoft Excel (Microsoft). The standard error of the mean (SEM) was calculated as standard deviation/$\sqrt{n}$.

PURIFICATION OF GST FUSION PROTEINS

GST-Pak5 was expressed in the Rosetta 2 strain of *E. coli* (EMD) by induction of log-phase cultures with 200 μM IPTG for 4 h at 30°C.

The fusion protein was obtained from the bacterial pellets by incubation with 40 μg/ml lysozyme in NETN lysis buffer (100 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, 0.5% Nonidet P-40, 5 mM benzamidine, 2 mM DTT plus protease inhibitors). After sonication, the lysates were cleared by centrifugation and incubated with glutathione–agarose beads (Sigma Life Sciences) or Glutathione Sepharose 4B (GE Healthcare) for 1 h at 4°C. After three washes with NETN lysis buffer, Pak5 was cleaved from GST by incubation with thrombin protease and dialyzed against two changes of either dialysis buffer (20 mM Tris, pH 7.6, 100 mM KCl, 0.2 mM EDTA, 2 mM DTT, 20% glycerol, 0.5 mM PMSF) or CNBr-activated Sepharose coupling buffer (0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl) containing 20% glycerol and 0.5 mM PMSF. GST-p120Δ was expressed in the BL21 strain of *E. coli* and purified as described above, then eluted into twice the bed volume of freshly made 20 mM glutathione, 50 mM Tris, pH 8, and dialyzed for storage against two changes of dialysis buffer (20 mM Tris, pH 7.6, 100 mM KCl, 0.2 mM EDTA, 2 mM DTT, 20% glycerol, 0.5 mM PMSF).

COVALENT COUPLING OF Pak5 TO CNBr-ACTIVATED SEPHAROSE

Pak5 protein was covalently linked to CNBr-activated Sepharose (GE Healthcare) according to the manufacturer's protocol. Control Sepharose was prepared by treating the resin according to the protocol in the absence of ligand.

ISOLATION OF Pak5-INTERACTING PROTEINS

To isolate Pak5-interacting proteins for analysis by mass spectrometry, preparative-scale affinity chromatography was performed. HeLa cells were grown in 15 cm tissue culture dishes until confluent, then harvested by scraping into PBS. Cell pellets were pooled and harvested into TL buffer. Five milliliters extract was precleared by incubation with 300 μl of a 50% slurry of GSH–Sepharose for 2 h at 4°C, then incubated with a 50% slurry of Pak5-Sepharose or control Sepharose. The resin was washed three times with TL buffer, and bound proteins were recovered by boiling the resin for 5 min with SDS–PAGE loading buffer. Results obtained from mass spectrometry were confirmed analytic-scale pulldowns followed by Western analysis.

PREPARATIVE SDS–POLYACRYLAMIDE GEL ELECTROPHORESIS AND SAMPLE PREPARATION FOR MALDI-TOF MS ANALYSIS

Proteins recovered from preparative affinity chromatography were separated by electrophoresis on 1 mm thick 10% SDS–polyacrylamide gels. The gels were fixed in 46% methanol, 7% acetic acid for 1 h at room temperature, then stained with filtered 46% methanol, 7% acetic acid, 0.1% Coomassie Brilliant Blue R-250 for 1 h at room temperature. After destaining, bands were excised using new, sterile scalpels in a laminar flow biosafety cabinet in order to minimize contamination. The excised bands were stored in sterile water in sterile 1.5 ml microcentrifuge tubes. Mass spectrometry was done at the Biological Mass Spectrometry Facility of the UMDNJ-Robert Wood Johnson Medical School and Rutgers, The State University of New Jersey. Results were analyzed by searching the proteomics database of the Global Proteomics Machine Organization (thegpm.org).

CO-IMMUNOPRECIPITATION ASSAY

Binding of Group B Pak proteins to p120 was assessed by co-immunoprecipitation assay. HEK 293 cells were transfected with vectors containing myc-tagged Group B Pak constructs or empty vector DNA. At 48 h, cells were harvested into TL buffer. Equal amounts of lysate protein were incubated with 0.5 μl anti-myc-tag antibody overnight at 4°C. Fifty microliters of a 50% slurry of protein A–Sepharose beads (GE Healthcare) were added, and the incubation continued for 1 h. Following three washes in TL buffer, the proteins were recovered by boiling in SDS–PAGE loading buffer and analyzed by Western blot.

To assess binding of endogenous Group B Pak proteins to p120, crude brain lysates were used. Brains from either C57/B6 or *Pak5−/−* mice were homogenized into CoIP lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing protease and phosphatase inhibitors), then rotated for 2 h at 4°C. The lysates were cleared by centrifugation for 15 min at 14,000 rpm at 4°C. One milligram of lysate protein was incubated overnight at 4°C with 5 μg anti-p120 antibody in 50 mM Tris, pH 7.5, 1 mM EDTA, 0.25% NP-40, for a final salt concentration of 32 mM. One hundred microliters of a 50% slurry of protein G–Sepharose beads (GE Healthcare) were added, and the incubation continued for 1 h. Following the incubation, the protein A–Sepharose beads were washed three times in CoIP wash buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA). Analysis of the bound proteins was as described above.

INDIRECT IMMUNOFLUORESCENCE

Cells were seeded either into 6-well plates containing 22-mm glass coverslips, or 12-well plates containing poly-D-lysine/laminin-coated 12-mm coverslips (BD Biosciences), then transfected as described. Cells were washed with PBS, then fixed in 4% paraformaldehyde on ice for 20 min. Cells were simultaneously blocked and permeabilized with 0.1% Triton X-100 and 5% donkey serum in PBS (blocking buffer) for 30–60 min at room temperature. Cells were incubated with primary antibody in blocking buffer overnight at 4°C. After washing with PBS, cells were incubated with secondary antibody in PBS-Triton containing 5% serum for 1–2 h at room temperature.

IN VITRO PHOSPHORYLATION ASSAY

Cell lysates for kinase assays were prepared by harvesting cells into M2 buffer (20 mM Tris, pH 7.6, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 0.5% NP-40 containing protease and phosphatase inhibitors). Equal amounts of protein were used for immunoprecipitation as described above. The immunoprecipitated proteins were washed twice in lysis buffer, then twice in kinase buffer (20 mM Hepes, pH 7.5, 10 mM $MgCl_2$). Kinase assays were performed in kinase buffer containing 20 mM β-glycerol phosphate, 20 mM *p*-nitrophenylphosphate, 1 mM DTT, 50 μM Na_3VO_4, 20 μM ATP for 20 min at 30°C in the presence of 5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol, Perkin-Elmer). Reactions were stopped by the addition of SDS-loading buffer, and denatured proteins were separated by SDS–PAGE. Gels were dried and exposed to X-ray films for the detection of radiolabeled proteins.

RESULTS

IDENTIFICATION OF p120 AS A BINDING PARTNER OF Pak5

In order to identify potential binding partners for Pak5, affinity chromatography was followed by matrix associated laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. HeLa cell lysates were incubated with either Pak5 immobilized on Sepharose, or with control resin. Proteins that remained bound to the resin after washing were eluted and separated on SDS–polyacrylamide gels and stained with Coomassie Blue. As MALDI-TOF mass spectrometry is extremely sensitive, bands were selected for analysis using criteria that would attempt to minimize contamination by proteins that were not bound to Pak5. Thus, bands were chosen if they were prominent, well separated from adjacent bands, and had no corresponding bands in the control lane. For the initial study, the selection was limited to 10 samples with molecular weights ranging between 175 and 32.5 kDa. These include five bands from the experimental lane (as indicated in Fig. 1), and five corresponding bands from the control lane. In screening the results of the MALDI-TOF analysis of the bands, any protein that appeared in the control lane as well as the experimental lane was immediately rejected. Other criteria for selection included reproducibility of the candidate by this assay, and whether the candidate had any documented association with Paks, Rho GTPases, or cytoskeletal proteins. Six potential proteins were chosen for further study, including p120-catenin, p114-Rho-GEF, and PKCδ. Of these, p120 emerged as a prominent candidate for further analysis. p120 was chosen because of the strength of the MALDI-TOF results, as well as its known role in cytoskeletal remodeling through regulation of Rac and Rho.

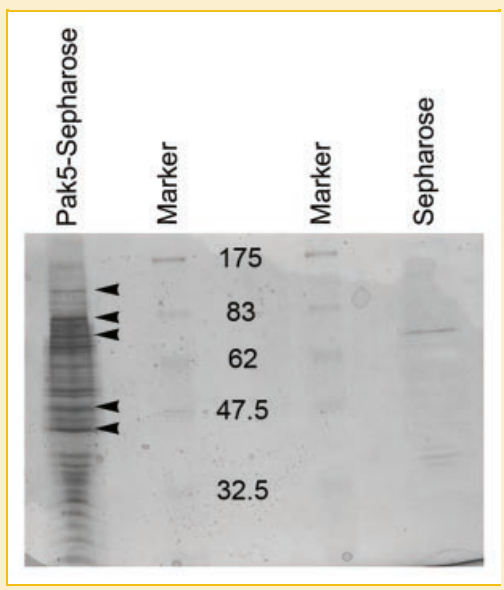

Fig. 1. Preparative SDS–polyacrylamide gel electrophoresis of proteins interacting with Pak5. Pak5 was covalently coupled to CNBr-activated Sepharose, then incubated with lysates from HeLa cells overnight at 4°C. HeLa cell lysates were also incubated with uncoupled Sepharose as a control. After washing in lysis buffer, proteins were eluted from the resin by boiling in SDS–gel loading buffer. The arrows indicate the positions of bands excised from the gel for MALDI analysis. Slices corresponding to the arrows were excised from the Sepharose in order to determine whether potential proteins of interest bind to the resin rather than Pak5.

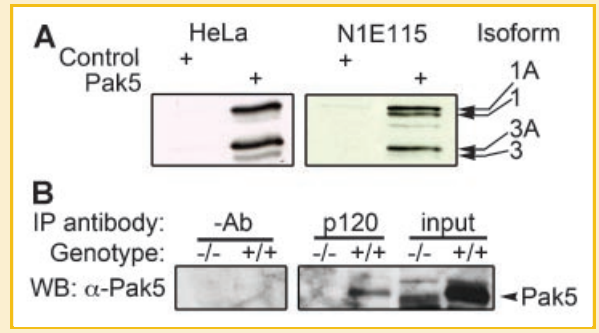

Fig. 2. Pak5 interacts with p120 in vitro and in tissues. A: Lysates prepared from HeLa or N1E115 cells were incubated with Pak5-coupled or control Sepharose. Recovered proteins were then separated by SDS–PAGE, immunoblotted, and probed with anti-p120 antibody. The various isoforms of p120 are indicated at right. B: Lysates prepared from the brains of wild-type or *Pak5−/−* mice were immunoprecipitated with antibody against p120, or protein G–Sepharose alone. Precipitated material was analyzed by immunoblotting with antibody against Pak5. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Pak5 INTERACTS WITH p120 IN VITRO AND IN VIVO

In vitro binding of p120 to Pak5 was confirmed by pull-down assays and Western blot analysis (Fig. 2A). Pak5-Sepharose was incubated with lysates from the neuroblastoma cell line N1E115 or Hela cells. N1E115 cells were chosen because Pak5 is expressed highly in the nervous system, and Hela cells were chosen to determine whether Pak5 and p120 also interact in the context of other cell types. p120 is expressed as a number of different isoforms resulting from differential splicing and use of alternative translational start sites. HeLa cells have been shown to express both of the predominant species of p120, isoforms 1 and 3, in roughly equal amounts [Mo and Reynolds, 1996] and are therefore useful to determine whether Pak5 preferentially binds to one or the other. Bound proteins were eluted and separated by SDS–PAGE, immunoblotted, and probed with anti-p120 antibody. The results from these experiments confirmed that Pak5 and p120 do interact in vitro and isoforms 1 and 3 bind equally well to Pak5. The binding assays were later repeated in HEK293 cells, with similar results (data not shown).

To determine whether endogenous Pak5 and p120 interact in tissues, we tested whether these proteins co-immunoprecipitate from mouse brain. Endogenous p120 was immunoprecipitated from an NP-40 soluble fraction of mouse brain, and then tested for co-immunoprecipitation of endogenous Pak5. As seen in Figure 2B, endogenous Pak5 co-precipitated with endogenous p120, but not in the absence of primary antibody. This result suggests that Pak5 and p120 also interact with each other in vivo.

p120 PREFERENTIALLY INTERACTS WITH Pak5 AMONG THE GROUP B PAKS

We next examined whether the interaction of p120 with Pak5 was unique among the Group B Paks. HEK293 cells were transfected with myc-tagged Pak4, Pak5, or Pak6 expression vectors. The Pak

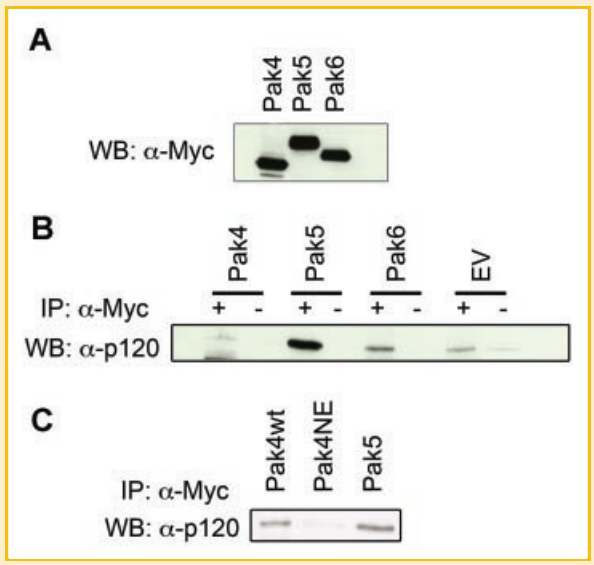

Fig. 3. p120 Preferentially interacts with Pak5. HEK293 cells were transfected with equal amounts of empty expression vector, or vectors containing myc-tagged Group B Pak constructs. Cells were harvested into lysis buffer. Equal amounts of lysate proteins were immunoprecipitated with antibody against the myc-tag. Precipitated material was analyzed by immunoblotting with antibody against p120. A: Input levels of Pak protein in transfected cell lysates were assessed by immunoblotting with antibody against the myc tag. B,C: Results of co-immunoprecipitation assays, blots were probed with anti-p120 antibody. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

proteins were immunoprecipitated with anti-myc epitope-specific antibody and tested for co-precipitation of endogenous p120 by Western blotting. As shown in Figure 3, although the input levels of all three Pak proteins were equivalent (Fig. 3A), p120 co-precipitated with Pak5 far more efficiently than either Pak4 or Pak6 (Fig. 3B). As it was subsequently shown that p120 is phosphorylated in the presence of constitutively active Pak4(S445N) (hereafter referred to as "activated Pak4" in the text or "Pak4NE" in the figures), we compared the level of interaction of p120 with Pak5 and both wild-type and activated Pak4. As shown in Figure 3C, the co-precipitation of p120 with activated Pak4 is significantly less efficient than with either wild-type Pak4 or Pak5.

p120 IS A SUBSTRATE FOR PHOSPHORYLATION BY Pak4 AND Pak5

p120 has been shown to be phosphorylated on numerous serine and threonine residues, making it a potential substrate for phosphorylation by Pak5. In vitro kinase assays were therefore carried out in order to determine whether Pak5 phosphorylates p120 directly. Furthermore, although a strong physical interaction between p120 and Pak4 was not seen, Pak4 has considerable sequence homology with Pak5 and like p120, it has important roles in Rho GTPase-mediated cytoskeletal reorganization, tumorigenesis, and metastasis. Therefore, Pak4 was also included in the in vitro kinase assays. HEK293 cells were transfected with either empty vector, myc-tagged wild-type Pak4 or Pak5 expression vectors, or myc tagged constitutively active Pak4 or Pak5(S573N) (hereafter referred to as "activated Pak5" in the text, or "Pak5NE" in the figures). Anti-myc tag antibody was used to immunopurify Pak protein from equal amounts of cell lysate. The immunopurified protein was incubated with a purified bacterially expressed GST fusion protein containing the first 330 amino acids of the 1A isoform of murine p120. This segment of p120, referred to as p120Δ, corresponds to the regulatory domain containing the majority of the serine/threonine phosphorylation sites. The mixture was incubated with kinase buffer containing [γ-^{32}P]ATP. Since GST-p120Δ and myc-Pak4 co-migrate on SDS–polyacrylamide gels, it was necessary to add thrombin protease to the kinase reactions containing Pak4, in order to cleave p120Δ from the GST tag. This allowed us to be able to distinguish between Pak4 autophosphorylation and substrate phosphorylation. Phosphorylation was analyzed after SDS–PAGE

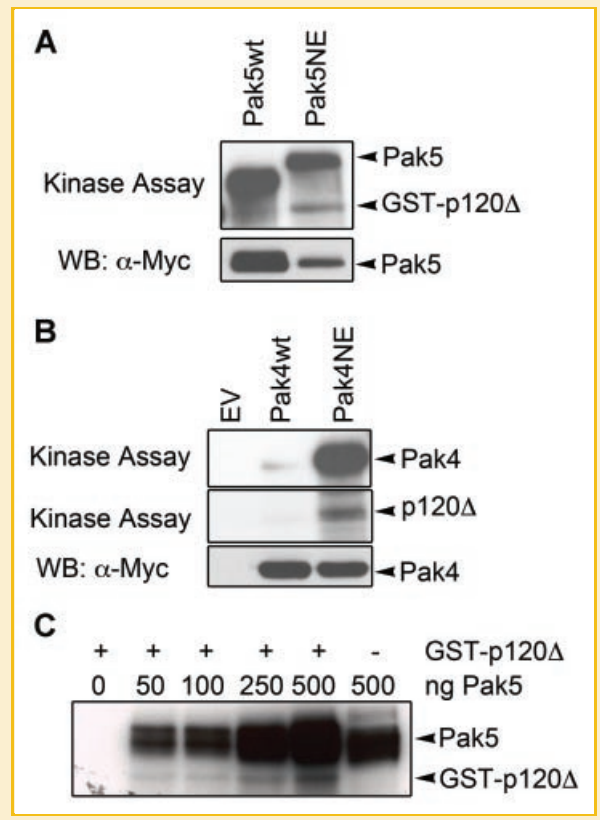

Fig. 4. p120 is phosphorylated in vitro by Pak4 and Pak5. A,B: HEK293 cells were transfected with equal amounts of empty vector or vectors containing myc-tagged wild-type or activated Pak5, or wild-type or activated Pak4. After transient expression, Pak4 and Pak5 were immunopurified from equal amounts of total whole cell lysate by using a mouse anti-myc tag antibody and protein A–Sepharose. The immune complexes were then incubated with GST-p120Δ and [γ-^{32}P]ATP in in vitro kinase buffer. A: Phosphorylation by Pak5. B: Phosphorylation by Pak4. In order to distinguish between autophosphorylation and substrate phosphorylation, thrombin was added to the kinase reaction of the Pak4 samples, releasing p120Δ from the GST tag. Phosphorylation was analyzed after SDS–PAGE (10% gel) and autoradiography. In both (A,B), autophosphorylation of Pak4 and Pak5 and substrate phosphorylation are indicated. A Western blot showing the expression of Pak4 or Pak5 is shown at the bottom of each panel. C: Phosphorylation of p120 by bacterially expressed Pak5. In vitro kinase reactions were carried out as described above, using the indicated amounts of purified bacterially expressed Pak5. Pak5 autophosphorylation and substrate phosphorylation are indicated.

and autoradiography (Fig. 4A,B). The results show that activated Pak5, and to a lesser extent wild-type Pak5, phosphorylate p120Δ in vitro. Interestingly, activated Pak4 also phosphorylates p120Δ. Unlike wild-type Pak5, the kinase activity of wild-type Pak4 is much lower than its activated counterpart. It is therefore not surprising that substrate phosphorylation by wild-type Pak4 could not be detected. To confirm that the observed phosphorylation of p120Δ could not be attributed to another interacting protein present in the immune complexes, we repeated the kinase assay using purified bacterially expressed Pak5 (see Fig. 4C). p120 phosphorylation could be detected when Pak5 was added to the reaction, but not in the absence of Pak5 or p120. These results confirm that p120 can be phosphorylated by Pak5.

p120 IS PHOSPHORYLATED IN VIVO WHEN CO-EXPRESSED WITH Pak5 OR CONSTITUTIVELY ACTIVE Pak4

p120 is phosphorylated on numerous serine and threonine residues, the majority of which are clustered in the amino terminal regulatory domain, which also contains a number of tyrosine phosphorylation sites. There are also two S/T phosphorylation sites at the carboxyl terminal end. To determine which residue(s) are phosphorylated by Pak4 and Pak5, anti-p120 phospho-specific antibodies were used. HEK293 cells were co-transfected with HA-tagged p120-isoform 1A (HA-p120-1A) and empty vector, along with expression vectors of myc-tagged wild-type or activated Pak4 or Pak5. p120 phosphorylation was then analyzed by Western blotting using antibodies specific for p120 phosphorylated on serine residues 268 or 288, or

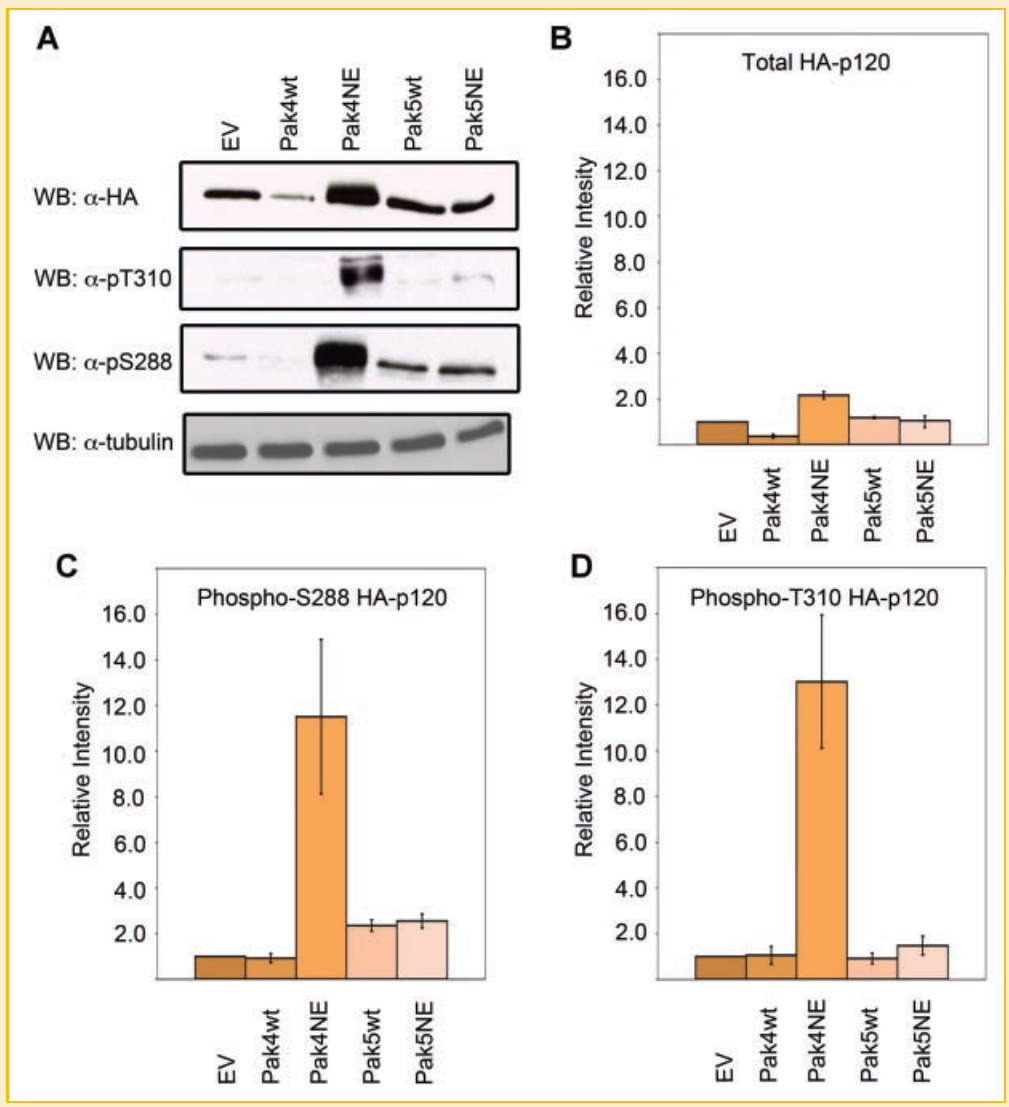

Fig. 5. p120 is phosphorylated in vivo on serine 288 and threonine 310 in the presence of activated Pak4 and Pak5. A: HEK293 cells were co-transfected with equal amounts of HA-p120-1A and either empty vector or vectors containing the indicated myc-tagged Pak constructs. Cells were harvested into lysis buffer, and equal amounts of whole cell lysate were examined by immunoblotting. The total amount of exogenous p120 was determined by probing with anti-HA antibody. Phosphorylation was determined by probing with anti-phospho-p120 antibodies. α-Tubulin was used as a loading control. B–D: Band intensities from the Western blots of triplicate transfections were quantitated using ImageJ software. The histograms show the relative band intensities. In all cases, error bars represent the SEM. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

threonine residues 310 or 916 (Fig. 5A). The results were quantified by densitometry analysis of the blots and the amount of p120 present in each sample was expressed as a multiple of the amount of p120 present in the sample transfected with the empty vector (therefore, empty vector always equals 1). Increased levels of p120 pS288 are clearly detected in cells transfected with wild-type Pak5, activated Pak5, and activated Pak4, but not with empty vector or wild-type Pak4. Phosphorylation on T310 was mainly confined samples transfected with activated Pak4. Interestingly, in all of the blots, the p120 migrates significantly more slowly in the activated Pak4 lane, suggesting phosphorylation on multiple sites.

No phosphorylation on S268 or T916 was detected by this assay, and no changes in phospho-tyrosine were seen (data not shown). Interestingly, in the presence of Pak5 or activated Pak4, there appeared to be an increase in the total amount of exogenous p120, which correlated with the increased levels of phosphorylated p120. However, as detected by densitometry analysis, the level of phosphorylated p120 was even higher than the overall increased protein level, indicating that there is a significant increase in p120 phosphorylation in response to Pak5 and activated Pak4 (see Fig. 5B–D).

p120 CO-LOCALIZES WITH Pak5 AND ACTIVATED Pak4 IN CELLS

To assess the cellular localization of total and phosphorylated p120, NIH-3T3 fibroblasts were transfected with EGFP-Pak5 or myc-tagged activated Pak4, then analyzed by immunofluorescence microscopy using anti-p120 phospho-specific antibodies as well as anti-total-p120 antibody. A vector expressing EGFP without any insert was used as a control. Figure 6A shows Pak5 expression in the cytoplasm as discrete granules. While total p120 shows a more diffuse pattern of expression throughout the entire cells, the merged images show many points of co-localization. As could be predicted from the Western blot results, p120 pS288 sharply co-localizes with Pak5 without the diffuse background seen with the total p120 antibody. In contrast, p120 pS268 and pT916 never co-localized with Pak5. Co-localization of Pak5 and p120 pT310 also could not be detected, but given the low level of pT310 detected by Western blot, that is not surprising.

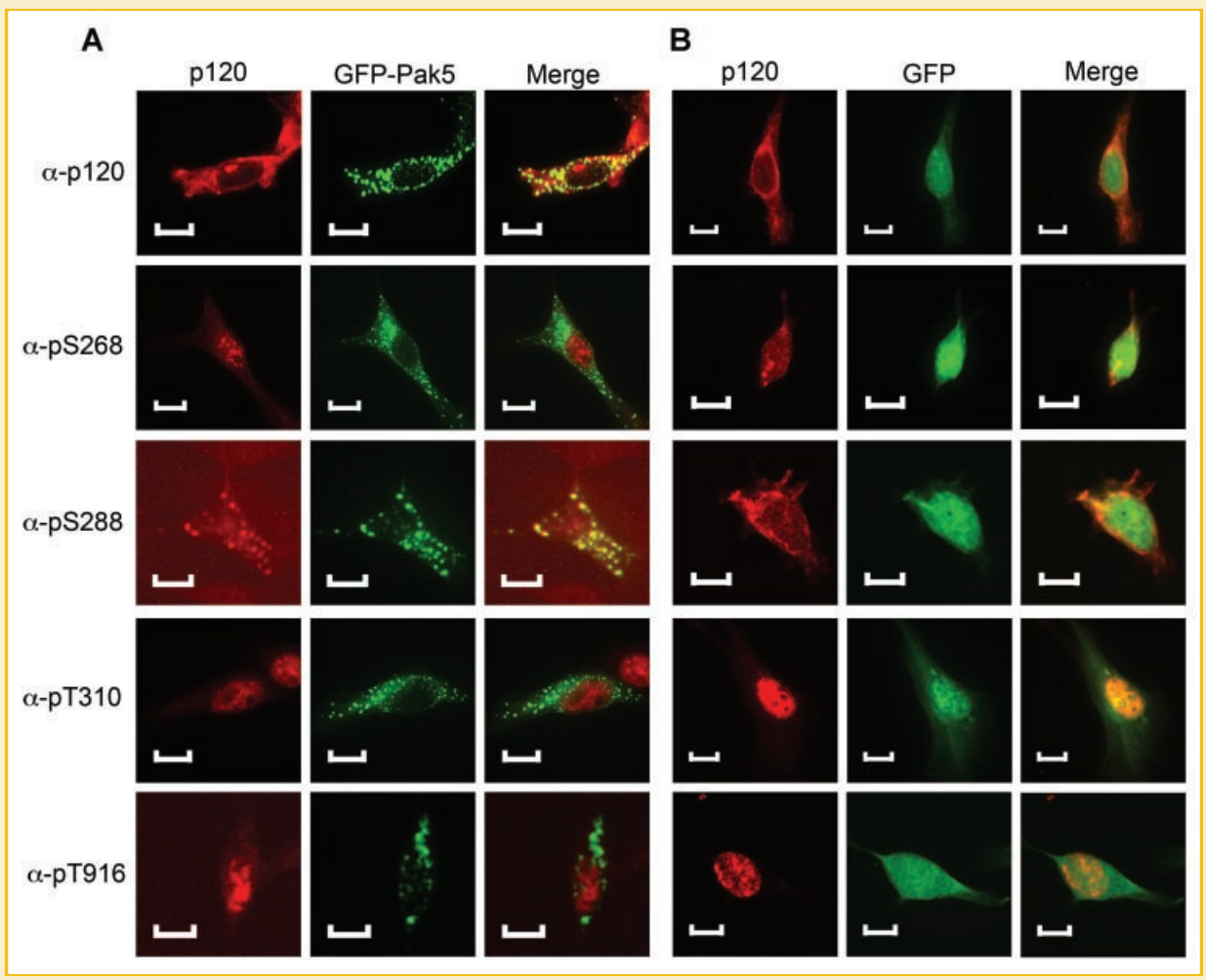

Fig. 6. NIH-3T3 cells were transfected with (A) EGFP-tagged Pak5 or (B) an EGFP control vector, then fixed and stained with antibodies specific for total p120 or p120 phosphorylated on S268, S288, T310, or T916. To illustrate co-localization, the p120 and Pak5 images were merged. In all cases, the scale bars represent 10 μm.

Since the level of phosphorylation of p120 seen in cells transfected with activated Pak4 is so high, we expected to also see high levels of co-localization of p120 with activated Pak4. In fact, the co-localization of Pak4 with both total p120 and p120 pS288 is quite striking, as shown in Figure 7. These results, as well as with the results from the pS268, pT310, and pT916 staining, are consistent with both the Western blot results, and the Pak5 co-localization results. In contrast to Pak5 and activated Pak4, transfection with the EGFP control plasmid does not cause any change in the localization of p120 (Fig. 6B). Taken together, these results suggest that Pak5 and activated Pak4 directly phosphorylate and co-localize with p120 on S288.

DISCUSSION

Pak5 is a member of the Group B family of p21-activated kinases, and is highly expressed in the brain [Dan et al., 2002; Pandey et al., 2002]. Like Pak4, the founding member of the Group B Paks, Pak5 interacts with the Rho GTPases Cdc42 and Rac, and is involved in the regulation of cytoskeletal remodeling [Dan et al., 2002]. However, the mechanisms by which Pak5 operates in neuronal tissue are not clearly understood. To investigate the function of Pak5, we sought to identify substrates and regulatory proteins by isolating Pak5-interacting proteins. We chose an affinity chromatography approach, in which purified Pak5 was covalently coupled to Sepharose and incubated with cell extracts. Proteins that bound to Pak5-Sepharose were separated by SDS–PAGE, then analyzed by MALDI-TOF mass spectrometry. A number of interesting candidate proteins were found by this method, including p120-catenin, PKCδ, and p114-Rho-GEF, a guanine exchange factor that interacts with Rho, but not Cdc42 or Rac [Blomquist et al., 2000]. p120-catenin (p120) is the founding member of the δ-catenin family. It was originally isolated as a substrate of Src [Reynolds et al., 1989]. Like β-catenin, it is a member of the Armadillo repeat family of proteins, characterized by an internal series of Arm repeats [Reynolds et al., 1992], which forms a superhelix of alpha helices. It is through this Arm repeat domain that p120 interacts with the juxtamembrane domain of classical cadherins [Daniel and Reynolds, 1995; Yap et al., 1998], where it is essential for cadherin stabilization [reviewed in Reynolds and Carnahan, 2004]. p120 regulates a number of processes through its action on Rho GTPases, including cell motility, metastasis, inflammation, and neural morphogenesis [reviewed in Anastasiadis, 2007]. p120 may also play a role in Wnt/β-catenin signaling through its interaction with the transcriptional repressor Kaiso [reviewed in Daniel, 2007]. In neuronal cells, p120 is required for Rho GTPase-dependent regulation of dendritic spine development in hippocampal pyramidal neurons [Elia et al., 2006]. p120 has also been hypothesized to regulate neurite outgrowth both through Rho GTPase regulation of cytoskeletal dynamics and through interaction with N-cadherin [Chauvet et al., 2003]. p120 is phosphorylated on multiple tyrosine, serine, and threonine residues, most of which are clustered within a 300 amino acid region at the N-terminus which has been termed the "regulatory domain" [Xia et al., 2003]. No distinct function has been assigned to any particular site, nor have any serine/threonine kinases that directly phosphorylate p120 been identified, although PKCα has been implicated in receptor tyrosine kinase-mediated phosphorylation of S879 [Brown et al., 2009]. Here we have found that Pak5 reproducibly interacts with and phosphorylates p120. This led us to the compelling hypothesis that Pak5 is a p120 kinase, and that phosphorylation of p120 by Pak5 on serine or threonine residues plays a role in its function in neuronal tissue.

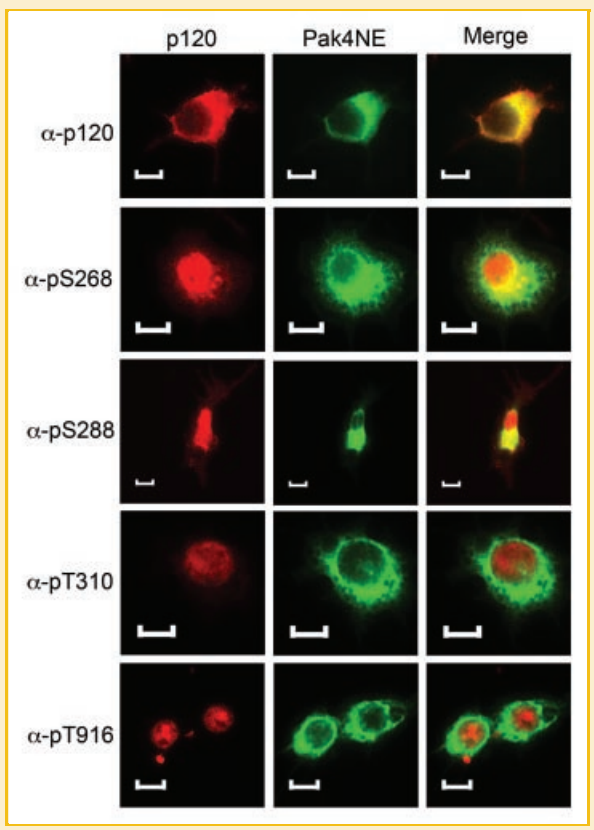

Fig. 7. NIH-3T3 cells were transfected with myc-tagged Pak4NE, then fixed and stained with antibodies specific for total p120 or p120 phosphorylated on S268, S288, T310, or T916 as well as anti-myc-tag antibody to enable visualization of Pak4. To illustrate co-localization, the p120 and Pak4 images were merged. In all cases, the scale bars represent 10 μm.

Pak5 interacts with p120 in a number of cell types. p120 bound to Pak5 could be isolated by affinity chromatography from HeLa, HEK293, and N1E115 murine neuroblastoma cells. Exogenously expressed Pak also co-localizes with p120 in cells, and in brain tissue, endogenous Pak5 co-immunoprecipitates with endogenous p120. Given the high degree of sequence homology among the Group B Paks, it is not surprising that p120 also interacts with Pak6, although that interaction is much weaker than the interaction with Pak5. In comparison, the interaction between p120 and Pak4 is negligible.

To determine whether p120 is phosphorylated by Pak5 and on what site, we created a bacterial vector expressing the N-terminal 330 amino acids of p120 fused to GST to use as a substrate for in vitro phosphorylation assays. When incubated with this substrate, activated Pak5 showed considerable phosphorylation activity towards p120, and wild-type Pak5 phosphorylated the p120 at a

lower level. Western blot analysis of p120 that had been co-expressed with wild-type or activated Pak5 showed phosphorylation takes place almost exclusively on S288. Only low-level phosphorylation on T310 could be observed in response to Pak5, and no phosphorylation of S268 or T916 could be detected. We concluded that S288 is the main p120 phosphorylation site in response to Pak5, although we cannot completely rule out the possibility that T310 is also an important site, but that the level is below the detection by this assay for these antibodies. Likewise, we cannot rule out the possible importance of S268 or T916. Further evidence for the importance of S288 could be seen, however, when immunofluorescence was carried out. When pS288 antibodies were used, the co-localization between p120 and Pak5 became much more dramatic than when antibodies against total p120 were used. This is further evidence that Pak5 phosphorylates p120 on S288 in cells. In contrast, p120 phosphorylated on T310 could not be detected by immunofluorescence.

If we compare the results of Pak5 phosphorylation in vitro and in vivo, it is obvious that the level of phosphorylation of p120 at S288 in the presence of wild-type Pak5 was much higher in vivo than in the in vitro assay. One important difference is that in the in vivo assay, phosphorylation of full-length p120 was observed. It is possible that although the site of phosphorylation is in the N-terminal regulatory domain, additional sequences downstream are required to stabilize the interaction between the two proteins and enable wild-type Pak5 to efficiently phosphorylate p120. Another possibility is that other proteins in the cell make a complex with Pak5 and p120, which may be necessary for efficient phosphorylation.

Our results are the first to demonstrate that p120 is a substrate and binding partner for Pak5. While the biological function of this interaction is still not understood, we propose that p120 plays a role in the regulation of cytoskeletal organization and cell morphology in response to Pak5. Pak5 has been shown to induce morphological changes in neuronal cells in culture. Specifically, expression of either wild-type or activated Pak5 induces the outgrowth of neurites in N1E115 mouse neuroblastoma cells [Dan et al., 2002]. Expression of a dominant negative mutant of Pak5 represses the induction of neurite outgrowth, demonstrating that Pak5 is also necessary for this process in N1E115 cells. Activated Rho has been shown to act antagonistically to Pak5 in neurite outgrowth assays, and Pak5 has been shown to inhibit RhoA activation [Dan et al., 2002]. The mechanism by which Pak5 induces neurite outgrowth still remains to be elucidated, but we speculate that it may involve downstream inactivation of RhoA by Pak5. The mechanism by which Pak5 downregulates RhoA, however, is incompletely understood. Interestingly, p120 has been shown to inhibit Rho activity [reviewed in Anastasiadis and Reynolds, 2001] and overexpression of p120 has been shown to induce the formation of dendrite-like processes in fibroblasts [Reynolds et al., 1996] in a manner that is dependent on RhoA inhibition [Anastasiadis et al., 2000]. We hypothesize that phosphorylation of p120 by Pak5 is an initial step in Pak5 signaling, followed by activation of p120, leading in turn to inhibition of RhoA, and leading finally to morphological changes required for neurite outgrowth. Interestingly, p120 has been shown to have a role in dendritic spine development [Elia et al., 2006]. Development and control of dendritic spines involves cytoskeletal changes such as the formation of filopodia, structures that that are regulated by Pak5 and other Group B Paks [Abo et al., 1998; Dan et al., 2002]. Rho GTPases also have a role in regulating dendritic spine growth and motility [Nakayama et al., 2000; Tashiro et al., 2000], and p120 regulation of dendritic spines has also been shown to be Rho dependent [Elia et al., 2006]. We therefore propose that p120 may function downstream to Pak5 in dendritic spine formation. Further studies will be required to clarify what roles p120 plays downstream to Pak5 in neuronal cells and other cell types. Future studies using *Pak5/Pak6* null mice [Nekrasova et al., 2008] should prove helpful in answering these questions. Future work will also involve determining the biological relevance of S288/T310 phosphorylation by Pak5. It will be interesting to determine whether these are key activation sites, and whether phosphorylation of these sites is necessary for p120-induced RhoA inhibition, or regulation of other signaling pathways. In future studies, it will also be interesting to determine whether there are other important sites that are specifically phosphorylated by the Group B Paks.

The relationship between Pak4 and p120 is somewhat different from that between Pak5 and p120. Immunoprecipitation of Pak4 does not reveal a strong physical interaction between the two. Wild-type Pak4 does not significantly phosphorylate p120 either in vitro or in vivo. However, the situation with activated Pak4 is dramatically different. In vitro and in vivo phosphorylation of p120 by activated Pak4 appears quite strong. In the in vitro assays it is difficult to make any quantitative comparisons between the kinase activity of activated Pak4 and Pak5 due to the fact that the GST-p120 fusion protein substrate co-migrates with Pak4, causing any substrate phosphorylation by Pak4 to be overshadowed by its autophosphorylation activity. For this reason the Pak4 kinase assays were carried out in the presence of thrombin, which cleaves the p120 from GST, but it is not clear that 100% of the p120 protein was cleaved. Western analysis suggests that Pak4 has the same target specificity as Pak5, phosphorylating primarily on S288, and on T310. Interestingly, in the activated Pak4 lane in the in vivo phosphorylation assays, the p120 band appears to migrate significantly more slowly than in all the other lanes (Figure 5B). This suggests that p120 may be phosphorylated on multiple sites in the presence of activated Pak4.

Interestingly, in Western blots of cell lysates there is an apparent increase in total exogenous p120 when activated Pak4 or Pak5 are present, as assessed by probing with anti-HA tag antibody (Fig. 5A). This correlates with the increase in phosphorylated p120. As the α-tubulin blot shows, this is not due to differences in the amount of total protein applied to the gels. Importantly, quantitation of the band intensities shows that the increase in p120 phosphorylation in the presence of Pak4 or Pak5 is significantly greater than the increase in total protein (Fig. 5B). Taken together with the in vitro phosphorylation data and the co-localization studies, these results show that Pak5 and activated Pak4 both directly phosphorylate p120. One possible explanation for the increase in total p120 levels is that phosphorylated p120 does not turn over as quickly in the cell, and therefore p120 is more stable when Pak4 or Pak5 are present.

Pak4 and Pak5 were first identified as Cdc42 and Rac-interacting proteins that are involved in cytoskeletal remodeling and regulation

of cell shape [Abo et al., 1998; Dan et al., 2002]. Both have also been shown to inhibit apoptosis and thereby promote cell survival [Gnesutta et al., 2001; Cotteret et al., 2003; Gnesutta and Minden, 2003; Li and Minden, 2005; Cotteret and Chernoff, 2006]. Pak4 has emerged as a key player in tumorigenesis. It is highly overexpressed in tumor cells [Callow et al., 2002; Liu et al., 2008]. Dominant negative Pak4 inhibits the formation of anchorage-independent foci in mouse fibroblasts in response to oncogenic Ha-Ras [Callow et al., 2002], oncogenic Dbl (an exchange factor for Rho GTPases) [Qu et al., 2001], but not oncogenic Src [Callow et al., 2002]. Like Cdc42, constitutively activated Pak4 promotes anchorage-independent growth in immortalized fibroblasts [Qu et al., 2001]. Recently, p120 has been shown to be required for Rac1 and Src, but not Ras-induced anchorage-independent growth in canine epithelial cells, although induction by all three oncogenes correlates with suppression of the Rho-ROCK-LIMK pathway [Dohn et al., 2009]. It would be of interest to determine if induction of anchorage-independent growth by Pak4 also involves p120-mediated Rho-ROCK-LIMK suppression, and if p120 plays a role in Pak4-mediated tumor formation.

It is interesting that that both Pak4 and Pak5 can phosphorylate p120, but only Pak5 binds to p120. While both Pak4 and Pak5 have similar cellular functions, they also have some differences. For example, while both promote formation of filopodia when overexpressed, Pak4 is much less efficient at promoting neurite outgrowth in cultured cells compared with Pak5 [Dan et al., 2002]. While Pak4 is highly transforming, and overexpressed in a wide range of tumor cell lines [Qu et al., 2001; Callow et al., 2002], the role for Pak5 in transformation is more elusive [Qu et al., 2001; Li and Minden, 2003; Gong et al., 2009]. There also appear to be differences in phosphorylation activity. Wild-type Pak4 both autophosphorylates and phosphorylates a substrate, LIM kinase, at a much lower level than wild-type Pak5 [Dan et al., 2002 and Fig. 4]. While the reason for the difference in kinase activity between Pak4 and Pak5 is not known, it may explain why we saw phosphorylation of p120 by activated Pak4 even when there was a negligible physical interaction between the two. Further investigation is required to determine if there is a true functional relationship between Pak4 and p120.

p120 has been shown to be involved in a variety of processes including cytoskeletal reorganization, cell–cell adhesion, and inflammation. A common element in these processes is the inhibition of RhoA by p120. Interestingly, Pak5 also inhibits RhoA [Dan et al., 2002]. The finding that Pak5 phosphorylates p120 raises the possibility that these two protein are part of the same pathway leading to Rho inhibition and cytoskeletal reorganization. Determining the role of serine/threonine phosphorylation of p120 in these processes has so far proven difficult, and functional studies have yet to reveal an upstream kinase for p120. By using an approach that relies on physical interactions between proteins, we have identified members of a kinase family, the Pak kinases that do indeed phosphorylate p120. These are the first kinases that have been shown to do so. p120 is one of the first substrates to be identified that could potentially connect Pak5 to inhibition of RhoA, leading to cytoskeletal reorganization and neurite outgrowth. The identification of p120 as a substrate for Pak5 and the identification of the specific residues upon which it is phosphorylated, is an important step for determining how Pak5 regulates downstream signaling pathways.

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