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Biochemical and Biophysical Research Communications 331 (2005) 153–158
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Identification of dynein light chain 2 as an interaction partner of p21-activated kinase 1

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Received 17 March 2005
Available online 30 March 2005

Abstract

p21-activated kinase 1 (PAK1), a member of the evolutionarily conserved PAK family of serine/threonine kinases, is essential for a variety of cellular functions. Our previous studies showed that PAK1 participated in the apoptotic pathway mediated by p110C. To further investigate its functions, we used the yeast two-hybrid system to screen a human fetal brain cDNA library and identified dynein light chain 2 (DLC2)/myosin light chain (MLC) as an interacting partner of PAK1. The association of PAK1 with DLC2 was further confirmed by in vitro binding assay. With the stimulation of EGF, PAK1 interacted with HA-DLC2 in vivo and relocalized in cytoplasm near the perinuclear location in confocal microscope analysis. The deletion analysis showed that the interaction of DLC2 with PAK1 occurred within the residues 210–332 of PAK1. For that studies showed that DLC2 was a subunit of myosin complex, so it is possible that PAK1 binds to DLC2 and transports by myosin complex.

Keywords: Dynein light chain 2/myosin light chain; p21-activated kinase 1; Myosin complex; Transport

The p21-activated kinases were initially identified in a screen for specific Rho GTPase (p21) binding partners in rat brain cytosol [1,2]. There are six known p21-activated kinase 1 (PAK) isoforms (PAK1–6) that are differentially expressed in mammalian tissues [2]. Based on their conserved structure, PAK1–3 are classified together as the Group I PAKs, whereas PAK4–6 are classified together as the Group II PAKs [2]. PAKs1–3 have highly conserved serine/threonine protein kinase and p21 binding domains, and can be activated by binding to the GTP bound form of Rac or Cdc42, two GTPases of the Rho subfamily, thus making PAKs good candidate effectors for these signaling molecules [3,4]. PAK1 (or α-PAK1) have been implicated as the downstream effectors for several Cdc42 and Rac1 regulated signaling pathways including modulation of the actin cytoskeleton and establishment of cell polarity [3,5–8], processes that are required for cell motility. PAK1 has two types of effects on cell morphology, one related to its protein kinase activity and one that is kinase independent [3,7]. Activated PAK1 could protect cells from apoptosis, mediated through the suppression of the pro-apoptotic activity of Bad, and promote apoptosis through activation of the JNK1 pathway [9–14]. Our previous studies have shown PAK1 inhibited its activity during anoikios mediated by p110C [2].

To further investigate the functions of PAK1, we used the full length cDNA of PAK1 to screen human fetal brain cDNA library by using yeast two-hybrid system. This analysis resulted in the identification of a substantial number of known PAK1 binding proteins (i.e., Cdc42, Rac1, Pix, and Nck) [1,15–18] and several previously uncharacterized PAK1-interacting proteins.

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doi:10.1016/j.bbrc.2005.03.128

Sequence analysis showed that the sequence of the novel interacting clones was identical to that of DLC2.

DLCs (dynein light-chain proteins), found as components of both cytoplasmic dynein [19] and myosin-V motor complexes [20], connect the complexes to their cargoes. DLC2 was found as a subunit of the myosin-V complex [20]. We provided the evidences that PAK1 was a partner of the DLC2, thus it implying that the interaction may be involved in the trafficking of PAK1 by motor protein.

Experimental procedures

Yeast two-hybrid assays. A genetic screen using the yeast interaction trap was performed as recommended by the manufacturers (according to Clontech Matchmaker LexA two-hybrid system user manual). The full length of PAK1 was amplified by polymerase chain reaction (PCR) and cloned in-frame into LexA-coding sequence to generate bait plasmid, pLexA-PAK1. A human fetal brain cDNA library in the pB42AD plasmid (Clontech) was screened for proteins that interact with PAK1 in EGY48 yeast strain. Yeast transformation was performed by the lithium acetate method. A total of 2×10^6 clones were screened. Positive interactors were verified by one-on-one transformations followed by selection of agar plates lacking histidine, leucine, and tryptophan (HLT), and also by β-galactosidase assay. Plasmid DNA from LEU2+/LacZ+ colonies was isolated and recovered, and the true positives were sequenced with dideoxy sequencing according to the manufacturer's instructions. The fish plasmid, pB42AD harboring DLC2, was re-transformed into yeast along with either the bait plasmid or other nonspecific bait plasmids to verify the specificity of the two-hybrid assay.

Plasmid construction. For the bait of two-hybrid system, the full length of PAK1 was cloned into the *Eco*RI/*Xho*I site of pLexA (Clontech) in-frame with the DNA binding domain of LexA. To generate epitope-tagged constructs of DLC2, the open reading frame of DLC2 was amplified by PCR and subcloned into pLexA (Clontech) and pcDNA 3-HA (Invitrogen) vectors. GST fusion vector of DLC2 was generated by PCR cloning into pGEX-4T-1 vector (Amersham Biosciences). PAK1 was generated by PCR cloning into pDsRed1-C1 (Clontech) vector. PAK1 mutants were also subcloned into pB42AD and pLexA vector. The deletion mutants of PAK1 were constructed by PCR with pLexA-PAK1 as the template using the primers: PAK1-N (1–270 aa) (a restriction endonuclease site for subsequent subcloning is underlined; sense 5′-gatgaattcatgtcaaataacggcc-3′; antisense 5′-gatgtcgactttatttcttcttaggatcgcc-3′), PAK1-C (271–545 aa) (sense 5′-cctgaattcatgtatacacggtttg-3′; antisense 5′-gtgctcgagttagtgattgttctttg-3′), PAK1-F1 (1–456 aa) (antisense 5′-ccctcgagttattcgatggccatgat-3′), PAK1-F2 (1–394 aa) (antisense 5′-gatctcgagttaattgtcactcttgatgtc-3′), PAK1-F3 (1–332 aa) (antisense 5′-gatctcgagttagtccaagtaattcacaat-3′), PAK1-F4 (210–332 aa) (sense 5′-gatgaattcatgcctgtcactccaa-3′), and PAK1-F5 (240–332 aa) (sense 5′-gatgaattcatggagaagcagaagaag-3′; antisense 5′-gatctcgagttagtccaagtaattcacaat-3′). The PAK2, PAK3 were constructed by Chen [2].

In vitro binding analysis. GST-DLC2 fusion protein was constructed by inserting GST-DLC2 fragment into pGEX-4T-1. The expression in *Escherichia coli* BL21 is under the control of the *tac* promoter, which was induced by the lactose analog isopropyl β-D-thiogalactoside (IPTG). GST-DLC2 fusion protein was purified with glutathione–Sepharose 4B (Amersham Biosciences). NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cells were washed three times with ice-cold PBS and solubilized with 1 ml lysis buffer. The binding reactions were performed in protein binding buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 10% glycerol, 10 mM NaF, 1% NP-40, 1 mM $NaVO_4$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF) at 4 °C for 2 h with constant mixing. The beads were washed three times with the same buffer, and the bound proteins were subjected to 12% SDS–PAGE analysis and transferred to PVDF (Roche) membranes. Membranes were blocked using fat-free milk, probed with antibodies, and analyzed as Western blots.

Cell cultures and in vivo interaction assay. NIH3T3 cells grown in DMEM supplemented with 10% bovine calf serum were plated in 60-mm dishes at a concentration of 6×10^5 cells/dish the day before transfection. Plasmid DNA (4 μg) was transfected into NIH3T3 cells with Lipofectamine (Invitrogen). Twenty four hours after transfection, NIH3T3 cells were serum starved for 48 h and treated with 100 ng/ml epidermal growth factor (EGF) (Cell Signal) for 30 min, washed three times with ice-cold PBS, and solubilized with 1 ml lysis buffer. Detergent-insoluble materials were removed by centrifugation at 13,000 rpm for 15 min at 4 °C. Whole cell lysates were incubated with relevant antibody at 4 °C for 2 h. Pre-equilibrated protein G–agarose beads (Roche) were then added and collected by centrifugation after 2 h of incubation and then gently washed three times with the lysis buffer. The bound proteins were eluted by boiling in SDS sample buffer and resolved on a 15% or 10% SDS–PAGE gel. The proteins were transferred onto a PVDF membrane and analyzed using Western blots. Membranes were blocked using fat-free milk, probed with antibodies. The following antibodies were used: rabbit polyclonal anti-PAK1 (N-20) from Santa Cruz, mouse monoclonal anti-HA (12CA5) antibody from Roche. Secondary antibodies were goat anti-rabbit and anti-mouse from Santa Cruz, goat anti-rat (IgM) from KPL.

Immunofluorescence and confocal studies. Cells grown on glass coverslips were fixed in methanol for 1 h and blocked in PBS containing 10% BSA. Cells were incubated with primary antibodies described above for 2 h, washed three times in PBS, and then incubated with FITC or rhodamine-conjugated secondary antibodies. The plasmid conjugated with pDsRed1-C1 was visualized by autofluorescence. Secondary IgG-R-antibodies were goat anti-rabbit from Santa Cruz. Secondary FITC-antibodies were goat anti-mouse from Santa Cruz, rabbit anti-rat from DAKO. Secondary antibodies were diluted 1:1000. Coverslips were visualized with a Zeiss laser-scanning confocal microscope. Colocalization of two proteins, PAK1 stained in red while DLC2 stained in green, was indicated by the development of yellow colors.

Results

Isolation of DLC2 as a PAK1-binding protein with the yeast two-hybrid system

To identify proteins that interact with PAK1, the yeast two-hybrid system was employed with PAK1-fused LexA DNA-binding domain as bait. The bait did not have any intrinsic activity of transcriptional activation for the two reporters (LEU2 and LacZ). A human fetal brain cDNA library was screened as described under Experimental procedures. In a screen of approximately 6×10^6 transformants, 18 clones were found to be positive for all reporters tested (LEU2 and LacZ). Sequencing of the positive clones identified many known PAK1-binding proteins (Cdc42, Rac1, Pix, and Nck) and several previously uncharacterized PAK1-interacting proteins. Sequence analysis of several isolated clones was identical to that of DLC2 (GenBank Accession No. NM_080677). Recently it was reported that DLC1 is a physiologic substrate of PAK1, so we use

DLC1 as a positive control. To further confirm the interaction between PAK1 and DLC2, two cloning vectors were exchanged by moving DLC2 from the activation domain (pB42AD) to the DNA-BD vector (pLexA) and PAK1 from the pLexA to pB42AD. The repeated two-hybrid assay was also positive for the two reporters (Fig. 1A). The fact that PAK1 interacted with DLC2 raised the question of whether DLC2 interacted preferentially with PAK1 or it also interacted with the other p21-activated kinases. To answer this question, DLC2 was co-transformed, respectively, with PAK1, PAK2, and PAK3 into EGY48 (p8op-lacZ). As shown in Fig. 1B, only PAK1 was able to bind to DLC2. Neither interaction between PAK2 and DLC2 nor interaction between PAK3 and DLC2 was observed.

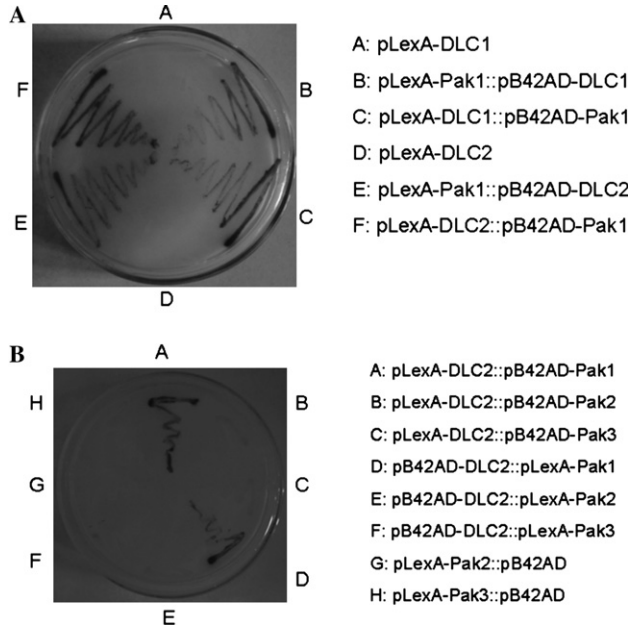

Fig. 1. Interaction between PAK1 and DLC2. (A) Yeast cells were co-transformed with pLexA-DLC1, pLexA-DLC2 along with pB42AD, or pB42AD-PAK1 (full length) and reversed. Co-transformants were plated on selection plates lacking histidine, leucine, and tryptophan. Growth was recorded after 72 h. For β-galactosidase assay, black color indicates specific interaction of two proteins. DLC1 was used as a positive control. (B) The specificity of PAKs binding with DLC2 in yeast two-hybrid system. PAK1, PAK2, and PAK3 were co-transformed, respectively, with DLC2 into EGY48.

Mapping of the PAK1 region that interacted with DLC2

Several PAK1 deletion mutants (Fig. 2A) were tested in order to map the specific sites required for the interaction (Fig. 2B). Direct two-hybrid tests were performed using the full-length DLC2 and the different PAK1 deletion constructs. As shown in Fig. 2B, the interaction was detected when amino acids 210–332 were present and was disrupted when the PAK1-F3 (aa 1–332) was further deleted into the PAK1-F5 (aa 240–332). This suggested that the region within PAK1 that directly interacted with DLC2 was between amino acids 210 and 332, which partly consisted of regulatory domain and partly kinase domain of PAK1.

DLC2 binds PAK1 in vitro and in vivo

To demonstrate the interaction of PAK1 and the DLC2 in vitro, we constructed the expression vector for the fusion protein of DLC2 (GST-DLC2), while the fusion protein of DLC1 (GST-DLC1) as a positive control. PAK1 expressed in NIH3T3 cells were incubated with GST, GST-DLC1 or GST-DLC2 immobilized on GSH–Sepharose beads. The protein mixtures were washed and run on SDS–PAGE and Western blot analysis was performed using anti-PAK1 antibody. A strong PAK1 signal was observed after the incubation of GST-DLC2 with PAK1 (Fig. 3, lane 4), as positive control GST-DLC1 (Fig. 3, lane 3). For negative

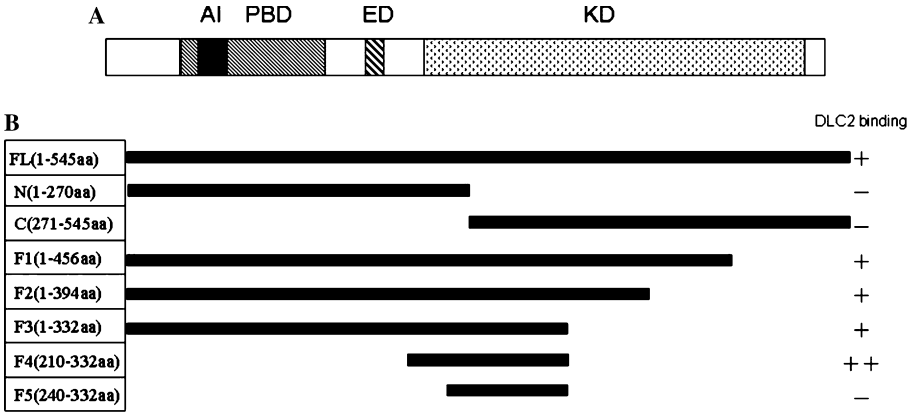

Fig. 2. Identification of PAK1 domains responsible for the interaction with DLC2. (A) Domain structures of PAK1: PBD, p21 binding domain; AI, autoinhibitory switch domain; ED, acidic domain; and KD, catalytic domain. (B) Two-hybrid interactions between DLC2 and the deletion mutants of PAK1. A series of PAK1 deletion mutants ((B) shown) constructed in pLexA or pB42AD vector were co-transformed with pB42AD-DLC2 or pLexA-DLC2.

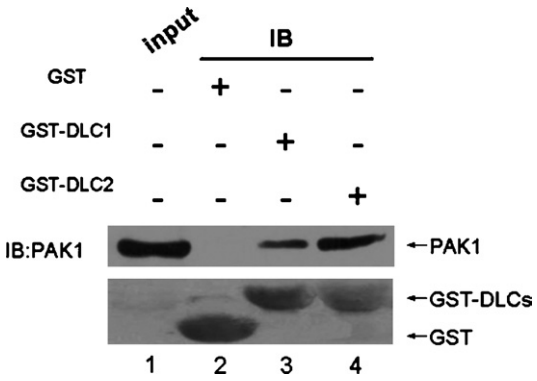

Fig. 3. PAK1 and DLC2 interact in vitro. GST-DLC2 was expressed in prokaryote. NIH3T3 cell lysates were immunoprecipitated with GST-DLC2 fusion protein and analyzed by Western blotting with PAK1.

control, no PAK1 was observed after the incubation with GST (Fig. 3, lane 2). These data showed that PAK1 interacted with DLC2 in vitro. To further characterize the in vivo interaction of PAK1 and DLC2, in vivo co-immunoprecipitation experiment was performed on NIH3T3 cells. NIH3T3 cells were transiently transfected with HA-DLC2 or without HA-DLC2 as indicated. Twenty hours after transfection, cells were serum starved for 48 h. Protein complexes were immunoprecipitated from lysates of NIH3T3 cells treated with or without epidermal growth factor (EGF), with a polyclonal antibody against PAK1. The immunoprecipitated protein complexes were run on SDS–PAGE and Western blot analysis was performed using anti-HA antibody. As shown in Fig. 4A, HA-DLC2 was co-immunoprecipitated with PAK1 (Fig. 4A, lane 5). Protein complexes were also immunoprecipitated with antibody against HA epitope (Fig. 4B) under the same condition and found PAK1 binding with HA-DLC2 (Fig. 4B, lane 4). Therefore, PAK1 and DLC2 can interact both in vitro and in vivo.

DLC2 and PAK1 colocalize in the cytoplasm

To further confirm the interactions in mammalian cells, HA-DLC2 with PAK1-DsRed was expressed in NIH3T3 cells and their subcellular localization was examined. From Fig. 5A, we found that PAK1-DsRed localized in the peripheral sites of the plasma membrane. Immunofluorescence microscopy using an anti-HA antibody revealed that DLC2 also localized both in nucleus and cytoplasm in NIH3T3 cells transfected with HA-DLC2 and no confocal fluorescence of PAK1-DsRed and HA-DLC2 was seen (Fig. 5A, merge). As shown in Fig. 5B, EGF treatment rapidly stimulated PAK1-DsRed localized in the whole of NIH3T3 cytoplasm. Under the stimulation of EGF, the co-localization of PAK1-DsRed (red) with HA-DLC2 (green) can be seen as yellow color (Fig. 5B, merge). Similarly, we further examined the colocalization of HA-DLC2 with endogenous PAK1. From Fig. 5C, which was not treated EGF after transfection, we found that the fluorescent signals of PAK1 and DLC2 were detected most in cytoplasm. Merging the separate fluorescent images we did not find that the transfected cells contained yellow (Fig. 5C, merge), indicating no co-localization of PAK1 and HA-DLC2. NIH3T3 cells were treated with EGF for 30 min as shown in Fig. 5D. In the merging image, transfected cells contained yellow, indicating the co-localization of PAK1 and HA-DLC2. These data suggested that ligand stimulation, such as EGF, was essential for the interaction of PAK1 and DLC2.

Discussion

In this study, we discovered that DLC2 was a PAK1-interacting protein. This is supported by the ability of PAK1 to interact with DLC2 in yeast two-hybrid, in vitro and in vivo immunoprecipitation assays. Deletion

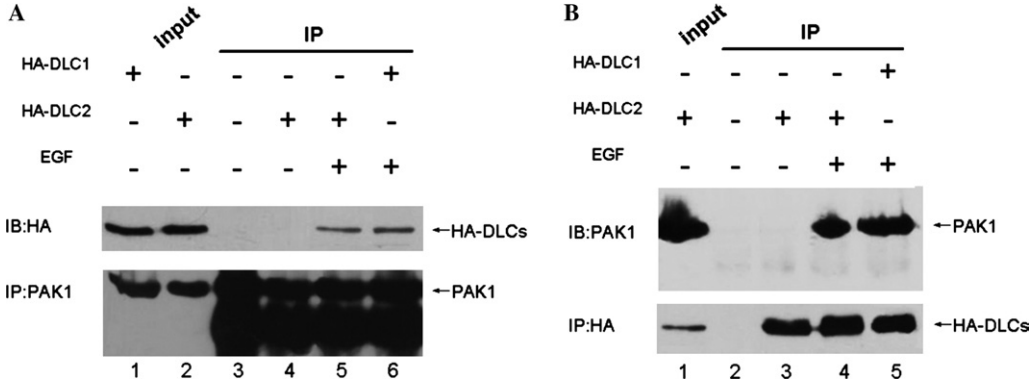

Fig. 4. PAK1 and DLC2 interact in vivo. (A) Expression of PAK1 (bottom) and HA-DLC2, HA-DLC1 (top) in transfected NIH3T3 cells by Western blot analysis. Twenty four hours after transfection, NIH3T3 cells were serum starved for 48 h and treated with EGF (100 ng/ml) for 30 min. PAK1 expression was detected using a specific anti-PAK1 antibody. The HA tag DLCs were detected using a monoclonal antibody against the HA tag. (B) Cell lysates were immunoprecipitated (IP) with an anti-HA monoclonal antibody. DLC2 association was analyzed by Western blotting with PAK1 antibody. The immunoprecipitates were immunoblotted (IB) with a specific anti-PAK1 antibody.

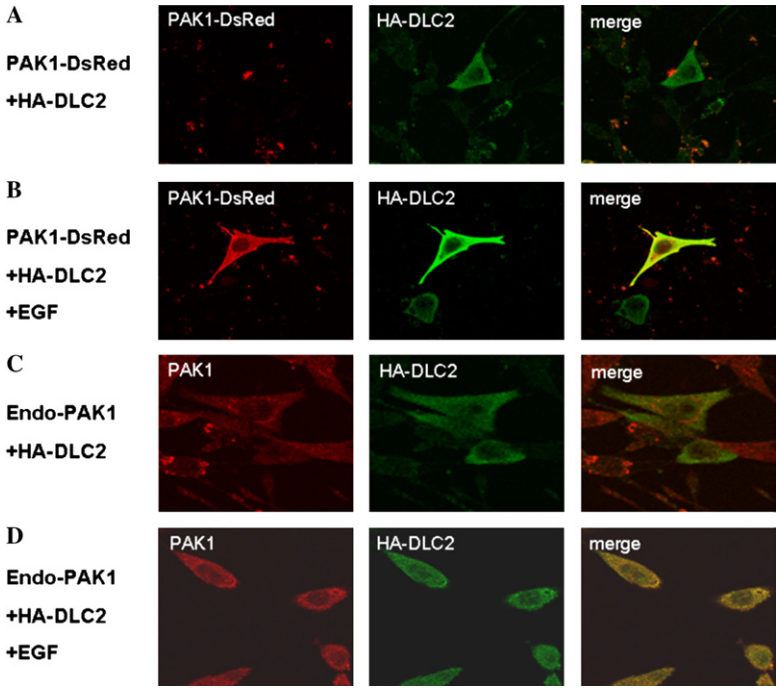

Fig. 5. Subcellular distribution of DLC2 and PAK1 determined by confocal laser scanning fluorescence microscopy. Full length of PAK1 was inserted into the pDsRed C1 as a fusion protein with DsRed and full length of DLC2 was inserted into the pcDNA3-HA as a fusion protein with HA. NIH3T3 were processed for immunofluorescence microscopy using an anti-HA monoclonal antibody. NIH3T3 cells were co-transfected with pcDNA3-HA-DLC2 and pDsRed-PAK1 (A,B). Twenty-four hours after transfection, 48 h serum-starved cells. (B) were stimulated with EGF (100 ng/ml) for 30 min. The co-localization (yellow, (B), merge) of green (HA-DLC2) and red (PAK1-DsRed) in NIH3T3 cells was analyzed by confocal microscopy. NIH3T3 cells transfected with HA-DLC2 (C,D) were serum starved for 48 h and then 100 ng/ml EGF was added into the medium (D). After incubation at 37 °C for 30 min, the co-localization (yellow, (B), merge) of endogenous PAK1 (red) and HA-DLC2 (green) was imaged using confocal microscopy.

analysis showed that this interaction was mediated by amino acids 210–332 of PAK1.

The p21-activated kinases (PAKs) are major targets for Rho-GTPases Rac and Cdc42 in regulation of actin dynamics and cell migration [21]. Their functions have been implicated in the regulation of directionality/persistence of movement [22], cell adhesion, and contractility [23]. They are localized to actin-rich structures such as pseudopodia and membrane ruffles [24], and autophosphorylated/activated forms are located to the leading edge of migrating cells [22]. PAK1 is the first mammalian member of PAKs and has also been demonstrated to be the regulators of myosin function through phosphorylation and inhibition of MLCK [25]. Active PAK1 increases stress fibers and focal adhesions, and these structures retained some degree of dynamic behavior consistent with increased cell contractility and phosphorylation of MLC [23]. It is shown that PAK1 plays a role in regulating directional cell motility and morphology through its effects on MLC.

DLC2/MLC is a cargo-binding domain of myosin-V complex, which can be phosphorylated by activated PAK1 [23]. Myosin-V was initially characterized as an unusual calmodulin binding protein from brain with a number of myosin-like biochemical properties. Subsequently, myosin-V heavy chain genes were cloned from mouse, yeast, and chicken, thus defining the fifth class of actin-based motors [26]. Myosins are a diverse protein family comprising 18 different classes that are structurally distinct based on comparisons of the primary structure of the motor domains of the known myosin heavy chain genes. Like its better-known cousin the myosin II of muscle, myosin-V is a molecular motor that moves along actin filaments powered by the hydrolysis of ATP [27] and has multiple functions in the cell ranging from mRNA transport, cell polarity, and membrane trafficking [26]. The activation of myosin-V complex is thought to be at least partially responsible for creating the traction force needed for cell movement [28]. DLC2, first co-purified from chicken myosin-V [29], a homolog of DLC1, has been shown to bind to a region of the tail domain carboxyl terminal of myosin-V complex [26]. Specifically, DLC1 is an integral component of the dynein motor complex and interacts with Bim. The two DLCs share 93% sequence identity yet show unambiguous in vivo specificity for their respective BH3-only ligands. In vivo DLC2 is found exclusively as a component of the myosin-V motor complex and Bmf binds DLC2 selectively [30]. As shown in Fig. 5, in resting cells, PAK1 is localized in the peripheral sites of the cell membranous structures within the cytoplasm.

While stimulated with EGF, PAK1 co-localized with DLC2 in the whole cytoplasm. These interacting proteins were transported from the peripheral sites to the perinuclear location. It is possible that there is a novel way, by binding myosin-V motor complex, for PAK1's transporting along actin filaments in cell and it will be a good explanation for the temporal and spatial distribution of PAK1 in cell motility and cell morphology. So it is investigated that DLC2 plays a role of transporting PAK1 as a co-operation for these functions.

Acknowledgments

This work was supported by 863 Program of China (2001AA234031), National Natural Scientific Foundation of China (30330320), and Mizutani Foundation for Glycoscience of Japan (040025). We thank Dr. Rong Yu for technical support.

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