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Dynamin Forms a Src Kinase–sensitive Complex with Cbl and Regulates Podosomes and Osteoclast Activity

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Podosomes are highly dynamic actin-containing adhesion structures found in osteoclasts, macrophages, and Rous sarcoma virus (RSV)-transformed fibroblasts. After integrin engagement, Pyk2 recruits Src and the adaptor protein Cbl, forming a molecular signaling complex that is critical for cell migration, and deletion of any molecule in this complex disrupts podosome ring formation and/or decreases osteoclast migration. Dynamin, a GTPase essential for endocytosis, is also involved in actin cytoskeleton remodeling and is localized to podosomes where it has a role in actin turnover. We found that dynamin colocalizes with Cbl in the actin-rich podosome belt of osteoclasts and that dynamin forms a complex with Cbl in osteoclasts and when overexpressed in 293VnR or SYF cells. The association of dynamin with Cbl in osteoclasts was decreased by Src tyrosine kinase activity and we found that destabilization of the dynamin-Cbl complex involves the recruitment of Src through the proline-rich domain of Cbl. Overexpression of dynamin increased osteoclast bone resorbing activity and migration, whereas overexpression of dynK44A decreased osteoclast resorption and migration. These studies suggest that dynamin, Cbl, and Src coordinately participate in signaling complexes that are important in the assembly and remodeling of the actin cytoskeleton, leading to changes in osteoclast adhesion, migration, and resorption.

INTRODUCTION

Osteoclasts are multinucleated bone-resorbing cells that are responsible for the degradation of bone. After the engagement of integrins with extracellular matrix proteins, osteoclasts undergo cytoskeletal reorganization and cellular polarization, resulting in the establishment of distinct apical and basolateral membrane domains (Baron *et al.*, 1985; Vaananen *et al.*, 2000). The domain facing the bone surface is comprised of a highly convoluted ruffled border membrane, circumscribed by a ring-like structure called the sealing zone. The sealing zone contains highly dynamic actin-containing adhesion structures known as podosomes, which mediate cell attachment and motility and which are also observed in leukocytes, macrophages, and v-Src or Rous sarcoma virus (RSV)-transformed cells (Marchisio *et al.*, 1987; Ochoa *et al.*, 2000). Within the core of individual podosomes, actin adopts a columnar structure perpendicular to the plasma membrane and undergoes continuous cycles of polymerization and depolymerization (Ochoa *et al.*, 2000; *Pfaff* and Jurdic, 2001; Destaing *et al.*, 2003). F-actin, cortactin, gelsolin, and the actin-regulatory proteins N-WASP and Arp2/3 are present within the podosome core, whereas integrins and the integrin-associated proteins paxillin, talin, and vinculin are localized around the actin core (Linder and Aepfelbacher, 2003). Although podosomes and focal adhesions have many of these proteins in common, podosome turnover occurs within minutes (Stickel and Wang, 1987; Chen, 1989; Destaing *et al.*, 2003), in contrast to the relatively stable structure of focal adhesions which serve to anchor cells to the extracellular matrix (Sastry and Burridge, 2000).

Osteoclasts express three major integrins, $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_2\beta_1$ (Helfrich *et al.*, 1992; Nesbitt *et al.*, 1993; Duong and Rodan, 1998, 1999), of which $\alpha_v\beta_3$ or vitronectin receptor (VnR) is the most highly expressed (Hughes *et al.*, 1993; Nesbitt *et al.*, 1993). Several studies suggest that $\alpha_v\beta_3$ plays a role in the initial attachment of osteoclasts to RGD-containing bone proteins, including vitronectin, osteopontin, and bone sialoprotein (Davies *et al.*, 1989; Horton *et al.*, 1991; Lakkakorpi *et al.*, 1991, 1993; Nesbitt *et al.*, 1993; Vaananen and Horton, 1995). Consistent with this function, $\alpha_v\beta_3$ participates in precursor spreading before osteoclast cell fusion (Boissy *et al.*, 1998) and osteoclasts from mice lacking the β_3 subunit exhibit abnormal spreading and disrupted actin ring and ruffled border formation, resulting in decreased bone resorption and osteosclerosis (McHugh *et al.*, 2000; Feng *et al.*, 2001). Engagement of $\alpha_v\beta_3$ leads to a complex series of intramolecular and intermolecular interactions between the nonreceptor tyrosine kinases Src and Pyk2, and the adaptor protein c-Cbl (Cbl), a substrate of Src (Tanaka *et al.*, 1996; Sanjay *et al.*, 2001; Chiusaroli *et al.*, 2003; Miyazaki *et al.*, 2004). The formation of a multiprotein signaling complex containing Cbl, Src, and Pyk2 is essential for podosome dynamics, osteoclast migration, and bone resorption, and its absence is believed to contribute to the decreased osteoclast motility observed in $Src^{-/-}$ and $Cbl^{-/-}$ mice, leading to

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Abbreviations used: IP, immunoprecipitate; mRIPA, modified radioimmune precipitation assay; OCL, osteoclast-like cell; VnR, vitronectin receptor; GTP, guanosine 5′ triphosphate; GED, GTPase effector domain; PTB, phosphotyrosine-binding domain; PRD, proline-rich domain. PH, pleckstrin homology; PBS, phosphate buffered saline; FCS, fetal calf serum.

osteopetrosis in $Src^{-/-}$ mice (Tanaka *et al.*, 1996; Schwartzberg *et al.*, 1997; Meng and Lowell, 1998; Sanjay *et al.*, 2001; Chiusaroli *et al.*, 2003).

In addition to its well-established role in endocytosis, several lines of evidence suggest that the large GTPase dynamin is a potential player in actin remodeling and thereby possibly in cell migration and bone resorption. First, dynamin's binding partners include the actin-regulating proteins profilin II, syndapin, intersectin, cortactin, and Abp1 (Qualmann *et al.*, 2000; Slepnev and De Camilli, 2000; Schafer, 2002). The GTPase activity of dynamin is also associated with actin comets generated by *Lysteria* and type I phosphatidylinositol phosphate kinase (PIPK) expression (Lee and De Camilli, 2002; Orth *et al.*, 2002). Second, dynamin is recruited to cortical ruffles in fibroblasts (Cao *et al.*, 1998) and dynamin as well as the dynamin-associated proteins endophilin (Ringstad *et al.*, 1997), cortactin (McNiven *et al.*, 2000), and Src (Gout *et al.*, 1993) have been found at podosomes of osteoclasts and in RSV-transformed or v-Src–transformed fibroblasts (Ochoa *et al.*, 2000). A dynamin mutant (dynK44A), which binds GTP with reduced affinity (Damke *et al.*, 1994; Warnock *et al.*, 1995), affected actin stress fiber formation and cell shape (Damke *et al.*, 1994) and delayed actin turnover at podosomes in BHK-RSV cells (Ochoa *et al.*, 2000). This is especially important given that podosomes sometimes contain narrow tubular invaginations of the plasma membrane (Ochoa *et al.*, 2000) that resemble membrane structures that form as a consequence of the activity of dynamin, amphiphysin, or endophilin (Takei *et al.*, 1995, 1999; Farsad *et al.*, 2001). Finally, dynamin is a substrate of the tyrosine kinase Src, which has been shown to regulate the strength of adhesive forces between $\alpha_v\beta_3$ and the extracellular matrix and to be required for osteoclast function (Felsenfeld *et al.*, 1999; Sanjay *et al.*, 2001). Thus, in parallel to its known role in endocytosis, dynamin may play a pivotal role in signaling events downstream of integrins and in the assembly and remodeling of the actin cytoskeleton at sites of cell attachment, which are essential for osteoclast adhesion and migration.

In this study, we characterized the intermolecular association of dynamin with the adaptor protein Cbl and identified Src as a major modulator of dynamin-Cbl interaction in osteoclasts. Furthermore, dynamin activity was found to affect podosome formation and osteoclast bone resorption. These studies highlight the important role of dynamin in actin dynamics associated with podosome turnover and with signaling downstream of Src and Cbl.

MATERIALS AND METHODS

Materials

Antibodies used for Western blot analyses against endogenous Cbl or dynamin 2 (dyn II; epitope 274–555) were obtained from Transduction Laboratories (Lexington, KY). Antibodies to human dynamin (hudy1 and hudy2) were purchased from Upstate Biotechnology (Lake Placid, NY) and were used for the immunoprecipitation of endogenous dynamin. Polyclonal and monoclonal anti-GFP antibodies (Living Colors) were from Clontech (Palo Alto, CA). Anti-avian Src, anti-phosphotyrosine (clone 4G10), and anti-vinculin were all from Upstate Biotechnology. Anti-v-Src antibody (clone antibody-1) was obtained from Calbiochem (La Jolla, CA). Anti-HA was from Bethyl Laboratories (Montgomery, TX). The anti-myc antibody (clone 9E10) and was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Both anti-rabbit HRP and anti-mouse HRP secondary antibodies were obtained from Fisher Scientific (Fairlawn, NJ). PP1 and PP2 were from Alexis Biochemicals (San Diego, CA) and genistein was from Sigma-Aldrich (St. Louis, MO). Immunofluorescent detection of dynamin in osteoclasts was performed using the dyn II monoclonal antibody from Transduction Laboratories (Cao *et al.*, 1998). Nuclei were stained with the nuclear fluorescent dye TO-PRO-3 (Molecular Probes, Eugene, OR). Fluorescein-conjugated secondary antibodies were from Molecular Probes.

SYF cells harboring functional null mutations in both alleles of the Src family protein tyrosine kinases Src, Yes, and Fyn were obtained from the American Tissue Culture Collection (CRL-2459; Manassas, VA).

Plasmids

Plasmids containing myc-tagged full-length c-Cbl (Cbl) were described previously (Bartkiewicz *et al.*, 1999; Sanjay *et al.*, 2001). The Cbl6PA mutant was generated using the QuickChange site-directed mutagenesis technique (Stratagene, La Jolla, CA). Cbl6PA was generated by PCR using the forward primer 5′-cacacttcgagatcttgcagcagcagcggctgcagaccggccatattctg-3′ in combination with the reverse primer 5′-cagaatatggccggtctgcagccgctgctgctgcaagatctcgaagtgtg-3′ and the product was subcloned into pcDNA3.1. The resulting Cbl6PA protein contains six proline-to-alanine substitutions (amino acids 543–548) within the proline-rich domain (PRD) of human Cbl and exhibits decreased binding of Cbl to the SH3 domain of Src (Sanjay *et al.*, unpublished results). Cbl-CT (residues 479–906) contained the C-terminal half of Cbl, including the PRD domain (Bartkiewicz *et al.*, 1999). The c-Src constructs were derived from avian c-Src as previously described and were a generous gift from Dr. P. Schwartzberg (National Institutes of Health, Bethesda, MD) and Dr. J. Brugge (Harvard University, Cambridge, MA). A construct of rat dynamin 2 (splice variant aa; Cao *et al.*, 1998) and the dynamin mutant dynK44A were generated as previously described (Ochoa *et al.*, 2000). GST-Dyn-PRD made from rat dynamin 2 (2271-end) fused to pGEX-4T-1 was a kind gift from Dr. E. Lee (Lee *et al.*, 1999). Dynamin 2-PRD-GFP (dynPRD; amino acids 735–869 of dynamin 2 splice variant aa) was fused to GFP in pCDNA3.1 (Lee *et al.*, 1999). Dynamin 2 that lacked the PRD domain (dynΔPRD) was made by inserting amino acids 1–745 of dynamin 2 into the pEGFP-N1 vector. The dynamin GED+PRD domain construct (amino acids 618–870) was subcloned into pEGFP-N1 (Lee *et al.*, 1999).

Transient Transfections

Transient transfections were performed using FuGENE 6 (Roche, Indianapolis, IN) according the manufacturer's specifications using a total of 10 μg of DNA per 100-mm^2 tissue culture plate. In all experiments, the total amount of cDNA transfected was normalized by the addition of empty vector pcDNA3.1. Transient expression was performed in human embryonal kidney (HEK293) cells stably expressing the $\alpha_v\beta_3$ vitronectin receptor (293VnR), the major integrin found on osteoclasts. This cell line has been used as a model system to elucidate Cbl-Src-Pyk2 intermolecular interactions, which were later confirmed in osteoclasts (Sanjay *et al.*, 2001; Miyazaki *et al.*, 2004). Transiently transfected cells were maintained in α-MEM containing 10% fetal calf serum (FCS) for 72 h after transfection.

Preparation of Authentic Osteoclasts and Osteoclast-like Cells

Mouse osteoclast-like cells (OCLs) were generated using the murine coculture system (Tanaka *et al.*, 1996) by culturing neonatal primary calvarial osteoblasts with spleen and marrow cells in the presence of 1,25-dihydroxy vitamin D_3 and PGE_2. After 4–5 d in culture, the osteoblast layer was removed by gentle pipetting of medium over the surface of the cell layer, leaving purified OCLs behind. Suspensions of serum-starved (1% FCS, 2 h) OCLs were obtained by treating the purified OCLs with 10 mM EDTA for 5 min at 37°C. Cells were then flushed off the culture dishes, washed once in serum-free medium, and then resuspended in serum-free medium (Tanaka *et al.*, 1998). Alternatively, bone marrow cells were plated on collagen gel (Miyazaki *et al.*, 2000) and differentiated with RANKL (100 ng/ml) and M-CSF (20 ng/ml) for 1 wk (Takayanagi *et al.*, 2002). Collagen was digested with 0.1% collagenase and the cells were replated onto coverslips or dentin.

Recombinant Adenovirus

Recombinant adenovirus expressing dynamin 2 (ba splice variant) or mutant dynamin 2 (dynK44A) containing hemagglutinin tags (HA) were made using the tetracycline-inhibited Cre-*lox* recombination system and were a kind gift from Dr. Sandra Schmid and are described elsewhere (Damke *et al.*, 2001). Overexpression of dynamin in osteoclasts required the cotransduction of adenovirus expressing the tetracycline transcription activator (tTA; 20 μl) and the dynamin-expressing adenoviruses (0–400 μl), in the absence of tetracycline (Altschuler *et al.*, 1998). Adenovirus expressing kinase-inactive SrcK295M (Miyazaki *et al.*, 2004) was prepared using the Adenovator Vector system (Qbiogene, Carlsbad, CA) as previously described (Tanaka *et al.*, 1989).

Infection of mouse OCLs using the adenovirus expression systems was performed on immature OCLs after 5 d of culture in differentiation medium containing RANKL and M-CSF. Cells were infected with adenovirus using a multiplicity of infection (MOI) score of 100, which results in a 2–3-fold overexpression of dynamin relative to endogenous dynamin. An MOI score of 10–20 is equivalent to the volume of virus required to infect 100% of 293VnR cells after 2 d of infection. The following day, cells were washed and differentiation with RANKL and M-CSF was continued for an additional 1–2 d before the cells were replated onto coverslips or dentin slices for 24 h and then either processed for confocal immunofluorescence microscopy or harvested for biochemical analyses.

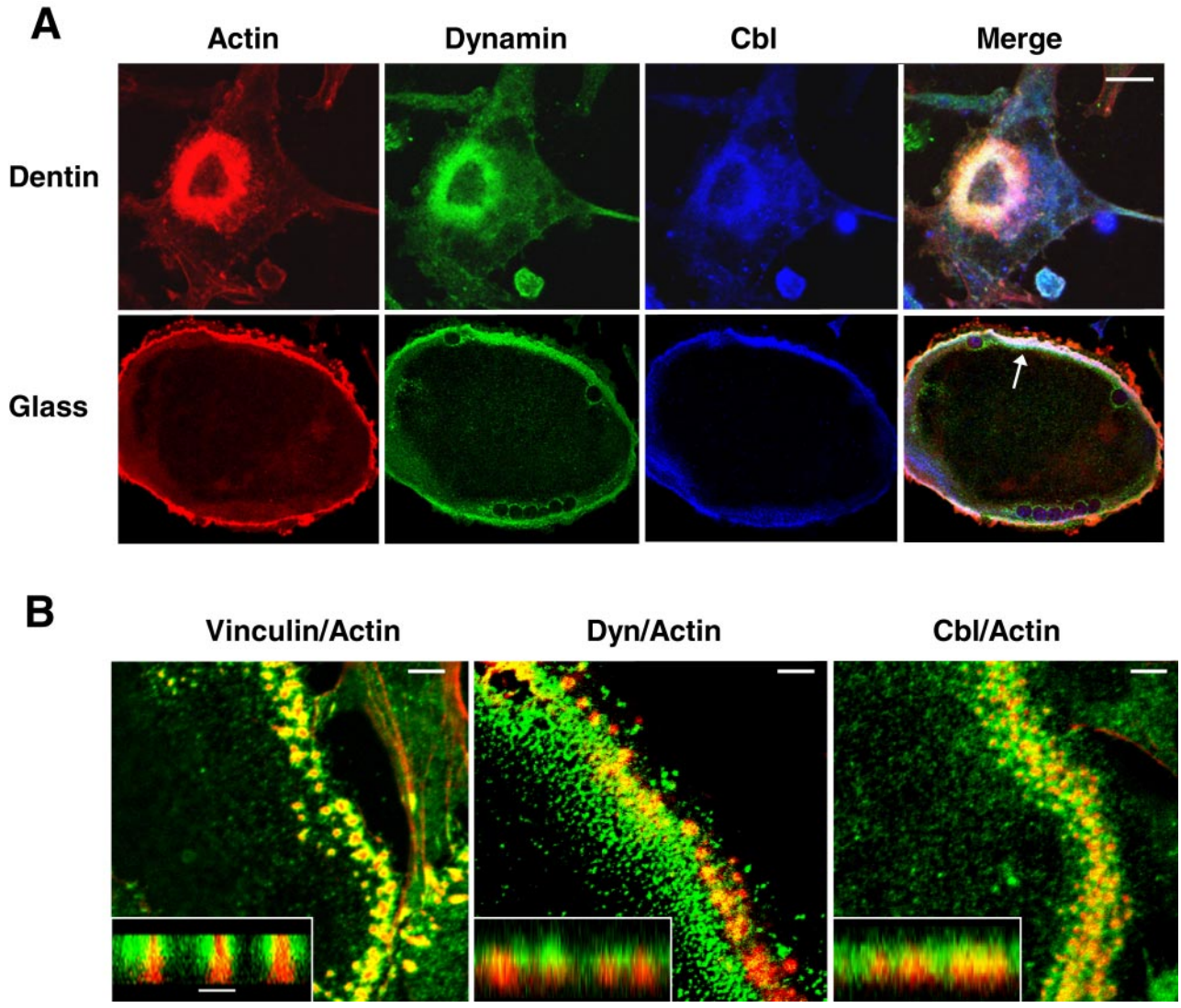

Figure 1. Dynamin is localized to the actin-rich sealing zone and podosome belt in osteoclasts. (A) Scanning confocal immunofluorescence microscopy of mouse osteoclasts plated on dentin or on serum-coated glass coverslips. Dynamin (green), labeled with anti-dyn II antibody, colocalizes with the actin podosome ring (red) and with Cbl (blue). The merged image was created by superimposing the actin, dynamin, and Cbl fluorescent spectra. The large circular areas devoid of dynamin staining are nuclei. (B) High-power confocal images showing distinct colocalization patterns of actin (red) with vinculin (green, left), dynamin (green, middle), and Cbl (green, right) at podosome adhesion structures in osteoclasts. Overlapping expression of actin with each protein is shown in yellow. Scale bar, 5 μm. Insets: high-magnification *Z*-axis images of individual podosomes showing the localization of vinculin, dynamin, and Cbl, relative to the actin core (red). Individual podosomes are ~0.5 μm in diameter. The apical domain of the osteoclast in contact with the coverslip is at the bottom of the image. Images shown are representative of osteoclast cultures.

Confocal Microscopy

Authentic osteoclasts and OCLs were seeded onto FCS-coated coverslips and incubated for 2–24 h at 37°C followed by fixing with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min. Fixed cells were visualized using a Zeiss 510 Meta laser scanning confocal microscope (Thornwood, NY). Coverslips for cytoskeletal labeling were extracted in ice-cold acetone for 3–5 min. All other coverslips were permeabilized in 0.05% saponin for 30 min. Coverslips for actin labeling were incubated in a 1:40 dilution of rhodamine phalloidin stock solution (Molecular Probes) for 1 h, washed with PBS, and mounted in FluorSave (Calbiochem). All other coverslips were blocked in 5% normal goat serum (Roche, Indianapolis, IN) for 30 min and incubated in appropriate primary antibodies, washed, incubated with fluorescent secondary antibody (Alexa Fluor 488, green; 568, red; 647, blue), washed again, and mounted in FluorSave. Nuclear labeling was with TO-PRO-3 (1:1000) in the secondary antibody solution. Images were recorded and composites compiled and total image enhancements were performed using Adobe Photoshop 6.0 (San Jose, CA).

Coimmunoprecipitation Assays

Coimmunoprecipitations were performed as previously described (Sanjay *et al.*, 2001). Briefly, cells were lysed in modified radioimmune precipitation assay (mRIPA) buffer containing 50 mM Tris-Cl, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, and protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM sodium orthovanadate, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin). Lysates were sonicated and the supernatant was clarified by centrifugation. Typically, 5 μg of primary antibody was used for 500 μg of cell lysates, and tubes were incubated at 4°C for 2 h-16 h, rotating. Immunoprecipitations were performed using protein G-agarose beads (Roche) for 1 h. Immunoprecipitation of dynamin-GFP and Cbl-myc was performed using a polyclonal antibody against GFP (Clontech) or a monoclonal anti-c-myc antibody (Santa Cruz Biotechnology), respectively. Nonspecific proteins were removed by washing four times in mRIPA buffer. Immunoprecipitations from mouse OCLs were performed in a RIPA solution containing 10 mM Tris-Cl, 150 mM NaCl, 0.2% NP-40, 2 mM EDTA using the hudy1 antibody or a monoclonal anti-Cbl antibody (Trans-

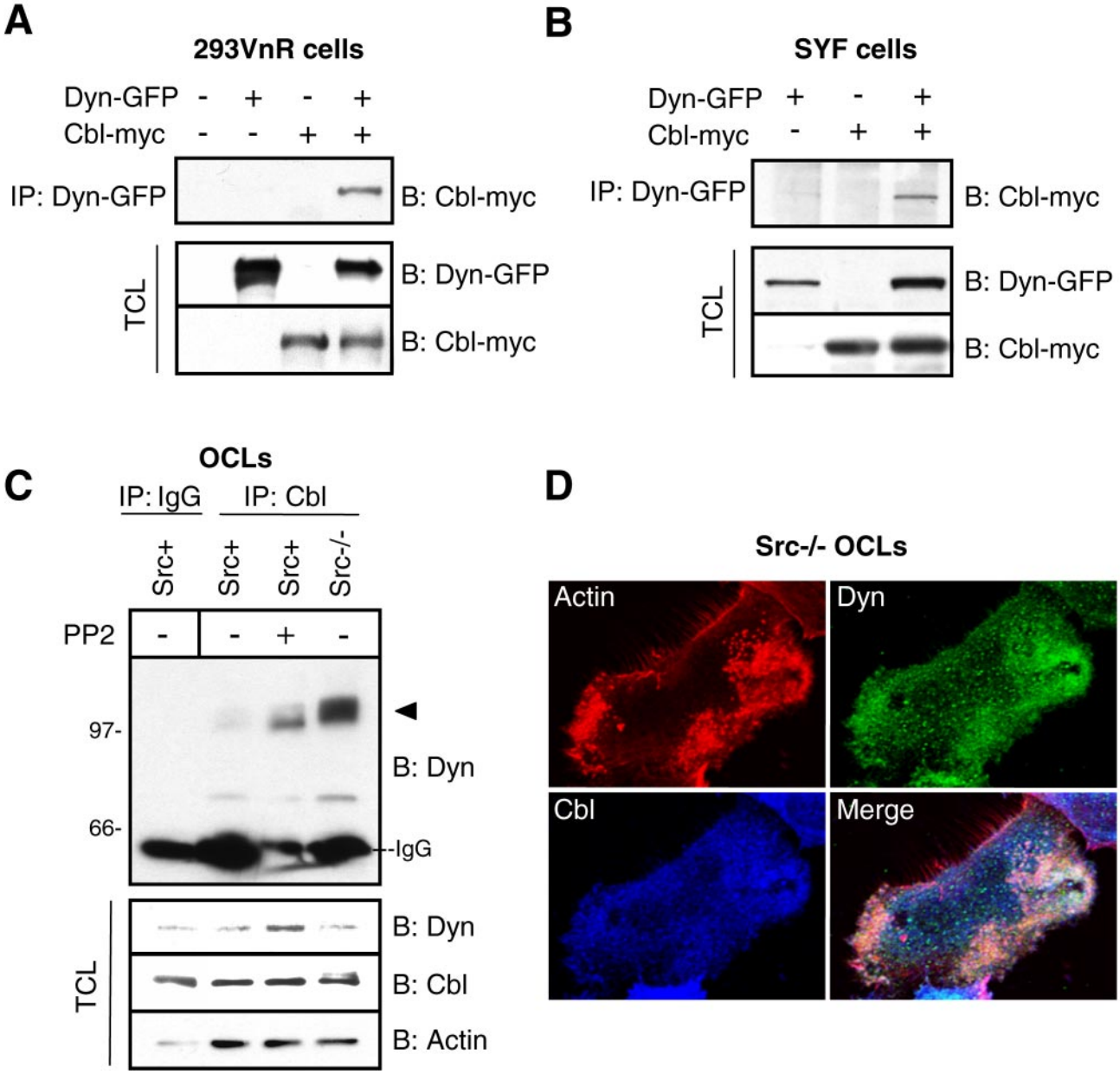

Figure 2. Dynamin associates with Cbl in osteoclasts. (A) Equivalent amounts of dyn-GFP, Cbl-myc, or empty vector were transiently transfected into 293VnR cells. Cell lysates were subjected to immunoprecipitation (IP) using a polyclonal anti-GFP antibody, and the precipitated proteins were resolved by SDS-PAGE and analyzed by Western blotting using a monoclonal anti-myc antibody. Total cell lysates were blotted with either anti-GFP or anti-myc antibodies to demonstrate levels of expression. (B) Dynamin and Cbl were cotransfected into SYF cells, which lack the Src family members Src, Yes, and Fyn. Cell lysates were processed by immunoprecipitation and Western blotting. (C) OCLs were differentiated from $Src^{+/?}$ (Src^{+}) or $Src^{-/-}$ bone marrow cells. Src^{+} OCLs were treated with 20 μM PP2 or vehicle control for 30 min before lysis. IPs were performed using an anti-Cbl antibody, and Western blotting was performed using the anti-dyn II antibody. Endogenous protein levels were determined by blotting of total cell lysates with anti-dynamin or anti-Cbl antibodies. As a control for nonspecific binding, IPs were also performed using an isotype-matched IgG1. (D) Labeling of osteoclasts derived from $Src^{-/-}$ mice with RITC-phalloidin (F-actin; red), anti-dynamin (green), or anti-Cbl (blue) revealed overlapping expression of actin, dynamin, and Cbl at distinct patches at the periphery of the cell. These F-actin–containing patches are commonly found in $Src^{-/-}$ osteoclasts which, unlike wild-type osteoclasts, fail to develop an organized actin belt at the cell periphery.

duction Laboratories). Beads were washed twice with the same RIPA solution and twice with RIPA containing a total of 225 mM NaCl. Samples were boiled in SDS-containing sample buffer under reducing conditions and proteins were resolved by SDS-PAGE, followed by Western blot analyses. Protein blots were visualized by ECL, films were scanned using FotolookPS 2.5 (Agfa, Ridgefield Park, NJ) and images manipulated with Adobe Photoshop.

GST-pulldown Assays

GST-pulldown assays were performed using the GST-tagged PRD domain of dynamin or with GST alone. Bacterial pellets were resuspended in B-Per buffer (Pierce, Rockford, IL), incubated for 15 min at room temperature, and pelleted. Cleared bacterial lysates were incubated overnight with prewashed

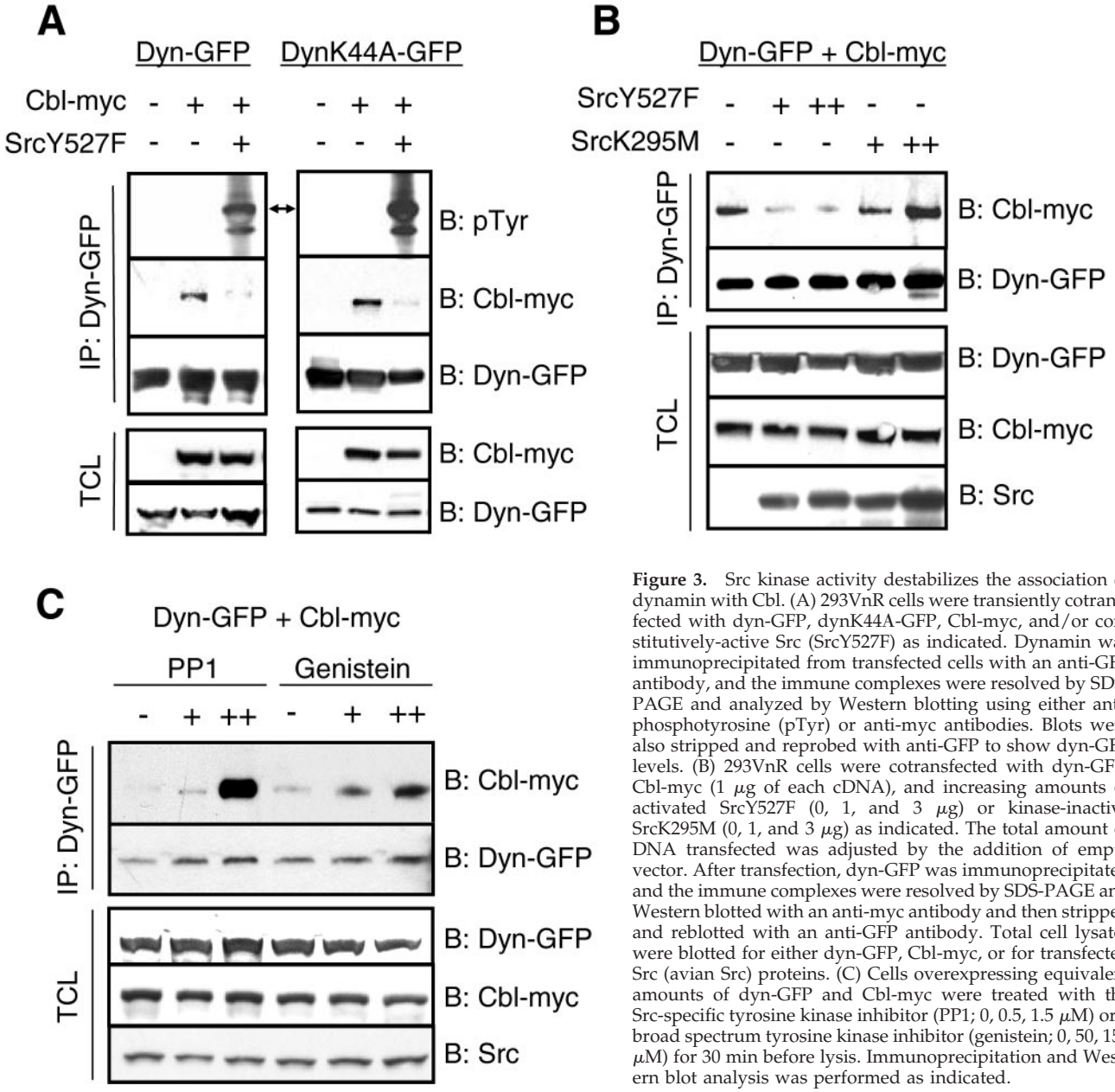

Figure 3. Src kinase activity destabilizes the association of dynamin with Cbl. (A) 293VnR cells were transiently cotransfected with dyn-GFP, dynK44A-GFP, Cbl-myc, and/or constitutively-active Src (SrcY527F) as indicated. Dynamin was immunoprecipitated from transfected cells with an anti-GFP antibody, and the immune complexes were resolved by SDS-PAGE and analyzed by Western blotting using either anti-phosphotyrosine (pTyr) or anti-myc antibodies. Blots were also stripped and reprobed with anti-GFP to show dyn-GFP levels. (B) 293VnR cells were cotransfected with dyn-GFP, Cbl-myc (1 μg of each cDNA), and increasing amounts of activated SrcY527F (0, 1, and 3 μg) or kinase-inactive SrcK295M (0, 1, and 3 μg) as indicated. The total amount of DNA transfected was adjusted by the addition of empty vector. After transfection, dyn-GFP was immunoprecipitated and the immune complexes were resolved by SDS-PAGE and Western blotted with an anti-myc antibody and then stripped and reblotted with an anti-GFP antibody. Total cell lysates were blotted for either dyn-GFP, Cbl-myc, or for transfected Src (avian Src) proteins. (C) Cells overexpressing equivalent amounts of dyn-GFP and Cbl-myc were treated with the Src-specific tyrosine kinase inhibitor (PP1; 0, 0.5, 1.5 μM) or a broad spectrum tyrosine kinase inhibitor (genistein; 0, 50, 150 μM) for 30 min before lysis. Immunoprecipitation and Western blot analysis was performed as indicated.

glutathione beads. Beads were then washed three times with Wash buffer (50 mM Tris.Cl, pH 7.5, 300 mM NaCl, 5 mM EDTA, 1% TX-100). OCL lysates prepared in mRIPA were incubated with dynamin-GST beads for 4 h followed by washing in Wash buffer. Proteins were resolved by SDS-PAGE and examined by Western blot analysis.

TRAP Staining

Cytological demonstration of tartrate-resistant acid phosphatase (TRAP) in osteoclasts was performed using the Leukocyte Acid Phosphatase labeling kit according to the manufacturer's instructions (Sigma).

Osteoclast Resorption Assay

Infected OCLs were plated onto dentin slices and cultured for 24–48 h. Dentin slices were washed, incubated in 6% NaOCl for 5 min, and sonicated for 10 s to remove cells. Resorption pits were stained with a solution containing 1% Toluidine Blue and 1% sodium borate for 3–4 min, washed with water, and air-dried. Pit surface area was quantified using the OsteoMeasure cell counting program (Osteometrics, Decatur, GA). Results were then normalized for osteoclast number, as measured by staining for TRAP activity and counting cells with more than three nuclei.

RESULTS

Dynamin Colocalizes with Cbl within the Podosome Actin Belt of Osteoclasts

We previously reported that dynamin was highly enriched in the actin podosome belt of RSV-transformed BHK cells and osteoclasts (Ochoa *et al.*, 2000). To further characterize the localization of dynamin in podosomes, we plated mouse osteoclasts on dentin or on serum-coated coverslips and examined dynamin expression by confocal immunofluorescence. In resorbing mouse osteoclasts plated on dentin, dynamin was enriched in the actin-rich sealing zone that delineates the resorptive space (Zambonin-Zallone *et al.*, 1988; Tanaka *et al.*, 1996; Figure 1A). Similarly, when plated on serum-coated coverslips, dynamin was highly concentrated at the periphery of the cell, where podosomes are localized as a belt. In some areas of the cell, dynamin tended to be

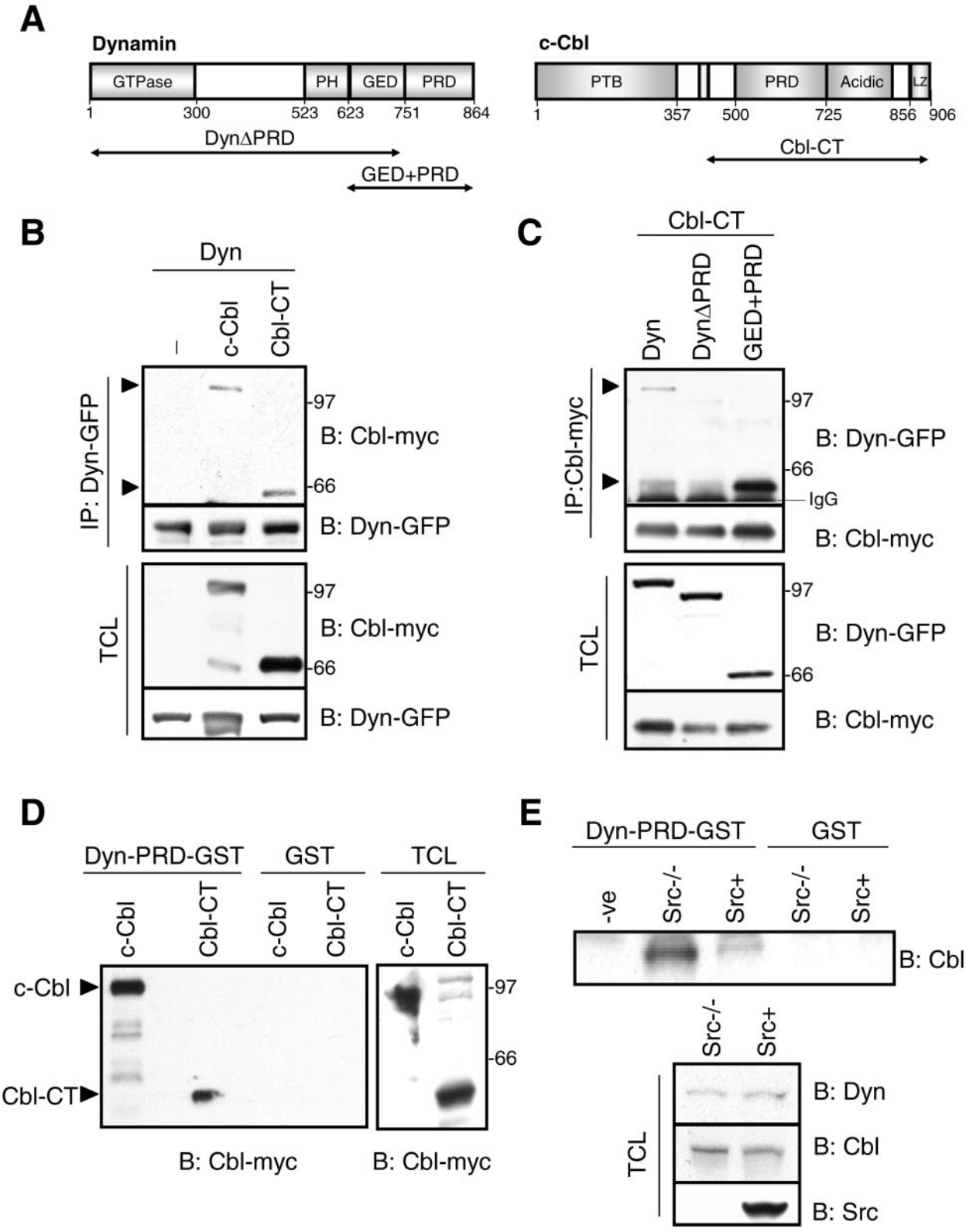

Figure 4. Dynamin-Cbl interaction is mediated via their respective PRD domains. (A) Schematic representation of the domain structures of dynamin and Cbl are shown. The phosphotyrosine-binding domain (PTB), proline-rich domain (PRD), acidic domain and leucine zipper (LZ) of Cbl are indicated. Dynamin contains a GTPase domain, a pleckstrin homology domain (PH), a GTPase effector domain (GED), and a PRD. (B) Full-length dyn-GFP was cotransfected with full-length Cbl-myc or the C-terminal half of Cbl (Cbl-CT), which was also myc-tagged. Immunoprecipitated dynamin-containing immune complexes were blotted for Cbl expression and subsequently stripped and reblotted for dyn-GFP. The arrows indicate the migration position of full-length Cbl and truncated Cbl-CT. (C) Myc-tagged Cbl-CT was coexpressed in 293VnR cells with GFP-tagged full-length dynamin, dynamin lacking only the PRD domain (dynΔPRD), or dynamin containing only the

found at the inner edge of the podosome belt (Figure 1, A and B). Closer examination of dynamin's distribution using high-power magnification and Z-series analyses revealed that dynamin was associated with a subset of podosomes in osteoclasts. Z-series analyses of individual podosome structures (Figure 1B, insets) further revealed that unlike the actin-binding protein vinculin (Figure 1B, left), which is an intrinsic component of podosomes and closely surrounds the actin core, dynamin is localized between individual podosomes (Figure 1B, middle).

Consistent with its role in signal transduction events downstream of integrins, Cbl is present within the actin-rich podosome belt of osteoclasts and affects cell migration and resorption (Tanaka *et al.*, 1996; Sanjay *et al.*, 2001; Chiusaroli *et al.*, 2003; Miyazaki *et al.*, 2004). Because both dynamin and Cbl are implicated in actin dynamics, we examined whether dynamin and Cbl exhibit overlapping subcellular localization in osteoclasts and might therefore be part of the same molecular signaling complex. Confocal analysis of the localization of Cbl in osteoclasts plated on dentin and on glass revealed that Cbl was expressed within the actin-rich sealing zone and podosome belt, respectively (Figure 1A). As was observed for dynamin, Cbl was uniformly distributed within and around individual podosomes (Figure 1B, left). However, although dynamin was associated with only some of the podosomes, Cbl was distributed throughout the podosome belt (Figure 1B, right).

Dynamin Forms a Molecular Complex with Cbl

The overlapping expression of dynamin and Cbl within the podosome belt of osteoclasts and the previously reported functional link of both proteins to actin dynamics suggested that dynamin and Cbl may functionally interact in a cellular context. We examined the molecular interaction of these proteins by overexpressing dynamin (dyn-GFP) and Cbl (Cbl-myc) in 293VnR cells. The association of the tagged proteins was then examined by coimmunoprecipitation and Western blot analysis. These studies demonstrated that dynamin and Cbl form a molecular complex in 293VnR cells (Figure 2A).

Src-induced phosphorylation of the pleckstrin homology domain (PH) of dynamin stimulates its GTPase activity (Ahn *et al.*, 2002), thereby affecting its function in receptor-mediated endocytosis (Herskovits *et al.*, 1993; Scaife *et al.*, 1994; Ahn *et al.*, 1999; Schmid *et al.*, 2000). Similarly, Src-induced phosphorylation of Cbl regulates the binding of Cbl to several of its downstream targets (Tanaka *et al.*, 1996; Yokouchi *et al.*, 2001; Kassenbrock *et al.*, 2002; Miyazaki *et al.*, 2004). We asked whether Src was required for the molecular association of dynamin and Cbl. Dynamin (dyn-GFP) and Cbl (Cbl-myc) were transiently expressed individually or in combination in SYF cells, a cell line that lacks the tyrosine kinases Src, Yes, and Fyn. Coimmunoprecipitations and Western blot analyses revealed that dynamin associates with Cbl even in the absence of the Src, Yes, and Fyn tyrosine kinases (Figure 2B), indicating that Src or other Src family kinases are not required to induce or mediate the formation of the dynamin- and Cbl-containing complex.

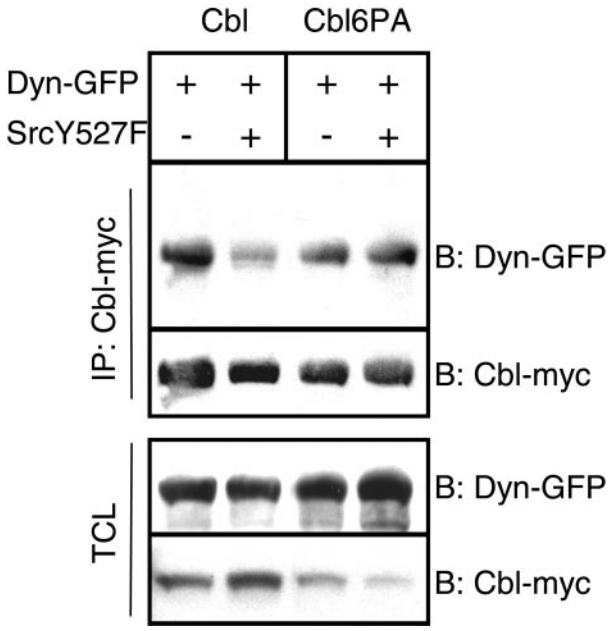

Figure 5. Binding of Cbl PRD by active Src destabilizes dynamin-Cbl association. Mutation of proline residues (amino acids 543–548) within the PRD domain of Cbl (Cbl6PA) decreases Cbl-Src binding and consequently reduces Src-dependent tyrosine phosphorylation of Cbl (Sanjay *et al.*, unpublished results). Here, dynamin was cotransfected with either Cbl or Cbl6PA (both myc-tagged) in the presence or absence of SrcY527F. Immunoprecipitation was performed with an anti-myc antibody, and Western blot analysis to determine levels of associated dynamin was performed using an anti-GFP antibody. Blots were then stripped and reprobed with anti-myc to determine immunoprecipitated Cbl. In the presence of activated SrcY527F, decreasing association of dynamin is seen with wild-type Cbl but not with Cbl6PA. Total cell lysates were blotted as indicated.

The Stability of the Dynamin-Cbl Complex Is Regulated by Src

To determine if endogenous dynamin and Cbl also interact, we performed coimmunoprecipitation experiments using lysates of $Src^{+/?}$ and $Src^{-/-}$ OCLs. Only a small amount of dynamin was coimmunoprecipitated with Cbl from the lysates of Src-expressing OCLs, but a much larger amount was coimmunoprecipitated from the $Src^{-/-}$ lysates (Figure 2C), suggesting that Src destabilizes the dynamin-Cbl complex. Consistent with this hypothesis, treating the Src-expressing OCLs with the Src family kinase inhibitor PP2 increased the amount of dynamin-Cbl complex recovered, although not to the level seen with the $Src^{-/-}$ OCLs.

The organization of podosomes into a circular actin belt at the periphery of the cell is dependent on Src kinase activity, because the podosome belt in osteoclasts derived from

Figure 4 (cont). GED plus PRD domains (GED+PRD). Coimmunoprecipitations were performed using anti-myc antibody followed by Western blot analysis with anti-GFP to detect dynamin, and later with anti-myc to detect Cbl. The arrows indicate the migration position of full-length dynamin and truncated dynamin (GED+PRD). (D) Myc-tagged full-length Cbl or Cbl-CT were transfected into 293VnR cells. GST-pulldown assays were performed using the GST-tagged PRD domain of dynamin or with GST alone. Proteins were resolved by SDS-PAGE and Western blot analysis was performed using an anti-myc antibody to detect Cbl proteins. (E) Lysates prepared from $Src^{-/-}$ or $Src^{+/?}$ (Src+) OCLs were subjected to GST-pulldown using dyn-PRD-GST or GST alone. Membranes were blotted for Cbl expression and total cell lysates were blotted for Cbl, dynamin, and Src protein expression. Dyn-PRD bound less Cbl in lysates from Src^{+} OCLs than in lysates from $Src^{-/-}$ OCLs, confirming that Src destabilizes the dynamin-Cbl association in osteoclasts.

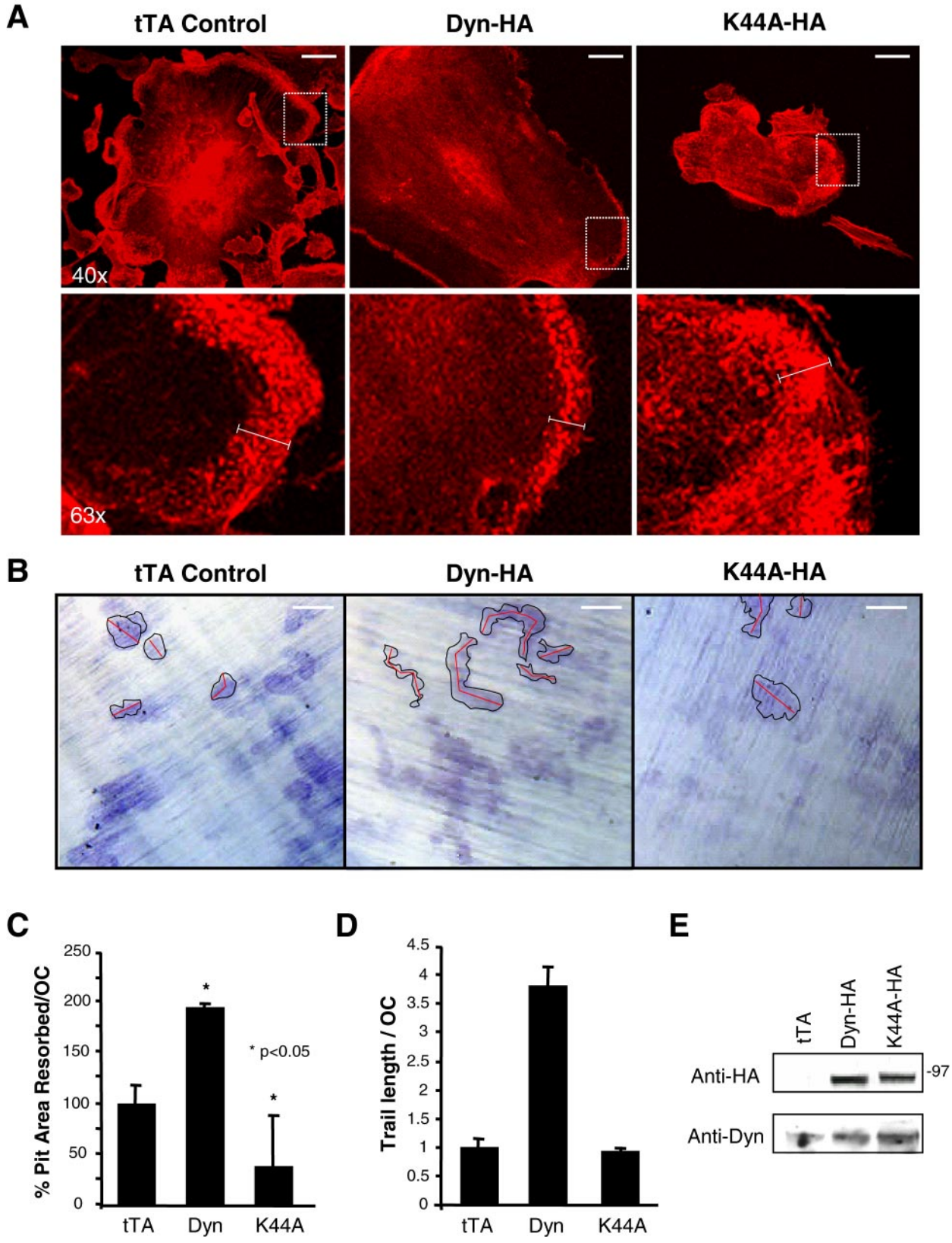

Figure 6. Dynamin GTPase activity stimulates osteoclast migration and resorption. (A) OCLs were infected with adenovirus encoding HA-tagged wild-type dynamin (dyn-HA) or mutant dynamin (dynK44A-HA) in the presence of the tTA transcription activator. Two days after infection, cells were replated on FCS-coated glass coverslips and incubated for an additional 24 h. Cells were stained with RITC-phalloidin and representative images are shown. Insets: high-power magnifications (63×) showing changes in the thickness of the podosome belt (hashed line). Scale bar, 5 μm. Note decreased width of the podosome band in dyn-HA–infected OCLs relative to tTA control, whereas

$Src^{-/-}$ mice is disorganized and is replaced by dense actin patches, resulting in impaired osteoclast migration and bone resorption (Tanaka *et al.*, 1996; Sanjay *et al.*, 2001). Because dynamin-Cbl binding was enhanced in $Src^{-/-}$ OCLs, we examined the distribution of dynamin and Cbl in authentic osteoclasts from $Src^{-/-}$ mice. Confocal microscopy revealed that dynamin was localized to dense F-actin–rich patches (Figure 2D). Analysis of the distribution of Cbl in the $Src^{-/-}$ osteoclasts also revealed the presence of Cbl in the F-actin–containing structures where it colocalized with dynamin. These findings demonstrate that the interaction of dynamin with Cbl occurs in Src-expressing OCLs and in $Src^{-/-}$ OCLs in which podosome organization is disrupted, suggesting that dynamin-Cbl interaction may be functionally significant in osteoclasts.

Dynamin-Cbl Interaction Is Destabilized by Src Kinase Activity but Is Independent of GTP Binding and Hydrolysis

The increased binding of dynamin with Cbl observed in lysates from $Src^{-/-}$ OCLs and in lysates from wild-type OCLs treated with PP2 suggested that Src kinase activity negatively regulates dynamin-Cbl complex formation. Moreover, our studies confirmed that dynamin and Cbl form a molecular complex when overexpressed in 293VnR cells. To further investigate whether Src-catalyzed phosphorylation of either dynamin or Cbl potentially affected the molecular association of these proteins, we overexpressed combinations of dynamin (dyn-GFP) and Cbl (Cbl-myc) and a constitutively active mutant of Src (SrcY527F) in 293VnR cells. In addition, we also compared the association of Cbl with dynK44A, which is impaired in its GTP binding and hydrolysis (Damke *et al.*, 1994; Warnock *et al.*, 1995).

As shown in Figure 3A, both dynamin and dynK44A were coimmunoprecipitated with Cbl in the absence of SrcY527F (lane 2). Constitutively active SrcY527F induced the phosphorylation of both dynamin and dynK44A, confirming the expression of activated Src in these transfected cells. However, overexpression of SrcY527F significantly decreased the association of Cbl with either dynamin or dynK44A, consistent with the low amount of dynamin-Cbl complexes that were immunoprecipitated from $Src^{+/?}$ OCLs (Figure 2C), which express large amounts of endogenous Src (Horne *et al.*, 1992). To verify the role of Src kinase activity on dynamin-Cbl association, we performed similar coimmunoprecipitation experiments using cells transfected with variable amounts of cDNA encoding kinase-active (SrcY527F) or kinase-inactive Src (SrcK295M; Figure 3B). SrcY527F decreased dynamin-Cbl association in a dose-dependent manner, whereas SrcK295M dose-dependently enhanced the interaction of dynamin with Cbl, presumably as a consequence of the displacement of endogenous c-Src. Similarly, a dose-dependent increase of the dynamin-Cbl complex was observed after inhibition of Src activity with the Src kinase inhibitor, PP1, or the nonspecific tyrosine kinase inhibitor, genistein (Figure 3C). Thus, the dynamin-Cbl interaction is negatively regulated by Src tyrosine kinase activity in OCLs and in 293VnR cells, suggesting that at steady state the interaction of dynamin with Cbl is regulated similarly in these cells. Moreover, neither the dynamin-Cbl association nor the Src-mediated destabilization of the dynamin-Cbl complex require the binding or hydrolysis of GTP by dynamin because both effects were observed using the dynK44A mutant.

Figure 6 (cont). dynK44A-HA–infected OCLs contain more actin stress fibers. (B) Mouse OCLs infected with dyn-HA or dynK44A-HA adenovirus (plus tTA virus) were replated on dentin slices and incubated for 24 h. Osteoclast resorption pits were stained with toluidine blue and photographed (representative resorption pits are outlined). Scale bar, 5 μm. (C) The surface area of toluidine blue–stained resorption pits/trails was determined using the OsteoMeasure program. Resorption activity of infected osteoclasts (arbitrary units) was normalized for the number of TRAP-positive, multinucleated osteoclast-like cells. Error bars, SEM; the differences were statistically significant ($p < 0.05$) as determined by Student's *t* test. (D) The average length of resorption trails formed by dyn-HA–infected and dynK44A-HA–infected OCLs, relative to control tTA-infected cells, was determined using the OsteoMeasure program, and the results were adjusted for the number of osteoclasts. (E) Dynamin expression after infection with dyn-HA or dynK44A-HA was determined by Western blot using an anti-HA antibody or the pan anti-dynamin antibody (Hudy2). As shown, adenovirus infection at an MOI of 100 led to a 2–3-fold increase in dynamin expression with comparable amounts of dyn-HA and dynK44A-HA being expressed.

Dynamin-Cbl Association Requires the Proline-rich Sequences of Both Proteins

Inspection of the domain structure of Cbl and dynamin does not reveal modules or domains predicted to interact with each other (Figure 4A), suggesting that dynamin is unlikely to interact directly with Cbl. To determine which molecular domains are likely to be involved in dynamin-Cbl complex formation, we examined the ability of several truncated dynamin and Cbl proteins to interact in coimmunoprecipitation studies. First, we transfected full-length dynamin with either full-length Cbl or the C-terminal half of Cbl (Cbl-CT; residues 479–906), which includes the proline-rich, acidic, and leucine zipper domains of Cbl. Coimmunoprecipitation experiments demonstrated that both full-length Cbl and the Cbl-CT interacted with full-length dynamin (Figure 4B). Next, we cotransfected Cbl-CT with either full-length dynamin, dynamin lacking only the PRD (ΔPRD) or a construct containing only the GTPase effector domain (GED) and the PRD of dynamin (GED+PRD). Both full-length dynamin and the dynamin GED+PRD protein coimmunoprecipitated with the C-terminal domain of Cbl, whereas dynΔPRD failed to bind the Cbl-CT (Figure 4C).

The above studies suggested that the dynamin PRD mediates its interaction with the C-terminal domain of Cbl. To test this conclusion, we transfected 293VnR cells with either Cbl or Cbl-CT and performed pull down assays using a dynamin-PRD–GST fusion protein (Figure 4D). These studies demonstrated an interaction of the dynamin PRD with the full-length Cbl as well as with the C-terminal domain of Cbl. To determine whether the interaction of Cbl with the PRD of dynamin, like Cbl's interaction with full-length dynamin, was modulated by Src activity, we performed dynamin PRD-GST-pulldown assays using cell lysates from both $Src^{+/?}$ and $Src^{-/-}$ OCLs (Figure 4E). While endogenous Cbl in lysates of $Src^{+/?}$ cells was only weakly associated with the PRD of dynamin, increased association of dynamin with Cbl was observed when lysates from $Src^{-/-}$ cells were used, providing further evidence that Src activity inhibits dynamin-Cbl association. These studies suggest that Src-catalyzed phosphorylation of endogenous Cbl contributes to the dissociation of the dynamin-Cbl complex.

Docking of Src to an SH3-binding Site in the PRD of Cbl Is Required for Destabilization of the Cbl-Dynamin Complex

Earlier studies revealed that Src binds to Cbl primarily via Src's SH3 domain (Tanaka *et al.*, 1996; Sanjay *et al.*, 2001).

Furthermore, Src-catalyzed phosphorylation of Cbl decreased the binding of Cbl to the ubiquitin conjugating protein, UbcH7 (Yokouchi *et al.*, 2001). As discussed above, the association of dynamin with Cbl most likely occurs via the proline-rich sequences of both proteins. We therefore asked whether the binding of Src and the subsequent tyrosine phosphorylation of Cbl affects the formation of the dynamin-Cbl complex. To this end, we created a Cbl mutant, Cbl6PA, which contains six proline-to-alanine substitutions (residues 543–548) within its proline-rich domain and which exhibits decreased binding to Src and as a consequence, decreased phosphorylation by Src (Sanjay *et al.*, unpublished results). The Cbl6PA mutant was coexpressed with dynamin in the presence or absence of activated SrcY527F. Activated SrcY527F decreased the association of dynamin with wild-type Cbl but had little effect on the binding of Cbl6PA to dynamin (Figure 5). These studies suggest that the recruitment of Src through the PRD of Cbl is required to destabilize the dynamin-Cbl complex, possibly due to phosphorylation of Cbl as demonstrated for the Cbl-UbcH7 interaction (Yokouchi *et al.*, 2001).

The GTPase Activity of Dynamin Regulates Osteoclast Morphology and Function

We previously reported that overexpression of dynK44A delayed actin turnover and podosome formation in BHK-RSV cells (Ochoa *et al.*, 2000). We also established that the Src-Cbl complex plays a key role in podosome formation, affecting both cell motility and bone resorption (Sanjay *et al.*, 2001). Therefore, we examined whether dynamin function affects the properties of osteoclasts in which bone resorbing activity is dependent upon actin remodeling. Using the adenovirus expression system, we introduced either wild-type dynamin (dyn-HA) or GTPase defective dynK44A (dynK44A-HA) into osteoclasts and examined changes in osteoclast morphology, bone resorption, and cell migration. OCLs were infected with a multiplicity of infection (MOI) of 100, resulting in a 2–3-fold higher expression of dynamin or dynK44A, relative to endogenous dynamin. OCLs that overexpressed dynamin were larger than control cells and exhibited a thinner podosome belt at the periphery of the cell (Figure 6A). Overall, in areas of the cell where podosomes were present, the width of the podosome belt was reduced by ~30% ($p < 0.05$) in dynamin-infected OCLs, relative to control-infected cells. In contrast, OCLs infected with dynK44A were smaller than control cells containing an equivalent number of nuclei and dynK44A-infected cells contained more actin-stress fibers than the cells infected with wild-type dynamin. Podosomes were also present in dynK44A-infected cells, although in many cells the podosomes were highly disorganized and were not clearly localized as a belt at the periphery of the cell. In dynK44A-infected cells in which a podosome belt was observed at the periphery of the cell, the average thickness of the podosome belt was similar to controls. tTA-infected cells (control virus) appeared similar to uninfected OCLs (unpublished data).

The effects of dynamin activity on osteoclast bone resorbing activity in vitro was examined by overexpressing wild-type dynamin and dominant-negative dynK44A in OCLs. Infected OCLs were then cultured on dentin, and resorption pits were stained with toluidine blue. The size and shape of the resorbed area was quantified as a measure of osteoclast activity. Dynamin-infected OCLs exhibited long serpentine-like resorption trails (Figure 6B, middle), rather than the circular pits or short resorption trails observed in control tTA-infected cells (Figure 6B, left). The average length of the pits formed by the dynamin-infected OCLs was fourfold greater than the length of the pits formed by either the tTA-infected or the dynK44A-infected OCLs (Figure 6D), suggesting that dynamin modulates the actin cytoskeleton in a manner that is dependent on dynamin's GTPase activity, leading to an increase in osteoclast migration on dentin.

To further examine the effect of dynamin on the resorbing activity of osteoclasts, we quantified the total surface area resorbed by adenovirus-infected OCLs. The results revealed a statistically significant increase in the area of resorption after expression of dynamin in OCLs, compared with control-infected cells (Figure 6C). In contrast, a decrease in total area of resorption per osteoclast was observed when OCLs were infected with the GTP-binding mutant dynK44A.

DISCUSSION

Podosomes are highly dynamic actin-containing adhesion structures found in motile cells such as osteoclasts and macrophages (Marchisio *et al.*, 1987; Ochoa *et al.*, 2000). Unlike focal adhesions, which are relatively stable structures, podosome clusters assemble and disassemble within minutes in a process involving the rapid polymerization and depolymerization of the central actin core (Destaing *et al.*, 2003). Of relevance to actin turnover in osteoclasts, dynamin has been linked to actin dynamics through its interaction with actin regulatory proteins such as cortactin and N-WASP (Schafer, 2002; Orth and McNiven, 2003; McNiven *et al.*, 2004), which are also components of podosomes (Linder and Aepfelbacher, 2003). Fluorescence recovery after photobleaching (FRAP) studies demonstrated that actin turnover in podosomes was significantly reduced in BHK-RSV cells expressing the GTP-binding dynamin mutant dynK44A, relative to wild-type dynamin (Ochoa *et al.*, 2000), indicating that dynamin promotes podosome disassembly.

Consistent with our previous study (Ochoa *et al.*, 2000), we found that dynamin and Cbl, like actin and vinculin, were enriched within the podosome belt of mouse osteoclasts. However, in contrast to vinculin, which closely surrounds the actin core, dynamin and the adaptor protein Cbl exhibited a partially overlapping distribution throughout the podosome belt. The localization of dynamin and Cbl at podosomes suggested that these two proteins may be part of the same signaling complex in osteoclasts. This was supported by the demonstration that dynamin and Cbl were coimmunoprecipitated from osteoclasts and from 293VnR cells. The association of dynamin with Cbl occurred in the absence of c-Src and was independent of the Src family kinases Yes and Fyn as shown in the $Src^{-/-}$ OCLs and SYF cells, respectively. Importantly, Src tyrosine kinase activity destabilized the association of dynamin with Cbl, whereas overexpression of the dominant-negative kinase-inactive Src mutant SrcK295M or chemical inhibition of Src activity enhanced the association of dynamin with Cbl. This was true for overexpressed proteins in 293VnR cells as well as for endogenous proteins expressed in OCLs. Furthermore, we found that the recruitment of Src to the proline-rich domain of Cbl was required for Src-induced destabilization of the dynamin-Cbl complex. Because Src-catalyzed phosphorylation of dynamin's PH domain is known to promote dynamin self-assembly, resulting in an increase in its GTPase activity (Ahn *et al.*, 2002), it is possible that Src-mediated phosphorylation of dynamin may also contribute to the disruption of the dynamin-Cbl complex. Our GST pulldown studies demonstrated that the dynamin PRD interacted more strongly with endogenous Cbl from $Src^{-/-}$ OCLs than from $Src^{+/?}$ OCLs. This suggests that phosphorylation of dynamin by

Src is not required for the destabilization of the dynamin-Cbl complex.

The association of dynamin with Cbl occurs via their respective PRD domains and is therefore likely to be indirect and mediated by a scaffolding protein containing multiple SH3 binding domains. While numerous SH3-containing proteins exist that could potentially fulfill this role, we have identified Grb2 as a possible Cbl-binding protein in osteoclasts (Sahni *et al.,* 1996). Furthermore, Grb2 is known to bind to dynamin (Gout *et al.,* 1993) and in osteoclasts, Grb2 was present in a molecular complex containing both dynamin and Cbl (our unpublished observations). Interestingly, binding of Grb2 to dynamin's PRD increases dynamin's GTPase activity (Herskovits *et al.,* 1993; Barylko *et al.,* 1998) and Grb2 also enhances WASP-mediated Arp2/3-dependent actin nucleation (Carlier *et al.,* 2000; Pantaloni *et al.,* 2000; Castellano *et al.,* 2001), therefore providing an additional molecular link between dynamin and actin dynamics in podosomes.

Although endogenous dynamin and Cbl form a complex in osteoclasts, this does not imply that the entire pool of Cbl associates with dynamin nor that the stoichiometry of the two proteins would allow this association to be exclusive of others. Instead, it is likely that the interaction of dynamin, Cbl, and Src with each other and with their respective binding partners is dynamic in nature. This would allow for the formation of distinct, temporally regulated signaling complexes with Src potentially acting as a molecular switch to coordinate Cbl-dependent and dynamin-dependent signaling events. For example, it was recently reported that Src activity decreases the association of dynamin with the SH3-containing adaptor protein endophilin (Wu *et al.,* 2004). Like dynamin, endophilin is implicated in receptor-mediated endocytosis (Verstreken *et al.,* 2002) and has been shown to localize to podosomes in v-Src–transformed fibroblasts (Ochoa *et al.,* 2000). A number of observations also suggest that Cbl's N- and C-terminal halves interact and that phosphorylation-induced conformational changes in Cbl mask or unmask binding sites at various locations on the protein (Bartkiewicz *et al.,* 1999; Take *et al.,* 2000; Kirsch *et al.,* 2001; Soubeyran *et al.,* 2002). Moreover, it has been shown that Cbl can form a complex with endophilin and with the multidomain adaptor protein CIN85, which mediates the internalization and ubiquitination of receptor tyrosine kinases (Take *et al.,* 2000; Watanabe *et al.,* 2000; Dikic, 2002; Haglund *et al.,* 2002; Petrelli *et al.,* 2002; Soubeyran *et al.,* 2002). Thus, Src-mediated dissociation of dynamin and Cbl would potentially enable dynamin and Cbl proteins to form alternate protein signaling complexes.

In osteoclasts, Cbl affects both migration (Sanjay *et al.,* 2001; Chiusaroli *et al.,* 2003) and bone resorbing activity in vitro (Tanaka *et al.,* 1996). Results in this study suggest that dynamin also plays a role in osteoclast function. Accordingly, overexpression of dynamin in OCLs increased OCL spreading in a way that was functionally linked to podosome number and to the bone resorbing activity of these cells. Conversely, expression of dynK44A, which exhibits reduced GTP binding and hydrolysis (Damke *et al.,* 1994; Warnock *et al.,* 1995), increased actin stress fiber formation and decreased bone resorption. It is tempting to speculate that modulation of the actin cytoskeleton, and more specifically podosome disassembly by dynamin regulates osteoclast shape, motility, and function.

Understanding the actions of key proteins involved in podosome assembly and disassembly is essential to our understanding of the mechanisms by which highly motile cells such as osteoclasts migrate, attach, and resorb bone. In this study, we identified a dynamic molecular signaling complex containing dynamin and Cbl that occurs independently of the GTPase activity of dynamin, but is negatively regulated by Src tyrosine kinase activity. Moreover, overexpression of dynamin resulted in increased osteoclast resorption and migration in vitro. This is consistent with earlier findings demonstrating that dominant-negative dynamin is targeted to podosomes and delays actin turnover in cells that express activated Src (Ochoa *et al.,* 2000). Based on these studies, we propose that dynamin and Cbl coordinately participate in a Src-regulated process that controls signaling pathways, leading to podosome turnover and, thereby, osteoclast motility and resorption.

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