Recruitment of Pyk2 and Cbl to lipid rafts mediates signals important for actin reorganization in growing neurites

Kaisa Haglund[1,2], Inga Ivankovic-Dikic[2], Noriaki Shimokawa[2], Gary D. Kruh[3] and Ivan Dikic[1,2,*]

[1]Institute for Biochemistry II, Building 75, Goethe University Medical School, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany
[2]Ludwig Institute for Cancer Research, Box 595, Husargatan 3, Uppsala, 75124, Sweden
[3]Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111, USA
*Author for correspondence (e-mail: ivan.dikic@biochem2.de)

Accepted 10 February 2004
Journal of Cell Science 117, 2557-2568 Published by The Company of Biologists 2004
doi:10.1242/jcs.01148

Summary

Protein tyrosine kinase Pyk2 and multifunctional adaptor protein Cbl are implicated in the regulation of the cytoskeleton in several cell types. We report that Pyk2 and Cbl form a signaling complex that is translocated to lipid rafts and is enriched in growth cones of differentiating PC12 cells following growth factor stimulation. We found that Pyk2 and Cbl interacted with the adaptor protein ArgBP2, which also bound to flotillin-1, a component of lipid raft microdomains. These interactions contributed to recruitment of the Pyk2/Cbl complex to lipid raft compartments. In addition, Pyk2, Cbl and ArgBP2 were found co-localized with actin in axons and growth cones of differentiated PC12 cells. Moreover, co-expression of Pyk2, ArgBP2 and Cbl facilitated growth factor-induced formation of lamellipodia at the tip of neurites. Formation of these growth cone lamellipodia was dependent on intact lipid rafts and the Cbl-associated effectors Crk and phosphatidylinositol 3 (PI 3)-kinase. Our results indicate that recruitment of Pyk2/Cbl complexes to lipid rafts participates in growth factor-induced regulation of the actin cytoskeleton in growing neurites.

Key words: Pyk2, Cbl, ArgBP2, PC12 cells, Neurite outgrowth, Lipid rafts

Introduction

During the formation of the nervous system, neurons extend axons and dendrites in order to form proper neuronal connectivity. The dynamic structures at the tip of neurites, known as growth cones, rapidly remodel the actin cytoskeleton through cyclical extension of filopodia and lamellipodia, which control the directionality of the growth and attachment to the substratum, respectively (Hall, 1998; Pollard et al., 2000). Recent studies have provided evidence that membrane receptors, including receptor tyrosine kinases (RTKs) and adhesion receptors, activate intracellular signal cascades that promote actin cytoskeletal changes in the growing neurite (da Silva and Dotti, 2002; Huang and Reichardt, 2001). Activated RTKs and associated protein complexes were shown to locally regulate the activity of Rho family GTPases (Rho, Rac and Cdc42), which are the major determinants of actin remodeling into stress fibers, lamellipodia and filopodia (Hall, 1998). During neuritogenesis, Rac and Cdc42 positively regulate neurite growth, whereas Rho induces growth cone collapse and inhibits neurite extension (Kozma et al., 1997; Luo, 2000). The neurite protrusion might be determined by the presence of specific areas of the plasma membrane, which cluster neurite-inducing signals (da Silva and Dotti, 2002). Recent studies indicate that specific membrane microdomains known as lipid rafts may act as platforms for concentration of signaling molecules important for cell adhesion, axon guidance and synaptic transmission in neuronal cells (Simons and Toomre, 2000; Tsui-Pierchala et al., 2002). Lipid rafts contain various growth factor receptors, including the PDGFR, EGFR and TrkA, as well as other signaling proteins such as heterotrimeric G proteins and Src family kinases and may respond to extracellular signals by inclusion or exclusion of signaling proteins (Simons and Toomre, 2000). Association of the actin cytoskeleton with lipid rafts is emphasized by the fact that variable amounts of cytoskeletal proteins, including integrins, actin and spectrin, are present in the lipid rafts and in raft-related caveolae (Baron et al., 2003; Leitinger and Hogg, 2002; Salzer and Prohaska, 2001).

Many proteins associated with RTKs are known regulators of cytoskeletal organization and cell adhesion, including adaptor proteins paxillin, Crk and p130Cas, and tyrosine kinases FAK and Pyk2 (Ivankovic-Dikic et al., 2000; Sieg et al., 2000; Turner, 2000). Studies in a variety of cell types have established that Pyk2 and FAK are activated by multiple cellular stimuli including cell attachment to the extracellular matrix and growth factor stimulation (Neet and Hunter, 1996) and are involved in the control of cell adhesion, migration and survival (Avraham et al., 2000; Dikic et al., 2002). In particular, Pyk2 and FAK have been shown to act as proximal linkers between growth factor receptors and integrins involved in the regulation of neurite formation in PC12 cells or motility of fibroblasts, respectively (Ivankovic-Dikic et al., 2000; Sieg et al., 2000). Recent studies also indicate that Pyk2 associates with Cbl, an adaptor protein with ubiquitin ligase activity (Dikic et al., 2003), and Src in osteoclasts, where the complex regulates osteoclast adhesion and motility upon integrin

stimulation (Sanjay et al., 2001). Cbl plays an important role in osteoclast motility and bone resorption during skeletal development in vivo (Chiusaroli et al., 2003). Cbl has also been shown to be involved in regulating cell morphology and in organization of the cytoskeleton in macrophages and fibroblasts (Feshchenko et al., 1999; Meng and Lowell, 1998; Ojaniemi et al., 1997; Scaife and Langdon, 2000). Tyrosine phosphorylation of Cbl is induced upon stimulation of several cell surface receptors and results in its association with SH2 domain-containing proteins, such as Crk, the p85 subunit of PI 3-kinase and guanine exchange factor Vav, which are all implicated in cytoskeletal regulation (Hartley and Corvera, 1996; Marengere et al., 1997; Ribon et al., 1996). Moreover, overexpression of Cbl facilitates lamellipodia formation via interactions with Crk signaling pathways (Scaife and Langdon, 2000). However, Cbl also acts as a RING type ubiquitin ligase (E3) that negatively regulates receptor tyrosine kinases by promoting their ubiquitination and lysosomal degradation (Dikic et al., 2003).

The aim of these studies was to analyze the functional significance of the interactions between Pyk2 and Cbl and in particular their role in actin reorganization in neuronal PC12 cells. In this report, we show that ArgBP2, an SH3- and SoHo-domain containing adaptor protein, links Pyk2 and Cbl in a signaling complex in mammalian cells. We also show that growth factor stimulation leads to translocation of Pyk2/Cbl complexes to lipid rafts and lamellipodia of growing neurites of PC12 cells. It appears that intact lipid rafts and signals initiated by the Pyk2/Cbl complex are critical for regulating actin cytoskeletal changes during neurite and growth cone lamellipodia formation.

Materials and Methods

Reagents and antibodies

PDGF-BB was from Amgen (Thousand Oaks, CA, USA), recombinant human EGF from Intergen (Oxford, UK), murine NGF 2.5S from Promega (Madison, WI, USA). Phorbol 12-myristate 13-acetate (PMA), LY294002, lovastatin (mevinolin) and methyl-β-cyclodextrin were from Sigma (St Louis, MO, USA). Geniticin, Dulbecco's modified Eagle's medium (DMEM), horse serum and fetal calf serum were from Gibco-BRL (Carlsbad, CA, USA). Chemicals of analytical grade were from Merck (Whitehouse Station, NJ, USA) or Sigma (St Louis, MO, USA). Rabbit polyclonal anti-Cbl (RF), anti-Pyk2 (600) and anti-ArgBP2 antibodies were described previously (Soubeyran et al., 2002; Ivankovic-Dikic, 2000; Wang et al., 1997). Mouse monoclonal anti-nArgBP2 antibodies were provided by Yoshimi Takai (Osaka, Japan). Mouse monoclonal anti-FLAG (M2 and M5) antibodies were from Sigma (St Louis, MO, USA). Mouse monoclonal anti-Cbl (TL), anti-flotillin-1 and anti-Crk antibodies were from Transduction Laboratories (Palo Alto, CA, USA). Rabbit polyclonal anti-Cbl (C-15), goat polyclonal anti-Pyk2 (N-19), mouse monoclonal anti-phosphotyrosine (PY99) and anti-Myc (9E10) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Src (Ab1) antibodies were from Oncogene Science (Cambridge, MA, USA). Rabbit polyclonal anti-Fyn antibodies were provided by Dominique Davidson (Montreal, Canada). Rabbit polyclonal anti-HA antibodies have been described previously (Szymkiewicz et al., 2002). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was from Pierce (Rockford, IL, USA) and donkey anti-goat polyclonal IgG-HRP from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-labeled protein A was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, ML, USA). TRITC-conjugated swine anti-rabbit IgG was from DAKO (Glostrup, Denmark) and AMCA-labeled goat anti-mouse antibodies from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Alexa-Fluor-488-conjugated donkey anti-goat IgG and Alexa-Fluor-350-conjugated phalloidin were from Molecular Probes (Eugene, OR, USA) and FITC-labeled phalloidin was from Sigma (St Louis, MO, USA).

Plasmids

The following plasmids have been described previously: pRK5-Pyk2, pRK5-PKM (Pyk2-K457A; kinase inactive mutant of Pyk2), pRK5-PKM-P717/859A (Ivankovic-Dikic et al., 2000) and pRK5-Cbl (Soubeyran et al., 2002). pFLAG-CMV2-CMS was provided by Kathrin Kirsch (New York, NY). pJ3M-Myc-ArgBP2 and pFLAG-ArgBP2 were described previously (Wang et al., 1997). pJ3M-ArgBP2-ΔSoHo (lacking amino acids 135-275) was generated by PCR. The expression vector for nArgBP2 was provided by Yoshimi Takai (Osaka, Japan). pCI-FLAG-CAP was provided by Alan R. Saltiel and Vered Ribon (Ann Arbor, MI). pSV-Src kinase and the pSV-Src kinase inactive mutant were described provided by Sara A. Courtneidge, Van Andel Research Institute, Grand Rapids, USA. Cbl constructs in pAlter max (Cbl, Cbl-Y700F, Cbl-Y700,774F, Cbl-Y700,731,774F) were provided by Elena Feschenko (Philadelphia, PA). pCAGGS-myc-CrkII, pCAGGS-myc-CrkII-SH2M(R38V) and pCAGGS-myc-CrkII-SH3M(W109C) were described previously (Blaukat et al., 1999). pGEX4T1-Cbl-NT (amino acids 2-451) and pGEX4T1-Cbl-CT (amino acids 451-907) were generated by PCR. pGEX-ArgBP2-SH3A, pGEX-ArgBP2-SH3B, and pGEX-ArgBP2-SH3C were described previously (Soubeyran et al., 2003). pGEX6P-1-Flotillin was provided by Alan R. Saltiel (Ann Arbor, MI).

Cell culture, stimulations and transfections

Cells were kept in a humid atmosphere with 5% CO_2 at 37°C. Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 Units/ml) and streptomycin (100 μg/ml). Rat pheochromocytoma PC12 cells, stably transfected with platelet-derived growth factor (PDGF) β-receptors (PC12-PDGFR), were grown in DMEM supplemented with 10% horse serum, 5% fetal calf serum, penicillin (100 Units/ml) and streptomycin (100 μg/ml). Parental rat pheochromocytoma PC12 cells (ATCC) were maintained in RPMI 1640 medium supplemented with 10% heat inactivated horse serum, 5% fetal calf serum, 2 mM L-glutamine, 10 mM Hepes, 1 mM sodium pyruvate, 4.5 g/l glucose, penicillin (100 Units/ml) and streptomycin (100 μg/ml) and PC12 cells stably expressing Myc-ArgBP2 in the same medium supplemented with 1 mg/ml geniticin. PC12 cells were starved for 16-24 hours in serum-free DMEM before stimulation for the indicated times with 100 ng/ml of PDGF-BB, EGF or 50 ng/ml NGF.

HEK 293T cells were transiently transfected according to the LipofectAMINE (Invitrogen) manufacturer's instructions. Transient transfections of PC12 cells were performed according to the LipofectAMINE Plus or LipofectAMINE 2000 protocols (Invitrogen). Stable PC12 clones of Myc-ArgBP2 were created according to the LipofectAMINE 2000 instructions and PC12-PDGFR cells were described before (Ivankovic-Dikic et al., 2000).

Cell lysis, immunoprecipitation, GST pulldown, SDS-PAGE and western blot

Cells were lysed on ice in lysis buffer containing 50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 1% (w/v) Triton X-100, 10% glycerol, 10 μM $ZnCl_2$ protease inhibitors (1 mM PMSF, 2.5 μg/ml leupeptin, 1 μg/ml aprotinin) and 1 mM sodium orthovanadate (a phosphatase inhibitor). The cell lysates were cleared by centrifugation for 15 minutes at 16,200 ***g*** at 4°C.

For immunoprecipitation, equal amounts of cell lysates were mixed with the indicated antibodies and incubated end-over-end at 4°C for 1 hour and 30 minutes. Protein A Sepharose beads (ZYMED Laboratories, South San Francisco, CA, USA) were added for 1 hour and subsequently washed three times in ice-cold lysis buffer. For GST pulldown assays, cell lysates of transiently transfected HEK 293T cells were incubated with either glutathione S-transferase (GST) alone or GST fusion proteins immobilized on glutathione-agarose beads for 2 hours at 4°C. Proteins were eluted and denatured in Laemmli sample buffer containing 5% β-mercapto-ethanol, separated by SDS-PAGE and transferred to nitrocellulose membranes (Osmonics, Minnetonka, MN, USA). Membranes were blocked in TBS containing 5% BSA at 4°C overnight. Immunoblotting was performed with the indicated primary antibodies diluted in TBS with 5% BSA for 1 hour and 30 minutes at room temperature. After washing in TBS containing 0.05% Triton X-100 for 45 minutes, membranes were incubated with anti-mouse IgG, protein A or anti-goat IgG coupled to HRP in TBS with 5% milk powder for 1 hour. After one more washing step, the blots were developed using enhanced chemiluminescence reagents (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Isolation of lipid rafts

Lipid rafts were isolated by sucrose gradient centrifugation. Briefly, cells were lysed for 15 minutes in lipid raft lysis buffer (25 mM Mes pH 6.5, 150 mM NaCl, 1% Triton X-100 including 1 mM sodium vanadate, 5 mM EDTA, 20 μg/ml leupeptin and 20 μg/ml aprotinin), scraped and homogenized (10 strokes in a glass Dounce homogenizer with a Teflon pestle). 1 ml of cell lysate was mixed with 1 ml of 80% sucrose in MNE buffer (25 mM Mes pH 6.5, 150 mM NaCl and 5 mM EDTA) in ultraclear tubes (Beckman, Palo Alto, CA, USA). A gradient was created by overlaying the mixture with 2 ml of 30% sucrose in MNE buffer followed by 1 ml of 5% sucrose in MNE buffer. The sucrose gradients were centrifuged at 4°C for 18 hours at 140,000 ***g*** in an SW50.1 rotor (Beckman Instruments, Palo Alto, CA, USA). Fractions of 400 μl each were collected from the top of the gradient. Fractions 2-4 contained detergent-insoluble lipid rafts and fractions 8-12 the Triton X-100 soluble fractions. The rafts were solubilized on ice in RIPA buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.2, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS).

Immunofluorescence

PC12 cells stably overexpressing PDGF β-receptors or Myc-ArgBP2 were plated on collagen I (10 μg/ml)-coated or poly-D-Lysine-coated coverslips and left untreated or stimulated with 100 ng/ml PDGF (or EGF) or 50 ng/ml NGF for 40-48 hours, respectively. The cells were either untransfected or transiently transfected with the cDNAs for Pyk2, Cbl, ArgBP2 and/or other cDNAs as indicated, and grown for 48 hours before fixation. Cells were fixed in 3% paraformaldehyde for 20 minutes, paraformaldehyde groups were blocked in 50 mM NH_4Cl in PBS for 10 minutes and the cells were permeabilized in 0.1% Triton-X 100 in PBS for 10 minutes. Thereafter, the cells were blocked in 5% FCS in PBS for 1 hour and incubated with the indicated primary antibodies in 5% FCS in PBS for 2 hours. After extensive washing in PBS, the cells were incubated with the appropriate fluorophore-labeled secondary antibodies. The preparations were mounted in Fluoromount G (Merck) and images were taken with the Zeiss Axioplan2 microscope.

For quantification of neurite lamellipodia formation, PC12 cells were transfected and treated as described in the legend to the appropriate figures. Cells were scored for the presence or absence of lamellipodia and the percentage of cells with lamellipodia was determined for each experimental point. In order to standardize the data, the results were subsequently presented as a percentage relative to the control showing 100% effect for each individual experiment.

Results

Constitutive association between Pyk2 and Cbl in mammalian cells

Pyk2 forms a complex with Cbl in adhesion structures of osteoclasts leading to regulation of cell attachment (Sanjay et al., 2001). In order to further investigate the presence of Pyk2/Cbl complexes and their importance in the regulation of cell morphology we used neuronal PC12 cells, where both Pyk2 and Cbl are highly expressed. We observed co-precipitation between endogenous Pyk2 and Cbl in both unstimulated and EGF- or PDGF-treated PC12 cells (Fig. 1A). In contrast, immunoprecipitation with preimmune sera did not lead to detectable amounts of co-precipitated Pyk2 or Cbl (Fig. 1A). Similar results were observed for NGF-treated PC12 cells (data not shown). Thus, Pyk2 and Cbl are constitutively associated in adherent cells.

It has previously been shown that Src may act as a linker between Pyk2 and Cbl by constitutively binding to Cbl via its SH3 domain, and interacting with Pyk2 via its SH2 domain following activation of Pyk2 (Sanjay et al., 2001). We therefore investigated the ability of Pyk2 and Cbl to form complexes in the presence of Src kinase in HEK 293T cells. Small amounts of endogenous Cbl present in HEK 293T cells precipitated both Pyk2 and a Pyk2 kinase inactive mutant (PKM), while further overexpression of Cbl led to an increase in their co-precipitation (Fig. 1B). However, the same amounts of Pyk2 or PKM were also found in a complex with Cbl in cell lysates expressing Src or a Src kinase inactive mutant (SrcK–) (Fig. 1B). In addition, by using GST binding assays we found that the carboxy-terminal part of Cbl is sufficient to bring down similar amounts of Pyk2 and the Pyk2 kinase inactive mutant (PKM) from lysates of transfected HEK 293T cells (Fig. 1C). The interaction between Pyk2 and Cbl-CT appeared to be indirect since GST-Cbl-CT did not bind Pyk2 in an overlay assay (data not shown). Taken together, these data demonstrate that Cbl and Pyk2 form constitutive complexes in mammalian cells that are independent of the activation of Pyk2 and growth factor stimulation.

Adaptor protein ArgBP2 links Pyk2 and Cbl in a complex

The above described experiments suggested that Pyk2 and Cbl may be indirectly linked in a functional complex. Since Pyk2 and Cbl contain proline-rich sequences in their carboxyl termini, we hypothesized that an adaptor protein, which contains multiple SH3 domains, could link these proteins in a ternary complex. Recent data indicated a functional role for adaptor proteins containing three SH3 domains, such as CAP, ArgBP2 and CMS to couple Cbl with tyrosine kinases FAK, Arg/Abl or the adaptor protein p130Cas, respectively (Baumann et al., 2000; Kirsch et al., 2001; Ribon et al., 1998; Soubeyran et al., 2003). We therefore analyzed whether CAP, ArgBP2 or CMS could serve as a bridge between Pyk2 and Cbl. As shown in Fig. 2A, ArgBP2 was able to bind and precipitate significant amounts of Pyk2 (Fig. 2A, left panel), whereas the amount of Pyk2 brought down by CAP or CMS was very similar to the background binding. In contrast, all three proteins associated with Cbl (Fig. 2A, left and right panels), as previously reported (Baumann et al., 2000; Kirsch et al., 2001; Ribon et al., 1998; Soubeyran et al., 2003). The presence of ArgBP2 also increased the amount of Pyk2 in

complex with Cbl (Fig. 2A, right panel), while neither CAP nor CMS enhanced co-precipitation between Cbl and Pyk2 (Fig. 2A, right panel). Therefore, ArgBP2 potentially acts as an adaptor between Pyk2 and Cbl. We further tested whether ArgBP2 is critical for Pyk2/Cbl association by co-expressing Pyk2 and Cbl together with increasing amounts of ArgBP2. The complex formed between Pyk2 and Cbl was enhanced in the presence of ArgBP2 (Fig. 2B, left and middle panels). Co-precipitation between these proteins was obtained even at low levels of co-expressed ArgBP2 (Fig. 2B, left and middle panels), while increasing amounts of ArgBP2 co-precipitated correspondingly more of either Pyk2 or Cbl from the same lysates (Fig. 2B, right panel). This shows that in addition to a ternary complex between Pyk2, Cbl and ArgBP2, there are also dimers present consisting of Pyk2/ArgBP2 or Cbl/ArgBP2 upon overexpression of these proteins. In addition, Pyk2 or Cbl could be co-precipitated from HEK 293T cell lysates even when ArgBP2 was not overexpressed (Fig. 1B, Fig. 2A,B), owning to the ability of ArgBP2 endogenously present in HEK 293T cells to link Pyk2 and Cbl in the complex. In order to elucidate the molecular mechanisms by which the ternary complex is formed, we asked whether ArgBP2 interacts with Pyk2 and Cbl via its individual SH3 domains. In a GST pull-down assay with each of the three SH3 domains of ArgBP2 (SH3A, SH3B and SH3C), Pyk2 preferentially interacted with SH3A and to a weaker extent with SH3B (Fig. 2C, left panel). We also found that Pyk2 and PKM have the same ability to interact with the SH3A of ArgBP2, whereas a proline-deficient mutant of PKM (P717/859A) failed to bind (Fig. 2C, right panel). However, the isolated SH3B and SH3C domains of ArgBP2 are sufficient for maximal binding to Cbl (Soubeyran et al., 2003). Furthermore, the interaction between PKM-P717/859A and Cbl in mammalian cells was greatly reduced when compared to the association between wild-type Pyk2 or PKM and Cbl (Fig. 2D). The residual binding detected between PKM-P717/859A and Cbl could be due to additional proline-rich binding sites in PKM-P717/859A for ArgBP2 SH3 domain B, which showed faint binding to Pyk2 in Fig. 2C. Moreover, the presence of endogenous Pyk2 in HEK 293T cells might also contribute to the precipitation of Cbl seen when PKM-P717/859A was overexpressed. Taken together these data indicate that, ArgBP2, via its three SH3 domains, is able to simultaneously associate with proline-rich motifs in both Pyk2 and Cbl, and thereby function as a bridge between the latter proteins.

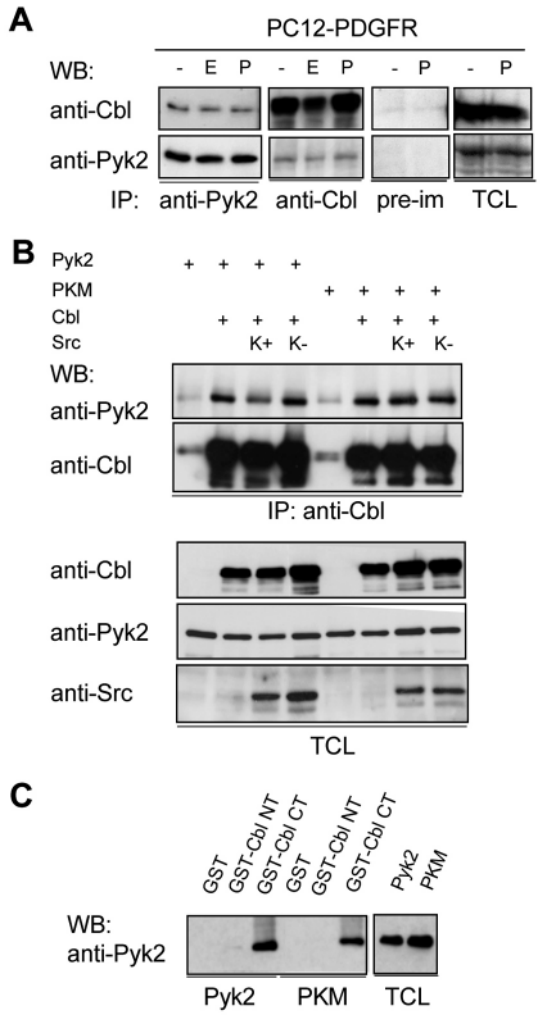

Fig. 1. Constitutive association between Pyk2 and Cbl in mammalian cells. (A) PC12-PDGFR cells were starved for 16 hours and were left untreated (–) or stimulated with 100 ng/ml EGF (E) or PDGF (P) for 5 minutes. Following cell lysis, equal amounts of cell lysates were subjected to immunoprecipitation (IP) with antibodies to Pyk2 (600) or Cbl (RF) or pre-immune sera (pre-im) and immunoprecipitates were analyzed by western blotting (WB) with anti-Cbl (TL) and anti-Pyk2 (N-19) antibodies. TCL, total cell lysate. (B) Pyk2 or a kinase inactive mutant of Pyk2 (PKM) were overexpressed in HEK 293T cells alone or together with Cbl, or with Cbl and Src kinase (K+) or Cbl and a kinase inactive mutant of Src (K–). Cbl was immunoprecipitated (IP) with anti-Cbl (RF) antibodies and co-precipitation was monitored by western blotting (WB) with anti-Pyk2 (N-19) antibodies. Levels of proteins in total cell lysates (TCL) were determined with anti-Cbl (TL), anti-Pyk2 (N-19) and anti-Src (Ab1) antibodies. (C) Pyk2 or PKM were overexpressed in HEK 293T cells and equal amounts of cell lysates were subjected to glutathione S-transferase (GST) pulldown with GST fusion proteins of the amino terminus of Cbl spanning the SH2 and RING finger domains (GST-Cbl NT) or the carboxyl terminus of Cbl containing the proline-rich sequences; the acidic box and the leucine zipper domain (GST-Cbl CT) and the western blot (WB) was probed with anti-Pyk2 (600) antibodies.

A Pyk2-ArgBP2-Cbl complex is recruited to lipid rafts following growth factor stimulation

In order to study biological functions of the Pyk2/ArgBP2/Cbl complex, we next investigated their subcellular localization in unstimulated and growth factor-differentiated PC12 cells. In resting PC12 cells, Pyk2 and Cbl were diffusely localized in the cytoplasm and in the perinuclear region, and partially co-localized with ArgBP2 in these structures (Fig. 3A). Interestingly, following growth factor-induced differentiation, ArgBP2 was found co-localized with F-actin throughout the neurites and in growth cones of differentiated PC12 cells (Fig. 3B). In addition, Pyk2 and Cbl were partially co-localized with actin in growth cones of neurites (Fig. 3B). This suggests that growth factor stimulation may regulate the cellular localization of the Pyk2-ArgBP2-Cbl complex, rather than its formation, in neuronal cells.

Close inspection of the localization of Pyk2 and Cbl in neurites indicated that the proteins are not evenly distributed

along actin but are rather co-localized in specific microdomains along axons as well as in growth cones. We hypothesized that these microdomains may include lipid rafts, which are enriched in growth factor receptors as well as different cytoskeletal proteins, including integrins, actin and spectrin (Baron et al., 2003; Leitinger and Hogg, 2002; Salzer and Prohaska, 2001; Simons and Toomre, 2000). The involvement of lipid rafts in regulation of cell adhesion, axon guidance and synaptic transmission in neuronal cells has previously been described (Tsui-Pierchala et al., 2002). Several studies in different cell types have visualized lipid rafts using FITC-labeled cholera toxin B, which binds to the GM1 ganglioside, a component of lipid raft microdomains in the outer leaflet of the plasma membrane (Brown and London, 1998; Gupta and DeFranco, 2003; Xavier et al., 1998). However, we were unable to detect significant co-localization between Pyk2, Cbl and FITC-labeled cholera toxin B in differentiated PC12 cells because of a lack of sensitivity in the staining (data not shown). We therefore decided to investigate Pyk2, Cbl and ArgBP2 in lipid rafts using sucrose density gradient centrifugation. Insoluble membrane rafts are found in the low-density portion of the gradient, as demonstrated by the presence of resident proteins including flotillin-1 (Fig. 4A), whereas soluble membrane and cytoplasmic proteins were found in the higher density fractions. The major part of Pyk2, Cbl and ArgBP2 were localized in detergent-soluble (Tx-soluble) fractions in both unstimulated and NGF-treated PC12 cells. However, Pyk2, Cbl, ArgBP2 and Crk were also recruited to flotillin-1-containing lipid raft fractions following stimulation with NGF or PDGF (Fig. 4A and data not shown). Similarly, Pyk2, Cbl and ArgBP2 were targeted to lipid rafts in HEK 293T cells treated with EGF and PDGF (Fig. 4B). Interestingly, the kinase-inactive mutant of Pyk2 (PKM) was also translocated to lipid rafts together with Cbl and ArgBP2 (Fig. 4B), indicating that the recruitment of the proteins to rafts is not dependent on the kinase activity of Pyk2.

ArgBP2 belongs to a family of Sorbin homology (SoHo)-containing proteins, which also includes Cbl-associated protein (CAP) and vinexin α. ArgBP2 and CAP are widely expressed and have been shown to co-localize with stress fibers in epithelial cells (Wang et al., 1997) and fibroblasts (Ribon et al., 1998), respectively. CAP was previously shown to associate with membrane rafts via its binding to flotillin-1, a resident component of lipid rafts (Kimura et al., 2001). We found that ArgBP2 and nArgBP2, an alternatively spliced form of ArgBP2 (Kawabe et al., 1999), can also interact with flotillin-1 in pull-down assays (Fig. 4C). Thus, by binding to flotillin-1 via its SoHo domain and to Pyk2 and Cbl via its SH3 domains, ArgBP2 is able to recruit Pyk2 and Cbl to lipid rafts following growth factor stimulation. However, expression of an ArgBP2 mutant lacking the SoHo domain (ArgBP2-ΔSoHo)

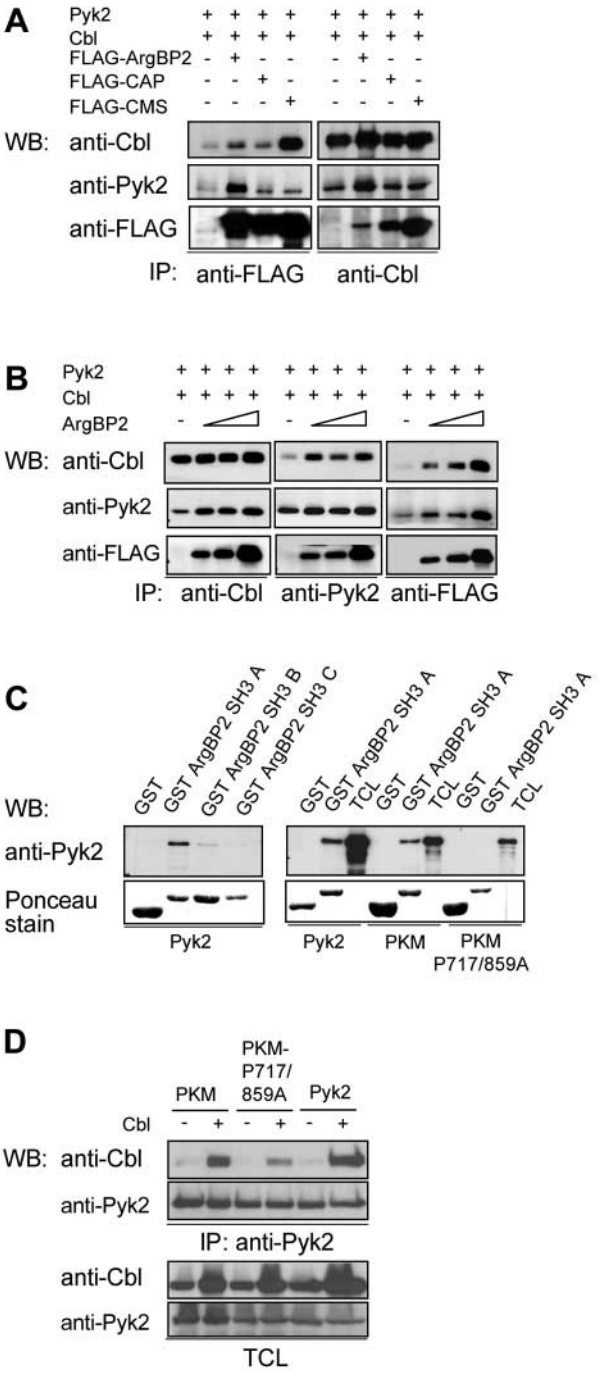

Fig. 2. Adaptor protein ArgBP2 links Pyk2 and Cbl in a complex. (A) Pyk2 and Cbl were co-expressed together with FLAG-tagged ArgBP2 (~88 kDa), CAP (~90 kDa) or CMS (~85 kDa) as indicated. Immunoprecipitation (IP) was performed with anti-FLAG (M2) or anti-Cbl (RF) antibodies. Western blotting (WB) was done as indicated with anti-Cbl (TL), anti-Pyk2 (N-19) and anti-FLAG (M5) antibodies. (B) Pyk2 and Cbl were co-expressed in HEK 293T cells with increasing amounts of ArgBP20 Cbl, Pyk2 or FLAG-tagged ArgBP2 were immunoprecipitated (IP) with anti-Cbl (RF), anti-Pyk2 (600) or anti-FLAG (M2) antibodies, respectively. Western blotting (WB) was performed as in A). (C) Glutathione S-transferase (GST) alone or GST fusion proteins of each of the three SH3 domains of ArgBP2 (SH3A, SH3B, SH3C) were incubated with equal amounts of lysates from HEK 293T cells overexpressing Pyk2. GST-ArgBP2-SH3A was also incubated with lysates expressing a kinase inactive mutant of Pyk2 (PKM) or a double mutant of PKM mutated at two prolines in major proline-rich motifs (PKM P717/859A). Western blotting (WB) was performed with anti-Pyk2 (N-19) antibodies. Levels of GST fusion proteins were examined by Ponceau staining. (D) PKM, PKM-P717/859A or Pyk2 were coexpressed with or without Cbl and cell lysates were subjected to immunoprecipitation (IP) with antibodies against Pyk2 (600). Western blotting (WB) was performed with anti-Cbl (RF) and anti-Pyk2 (N-19) antibodies. TCL, total cell lysate.

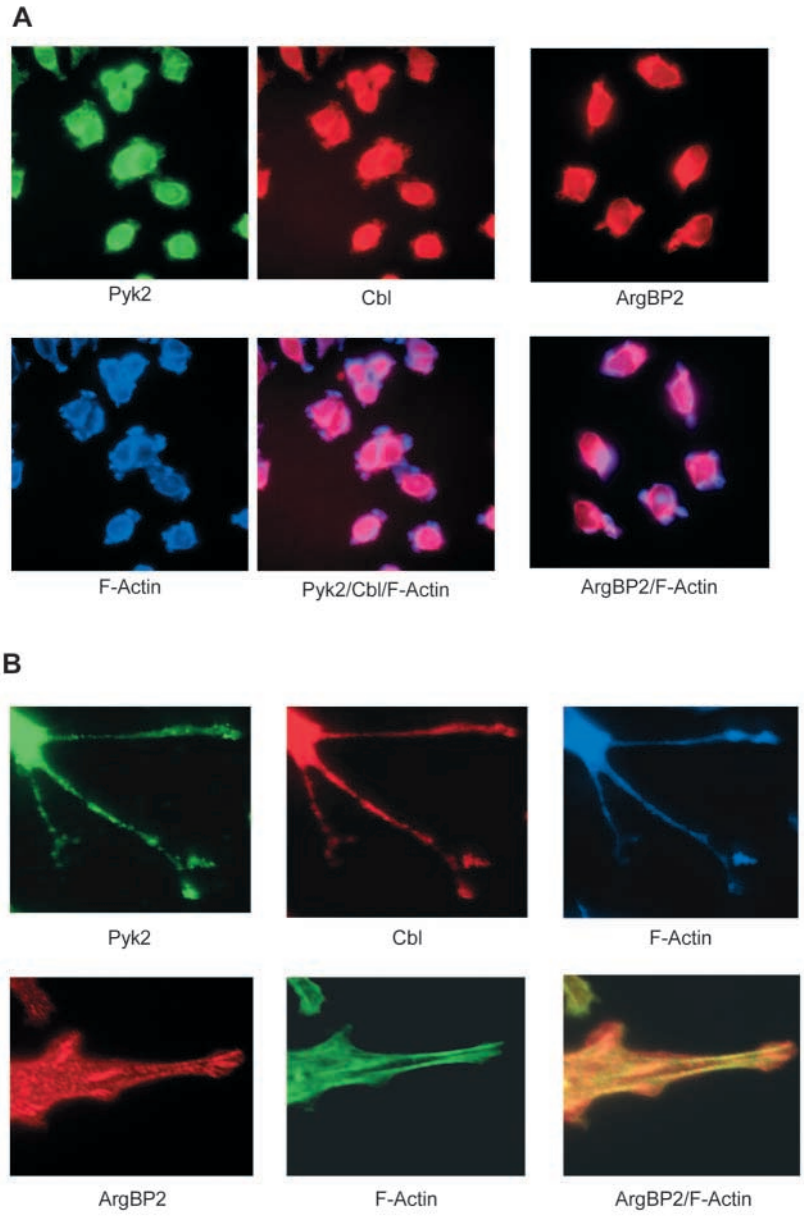

Fig. 3. Cellular localization of Pyk2, ArgBP2 and Cbl in PC12 cells. (A) PC12 cells stably expressing PDGF β-receptors were grown on collagen I-coated coverslips and subjected to immunofluorescence with anti-Pyk2 (N-19), anti-Cbl antibodies (C-15) and anti-ArgBP2 antibodies. In the upper panel, Pyk2 is visualized in green by Alexa 488-labeled secondary donkey anti-goat antibodies (left), Cbl (middle) and ArgBP2 (right) are stained red with TRITC-labeled swine anti-rabbit secondary antibodies. In the lower panel, F-actin is stained blue by Alexa 305-labeled phalloidin (left), the merged picture between Pyk2, Cbl and F-actin is in the middle and the superimposed image of ArgBP2 and F-actin is on the right. Left and middle panels are from one experiment and the right panel from a separate experiment. (B) PC12 cells stably expressing PDGF β-receptors were grown on collagen I-coated coverslips and subjected to immunofluorescence after 48 hours of PDGF-BB (50 ng/ml) stimulation. F-actin is stained with Alexa 305-labeled phalloidin (upper panel) or FITC-phalloidin (lower panel). Immunofluorescence was performed using goat polyclonal anti-Pyk2 (N-19), rabbit polyclonal anti-Cbl (C-15) and anti-ArgBP2 as indicated. Pyk2 was visualized by Alexa 488-labeled donkey anti-goat antibodies and Cbl (upper panel) and ArgBP2 (lower panel) by TRITC-labeled swine anti-rabbit antibodies. Upper and lower panels are form two different experiments.

was partially localized to lipid rafts and did not completely block translocation of Pyk2 and Cbl to lipid rafts upon EGF stimulation (Fig. 4D). These results support a model where Pyk2, Cbl and ArgBP2 are recruited to membrane rafts, via ArgBP2-SoHo domain-dependent as well as -independent pathways following growth factor stimulation.

Co-expression of Pyk2, Cbl and ArgBP2 induces membrane ruffles and lamellipodia formation in PC12 cells, a process dependent on intact lipid rafts

In order to further investigate the function of the complex between Pyk2, Cbl and ArgBP2, we overexpressed these proteins in PC12 cells and analyzed neurite formation in response to growth factor stimulation. Expression of each of these proteins individually did not significantly affect the morphology of cells bearing neurites following either PDGF or NGF stimulation (data not shown). However, PC12 cells expressing Pyk2, Cbl and ArgPB2 contained shorter and broader neurites with very developed lamellipodia and membrane ruffles at neurite growth cones (Fig. 5A). Pyk2, Cbl and ArgBP2 were co-localized in lamellipodia at the edges of the growing neurites (Fig. 5A, lower panel). This effect was very prominent for PDGF- and NGF-stimulated PC12 cells. Thus, growth factor-induced translocation of Pyk2, Cbl and ArgBP2 to growth cone lamellipodia may be involved in the actin reorganization of growing neurites.

In order to investigate whether localization of Pyk2, Cbl and ArgPB2 to lipid rafts is critical for the formation of neurites and lamellipodia at the growth cones, we treated PC12-Myc-ArgBP2 cells overexpressing Pyk2 and Cbl with the lipid raft inhibitors methyl-β-cyclodextrin and lovastatin. Indeed, lipid raft inhibition decreased the overall neurite outgrowth to about half, i.e. fewer cells differentiated and neurites that formed were thinner, shorter and with less dendrites (Fig. 5B), consistent with previous reports (Peiro et al., 2000; Tsui-

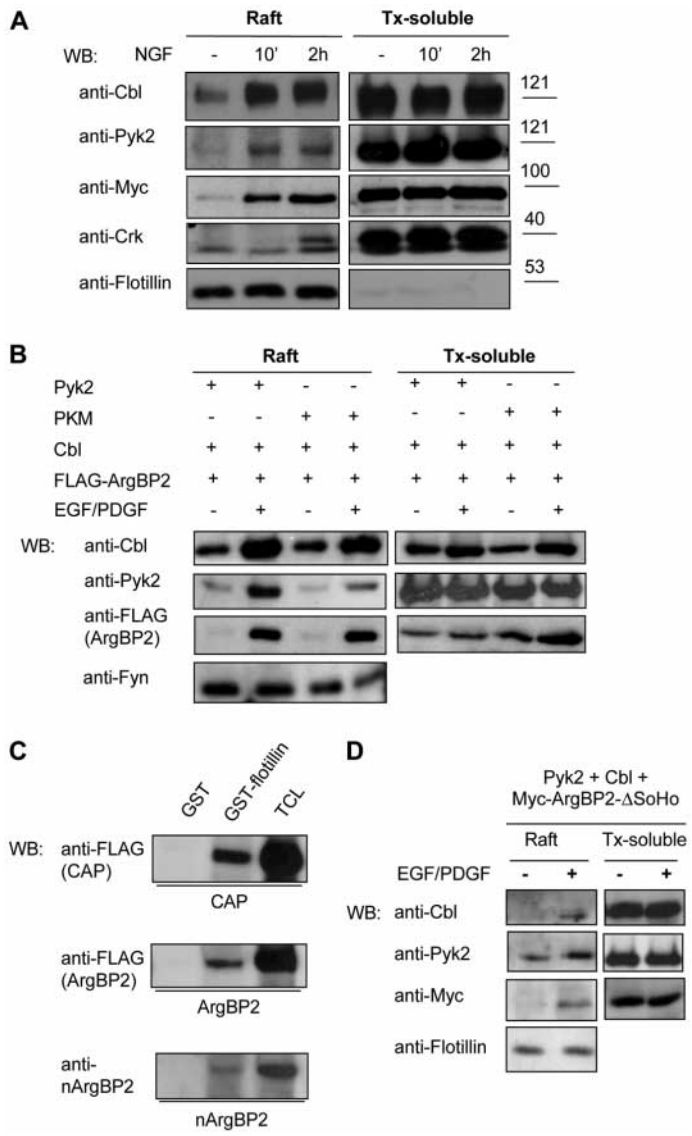

Fig. 4. A Pyk2-ArgBP2-Cbl complex is recruited to lipid rafts following growth factor stimulation. (A) Serum-starved PC12-Myc-ArgBP2 cells were mock-stimulated or treated for 10 minutes or 2 hours with 50 ng/ml NGF and homogenized lysates were subjected to sucrose gradient centrifugation as described in the Materials and Methods. Immunoprecipitates of Cbl, Pyk2, Myc-ArgBP2, Crk and flotillin-1 from lipid rafts and Triton X-100 soluble fractions were subjected to western blot with the following antibodies: anti-Cbl (RF), anti-Pyk2 (N-19), anti-Myc (9E10), anti-Crk and anti-flotillin-1. (B) HEK 293T cells were transfected with Pyk2 or PKM together with Cbl and FLAG-ArgBP2, serum-starved and stimulated with a mixture of 50 ng/ml EGF and 50 ng/ml PDGF-BB for 20 minutes. Immunoprecipitates of Cbl, Pyk2 and ArgBP2 from lipid rafts and Triton X-100-soluble fractions were analyzed with antibodies against Cbl (RF), Pyk2 (N-19), FLAG(M2). Fyn was detected in total cell lysates. (C) Lysates of HEK 293T cells overexpressing FLAG-CAP, FLAG-ArgBP2 or nArgBP2 were subjected to GST pulldown with GST alone or GST-flotillin-1. Western blotting was done with anti-FLAG and anti-nArgBP2 antibodies. (D) HEK 293T cells overexpressing Pyk2, Cbl and Myc-ArgBP2-ΔSoHo were serum-starved and stimulated with a mixture of 50 ng/ml EGF and 50 ng/ml PDGF-BB for 20 minutes. Immunoprecipitates of Cbl (RF), Pyk2 (600), Myc-ArgBP2-ΔSoHo (9E10) and flotillin-1 from lipid rafts and Triton X-100 soluble fractions are shown. Western blotting was performed with antibodies against Cbl (RF), Pyk2 (N-19), Myc (9E10) and Flotillin-1.

Pierchala et al., 2002). Moreover, lipid raft inhibition dramatically decreased the formation of lamellipodia at the growth cones of the neurites that formed (Fig. 5B). These data suggest that intact lipid rafts are required for Pyk2/Cbl-induced lamellipodia formation in growing neurites.

Binding of Crk and p85/PI 3-kinase to phosphorylated Cbl mediates lamellipodia formation in PC12 cells

In order to test in more detail which domains of Cbl or Pyk2 are responsible for this effect, we co-expressed Pyk2 or different mutants of Pyk2 with different parts of Cbl (Cbl-NT and Cbl-CT) in PC12 cells. Co-expression of the carboxyl terminus of Cbl and Pyk2 led to even stronger induction of lamellipodia formation than the presence of wild-type Cbl (Fig. 6A), most probably due to the deletion of the tyrosine kinase binding and RING finger domains in the amino-terminal part of Cbl that are implicated in negative regulation of RTK signaling (Dikic and Giordano, 2003). However, expression of the amino terminus of Cbl together with Pyk2 or expression of PKM or Pyk2-CT (PRNK) and Cbl did not induce any membrane ruffling and was rather inhibitory for the formation of neurites in transfected cells (Ivankovic-Dikic et al., 2000 and data not shown). Therefore, in addition to binding between Pyk2 and Cbl, further signals appear important for the formation of growth cone lamellipodia in PC12 cells. Since the carboxyl terminus of Cbl contains principal phosphorylated tyrosine residues, which bind to the adaptor protein Crk, the guanine exchange factor Vav and the p85 subunit of PI 3-kinase (Andoniou et al., 1996; Hunter et al., 1999; Liu et al., 1997; Marengere et al., 1997), it is possible that Cbl recruits these effectors in the complex with Pyk2 and thus regulates the actin cytoskeleton in PC12 cells. We therefore transfected Pyk2 together with Cbl or Cbl with mutations in principal phosphorylation sites and quantified immunofluorescence-stained transfected cells for the presence of lamellipodia and membrane ruffles. Co-expression of Pyk2 with the Cbl Y3F mutant (the three tyrosine residues Y700, Y731 and Y774 mutated to phenylalanine), promoted lamellipodia formation to a much smaller extent than wild-type Cbl or a Cbl mutant containing an individual mutation of Y700 (Fig. 6B). Moreover, the double mutation Y700/774F partially reduced the ability of Cbl to promote formation of lamellipodia when co-expressed with Pyk2 (Fig. 6B). Previous studies have shown that phosphorylated Y700 and Y774 are binding sites for the adapter protein Crk, while the p85 subunit of PI 3-kinase binds to phosphotyrosine 731 (Andoniou et al., 1996; Hunter et al., 1999; Liu et al., 1997). Thus, our results suggest that binding of Crk and PI 3-kinase to Cbl is involved in the rearrangements of the actin cytoskeleton initiated by the Pyk2/ArgBP2/Cbl complex. To further test this hypothesis we treated PC12 cells, transiently overexpressing Pyk2 and Cbl, with the PI 3-kinase inhibitor LY294002 or co-transfected dominant interfering forms of CrkII. Prolonged inhibition of PI 3-kinase gradually decreased Pyk2/Cbl-induced lamellipodia formation (Fig. 6C). In addition, overexpression of dominant interfering mutants of CrkII together with Pyk2 and Cbl also led to a reduction in lamellipodia formation at the tip of neurites (Fig. 6D). These results further strengthen the observation

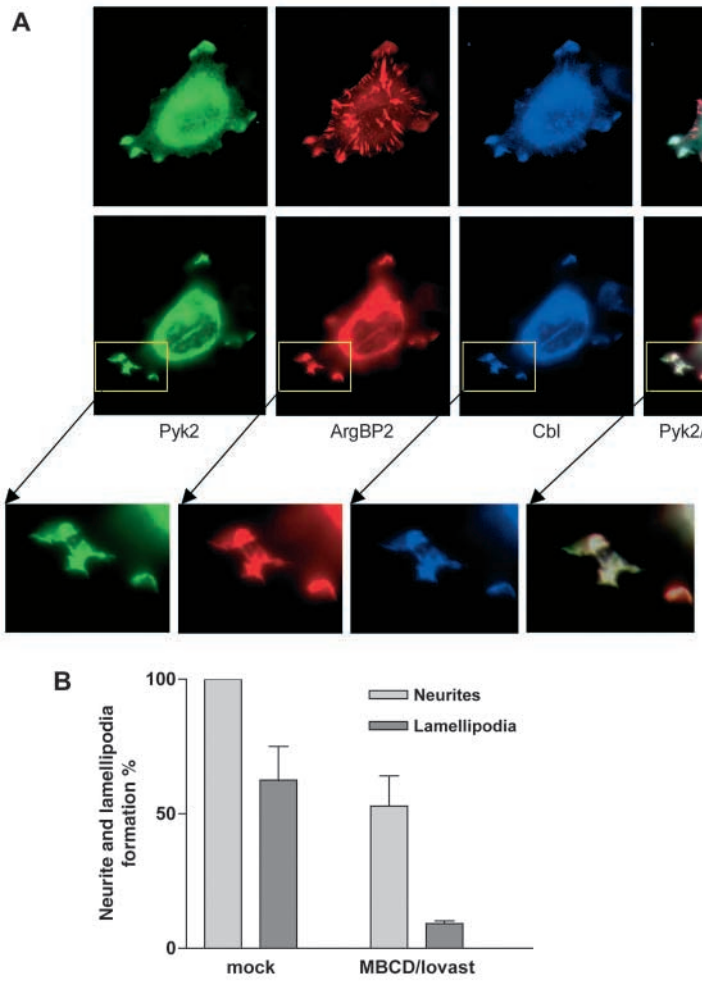

Fig. 5. Co-expression of Pyk2, Cbl and ArgBP2 induces membrane ruffles and lamellipodia formation in PC12 cells, a process dependent on intact lipid rafts. (A) Pyk2, Cbl and ArgBP2 were transiently transfected into PC12-PDGFR cells. Cells were stimulated with 100 ng/ml PDGF-BB for 48 hours and subjected to immunofluorescence with goat polyclonal anti-Pyk2 (N-19), mouse monoclonal anti-Cbl (TL) and rabbit polyclonal anti-ArgBP2 antibodies. Pyk2 was stained with Alexa 488-labeled donkey anti-goat, Cbl with AMCA-labeled goat anti-mouse and ArgBP2 with TRITC-labeled swine anti-rabbit antibodies. Shown is a cell with developed lamellipodia with the focus on two different layers of the cell, as indicated by the cell drawings. The merged pictures from the three channels are also shown. (B) PC12-Myc-ArgBP2 cells, grown in the presence of 50 ng/ml NGF for 78 hours after transfection with Pyk2 and Cbl, were mock-treated (mock) or treated with 4 μM lovastatin (lovast) for 24 hours and 0.2 M methyl-β-cyclodextrin (MBCD) for 4 hours at 37°C. Transfected cells were detected by immunofluorescence with antibodies against Pyk2 (N-19) and Cbl (C-15), which were visualized with donkey anti-goat-Alexa 488 and TRITC-labeled swine anti-rabbit antibodies. Cells showing neurites in the absence (mock) or presence of lipid raft inhibitors (MBCD/lovast) were quantified and the percentages of cells with neurites were calculated as described in Materials and Methods. Moreover, the percentage of cells showing lamellipodia at growth cones out of those cells with neurites was also determined for both untreated (mock) and treated (MBCD/lovast) cells. Error bars represent the standard error of the mean.

that the Pyk2/Cbl complex controls the actin cytoskeleton during neurite outgrowth via pathways involving PI 3-kinase and Crk.

Discussion

Pyk2 and Cbl have been implicated in the reorganization of the cytoskeleton and the regulation of cell morphology in several cell types (Avraham et al., 2000; Dikic et al., 2003). In particular, Pyk2 is present in neurites and growth cones of differentiated PC12 cells (Park et al., 2000) and studies of cells derived from Pyk2-deficient mice indicate that Pyk2 is important for the contractile activity of lamellipodia in migrating macrophages (Okigaki et al., 2003). Moreover, tyrosine-phosphorylated Cbl has previously been shown to translocate to the plasma membrane upon integrin-dependent adhesion and to co-localize with actin in lamellipodia of migrating cells (Kirsch et al., 2001; Meng and Lowell, 1998; Ojaniemi et al., 1997; Scaife and Langdon, 2000). Yet, the molecular mechanisms by which Pyk2 and Cbl are translocated to the plasma membrane to control these processes are incompletely understood. We show that Pyk2 and Cbl form a complex via the adaptor protein ArgBP2 and that the recruitment of the Pyk2/Cbl complex to growth cone lamellipodia and lipid rafts is critical for cytoskeletal rearrangements in growing neurites.

During differentiation of PC12 cells, Pyk2, Cbl and ArgBP2 co-localized with actin in neurites and at the leading edge of growth cones (Fig. 3), suggesting that these proteins may participate in regulating the cytoskeleton during neurite outgrowth. Indeed, overexpression of Pyk2, Cbl and ArgBP2 led to pronounced morphological changes resulting in unusually broad neurite extensions with pronounced growth cone lamellipodia and membrane ruffles (Fig. 5), emphasizing the positive effect of these proteins during neuronal differentiation. In addition, overexpression of the carboxyl terminus of Cbl together with Pyk2 had an even stronger effect than wild-type Cbl (Fig. 6), suggesting that the Cbl carboxyl terminus is sufficient to mediate these morphological changes.

The molecular mechanisms by which Pyk2 and Cbl transmit signals critical for the formation of lamellipodia in neurites appear to be complex and may involve recruitment of specific effectors to the sites of dynamic actin reorganization. We have shown that Pyk2 kinase activity (Ivankovic-Dikic et al., 2000) and tyrosine phosphorylation of carboxyl terminal tyrosines of Cbl (Fig. 6B) are required for neurite and lamellipodia formation. Furthermore, tyrosine phosphorylation of Pyk2 and Cbl is known to promote their association with several regulators of the cytoskeleton (Astier et al., 1997a; Dikic et al., 2002; Hiregowdara et al., 1997; Thien and Langdon, 2001). In particular, Cbl recruits PI 3-kinase and Crk, which are known to mediate growth factor-induced, Rac-dependent formation of

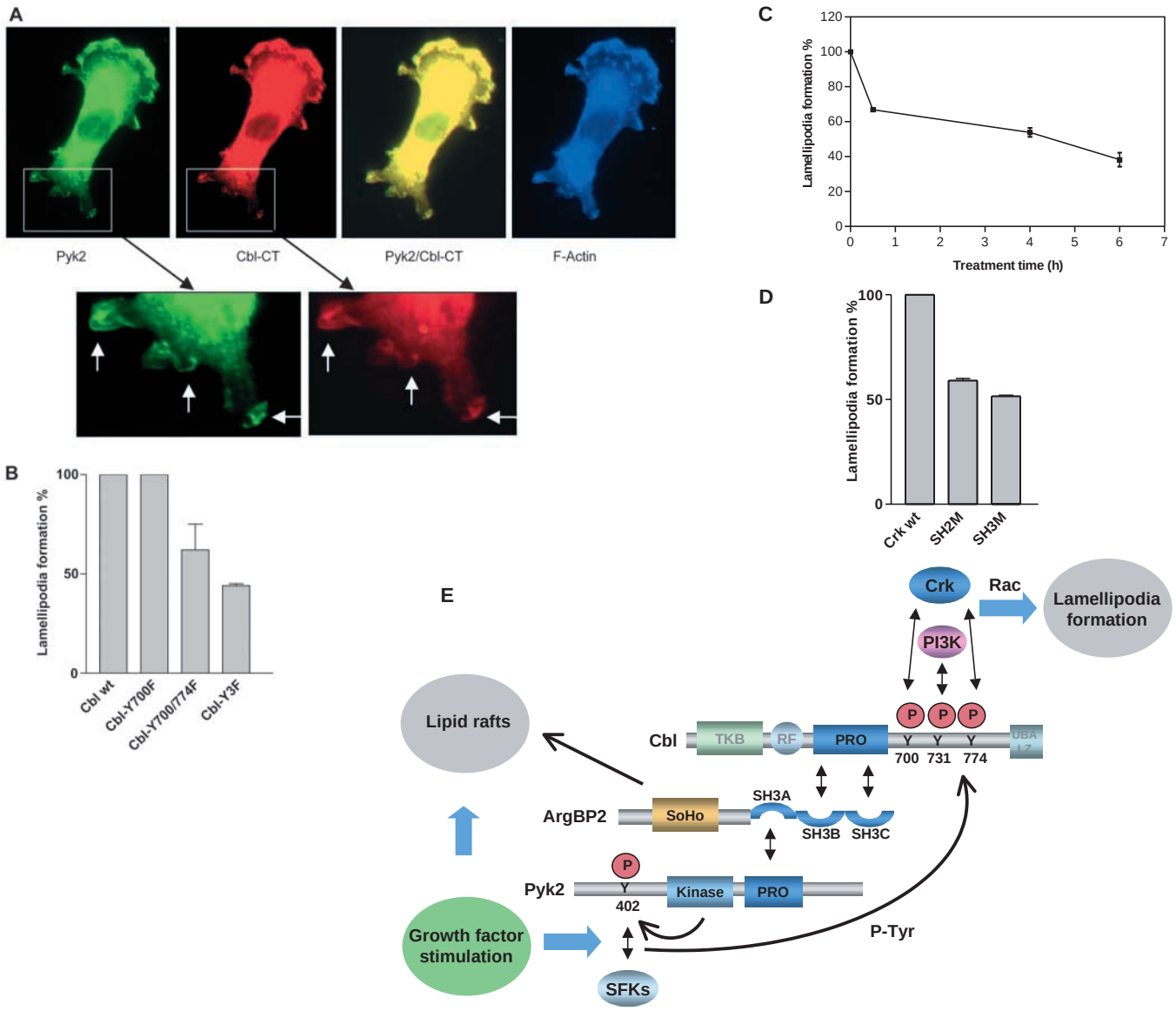

Fig. 6. Binding of Crk and p85/PI 3-kinase to phosphorylated Cbl mediates lamellipodia formation in PC12 cells. (A) PC12 cells stably expressing the PDGFR β-receptor and overexpressing Pyk2 and Cbl-CT (proline-rich regions, acidic box and leucine zipper domain) were analyzed for the formation of lamellipodia following stimulation with PDGF-BB for 48 hours. Cbl was detected with anti-Cbl (C-15) and Pyk2 with anti-Pyk2 (NT) antibodies. Cbl was visualized with swine anti-rabbit TRITC secondary antibodies, Pyk2 with donkey anti-goat Alexa 488 and F-actin with AMCA-labeled phalloidin. The boxed regions are enlarged in the panels below to show lamellipodia. (B) PC12-PDGFR cells were transiently transfected with Pyk2 and wild-type Cbl or Cbl mutants (Cbl-Y700F, Cbl-Y700/774F or Cbl-Y700,731,774F=Cbl-3YF) and stimulated with 100 ng/ml PDGF-BB for 48 hours. Cells were then subjected to immunofluorescence with goat polyclonal anti-Pyk2 (N-19) and rabbit polyclonal anti-Cbl (C-15) antibodies followed by incubation with Alexa-488-conjugated donkey anti-goat and TRITC-labeled swine anti-rabbit antibodies. Cells overexpressing Pyk2 and Cbl or Cbl mutants were quantified for the presence of neurite lamellipodia. Percentages for each construct relative to the wild-type control were determined as described in Materials and Methods. The error bars represent the standard error of the mean. (C) PC12-PDGFR cells overexpressing Pyk2 and Cbl were stimulated with 100 ng/ml PDGF-BB for 48 hours and treated with 100 mM LY294002 (a PI 3-kinase inhibitor) for 30 minutes, 4 hours or 6 hours. Cells overexpressing Pyk2 and Cbl were visualized by immunofluorescence as in B. Transfected cells showing neurite lamellipodia were quantified for each time period and compared to untreated cells. Percentages were determined as described in Materials and Methods. The error bars represent the standard error of the mean. (D) PC12-PDGFR cells overexpressing Pyk2, Cbl and wild-type CrkII, CrkII-SH2M or CrkII-SH3M were stimulated for 48 hours with PDGF-BB and quantified for the presence of neurite lamellipodia. Percentages for each construct were determined as in Materials and Methods. The error bars represent the standard error of the mean. (E) Model of interactions between Cbl, ArgBP2 and Pyk2 in lipid rafts and of how growth factor signals might regulate lamellipodia formation via this complex. ArgBP2 associates via its SH3 domains with proline-rich motifs in Pyk2 (via SH3A) and Cbl (via SH3B and SH3C) and is able to recruit Pyk2 and Cbl to lipid rafts via its SoHo domain. Growth factor stimulation promotes Pyk2 activation and its autophosphorylation on Y402. This leads to activation of Src family kinases, which are able to phosphorylate tyrosines in the carboxyl termini of Pyk2 and Cbl (Y700, Y731 and Y774). This in turn allows Pyk2 and Cbl to recruit several effectors involved in regulating the cytoskeleton. In particular, Cbl recruits Crk and the p85 subunit of PI 3-kinase, which are involved in Rac-dependent lamellipodia formation in the neurite growth cone.

lamellipodia and membrane ruffles in different cell types, including neuronal cells (Hall, 1998; Hawkins et al., 1995; Heldin et al., 1998; Kanda et al., 2003; Wennstrom et al., 1994). In our model of neurite growth of PC12 cells, overexpression of tyrosine mutants of Cbl (Cbl-Y700,774F and Cbl-Y700,731,774F), treatment with the PI 3-kinase inhibitor LY294002 or overexpression of dominant negative forms of CrkII (Fig. 6) reduced the growth cone lamellipodia formation. This implies that PI 3-kinase and CrkII are downstream effectors of the Pyk2/ArgBP2/Cbl complex (Fig. 6E). The ability of CrkII to enhance lamellipodia formation and cell spreading, is correlated with increased association of CrkII with paxillin, GIT2 (an ARF-GAP) and β-PIX (a Rac1 exchange factor) (Lamorte et al., 2003). Interestingly, recent data indicate that Cbl can directly associate with PIX family exchange factors following activation of Cdc42, leading to inhibition of EGFR ubiquitination and degradation (Wu et al., 2003).

Our data also indicate that recruitment of these complexes to distinct subcellular compartments, such as lipid rafts, may play important roles in signals governing cytoskeletal changes during neurite growth. We found that Pyk2, Cbl, ArgBP2 as well as the Cbl effector Crk were translocated to lipid rafts following growth factor stimulation of PC12 cells (Fig. 4A). Since lipid rafts are connected to the cytoskeleton and enriched in growth factor receptors, integrins and Src family kinases, they provide an environment where both Pyk2 and Cbl can become activated, tyrosine-phosphorylated and participate in cytoskeletal reorganization. A model of how this could occur is presented in Fig. 6E. In rafts, Pyk2 can become activated in growth factor/integrin receptor complexes (Ivankovic-Dikic et al., 2000), leading to its autophosphorylation on Y402, which may in turn promote Src binding and its local activation (Dikic et al., 1996). Src family kinases are in turn able to phosphorylate tyrosine residues in the carboxyl termini of both Pyk2 and Cbl, leading to recruitment of effectors that mediate Rac-dependent changes of the cytoskeleton (Duong et al., 1998; Qian et al., 1997; Thien and Langdon, 2001). Moreover, localization of Rac1 to sites of membrane ruffling and actin reorganization has been shown to require intact lipid rafts (Grimmer et al., 2002). Importantly, lipid raft inhibition dramatically decreased the neurite outgrowth as well as lamellipodia formation in cells overexpressing Pyk2, Cbl and ArgBP2 (Fig. 5B). In accordance with this, previous studies have shown that disruption of lipid rafts by depletion of cholesterol leads to inhibition of NGF receptor signaling as well as retraction of neurite extensions (Huang et al., 1999; Peiro et al., 2000).

How are Pyk2/Cbl complexes recruited to the sites of actin reorganization of growth cone lamellipodia? One possibility is that growth factor receptors are involved in this process, since ligand stimulation lead to redistribution of Pyk2/Cbl complexes to neurites and growth cones (Figs 3, 5 and 6). It is well established that Cbl is recruited to a number of RTKs upon growth factor stimulation (Thien and Langdon, 2001). Moreover, Pyk2/FAK induce neurite outgrowth by acting as proximal linkers between growth factor receptors and integrins (Ivankovic-Dikic et al., 2000). Pyk2 may associate with integrin/growth factor receptor complexes via its interaction with paxillin, vinculin, p130Cas and other cytoskeletal proteins and promote actin reorganization via Rho- and WASP-Arp2/3-dependent pathways (Astier et al., 1997b; Avraham et al., 2000; Dikic et al., 2002; Gismondi et al., 1997; Lakkakorpi et al., 1999; Ohba et al., 1998). Another possibility is that lipid rafts have a role in recruiting Pyk2/Cbl complexes to the edges of growth cone lamellipodia. Endogenous Pyk2 and Cbl were rapidly translocated to lipid rafts upon NGF stimulation and remained there during prolonged ligand stimulation (Fig. 4A). The same recruitment kinetics was observed for ArgBP2 (Fig. 4A), suggesting that ArgBP2 participates in localizing Pyk2 and Cbl to lipid rafts by associating with flotillin-1 via its SoHo domain (Fig. 4C). Interestingly, flotillins are upregulated in regenerating retinal ganglion axons (thus they are also called reggie proteins), and co-localize with lamellipodia and filopodia of growth cones (Lang et al., 1998; Schulte et al., 1997). However, flotillin-1/ArgBP2-independent recruitment pathways must also exist, since an ArgBP2 mutant lacking the SoHo domain, did not completely block the translocation of Pyk2 and Cbl to lipid rafts (Fig. 4D). Alternatively, recruitment of Pyk2/Cbl complexes to lipid rafts might therefore be, in part, mediated by their association with integrin/growth factor receptor complexes.

Cbl has previously been shown to positively regulate the cytoskeleton via signals initiated by its carboxyl terminus (Dikic et al., 2003). However, it is also well established that Cbl mediates ubiquitination and downregulation of several proteins, including receptor and non-receptor tyrosine kinases (Dikic and Giordano, 2003; Thien and Langdon, 2001). Does Cbl-mediated ubiquitination play a role in the processes described here? We noted that overexpression of the isolated carboxyl terminus of Cbl lead to enhanced lamellipodia formation (Fig. 6A), suggesting that the amino-terminal part of Cbl, which contains the RING finger domain, might indeed negatively regulate signals initiated by the Pyk2/Cbl/ArgBP2 complex. ArgBP2 has previously been shown to undergo Cbl-mediated ubiquitination (Soubeyran et al., 2003) and preliminary data indicate that Pyk2 is ubiquitinated by Cbl (unpublished observations). This negative regulation might be required, for instance, during lamellipodium disassembly. However, the availability of ubiquitin-activating (E1) and ubiquitin-conjugating (E2) enzymes in the growth cones is not known and may limit the activity of Cbl as a ubiquitin ligase (E3). In lipid rafts, Cbl has been shown to mediate both ubiquitin-dependent and -independent functions. For instance, Cbl and Nedd4 ubiquitin ligases co-localize with ubiquitinated FcεRI receptors in lipid rafts upon IgE-triggered cell signaling, probably leading to attenuation of receptor-induced signaling (Lafont and Simons, 2001). However, insulin stimulation of adipocytes leads to recruitment of tyrosine-phosphorylated Cbl to caveolae/lipid rafts via its binding to CAP and flotillin (Baumann et al., 2000). This induces translocation of CrkII/C3G/TC10 complexes, which promote GLUT4 translocation to the plasma membrane seemingly independently of the ubiquitin ligase activity of Cbl (Baumann et al., 2000). Apparently, Cbl is able to mediate both positive and negative regulation of cellular processes.

The formation of a Pyk2/Cbl complex may have a general role in the regulation of cell morphology in other cell types as well. We have also detected association between Pyk2 and Cbl in macrophages (data not shown). Previous reports have shown that integrin-induced tyrosine phosphorylation of Cbl and its association with PI 3-kinase and Src is involved in macrophage

spreading and migration (Meng and Lowell, 1998; Ojaniemi et al., 1997). In addition, a Pyk2/Cbl complex is required for Src-dependent adhesion and migration of osteoclasts (Sanjay et al., 2001). Whether recruitment of Pyk2/ArgBP2/Cbl complexes to lamellipodia and lipid rafts is a general mechanism by which signals initiated by cell surface receptors are integrated to control the actin cytoskeleton during cell migration and differentiation remains to be determined.

We thank Sara A. Courtneidge, Dominique Davidson, Elena Feschenko, Kathrin Kirsch, Vered Ribon, Alan Saltiel, Yoshimi Takai and Kristiina Vuori for providing reagents used in these studies. This work was supported by grants from the Swedish Cancer Foundation, Swedish Strategic Funds, German Science Foundation (DFG) and Boehringer Ingelheim Fonds to I.D. and by a grant-in-aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology to N.S.

References

Andoniou, C. E., Thien, C. B. and Langdon, W. Y. (1996). The two major sites of cbl tyrosine phosphorylation in abl-transformed cells select the crkL SH2 domain. *Oncogene* **12**, 1981-1989.

Astier, A., Avraham, H., Manie, S. N., Groopman, J., Canty, T., Avraham, S. and Freedman, A. S. (1997a). The related adhesion focal tyrosine kinase is tyrosine-phosphorylated after beta1-integrin stimulation in B cells and binds to p130cas. *J. Biol. Chem.* **272**, 228-232.

Astier, A., Manie, S. N., Avraham, H., Hirai, H., Law, S. F., Zhang, Y., Golemis, E. A., Fu, Y., Druker, B. J., Haghayeghi, N. et al. (1997b). The related adhesion focal tyrosine kinase differentially phosphorylates p130Cas and the Cas-like protein, p105HEF1. *J. Biol. Chem.* **272**, 19719-19724.

Avraham, H., Park, S. Y., Schinkmann, K. and Avraham, S. (2000). RAFTK/Pyk2-mediated cellular signalling. *Cell Signal* **12**, 123-133.

Baron, W., Decker, L., Colognato, H. and ffrench-Constant, C. (2003). Regulation of integrin growth factor interactions in oligodendrocytes by lipid raft microdomains. *Curr. Biol.* **13**, 151-155.

Baumann, C. A., Ribon, V., Kanzaki, M., Thurmond, D. C., Mora, S., Shigematsu, S., Bickel, P. E., Pessin, J. E. and Saltiel, A. R. (2000). CAP defines a second signalling pathway required for insulin-stimulated glucose transport. *Nature* **407**, 202-207.

Blaukat, A., Ivankovic-Dikic, I., Gronroos, E., Dolfi, F., Tokiwa, G., Vuori, K. and Dikic, I. (1999). Adaptor proteins Grb2 and Crk couple Pyk2 with activation of specific mitogen-activated protein kinase cascades. *J. Biol. Chem.* **274**, 14893-14901.

Brown, D. A. and London, E. (1998). Functions of lipid rafts in biological membranes. *Annu. Rev. Cell. Dev. Biol.* **14**, 111-136.

Chiusaroli, R., Sanjay, A., Henriksen, K., Engsig, M. T., Horne, W. C., Gu, H. and Baron, R. (2003). Deletion of the gene encoding c-Cbl alters the ability of osteoclasts to migrate, delaying resorption and ossification of cartilage during the development of long bones. *Dev. Biol.* **261**, 537-547.

da Silva, J. S. and Dotti, C. G. (2002). Breaking the neuronal sphere: regulation of the actin cytoskeleton in neuritogenesis. *Nat. Rev. Neurosci.* **3**, 694-704.

Dikic, I. and Giordano, S. (2003). Negative receptor signalling. *Curr. Opin. Cell Biol.* **15**, 128-135.

Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A. and Schlessinger, J. (1996). A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* **383**, 547-550.

Dikic, I., Haglund, K. and Ivankovic-Dikic, I. (2002). Regulation of cell morphology by proline-rich tyrosine kinase Pyk2. *Period. Biol.* **104**, 11-18.

Dikic, I., Szymkiewicz, I. and Soubeyran, P. (2003). Cbl signaling networks in the regulation of cell function. *Cell Mol. Life Sci.* **60**, 1805-1827.

Duong, L. T., Lakkakorpi, P. T., Nakamura, I., Machwate, M., Nagy, R. M. and Rodan, G. A. (1998). PYK2 in osteoclasts is an adhesion kinase, localized in the sealing zone, activated by ligation of alpha(v)beta3 integrin, and phosphorylated by src kinase. *J. Clin. Invest.* **102**, 881-892.

Feshchenko, E. A., Shore, S. K. and Tsygankov, A. Y. (1999). Tyrosine phosphorylation of C-Cbl facilitates adhesion and spreading while suppressing anchorage-independent growth of V-Abl-transformed NIH3T3 fibroblasts. *Oncogene* **18**, 3703-3715.

Gismondi, A., Bisogno, L., Mainiero, F., Palmieri, G., Piccoli, M., Frati, L. and Santoni, A. (1997). Proline-rich tyrosine kinase-2 activation by beta 1 integrin fibronectin receptor cross-linking and association with paxillin in human natural killer cells. *J. Immunol.* **159**, 4729-4736.

Grimmer, S., van Deurs, B. and Sandvig, K. (2002). Membrane ruffling and macropinocytosis in A431 cells require cholesterol. *J. Cell Sci.* **115**, 2953-2962.

Gupta, N. and DeFranco, A. L. (2003). Visualizing lipid raft dynamics and early signaling events during antigen receptor-mediated B-lymphocyte activation. *Mol. Biol. Cell* **14**, 432-444.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279**, 509-514.

Hartley, D. and Corvera, S. (1996). Formation of c-Cbl. phosphatidylinositol 3-kinase complexes on lymphocyte membranes by a p56lck-independent mechanism. *J. Biol. Chem.* **271**, 21939-21943.

Hawkins, P. T., Eguinoa, A., Qiu, R. G., Stokoe, D., Cooke, F. T., Walters, R., Wennstrom, S., Claesson-Welsh, L., Evans, T., Symons, M. et al. (1995). PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase. *Curr. Biol.* **5**, 393-403.

Heldin, C. H., Ostman, A. and Ronnstrand, L. (1998). Signal transduction via platelet-derived growth factor receptors. *Biochim. Biophys. Acta Mol. Cell Res.* **1378**, F79-F113.

Hiregowdara, D., Avraham, H., Fu, Y., London, R. and Avraham, S. (1997). Tyrosine phosphorylation of the related adhesion focal tyrosine kinase in megakaryocytes upon stem cell factor and phorbol myristate acetate stimulation and its association with paxillin. *J. Biol. Chem.* **272**, 10804-10810.

Huang, E. J. and Reichardt, L. F. (2001). Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci.* **24**, 677-736.

Huang, X., Gaballa, A., Cao, M. and Helmann, J. D. (1999). Identification of target promoters for the Bacillus subtilis extracytoplasmic function sigma factor, sigma W. *Mol. Microbiol.* **31**, 361-371.

Hunter, S., Burton, E. A., Wu, S. C. and Anderson, S. M. (1999). Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphatidylinositol 3-kinase. *J. Biol. Chem.* **274**, 2097-2106.

Ivankovic-Dikic, I., Gronroos, E., Blaukat, A., Barth, B. U. and Dikic, I. (2000). Pyk2 and FAK regulate neurite outgrowth induced by growth factors and integrins. *Nat. Cell Biol.* **2**, 574-581.

Kanda, E., Jin, Z. H., Mizuchi, D., Arai, A. and Miura, O. (2003). Activation of Rac and tyrosine phosphorylation of cytokine receptors induced by cross-linking of integrin alpha4beta1 and cell adhesion in hematopoietic cells. *Biochem. Biophys. Res. Commun.* **301**, 934-940.

Kawabe, H., Hata, Y., Takeuchi, M., Ide, N., Mizoguchi, A. and Takai, Y. (1999). nArgBP2, a novel neural member of ponsin/ArgBP2/vinexin family that interacts with synapse-associated protein 90/postsynaptic density-95-associated protein (SAPAP). *J. Biol. Chem.* **274**, 30914-30918.

Kimura, A., Baumann, C. A., Chiang, S. H. and Saltiel, A. R. (2001). The sorbin homology domain: a motif for the targeting of proteins to lipid rafts. *Proc. Natl. Acad. Sci. USA* **98**, 9098-9103.

Kirsch, K. H., Georgescu, M. M., Shishido, T., Langdon, W. Y., Birge, R. B. and Hanafusa, H. (2001). The adapter type protein CMS/CD2AP binds to the proto-oncogenic protein c-Cbl through a tyrosine phosphorylation-regulated Src homology 3 domain interaction. *J. Biol. Chem.* **276**, 4957-4963.

Kozma, R., Sarner, S., Ahmed, S. and Lim, L. (1997). Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. *Mol. Cell Biol.* **17**, 1201-1211.

Lafont, F. and Simons, K. (2001). Raft-partitioning of the ubiquitin ligases Cbl and Nedd4 upon IgE-triggered cell signaling. *Proc. Natl. Acad. Sci. USA* **98**, 3180-3184.

Lakkakorpi, P. T., Nakamura, I., Nagy, R. M., Parsons, J. T., Rodan, G. A. and Duong, L. T. (1999). Stable association of PYK2 and p130(Cas) in osteoclasts and their co- localization in the sealing zone. *J. Biol. Chem.* **274**, 4900-4907.

Lamorte, L., Rodrigues, S., Sangwan, V., Turner, C. E. and Park, M. (2003). Crk associates with a multimolecular Paxillin/GIT2/beta-PIX complex and promotes Rac-dependent relocalization of Paxillin to focal contacts. *Mol. Biol. Cell* **14**, 2818-2831.

Lang, D. M., Lommel, S., Jung, M., Ankerhold, R., Petrausch, B., Laessing, U., Wiechers, M. F., Plattner, H. and Stuermer, C. A. (1998). Identification of reggie-1 and reggie-2 as plasmamembrane-associated proteins which cocluster with activated GPI-anchored cell adhesion molecules in non-caveolar micropatches in neurons. *J. Neurobiol.* **37**, 502-523.

Leitinger, B. and Hogg, N. (2002). The involvement of lipid rafts in the regulation of integrin function. *J. Cell Sci.* **115**, 963-972.
Liu, Y. C., Elly, C., Langdon, W. Y. and Altman, A. (1997). Ras-dependent, Ca2+-stimulated activation of nuclear factor of activated T cells by a constitutively active Cbl mutant in T cells. *J. Biol. Chem.* **272**, 168-173.
Luo, L. (2000). Rho GTPases in neuronal morphogenesis. *Nat. Rev. Neurosci.* **1**, 173-180.
Marengere, L. E., Mirtsos, C., Kozieradzki, I., Veillette, A., Mak, T. W. and Penninger, J. M. (1997). Proto-oncoprotein Vav interacts with c-Cbl in activated thymocytes and peripheral T cells. *J. Immunol.* **159**, 70-76.
Meng, F. and Lowell, C. A. (1998). A beta 1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. *EMBO J.* **17**, 4391-4403.
Neet, K. and Hunter, T. (1996). Vertebrate non-receptor protein-tyrosine kinase families. *Genes Cells* **1**, 147-169.
Ohba, T., Ishino, M., Aoto, H. and Sasaki, T. (1998). Interaction of two proline-rich sequences of cell adhesion kinase beta with SH3 domains of p130Cas-related proteins and a GTPase-activating protein, Graf. *Biochem. J.* **330**, 1249-1254.
Ojaniemi, M., Martin, S. S., Dolfi, F., Olefsky, J. M. and Vuori, K. (1997). The proto-oncogene product p120(cbl) links c-Src and phosphatidylinositol 3′-kinase to the integrin signaling pathway. *J. Biol. Chem.* **272**, 3780-3787.
Okigaki, M., Davis, C., Falasca, M., Harroch, S., Felsenfeld, D. P., Sheetz, M. P. and Schlessinger, J. (2003). Pyk2 regulates multiple signaling events crucial for macrophage morphology and migration. *Proc. Natl. Acad. Sci. USA* **100**, 10740-10745
Park, S. Y., Avraham, H. and Avraham, S. (2000). Characterization of the tyrosine kinases RAFTK/Pyk2 and FAK in nerve growth factor-induced neuronal differentiation. *J. Biol. Chem.* **275**, 19768-19777.
Peiro, S., Comella, J. X., Enrich, C., Martin-Zanca, D. and Rocamora, N. (2000). PC12 cells have caveolae that contain TrkA. Caveolae-disrupting drugs inhibit nerve growth factor-induced, but not epidermal growth factor-induced, MAPK phosphorylation. *J. Biol. Chem.* **275**, 37846-37852.
Pollard, T. D., Blanchoin, L. and Mullins, R. D. (2000). Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu. Rev. Biophys. Biomol. Struct.* **29**, 545-576.
Qian, D., Lev, S., van Oers, N. S., Dikic, I., Schlessinger, J. and Weiss, A. (1997). Tyrosine phosphorylation of Pyk2 is selectively regulated by Fyn during TCR signaling. *J. Exp. Med.* **185**, 1253-1259.
Ribon, V., Hubbell, S., Herrera, R. and Saltiel, A. R. (1996). The product of the cbl oncogene forms stable complexes in vivo with endogenous Crk in a tyrosine phosphorylation-dependent manner. *Mol. Cell Biol.* **16**, 45-52.
Ribon, V., Herrera, R., Kay, B. K. and Saltiel, A. R. (1998). A role for CAP, a novel, multifunctional Src homology 3 domain-containing protein in formation of actin stress fibers and focal adhesions. *J. Biol. Chem.* **273**, 4073-4080.
Salzer, U. and Prohaska, R. (2001). Stomatin, flotillin-1, and flotillin-2 are major integral proteins of erythrocyte lipid rafts. *Blood* **97**, 1141-1143.
Sanjay, A., Houghton, A., Neff, L., DiDomenico, E., Bardelay, C., Antoine, E., Levy, J., Gailit, J., Bowtell, D., Horne, W. C. et al. (2001). Cbl associates with Pyk2 and Src to regulate Src kinase activity, alpha(v)beta(3) integrin-mediated signaling, cell adhesion, and osteoclast motility. *J. Cell Biol.* **152**, 181-195.
Scaife, R. M. and Langdon, W. Y. (2000). c-Cbl localizes to actin lamellae and regulates lamellipodia formation and cell morphology. *J. Cell Sci.* **113**, 215-226.
Schulte, T., Paschke, K. A., Laessing, U., Lottspeich, F. and Stuermer, C. A. (1997). Reggie-1 and reggie-2, two cell surface proteins expressed by retinal ganglion cells during axon regeneration. *Development* **124**, 577-587.
Sieg, D. J., Hauck, C. R., Ilic, D., Klingbeil, C. K., Schaefer, E., Damsky, C. H. and Schlaepfer, D. D. (2000). FAK integrates growth-factor and integrin signals to promote cell migration. *Nat. Cell Biol.* **2**, 249-256.
Simons, K. and Toomre, D. (2000). Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1**, 31-39.
Soubeyran, P., Kowanetz, K., Szymkiewicz, I., Langdon, W. Y. and Dikic, I. (2002). Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors. *Nature* **416**, 183-187.
Soubeyran, P., Barac, A., Szymkiewicz, I. and Dikic, I. (2003). Cbl-ArgBP2 complex mediates ubiquitination and degradation of c-Abl. *Biochem. J.* **370**, 29-34.
Szymkiewicz, I., Kowanetz, K., Soubeyran, P., Dinarina, A., Lipkowitz, S. and Dikic, I. (2002). CIN85 participates in Cbl-b-mediated down-regulation of receptor tyrosine kinases. *J. Biol. Chem.* **277**, 39666-39672.
Thien, C. B. and Langdon, W. Y. (2001). Cbl: many adaptations to regulate protein tyrosine kinases. *Nat. Rev. Mol. Cell Biol.* **2**, 294-307.
Tsui-Pierchala, B. A., Encinas, M., Milbrandt, J. and Johnson, E. M., Jr (2002). Lipid rafts in neuronal signaling and function. *Trends Neurosci.* **25**, 412-417.
Turner, C. E. (2000). Paxillin interactions. *J. Cell Sci.* **113**, 4139-4140.
Wang, B., Golemis, E. A. and Kruh, G. D. (1997). ArgBP2, a multiple Src homology 3 domain-containing, Arg/Abl-interacting protein, is phosphorylated in v-Abl-transformed cells and localized in stress fibers and cardiocyte Z-disks. *J. Biol. Chem.* **272**, 17542-17550.
Wennstrom, S., Hawkins, P., Cooke, F., Hara, K., Yonezawa, K., Kasuga, M., Jackson, T., Claesson-Welsh, L. and Stephens, L. (1994). Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. *Curr. Biol.* **4**, 385-393.
Wu, W. J., Tu, S. and Cerione, R. A. (2003). Activated Cdc42 sequesters c-Cbl and prevents EGF receptor degradation. *Cell* **114**, 715-725.
Xavier, R., Brennan, T., Li, Q., McCormack, C. and Seed, B. (1998). Membrane compartmentation is required for efficient T cell activation. *Immunity* **8**, 723-732.