Clik1: a novel kinase targeted to actin stress fibers by the CLP-36 PDZ-LIM protein

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Summary

In this report we have characterized a novel, ubiquitously expressed kinase, Clik1, that is predominantly nuclear and undergoes autophosphorylation. Yeast two-hybrid analysis indicated a highly specific association between Clik1 and CLP-36, which was identified in 36 out of 37 Clik1-interacting clones. CLP-36 is a PDZ-LIM protein that localizes to actin stress fibers in nonmuscle cells and associates with α-actinin via its PDZ-domain. The association of CLP-36 with Clik1, in turn, is mediated by the C-terminal part of CLP-36 containing the LIM domain, and association was not noted with the closely related ALP PDZ-LIM protein. Interestingly, the association with CLP-36 led to relocalization of the otherwise nuclear Clik1 kinase to actin stress fibers, where it disrupted the periodic staining pattern of CLP-36. Taken together these results establish the CLP-36 PDZ-LIM protein as an adapter, recruiting the Clik1 kinase to actin stress fibers in nonmuscle cells, and suggest that Clik1 represents a novel regulator of actin stress fibers.

Key words: PDZ, LIM, Stress fibers, Kinase

Introduction

Kinases involved in intracellular signaling are regulated at multiple levels through phosphorylation, subunit binding, inhibitor binding and subcellular targeting by associated proteins (Hunter, 2000). A group of proteins containing one N-terminal PDZ domain and one to three C-terminal LIM domains has been proposed to act in targeting intracellular kinases (Guy et al., 1999; Kuroda et al., 1996). This suggested function for PDZ-LIM proteins is based on separate observations of association with the actin cytoskeleton on one hand and with various kinases on the other.

Interactions between kinases and PDZ-LIM proteins are mediated via their LIM domains. Thus the third LIM domain of Enigma has been reported to associate with the cytoplasmic tail of the insulin receptor, and this interaction is apparently important for the endocytosis of the receptor (Wu and Gill, 1994). Association of the second LIM domain of Enigma with Ret/ptc2 is required for the plasma membrane localization and mitogenic activity of Ret/ptc2 (Durick et al., 1998; Durick et al., 1996). The LIM domains of ENH bind to PKC (Kuroda et al., 1996), and subsequently Cypher1 was also found to associate with PKC via its LIM domain (Zhou et al., 1999).

PDZ domains of this family were first found associated with the Z lines of striated muscle (Xia et al., 1997). Thus the PDZ domains of Enigma, ALP and ZASP/Cypher1 proteins mediate localization to the Z line of striated muscle, where Enigma interacts with β-tropomyosin (Guy et al., 1999) and ALP (Xia et al., 1997) as well as ZASP/Cypher1 (Faulkner et al., 1999; Zhou et al., 1999) with α-actinin-2. Accordingly, both ALP and Cypher1 appear to be muscle specific on the basis of their expression patterns as well as of the phenotypes of mice with targeted mutations of these genes. $Alp^{-/-}$ mice gradually develop a cardiomyopathy (Pashmforoush et al., 2001), and $Cypher1^{-/-}$ mice die postnatally with a severe form of congenital myopathy (Zhou et al., 2001). On the basis of the histology, it was suggested that Cypher1 is not required for formation but rather maintenance of sarcomeres (Zhou et al., 2001).

The association of PDZ-LIM proteins with the cytoskeleton is not limited to striated muscle. The CLP-36, also called CLIM1, (Kotaka et al., 1999; Wang et al., 1995) PDZ-LIM protein is expressed in several epithelial tissues (Vallenius et al., 2000). Furthermore, the PDZ domain of CLP-36 mediates its stable association with nonmuscle forms of α-actinin (α-actinin-1 and α-actinin-4) (Vallenius et al., 2000), and CLP-36 is accordingly localized to actin stress fibers in nonmuscle cells.

Actin stress fibers form a dynamic organelle that is important in processes involving cell shape, such as cell migration, cell polarity and cytokinesis. These functions are thought to be mediated by actin filaments in response to several only partially characterized signal transduction pathways. One of these involves the LIM-kinase that acts downstream of Rac and Rho, and phosphorylates cofilin, thereby inactivating its F-actin depolymerization activity (Arber et al., 1998; Yang et al., 1998b). Biochemical purification of stress fibers has revealed that it is composed of at least 20 polypeptides (Katoh et al., 1998), one of which is likely to be CLP-36. However, a number of other potential novel regulators of stress fibers remain to be identified. In this report we have characterized a novel kinase targeted to actin stress fibers by the CLP-36 PDZ-LIM protein.

Materials and Methods

Cloning of Clik1

Expressed sequence tags (EST) H85389 and H29877 with similarity to the fission yeast Csk1 kinase (Molz and Beach, 1993) were

identified using TBLASTN from the dbEST database (Altschul et al., 1997). A cDNA fragment was PCR amplified from a WI-38 human fibroblast cDNA library (a kind gift from Claude Sardet) with primers from the H85389 and H29877 contig: 5′-TTA CAG CCT GTT GGC GAG GA-3′ and 5′-GGA CAG CCA GTA CTT ATT CAC-3′. Subsequently, the radiolabeled PCR product was used to screen $1{\times}10^6$ clones of a human fetal liver cDNA library in lambda-DR2 (Clontech Laboratories, Palo Alto, CA). A single cDNA clone (C1) was isolated, and the plasmid form (C1/pDR2) subjected to automated sequence analysis.

Cell culture and antibodies

The U2OS osteosarcoma cell line was propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin and streptomycin and glutamine at 37°C in 5% CO_2. The mouse monoclonal anti-Myc antibody (9E10) was from Babco Inc., Berkeley, CA. The rabbit polyclonal anti-CLP-36 antibody was raised against purified full-length GST-CLP-36. The ALP antibody is described by Pomies et al. (Pomies et al., 1999).

Mammalian expression vectors and transfections

The open reading frame of Clik1 was PCR amplified from (C1/pDR2) with the primers 5′-GGA ATT CCG TCG ACT GGC AGC CAT GGA AAC GGG GA-3′and 5′-TCT AGA TTA AGC GGC ACA TGT GAC CTG GTC-3′, then subcloned further to pAMC (Tiainen et al., 1999) as an *Eco*R1-*Xba*I insert to generate Myc-Clik1. The mutagenesis kit (Promega) was used to generate the ATP-binding site mutant of Clik1 (Clik1-K98M/pAMC) by mutating lysine 98 to methionine using primer 5′-GGC GGT CAT GAA GAT CCG-3′. The Myc-tagged or HA-tagged CLP-36 plasmids were generated by subcloning an *Eco*RI-*Xho*I fragment from 38/JG4-5 (Vallenius et al., 2000) into *Eco*RI-*Sal*I sites of pAMC or pAHC (Tiainen et al., 1999). Myc-CDK7 was expressed from MO15-3M (Makela et al., 1994). HA-tagged ALP was PCR amplified from smALP/pBS (a kind gift from Pascal Pomies) with primers 5′-CG ACG CGT ACC ACC ATG CCA CAG AAC GTT ATT-3′ and 5′-GC GTC GAC CTA GCA TTT TGG GTA AAC AG-3′, followed by digestion with *Mlu*I and *Sal*I and subcloned into *Mlu*I-*Sal*I sites of pAHC. EGFP-CLP-36 plasmids were generated by subcloning an *Eco*RI-*Xho*I fragment from 38/JG4-5 (Vallenius et al., 2000) into *Eco*RI-*Sal*I sites of EGFP-C2 (Clontech). EGFP-CLP1-231 was generated from EGFP-CLP-36 by *Bam*H1 digestion to delete the C-terminal amino acids 232-329. U2OS osteosarcoma cells were transfected by using the calcium phosphate transfection method as described previously (Sambrook, 1989).

Cell extracts, immunoprecipitation and western blotting

Adult mouse testes were frozen in liquid nitrogen, homogenized, lysed in ELB (150 mM NaCl, 50 mM HEPES pH 7.4, 5 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol (DTT), 2.5 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerol-phosphate and 1 μg/ml leupeptin) and subsequently treated the same as the U2OS cell extracts (see below). 48 hours after transfection, U2OS cells were washed in PBS and lysed in ELB buffer 40 minutes on ice. The lysates were centrifuged at 14 000 *g* for 20 minutes at +4°C. Equal amounts (200-400 μg) of cleared supernatant was incubated with 3 μl monoclonal anti-Myc antibody or 2μl anti-CLP-36 antibody for 2 hours at +4°C prior to adding 25% protein A-sepharose beads for 1 hour. Subsequently beads were washed four times with ELB. Western blotting analysis was performed according to standard procedures (Sambrook, 1989).

Immunofluorescence

Transfected cells on coverslips were fixed with 3.5% (w/v)

paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100 for 5 minutes, blocked with 5% goat serum in PBS for 30 minutes, labeled with primary antibody for 30 minutes, washed three times with PBS, labeled with secondary antibody for 30 minutes, washed, labeled with Hoechst, mounted with Elvanol and analyzed with Zeiss Axiovert microscope. The 0.5% Triton X-100 treatment was performed as described previously (Sainio et al., 1997). The expression of the fusion proteins used in immunofluorescence studies was confirmed using western blotting analysis. Frozen sections were sliced from adult mouse testis, fixed with 94% ethanol overnight at +4°C and labeled as transfected coverslips (see above).

In vitro kinase assay

Anti-Myc immunoprecipitates from Myc-Clik1 overexpressing lysates were washed twice in kinase buffer (20 mM Tris pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 1 mM DTT) and resuspended in 15 μl kinase buffer with 10 μCi [γ^{32}P]ATP without any added substrates (Fig. 1B) or with added substrates including HistoneH1, MBP, CTD, GST-CDK2(K33R), α-actinin and GST-CLP-36 for 30 minutes at 30°C. Samples were resolved on SDS-PAGE, transferred to nitrocellulose membranes and stained with Ponceau to detect substrates. Subsequently radiolabeled bands were detected with autoradiography.

Yeast two-hybrid screening

A LexA-Clik1 fusion protein encoding amino acids 40-406 of Clik1 was used to screen a human fetal liver cDNA library in pJG4-5 (Clontech Laboratories, cat. HL4507AK), essentially as described (Gyuris et al., 1993). Positive clones were subjected to sequence analysis or analyzed by digestion, and representative clones (38/pJG4-5, amino acids 1-329; 51/pJG4-5, amino acids 24-329; and 47/pJG4-5, amino acids 158-329) were used to subclone various forms of CLP-36 into other vectors.

Northern blot analysis

Clontech Multiple Tissue Northern Blot filters (7757-1, 7760-1, 7766-1) were probed with 630 bp (nucleotides 404-1034) probe of Clik1 cDNA according to standard protocols (Sambrook, 1989). The Raji (Burkitt's lymphoma) lane that was present in the Northern filter was excluded from the analysis because of low levels of mRNA.

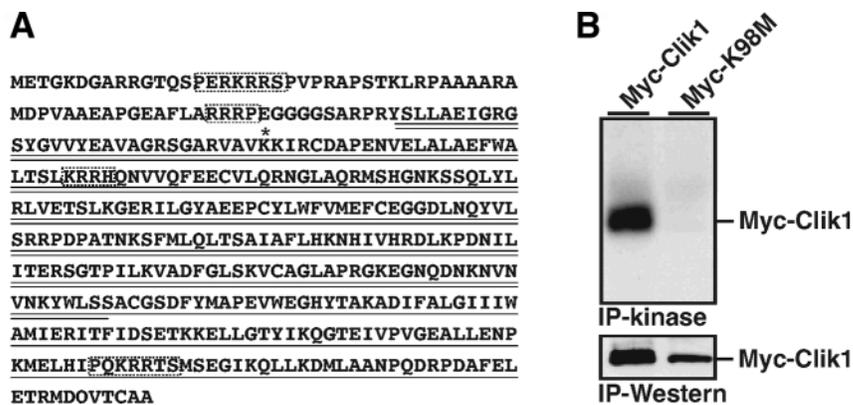### Recombinant proteins and solution binding assays

GST and GST-CLP-36 proteins were purified as described previously (Vallenius et al., 2000). For detection of association between cellular proteins and GST-CLP-36, 300 μg of U2OS cell extracts in ELB were incubated with 5 μg of purified GST or GST-CLP-36 for 2 hours at +4°C prior to adding 7.5 μl (packed volume) glutathione sepharose beads for 1 hour. Subsequently the beads were washed four times with ELB and subjected to SDS-PAGE, Ponceau staining and western blot analysis.

Results

Identification and cloning of Clik1

In an attempt to identify human homologues of fission yeast Csk1 kinase (Molz and Beach, 1993) or related Cdk-like kinases through database searches, a sequence contig generated from ESTs H85389 and H29877 was identified as a candidate. Using primers at the ends of the contig, a 654 bp fragment was PCR amplified from a human fibroblast cDNA library and was used to screen a human fetal liver cDNA library, resulting in the purification of a single 3.8 kb cDNA clone (C1). Sequence

Fig. 1. Characterization of the Clik1 kinase. (A) The amino-acid sequence of Clik1, demonstrating the kinase domain (underlined), the EST contig (double underlined), potential nuclear localization signals (dotted boxes) and the conserved lysine residue (asterisk) that was mutated to methionine to generate Myc-K98M. The GenBank Accession number is AF195026. (B) Immunoprecipitates from U2OS cells transfected with Myc-tagged Clik1 (Myc-Clik1) or with an ATP-binding site mutant Myc-tagged Clik1 (Myc-K98M) were subjected to an in vitro kinase reaction followed by SDS-PAGE, transfer to a nitrocellulose membrane and autoradiography (IP-kinase). Subsequently anti-Myc western blot analysis was used to visualize the 46 kDa Myc-Clik1 protein (IP-Western).

A

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METGKDGARRGTQSPERKRRSPVPRAPSTKLRPAAAARA
MDPVAAEAPGEAFLARRRRPEGGGGSARPRYSLLAEIGRG
SYGVVYEAVAGRSGARVAVKKIRCDAPENVELALAEFWA
                    *
LTSLKRRHQNVVQFEECVLQRNGLAQRMSHGNKSSQLYL
RLVETSLKGERILGYAEEPCYLWFVMEFCEGGDLNQYVL
SRRPDPATNKSFMLQLTSAIAFLHKNHIVHRDLKPDNIL
ITERSGTPILKVADFGLSKVCAGLAPRGKEGNQDNKNVN
VNKYWLSSACGSDFYMAPEVWEGHYTAKADIFALGIIIW
AMIERITFIDSETKKELLGTYIKQGTEIVPVGEALLENP
KMELHIPQKRRTSMSEGIKQLLKDMLAANPQDRPDAFEL
ETRMDOVTCAA
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B

Myc-Clik1 Myc-K98M

— Myc-Clik1

IP-kinase

— Myc-Clik1

IP-Western

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analysis of this clone and of the over 70 human ESTs identified as representing the same human gene (termed *CLIK1*) revealed an open reading frame of 1203 bp starting with an ATG surrounded by a Kozak consensus sequence (Kozak, 1991) predicted to encode a protein of 401 amino acids (Fig. 1A). This protein is predicted to contain the translated sequences from the ESTs H85389 and H29877 (see the double underline in Fig. 1A). *CLIK1* is located on chromosome 20p13 between markers D20S117 and D20S113 on the basis of localization of several of the ESTs (GeneMap99). A number of closely related ESTs were identified from other species including mouse, rat, cow, zebrafish and frog, and a potential full-length mouse homologue has been identified as part of the RIKEN Mouse Gene Encyclopaedia project (Kawai et al., 2001).

The predicted 44.6 kDa Clik1 protein contained a serine-threonine kinase domain between amino acids 69 and 390 (underlined in Fig. 1A), with the serine-threonine active site signature between amino acids 223-235. Other functional domains in Clik1 included four potential NLS signals (dotted boxes in Fig. 1A), yielding an NLS score of 1.14 by PSORT (Nakai and Kanehisa, 1992), and a proline-rich region in its N-terminal end. Despite our original observation that EST H85389 was similar to Csk1, full-length Clik1 shares only 20-30% identity to any known serine/threonine kinases, including Csk1, and thus does not clearly belong to characterized kinase families. Similar homologies were observed with a group of uncharacterized CaM-kinase related kinases (e.g. GenBank CAC29064), with several CDKs, and with the PAK family of kinases.

Autophosphorylation of Clik1

To determine whether the predicted protein encoded a kinase, a plasmid expressing a Myc-epitope tagged Clik1 (Myc-Clik1) was transiently transfected into COS-7 or U2OS osteosarcoma cells. At the same time, we also transfected a Clik1 mutant (Myc-K98M) with a methionine substitution of the conserved ATP-binding lysine (asterisk in Fig. 1A) to disrupt kinase activity, but this mutant behaved the same as the wild-type Clik1 in other regards (Fig. 5D). Anti-Myc immunoprecipitates from Myc-Clik1- or Myc-K98M-transfected cells were subsequently subjected to an in vitro kinase reaction. Autoradiography of the samples following SDS-PAGE and transfer to nitrocellulose membranes

indicated the presence of a broad radiolabeled band at 46-48 kDa in the wild-type Clik1-transfected but not in the Clik1-K98M lane (Fig. 1B, IP-kinase). This band co-migrated with the wild-type Myc-Clik1 protein detected as a tight doublet in western blot analysis (Fig. 1B, IP-Western). A band co-migrating with the faster migrating band was noted in the Clik1-K98M lane, indicating similar expression of the mutant and suggesting that the slower migrating band in wild-type Clik1 represented a phosphorylated form of Clik1. These results indicate that Clik1 undergoes autophosphorylation in vitro. Moreover, analysis of wild-type Clik1 and Clik1-K98M by western blot analysis from cell lysates showed the same difference in the bands, strongly suggesting that Clik1 undergoes partial autophosphorylation in cells (data not shown). The immunopurified Clik1 kinase did not phosphorylate any tested substrates including CDK2 (as GST-CDK2), Histone H1, myelin basic protein (MBP), the YSPTSPS heptapeptide C-terminal repeat of RNA Pol II, α-actinin, actin or CLP-36 (data not shown).

Clik1 associates with the PDZ-LIM protein CLP-36

To identify cellular proteins interacting with the Clik1 kinase, a yeast two-hybrid screen with LexA-Clik1 (amino acids 40-406) was performed. 36 out of 37 identified clones represented CLP-36, of which the longest (full length, clone 38) and the shortest (amino acids 158-329; clone 47) clones are shown in Fig. 2A. All clones contained the C-terminal LIM domain, suggesting that the association between CLP-36 and the Clik1 kinase is mediated by this protein-protein interaction motif. To investigate whether CLP-36 also interacts with cellular Clik1, a U2OS cell extract overexpressing Myc-Clik1 was incubated with baculovirus-produced GST-CLP-36 or GST alone. Subsequent purification of the GST proteins and analysis of associated proteins by western blot analysis indicated that Myc-Clik1 specifically interacted with GST-CLP-36 (Fig. 2B, anti-Myc, lane 2) and with GST-CLPΔ1-158 generated from clone 47 (data not shown). GST-CLP-36 did not associate with cellular Myc-CLP-36 under the same conditions (data not shown), suggesting that the PDZ and LIM domains of CLP-36 do not dimerize unlike the related PDZ-LIM protein Ril (Cuppen et al., 1998).
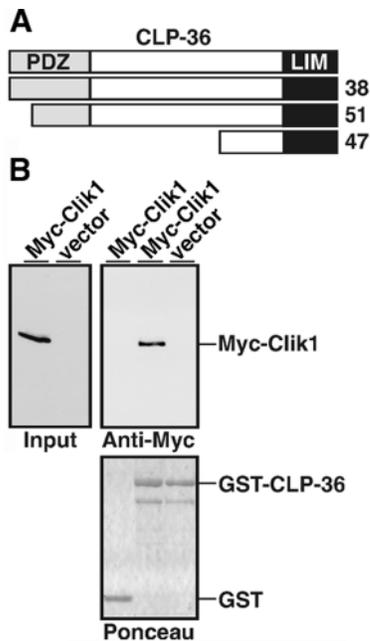
Fig. 2. Clik1 associates with CLP-36. (A) A yeast two-hybrid screen from a human fetal liver cDNA library with LexA-Clik1 identified 37 clones of which 36 represented CLP-36. Three representative clones (38, 51 and 47) with their PDZ (grey) and LIM (black) domains are shown. (B) To demonstrate the association of CLP-36 with cellular Clik1, 300 micrograms of U2OS cell extracts overexpressing Myc-Clik1 or vector control were incubated with GST-CLP-36 or GST proteins. Subsequently proteins associated with the GST proteins were purified and analyzed by anti-Myc western blotting (anti-Myc), demonstrating that CLP-36 associated with Myc-Clik1. Ponceau staining (lower panel) was used to control levels of GST-CLP-36 and GST proteins. The faster migrating bands in Ponceau staining in GST-CLP-36 lanes are caused by degradation of the purified protein. The input panel demonstrates amounts of Clik1 in 50 μg of extracts subsequently used for the pull-down assay.

Coexpression of CLP-36 leads to relocalization of the nuclear Clik1 kinase

When transfected alone, Myc-Clik1 was localized mainly in the nucleus of U2OS osteosarcoma cells by immunofluorescence analysis (Fig. 3A). Although these cells express no detectable endogenous CLP-36 (Fig. 3B and data not shown), transfected CLP-36 is localized to the cytoplasm and to actin stress fibers (Fig. 3E). Strikingly, in cells expressing both Clik1 and CLP-36, the majority of Clik1 was relocalized to the cytoplasm and colocalized with CLP-36 (Fig. 3G). This redistribution of nuclear Clik1 by CLP-36 was specific to Clik1, as coexpression of CLP-36 did not lead to relocalization of another nuclear kinase, CDK7 (Fig. 3J). The redistribution of Clik1 was not kinase dependent, as it was also observed with the Clik1-K98M mutant (Fig. 5D). Taken together with the physical association of Clik1 and CLP-36, these results indicate that when in association with CLP-36, Clik1 is mostly cytoplasmic and suggest that Clik1 may have distinct functions in the nucleus and the cytoplasm.

CLP-36 targets Clik1 kinase to actin stress fibers

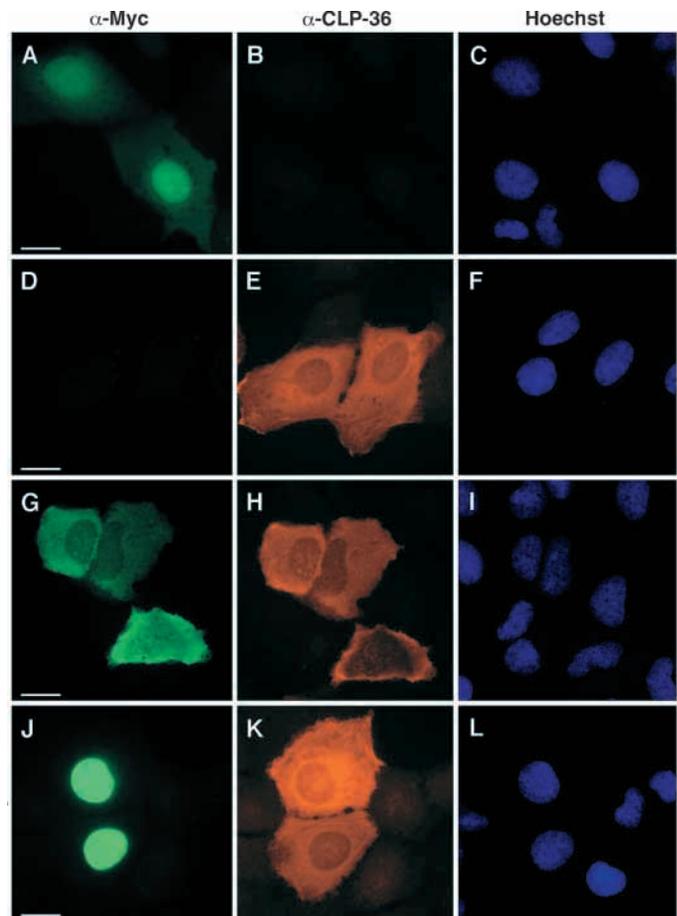The weak actin filament-like staining pattern of Clik1 in cells

Fig. 3. Nuclear Clik1 is relocalized when coexpressed with CLP-36. U2OS osteosarcoma cells were transfected with the following expression vectors: Myc-Clik1 (A-C), HA-CLP-36 (D-F), Myc-Clik1 and HA-CLP-36 (G-I), Myc-CDK7 and HA-CLP-36 (J-L). The subcellular localization of Clik1, CDK7 and CLP-36 was analyzed by double-label immunofluorescence with a mouse monoclonal anti-Myc (A,D,G,J) and a rabbit polyclonal CLP-36 antibody (B,E,H,K). Hoechst staining was used to visualize nuclei (C,F,I,L). Bars, 15 μm.

expressing CLP-36 (Fig. 3G) prompted us to study whether CLP-36 is able to target the Clik1 kinase to actin stress fibers. To this end, coverslips were subjected to detergent extraction prior to fixation to remove soluble material and to visualize actin stress fibers. As demonstrated in Fig. 4, the Clik1 kinase is colocalized with CLP-36 in actin stress fibers (Fig. 4A). In these cells, Clik1 staining was also detected in the nucleus where it accumulated into bright clusters. The significance of this observation is still unclear, but we could not detect these clusters with other tested kinases. The more diffuse nuclear staining was caused by background after detergent extraction as it was detected also with other constructs (data not shown) To get more insight into the specificity of the ability of CLP-36 to relocalize Clik1 from the nucleus to actin stress fibers, a similar experiment was carried out with ALP (Pomies et al., 1999; Xia et al., 1997), a close relative of CLP-36. Interestingly, whereas ALP did localize to actin stress fibers, as does CLP-36 (Fig. 4E), ALP failed to relocalize Clik1 from its nuclear localization (Fig. 4D), indicating that these proteins
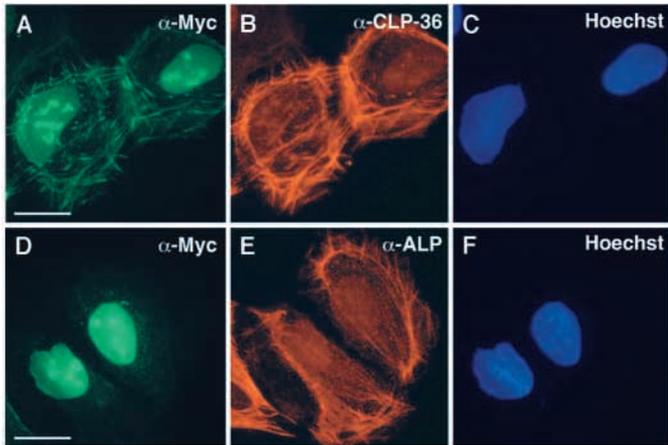
Fig. 4. CLP-36 targets Clik1 kinase to the actin stress fiber. U2OS osteosarcoma cells expressing Myc-Clik1 together with HA-CLP-36 (A-C) or HA-ALP (D-F) were treated with Triton X-100 (Triton) prior to fixation. Subsequently coverslips were analyzed by immunofluorescence with monoclonal anti-Myc to detect Myc-Clik1 (A,D) and with rabbit polyclonal CLP or ALP antibodies to detect CLP (B) and ALP (E), and Hoechst staining was used to visualize nuclei (C,F). Bars, 15 μm.

do not interact. These results indicate that CLP-36 specifically targets Clik1 to actin stress fibers.

C-terminal part of CLP-36 is essential to relocalize Clik1

The yeast two-hybrid analysis as well as the GST-binding assay suggested that the C-terminal part of CLP-36 is needed for the association between CLP-36 and Clik1. To test whether this was also the case in living cells, U2OS cells were transfected either with GFP-tagged CLP-36 (EGFP-CLP-36) or a mutant CLP-36 lacking the C-terminal LIM domain (EGFP-CLP1-231) together with Clik1. The results demonstrate that in cells expressing the full-length CLP-36, Clik1 was relocalized to the cytoplasm (Fig. 5B), whereas the C-terminal truncated CLP-36 that also localized to actin stress fibers was unable to relocalize nuclear Clik1 (Fig. 5F). Owing to the low background of GFP-constructs, we were able to study the localization of CLP-36 in actin stress fibers in more detail. Interestingly, in cells expressing both Clik1 and CLP-36, the continuous periodic staining pattern of CLP-36 was partly disrupted (Fig. 5A,I). Previous studies demonstrate that CLP-36 localizes to actin stress fibers (Vallenius et al., 2000), and therefore it was of interest to study the actin filaments in cells expressing both CLP-36 and Clik1. In cells
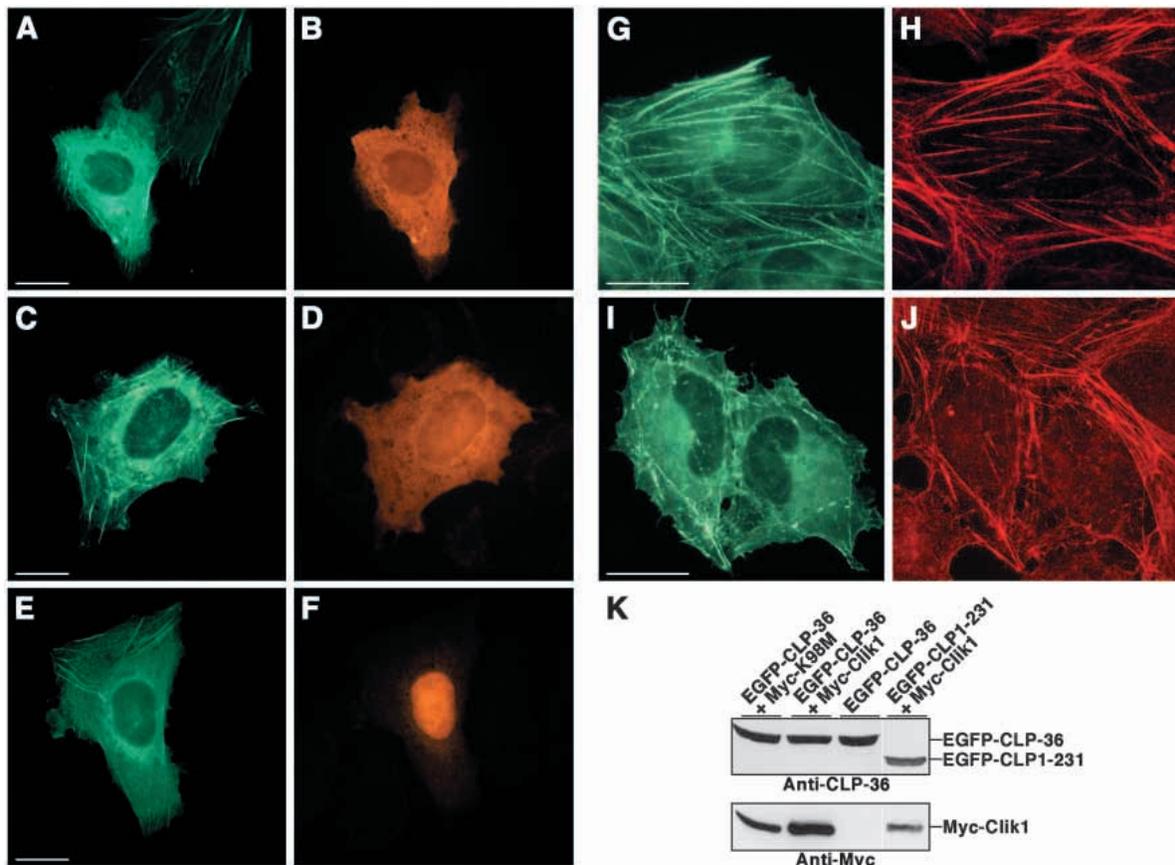

Fig. 5. The C-terminal LIM domain of CLP-36 is essential for Clik1 relocalization. U2OS cells expressing EGFP-CLP-36 and myc-Clik1 (A,B,I,J), EGFP-CLP-36 and the kinase-deficient myc-ClikK98M (C,D), a C-terminal mutant lacking the LIM domain EGFP-CLP1-231 and myc-Clik1 (E,F) or EGFP-CLP-36 alone (G,H) were immunostained with mouse monoclonal anti-myc to detect Clik1 (B,F) and ClikK98M (D) or were immunostained with Texas-Red-conjugated phalloidin (H,J) to demonstrate actin filaments. Bars, 15 μm. Anti-CLP-36 (upper panel) and anti-Myc (lower panel) western blot analyses were used to control the expression of fusion proteins used for immunofluorescence studies (K).

expressing GFP-tagged CLP-36 alone the actin filaments were intact when stained with phalloidin (Fig. 5H), and CLP-36 localized along actin filaments as expected (Fig. 5G). However, in cells expressing both CLP-36 and Clik1, the actin filament bundles were partially disorganized, suggesting that in those cells actin stress fibers is also altered (Fig. 5J) (data not shown). These results suggest a role for CLP-36 in maintaining normal stress fibers. The exact role of the Clik1 kinase in this regard is difficult to assess as a similar disorganization was noted even when a kinase-deficient Clik1 was used (Fig. 5C).

Both Clik1 and CLP-36 are expressed in adult testis

To investigate the possible physiological role of the association of Clik1 and CLP-36, the expression patterns of these proteins were compared. CLP-36 mRNA has been detected at variable levels in a wide variety of tissues (Kotaka et al., 2001; Kotaka et al., 1999; Vallenius et al., 2000). To determine the tissue distribution of the Clik1 mRNA, northern blotting analysis using various adult tissues was performed (Fig. 6A). A 7.0 kb band was present at low levels in all tissues and cell lines examined except for the testis, where strong expression of Clik1 mRNA was noted.

Subsequently, analysis of expression levels of the corresponding proteins was attempted. Unfortunately, we were not been able to observe any endogenous Clik1 protein with the polyclonal peptide antibodies that were generated against Clik1, although they did recognize Clik1 overexpressed following transfections. By contrast, a rabbit polyclonal CLP-36 antiserum raised against GST-CLP-36 detected a 38 kDa band in total cell extracts from a variety of tissues, including adult testes (Fig. 6B, lane 1). This antiserum was also capable of immunoprecipitating a 38 kDa band from non-denaturing testes lysates (Fig. 6B, lane 2), and this band was not observed with pre-immune serum or after blocking of the immune serum with antigen (Fig. 6B, lane 3). The specificity of the CLP-36 antibody was further demonstrated by its inability to detect overexpressed ALP or RIL, whereas a strong band was seen with overexpressed CLP-36 (data not shown). On the basis of these results, we conclude that our antiserum is capable of recognizing endogenous CLP-36. Also the results indicate that the testis represents a tissue where both Clik1 and CLP-36 are expressed.

The availability of the CLP-36 antiserum further allowed characterization of the cell types expressing CLP-36 in the testis. Immunofluorescence analysis indicated strong CLP-36 staining in the peritubular contractile cells surrounding the seminiferous tubuli and in elongating spermatids (Fig. 6C). In addition, a weaker but specific staining was noted throughout the seminiferous epithelium when compared with the block control.

Discussion

In this study we have characterized a novel kinase that associates with CLP-36 PDZ-LIM protein, leading to the naming of the kinase Clik1 (CLP-36 interacting kinase). The association between Clik1 and CLP-36 was first observed in yeast two-hybrid screens, where practically every Clik1-interacting clone was identified as CLP-36 from two different libraries. These results indicated a strong and specific
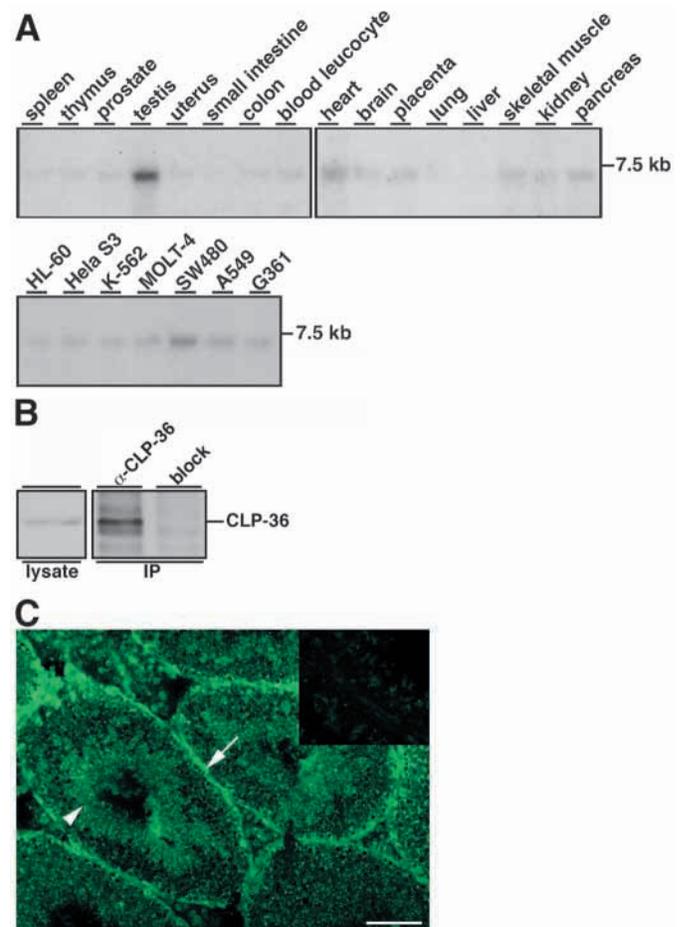

Fig. 6. Both Clik1 and CLP-36 are expressed in adult testis. (A) Human multiple-tissue northern blot filters containing the indicated mRNAs (Clontech, no. 7760-1, 7766-1 and 7757-1) were probed with Clik1. The positions of the 7.5 kb mRNA standards are indicated on the right. (B) Western blot analysis from adult mouse testis extract with rabbit polyclonal CLP-36 antibody: total testis extract (lysate), anti-CLP-36 immunoprecipitate from testis extract (IP, α-CLP-36) and blocked anti-CLP-36 immunoprecipitate (IP, block). (C) Frozen adult mouse testis sections were fixed with ethanol and stained with rabbit polyclonal anti-CLP-36 or with blocked anti-CLP-36 (inset). The white arrow indicates the peritubular contractile cells surrounding the seminiferous tubuli, and the white arrowhead indicates the elongating spermatids. Bar, 40 μm.

association between Clik1 and the C-terminal domain of CLP-36, which contains the LIM domain, and indeed this association was subsequently observed in cellular contexts as well.

Transfected Clik1 was found to localize predominantly to the nucleus, suggesting a nuclear function that will require further studies. However, a dramatic redistribution of Clik1 was noted following coexpression with CLP-36, where Clik1 shifted to the cytoplasm and into actin stress fibers, suggesting that CLP-36 levels determine the subcellular localization of Clik1. This was supported by a slightly less nuclear localization of Clik1 in COS-7 cells expressing higher levels of endogenous CLP-36 than U2OS cells used for the studies in Fig. 3 (data not shown).

Regarding the mechanism of this relocalization, it is

interesting to note that some wild-type CLP-36 was detected in the nucleus (Fig. 3), and this localization was more pronounced in the CLP-36 mutant with a disrupted PDZ domain (Vallenius et al., 2000). This suggests that CLP-36 may shuttle between the cytoplasm and the nucleus as has been reported for LIMK-1 containing both PDZ and LIM domains (Yang et al., 1998a). In this case, the observed relocalization of Clik1 could be caused by nuclear export following association with CLP-36.

Our results demonstrate the ability of the CLP-36 PDZ-LIM protein to recruit the Clik1 kinase to actin cytoskeleton. Thus, we provide direct evidence for an adapter role for the PDZ-LIM proteins. Several previous studies have suggested similar roles, especially for the more distantly related PDZ-LIM proteins (Enigma/LMP-1, ENH and Zasp/Cypher1 with 28-36% identity in LIM domains with CLP-36), whose LIM domains have all been found to associate with various isoforms of PKC (Kuroda et al., 1996; Zhou et al., 1999). However, the PDZ-LIM subfamily with a higher overall similarity and domain structure to CLP-36 (ALP and Ril with 60-67% identity in LIM domains with CLP-36) have not been reported to associate with kinases. Our results definitely indicate that within this subfamily the observed kinase interaction demonstrates a high specificity, as no interaction was observed between Clik1 and either ALP (Fig. 4) or Ril (data not shown).

Taken together with our previous data (Vallenius et al., 2000), the results presented in this report demonstrate an association of the Clik1 kinase with actin stress fibers. This association is mediated through a bridge formed by the PDZ-LIM protein CLP-36 and α-actinin (Vallenius et al., 2000). The observed changes in CLP-36 localization in actin stress fibers in cells expressing Clik1 kinase suggest that Clik1 may represent a novel regulator of the actomyosin cytoskeleton in nonmuscle cells.

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