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Pak6 Protein Kinase Is a Novel Effector of an Atypical Rho Family GTPase Chp/RhoV

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Abstract—Chp/RhoV is an atypical Rho GTPase whose functions are far from being fully understood. To date several effector proteins of Chp have been identified, including p21-activated kinases Pak1, Pak2, and Pak4. Using a yeast two-hybrid system and co-immunoprecipitation, here we show that another p21-activated kinase, Pak6, is a novel Chp-binding protein. Interaction between Chp and Pak6 depends on the activation state of the GTPase, suggesting that Pak6 is an effector protein for Chp. Point mutations in the effector domain of Chp or in the CRIB motif of Pak6 significantly impair the interaction between Chp and Pak6 upon co-immunoprecipitation, suggesting that the binding interface involves the effector domain of Chp and the CRIB motif in Pak6. We found that Chp does not affect the phosphorylation status of the S560 residue in the catalytic domain of Pak6 when Chp and Pak6 are co-expressed in HEK293 cells. Therefore, similarly to Cdc42, Chp is not likely to activate Pak6. In NCI-H1299 cells, Chp co-localizes with Pak6 on vesicular structures in activation state-dependent manner. Taking the data together, we report here the identification of p21-activated kinase Pak6 as a novel effector of the atypical Rho GTPase Chp. Our data suggest further directions in elucidating biological functions of these proteins.

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Key words: Chp/RhoV GTPase, Pak6 protein kinase, protein–protein interactions

Chp/RhoV (Cdc42 homologous protein) is an atypical Rho family GTPase whose functions are poorly understood [1]. Rho GTPases act as molecular switches that in their active GTP-bound state interact with protein targets called "effectors". Effectors transduce the signal downstream from the Rho GTPases to regulate diverse cellular functions ranging from cytoskeleton dynamics and cell polarity to gene expression, apoptosis, and many others [2]. Chp induces lamellipodia formation and is localized to focal adhesions in endothelial cells [3]. In PC12 cells Chp induces apoptosis via activation of the JNK pathway [4], while in mouse fibroblasts Chp acts as an oncogene inducing malignant transformation [5, 6]. Chp is expressed in *Xenopus laevis* embryos and plays a role in the differentiation of neural crest cells [7]. In zebrafish (*Danio rerio*) embryos Chp regulates E-cadherin localization at adherens junctions via a pathway involving activation of Pak1 and guanine nucleotide exchange factor βPIX [8]. To date, several effectors of Chp have been identified including N-WASP, Par6, Mlk3, and p21-activated kinases Pak1, Pak2, and Pak4 [3, 9, 10].

The family of Pak kinases in humans includes six members that are classified into two subfamilies, Pak I (Pak1, Pak2, Pak3) and Pak II (Pak4, Pak5, Pak6) [11, 12]. The Pak kinases are serine/threonine kinases that regulate a wide variety of cellular activities downstream of Rho GTPases Cdc42 and Rac1 [12-14]. Pak kinases contain an *N*-terminal regulatory domain and *C*-terminal catalytic domain. Unlike Pak I group kinases, Pak II kinases show strong preference in binding to Cdc42 compared to Rac1 [12]. Cdc42, which is a close homolog of Chp, binds to all members of the Pak family [13, 15-19]. Thus, we tested the possibility that Chp is able to interact with other members of Pak family, namely Pak6. Here we report that Pak6 is a binding partner and a putative effector protein for the atypical Rho GTPase Chp.

Abbreviations: 3AT, 3-amino-1,2,4-triazole; co-IP, co-immunoprecipitation; EGFP, enhanced green fluorescent protein; GAL4AD, transcription activation domain of GAL4 transcription factor; GAL4BD, DNA-binding domain of GAL4; SD, synthetic dextrose medium.

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MATERIALS AND METHODS

Plasmids. The pCan plasmids encoding *N*-terminally myc-tagged wild type Chp, its constitutively active GTPase-deficient mutant (Chp G40V), and its GDP-bound inactive mutant (Chp S45N) were provided by A. Abo (Nuvelo, Inc., USA) and are described elsewhere [9]. The pRK5-myc-Cdc42 Q61L plasmid encoding the active form of human Cdc42 was a gift from A. Hall (Memorial Sloan Kettering Cancer Center, NY, USA), and pcDNA-myc-Wrch-1 Q107L encoding active form of human Wrch-1 was kindly provided by M. Symons (Albert Einstein College of Medicine, NY, USA). The pFLAG-CMV2-Chp G40V and pFLAG-CMV2-Chp S45N plasmids were constructed by PCR and subsequent cloning of amplified cDNAs into *Hind*III/*Bgl*II sites of pFLAG-CMV2 vector (Sigma, USA). A plasmid encoding *N*-terminally HA-tagged human Pak6 was kindly provided by J. Chernoff (Fox Chase Cancer Center, Philadelphia, USA) and was used as a template in PCR. To produce pFLAG-CMV2-Pak6 plasmid encoding *N*-terminally FLAG-tagged Pak6 and pEGFP-C2-Pak6 encoding *N*-terminally EGFP-tagged Pak6, cDNA was amplified by PCR and cloned into pCR-Blunt vector (Invitrogen, USA) to produce pCR-Pak6. Then Pak6 cDNA was subcloned as *Hind*III/*Xho*I fragment into *Hind*III/*Sal*I sites of pFLAG-CMV2 and as *EcoR*I/*Xho*I fragment into *EcoR*I/*Sal*I sites of pEGFP-C2 (Clontech, USA), respectively. Mutations in the effector domain of Chp were introduced with the QuikChange site-directed mutagenesis kit (Agilent Technologies, USA) using the following oligonucleotides: T63A (5-ccctcgcgctatcggcctgcagcactggacactttctccg-3 and 5-cggagaaagtgtccagtgctgcaggccgatagcgcgaggg-3), L65A (5-ctatcggcctacagcagcggacactttctccgtg-3 and 5-cacggagaaagtgtccgctgctgtaggccgatag-3), D66A (5-cggcctacagcactggccactttctccgtgcaag-3 and 5-cttgcacggagaaagtggccagtgctgtaggccg-3), F68C (5-cggcctacagcactggacacttgctccgtgcaagtcctgg-3 and 5-ccaggacttgcacggagcaagtgtccagtgctgtaggccg-3). Mutations H20,23L in Pak6 cDNA were introduced into pCR-Pak6 plasmid with QuikChange site-directed mutagenesis kit using oligonucleotides 5-gtcgaaggaggtgaggacacggagctggaagttctg-3 and 5-cagaacttccagctccgtgtcctcacctccttcgac-3. pFLAG-CMV2-Pak6 H20,23L and pEGFP-C2-Pak6 H20,23L plasmids were constructed similarly to as described above for the wild type Pak6 using pCR-Pak6 H20,23L. The pPC86 and pPC97 vectors were described before [20]. Plasmids pPC86-Pak6 and pPC-Pak6 H20,23L encoding human Pak6 fused to transcription activation domain of GAL4 transcription factor (GAL4AD) and plasmids pPC-97-Cdc42 Q61L, pPC97-Wrch-1 Q107L, pPC97-Chp G40V, pPC97-Chp, pPC97-Chp S45N encoding Rho GTPases fused to DNA-binding domain of GAL4 (GAL4BD) were constructed by PCR and subsequent cloning of amplified cDNAs into *Sal*I/*Not*I sites of pPC86 or pPC97, respectively. Nucleotide sequences of vectors were confirmed by double strand DNA sequencing at the Genome Center (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia).

Cell culture, reagents, and transfection. HEK293 cells and HeLa B cells (European Collection of Cell Cultures, UK) were cultured on Dulbecco's modified Eagle's medium (DMEM) (HyClone, USA) supplemented with 10% fetal bovine serum (HyClone), 100 μg/ml streptomycin, and 100 U/ml penicillin (Gibco, UK). Human non-small cell lung cancer cell line NCI-H1299 (American Type Culture Collection, USA; ATCC Number: CRL-5803) was cultured on DMEM/F12 medium (HyClone) supplemented with 10% fetal bovine serum (HyClone), 100 μg/ml streptomycin, and 100 U/ml penicillin (Gibco). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 and transfected using Unifectin-56 transfection reagent (Unifect Group, Russia). Phosphate-buffered saline (PBS), Tris-buffered saline (TBS), BSA, 3AT, protease inhibitor cocktail, and phosphatase inhibitor cocktails I and II were purchased from Sigma.

Analysis of protein–protein interactions in a yeast two-hybrid system. The GAL4-based yeast two-hybrid system was used for analysis [20]. *Saccharomyces cerevisiae* strain Y153 (MATa, ura3-52, his3Δ200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, URA3::GAL-lacZ, LYS2::GAL-HIS3) was co-transformed with pPC86-based plasmid expressing GAL4AD fused to human Pak6 cDNA and with pPC97-based plasmid expressing GAL4BD fused to Rho GTPase cDNAs. Co-transformants were selected on SD medium lacking leucine and tryptophane. Protein–protein interactions were monitored by activation of the *HIS3* reporter gene in yeast growing on SD medium supplemented with 25 mM 3AT for 3-4 days at 30°C.

Co-immunoprecipitation. HEK293 cells were seeded at a density of $1.4 \cdot 10^6$ cells per 60 mm plate. Next day cells were transfected with 0.5 μg of FLAG-Pak6 or FLAG-Pak6 H20,23L expression plasmid together with 0.5 μg of the respective myc-tagged GTPase expression plasmid or empty vector. Forty-eight hours post-transfection cells were washed with ice-cold PBS and lysed in 300 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1% Triton X-100, 1× protease inhibitors cocktail). For analysis of FLAG-Pak6 Ser560 phosphorylation status, lysates were obtained in a similar manner except for the addition of 1× phosphatase inhibitor cocktails I and II to the lysis buffer. The lysates were cleared by centrifugation at 16,000*g* for 15 min at 4°C and incubated overnight with 2 μl of mouse monoclonal anti-c-myc antibodies, clone 9E10 (Sigma) at 4°C with continuous agitation. Next day, immune complexes were precipitated with 4Fast Flow Protein G-Sepharose beads (GE Healthcare, UK) for 1 h at 4°C. The beads were washed three times at 4°C with lysis buffer, and

bound proteins were eluted with 1× SDS-PAGE sample buffer and analyzed by Western blotting with the appropriate antibodies.

Western blot analysis and antibodies. Proteins were separated by SDS-PAGE and transferred to Hybond-P membrane (GE Healthcare). Blots were blocked with TBS supplemented with 0.1% Tween-20 (BioRad, USA) (TBS-T) and 5% nonfat dry milk for 1 h at room temperature and incubated with primary antibodies overnight at 4°C. Blots were washed three times with TBS-T and incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies as appropriate (GE Healthcare), which were detected using Immobilon Western Chemiluminescence HRP Substrate (Millipore, USA). Chemiluminescence was imaged with the ChemiDoc XRS gel documentation system (BioRad) and quantified with Quantity One 4.6.2 software (BioRad). Rabbit polyclonal anti-FLAG, mouse monoclonal anti-c-myc (clone 9E10), and mouse monoclonal anti-α-tubulin (clone DM1α) antibodies were from Sigma. Rabbit polyclonal anti-phospho-Ser474-Pak4/-Ser602-Pak5/-Ser560-Pak6 antibody was from Cell Signaling, USA.

Immunofluorescence. NCI-H1299 cells were seeded on glass coverslips at a density of $0.4 \cdot 10^5$ cells per well of a 12-well plate. Next day cells were transfected with 0.1 μg of EGFP-Pak6 expression plasmid together with 0.1 μg of FLAG-Chp G40V or FLAG-Chp S45N expression plasmids. Twenty-one hour post-transfection cells were washed with ice-cold PBS and fixed on ice in neutral-buffered 4% paraformaldehyde for 15 min. The coverslips were washed three times with PBS, and free aldehyde groups were quenched with 50 mM ammonium chloride for 10 min. Cells were permeabilized with PBS/0.5% Triton X-100 for 2 min and nonspecific binding sites were blocked with PBS supplemented with 5% BSA and 0.1% Tween-20. Next, the coverslips were stained for 1 h at room temperature with rabbit polyclonal anti-FLAG antibodies (Sigma) (dilution 1 : 500) in the same buffer. The coverslips were washed three times with PBS and stained for 1 h at room temperature with goat anti-rabbit IgG conjugated with Alexa Fluor 546 (Molecular Probes, USA) in PBS supplemented with 5% nonfat dry milk and 0.1% Tween-20. The coverslips were washed three times with PBS and mounted in Mowiol 4-88 (Calbiochem, Germany). Images were acquired on Leica DMR microscope with ×100 objective equipped with a Leica DC350F cooled CCD camera (Leica, Germany). The acquired images were processed using Photoshop 7.0 software (Adobe Systems, USA).

RESULTS

Chp interacts with Pak6 in yeast two-hybrid system. Chp was shown to bind to Pak1 [3, 10], Pak2 [9], and Pak4 [3], but its interaction with other Pak kinases has not been investigated. Therefore, we tested the possibility that Chp interacts with Pak6, a member of Pak II subfamily. To test this hypothesis we exploited a yeast two-hybrid system. Rho GTPases fused to GAL4BD and Pak6 fused to GAL4AD were co-expressed in *S. cerevisiae* Y153 strain and assayed for protein–protein interactions. For the assay we used three forms of Chp: wild type protein, its active GTP-bound form Chp G40V, and inactive GDP-bound form Chp S45N. The active form of Cdc42 Q61L was included as a positive control since it was shown to interact with Pak6 [21]. In addition, we assayed the binding between Pak6 and active form of Wrch-1/RhoU Q107L, which is the closest homolog of Chp [22]. We found that both active and wild type Chp, but not inactive GDP-bound Chp, interacted with Pak6 (Fig. 1). These findings suggest that Pak6 might be an effector of Chp. As expected, Cdc42 Q61L bound to Pak6, confirming the validity of our assay. Despite the high degree of homology to Chp as well as to Cdc42, Wrch-1 Q107L failed to interact with Pak6 (Fig. 1).

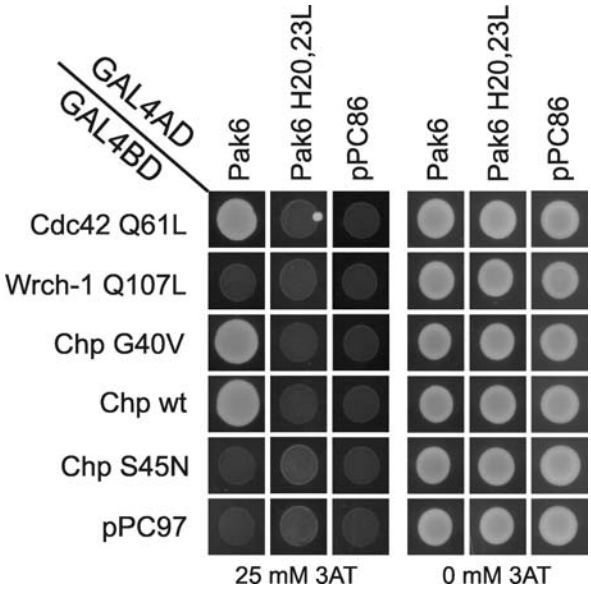

Fig. 1. Chp interacts with Pak6 in a yeast two-hybrid system. Yeast co-expressing Pak6 and Pak6 H20,23L fused to GAL4AD or empty vector (pPC86) and indicated Rho GTPases fused to GAL4BD or empty vector (pPC97) were grown in the presence (25 mM 3AT) or in the absence (0 mM 3AT) of 3-amino-1,2,4-triazole for 3 days. Activation of the *HIS3* reporter gene was monitored by the ability of the yeast to grow in the presence of 3AT.

Chp interacts with Pak6 in mammalian cells. To confirm that Chp is able to interact with Pak6 in mammalian cells, we performed co-immunoprecipitation (co-IP) experiments. FLAG-tagged Pak6 and myc-tagged Chp in its active state as well as Wrch-1 Q107L and Cdc42 Q61L were co-expressed in HEK293 cells, and myc-tagged proteins were precipitated with anti-c-myc antibodies. The precipitates were analyzed by Western blotting for the

presence of FLAG-Pak6. We found that Chp G40V and wild type Chp precipitated FLAG-Pak6 (Fig. 2). Again, we found that Cdc42 Q61L interacted with Pak6, while interaction between Wrch-1 Q107L and Pak6 was significantly weaker compared with Cdc42 Q61L or Chp G40V (Fig. 2). Together the data from the yeast two-hybrid assays and co-IP experiments strongly imply that Chp interacts with Pak6 only in its active, GTP-bound state. Therefore, Pak6 is likely to be an effector of Chp.

Chp binds to the CRIB motif in Pak6. Next we analyzed the molecular basis of interaction between Chp and Pak6. Cdc42 interacts with the CRIB motif found in many effectors of Cdc42 including Pak kinases [23]. Substitution of conserved histidine residues for leucine residues in the CRIB motif of Pak1 (Pak1 H83,86L) was shown to impair interaction of Pak1 with Cdc42 and Rac1 [24] as well as with Chp [10]. Therefore, we assayed the ability of Chp to interact with Pak6 containing homologous substitutions in the CRIB motif. In a yeast two-hybrid assay Pak6 H20,23L did not interact with either Chp G40V or Cdc42 Q61L (Fig. 1). Results of co-IP showed that mutations in the CRIB motif of Pak6 significantly reduce binding to active Cdc42 and Chp compared to wild type Pak6 (Fig. 3). Together, these data indicate that the CRIB motif of Pak6 is involved in binding to Chp.

Effector domain of Chp is required for binding to Pak6. Rho GTPases interact with their effector proteins via the so called "effector domain" that spans amino acids 26-50 in Cdc42 and amino acids 54-78 in Chp. A number of mutations in the effector domain of Cdc42 that affect binding of different effectors including Pak kinases have been reported [23]. Thus, we introduced the equivalent mutations (T63A, L65A, D66A, and F68C) in the effector domain in a context of activated Chp G40V and validated their ability to affect interaction with Pak6 in co-IP assays. We found that T63A, L65A, and D66A mutations significantly reduced FLAG-Pak6 binding to myc-Chp G40V, although the binding was not completely abolished (Fig. 4). In contrast, the F68C mutation did

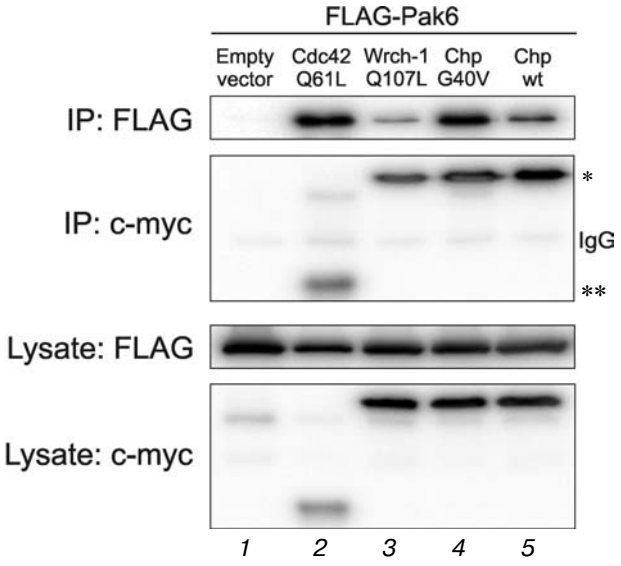

Fig. 2. Pak6 interacts with Chp in mammalian cells. Plasmid expressing FLAG-Pak6 was transfected into HEK293 cells together with plasmids expressing active forms of myc-tagged Rho GTPases (Cdc42 Q61L (*2*), Wrch-1 Q107L (*3*), Chp G40V (*4*), Chp wt (*5*)) or with empty vector (*1*). After 48 h the cells were lysed, and myc-tagged proteins were immunoprecipitated with anti-c-myc antibody. Precipitates (IP) along with input lysates (Lysate) were analyzed by Western blotting with anti-FLAG and anti-c-Myc antibodies. * Chp and Wrch-1; ** Cdc42; IgG, light chains of antibodies that were used for IP.

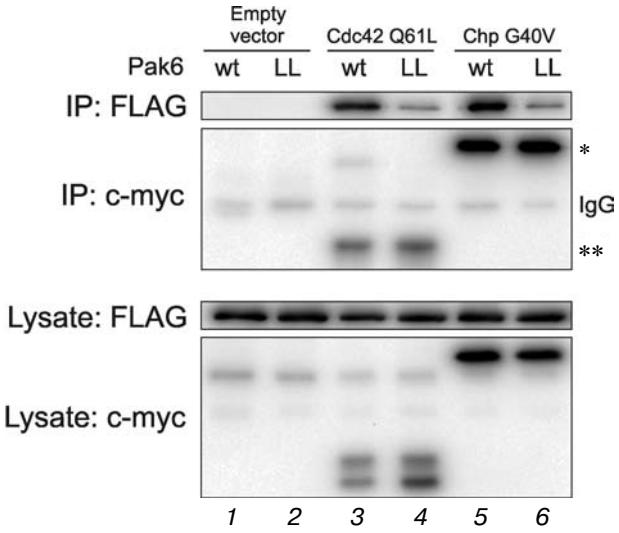

Fig. 3. Chp binds to the CRIB motif in Pak6. HEK293 cells were transfected with plasmid expressing FLAG-Pak6 (wt) and FLAG-Pak6 H20,23L (LL) together with empty vector (*1*, *2*) or with plasmids expressing myc-Cdc42 Q61L (*3*, *4*) and myc-Chp G40V (*5*, *6*). Co-IPs were performed and analyzed as described in Fig. 2. * Chp; ** Cdc42; IgG, light chains of antibodies that were used for IP.

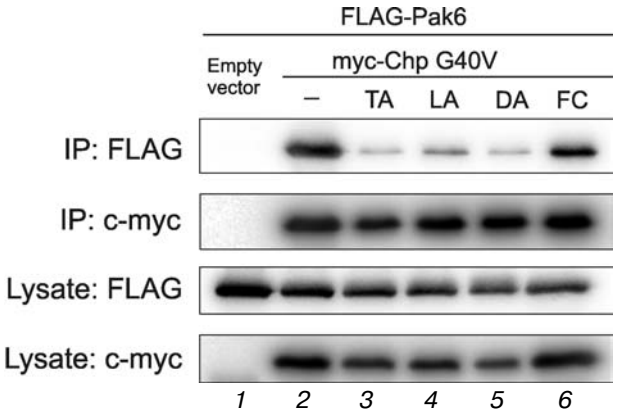

Fig. 4. Mutations of the effector domain in Chp affect the interaction with Pak6. Plasmid expressing FLAG-Pak6 was transfected into HEK293 cells together with empty vector (*1*) or with plasmids expressing myc-Chp G40V (*2*) or myc-Chp G40V with mutations in the effector domain (T63A (*3*), L65A (*4*), D66A (*5*), F68C (*6*)). Co-IPs were performed and analyzed as described in Fig. 2.

not affect the interaction of Chp with Pak6. Together these data indicate that Chp interacts with Pak6 via its effector domain.

Chp does not enhance the level of Ser560 phosphorylation in Pak6. Pak6 interacts only with active, GTP-bound Cdc42, but this binding does not stimulate the kinase activity of Pak6 [21]. It was reported that the phosphorylation status of Ser560 residue in the catalytic domain of Pak6 could be used as an indicator of Pak6 activity [21]. Therefore, we asked if Chp could regulate the activity of Pak6 by affecting the phosphorylation of Ser560 in Pak6. FLAG-Pak6 was transiently overexpressed in HEK293 cells together with myc-tagged Chp G40V and Cdc42 Q61L, and the status of Ser560 phosphorylation of FLAG-Pak6 was assessed with phospho-Pak6-specific antibody. We found that the level of Ser560 phosphorylation of FLAG-Pak6 expressed together with the empty vector was similar to that of FLAG-Pak6 expressed together either with active Cdc42 or Chp (Fig. 5). Based on these data, we conclude that similarly to Cdc42, Chp is not likely to activate Pak6, at least in terms of increasing Ser560 phosphorylation level.

Chp co-localizes with Pak6 in NCI-H1299 cells. To further corroborate the data of protein–protein interaction assays, we performed immunofluorescent staining of NCI-H1299 cells co-expressing EGFP-tagged Pak6 and FLAG-tagged Chp. It was shown earlier that Pak6 is localized mainly in cytosol and shuttles to the nucleus upon steroid hormone stimulation [15]. In contrast, we found that transiently expressed EGFP-tagged Pak6 is often localized to punctate structures in cytosol of HeLa B and NCI-H1299 cells (data not shown). Next, we assessed the co-localization between EGFP-Pak6 and FLAG-Chp and found that the expressed proteins indeed co-localize at the vesicular structures (Fig. 6; see color insert). The inactive form of Chp S45N did not co-localize with Pak6, although it was also targeted to punctate structures in cytosol (Fig. 6). These findings support the data of protein–protein interaction assay in yeast two-hybrid system and results of co-IP experiments and open a perspective to further gain an insight into functional consequences of Chp-Pak6 interaction by characterizing vesicular structures on which Chp and Pak6 co-localize.

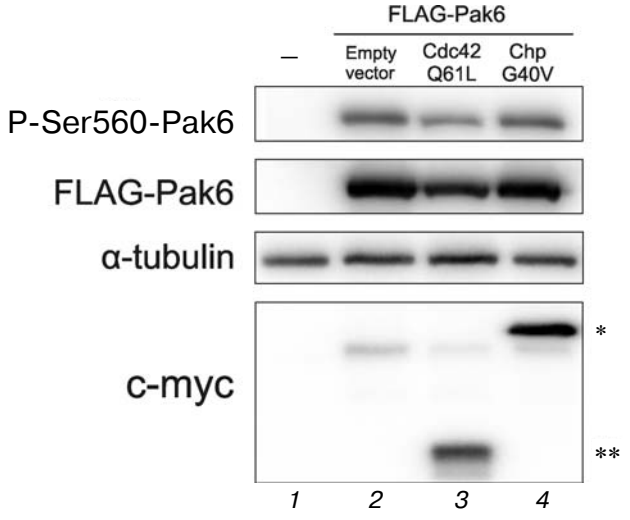

Fig. 5. Chp does not affect the level of Ser560 phosphorylation in Pak6. HEK293 cells were transfected with plasmid expressing FLAG-Pak6 together with empty vector (*2*) or with plasmids expressing myc-Cdc42 Q61L (*3*) and myc-Chp G40V (*4*) or left non-transfected (–) (*1*). Forty-eight hours post-transfection cell lysates were obtained and analyzed by Western blotting with the indicated antibodies. Equal protein loading was monitored by staining blots with anti-α-tubulin antibodies. * Chp; ** Cdc42.

DISCUSSION

In the present work we identified Pak6 as a novel binding partner for Chp/RhoV atypical Rho GTPase. Although we failed to demonstrate that Pak6 does not form a complex with inactive Chp S45N protein in co-IP experiments due to impaired solubility of Chp S45N protein expressed in mammalian cells under the used experimental conditions (data not shown), nevertheless the results of protein–protein interaction assay in a yeast two-hybrid system and immunocytochemical analysis clearly show that interaction between Pak6 and Chp is specific for the active state of GTPase (Chp G40V and wild type Chp), but not for inactive state (Chp S45N). These observations strongly imply that Pak6 is an effector of Chp. The closely related GTPase Wrch-1 did not interact with Pak6 in the yeast two-hybrid assays and showed significantly reduced binding to Pak6 in co-IP. It has been shown that Chp but not Wrch-1 interacts in the yeast two-hybrid system with another Pak II kinase, Pak4 [3]. Together, these data suggest that Chp is able to interact with both Pak I and Pak II kinases, while Wrch-1 specifically binds to Pak I kinases.

Cdc42 binds to the conserved CRIB motif found at the *N*-terminus of Pak kinases [23]. Schrantz et al. have shown that Cdc42 does not bind Pak6 H20,23L and, unlike Pak1, these substitutions do not increase Pak6 kinase activity [21]. Accordingly, we found that interaction of both Chp and Cdc42 with Pak6 is significantly diminished by introduction of H20,23L mutations, indicating that Chp, like Cdc42, binds to the CRIB motif of Pak6.

The effector domain of Rho GTPases is essential for effector binding. Point mutations in the effector domain are sufficient to abrogate the interaction with a particular effector. For example, the Y40C mutation impairs Cdc42 binding to Pak1 [25], while the F37A mutation has no effect. In a yeast ortholog of Cdc42, T35A substitution impairs binding to Pak1p [26, 27]. The F86C mutation in Wrch-1 Q107L completely abolishes interaction with polarity protein Par6 [28]. Ruusala et al. have shown that P80G, F83G, and F86C mutations in wild type Wrch-1

impair its binding to Pyk2, while binding to Pak1 is affected by P80G and F86C but not the F83G mutation [29]. The T81S, F83A, and F86C mutations in the context of Wrch-1 Q107L had no effect on binding to the GST-CRIB domain of Pak1 in *in vitro* pull-down assay. The binding was completely abolished only by the combination of the three above mutations [30]. We found that substitutions T63A, L65A, and D66A significantly reduced binding of activated Chp to Pak6, while the F68C mutation had no effect. It is interesting to note that the Y40C mutation in Cdc42, which is equivalent to F68C, does not affect binding of Cdc42 to Pak4 and Pak5 [19, 31]. Taken together, this is the first report characterizing the effector domain mutants of Chp GTPase. Our data identify T63, L65, and D66 residues in the effector domain of Chp as critical for mediating binding to Pak6 and suggest that the effector domain of Chp is involved in effector binding. In addition, these data confirm the specificity of interaction between Chp and Pak6. Chp mutants that have lost the ability to interact with the specific effector are valuable tools for dissecting the biological significance of a given effector downstream of Chp.

Activation of Pak I group kinases by Cdc42 GTPase is a well-established paradigm of signal transduction [13]. Similarly, Chp was shown to activate Pak1 kinase [10]. In contrast to the Pak I group, Pak6 kinases possess a high level of basal catalytic activity, which is not further enhanced by Cdc42 binding [21]. Currently, the only known mechanism of Pak6 activation is phosphorylation of the S165 and Y566 residues in a MKK6- and p38 MAPK-dependent manner [32]. In addition, it has been proposed that the level of Ser560 phosphorylation can be used to trace Pak6 activity [21]. Therefore, we asked if Chp affects Ser560 phosphorylation and found that neither active Chp nor active Cdc42 affected the phospho-Ser560 level. Our data are in line with the previously published observation that Cdc42 does not enhance already high basal activity of Pak6 [21]. Chp induces ubiquitin mediated degradation of Pak1, and this effect seems to be specific for Pak I kinases since Chp did not affect the level of Pak4 [10]. In line with this, we did not observe an effect of Chp on FLAG-Pak6 protein level upon transient co-expression in HEK293, suggesting that Chp does not regulate stability of Pak6. Although our data strongly suggest that Pak6 is an effector of Chp, nevertheless it is a future challenge to determine the functional consequences of interaction of Chp with Pak6.

It has been shown that Pak6 is localized mainly in cytosol of CV-1 cells and shuttles to the nucleus upon steroid hormone stimulation [15]. In HeLa cells the nuclear translocation of EGFP-Pak6 upon hormone stimulation was not observed, and Pak6 was localized to cytosol and plasma membrane [16, 21]. We assessed the subcellular localization of EGFP-Pak6 and found that, in contrast to previous reports, transiently expressed EGFP-tagged Pak6 is often localized to punctate structures in cytosol in HeLa B cells and NCI-H1299 lung carcinoma cells. Since Chp is localized to endosomal compartment in fibroblasts [5], we next assessed co-localization of Chp with Pak6 in NCI-H1299 cells and found that active but not GDP-bound Chp co-localized with EGFP-Pak6 at the vesicular structures. Upon activation, Rho GTPases act to recruit their effectors to a membrane compartment. Since Pak6 H20,23L mutant, which has an impaired ability to bind Chp, was also localized to vesicular structures when expressed in HeLa B or NCI-H1299 cells (data not shown), then it is unlikely that Chp recruits Pak6 to vesicles. We hypothesize that an interaction of Chp and Pak6 might occur at endosomes via a hypothetic scenario involving activation of Chp at endosomes, thus allowing for interaction with Pak6 that is already localized to endosomes. Co-localization of Chp and Pak6 in NCI-H1299 cells further corroborates the data of protein–protein interaction assays.

In conclusion, this work identifies Pak6 as novel effector protein of an atypical Rho GTPase Chp/RhoV, hence it might have an impact on the elucidation of signaling pathways downstream of Chp as well as novel functions of Pak6 and mechanisms regulating its activity and/or subcellular localization. Pak6 was shown to be overexpressed in prostate cancer cell lines and human cancer samples [33], and its inhibition improves radiosensitivity and chemosensitivity of prostate cancer cells [34, 35]. Given that Chp is able to promote malignant transformation of mouse fibroblasts and is implicated in the regulation of actin cytoskeleton, it raises the possibility that both proteins might be involved in pathways regulating cancer progression and increased cancer cell motility.

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