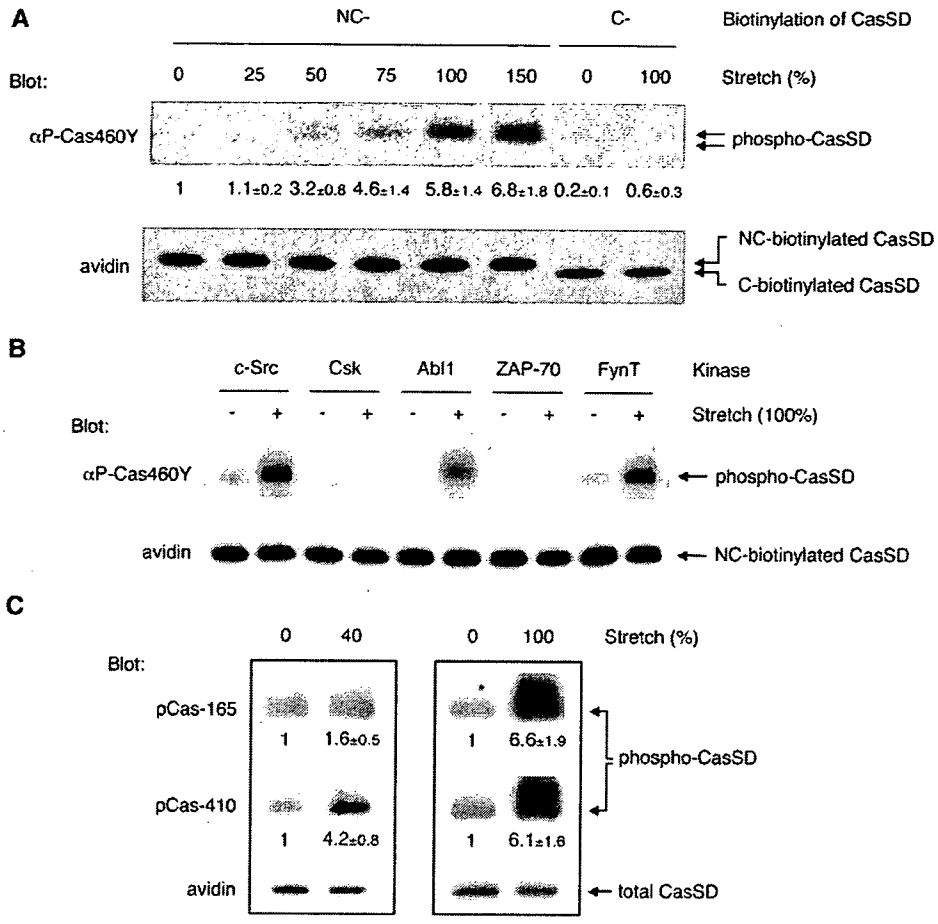

Figure 4. Extension-Dependent Phosphorylation of CasSD by Tyrosine Kinases In Vitro

(A) CasSD is tyrosine phosphorylated by recombinant c-Src in an extension-dependent manner. NC-biotinylated or C-biotinylated CasSD was either extended or left unextended on latex membrane, incubated with recombinant c-Src for 2 min, washed, solubilized, and analyzed for tyrosine phosphorylation by anti-phospho-Cas (αP-Cas460Y) immunoblotting and avidin affinity blotting. The magnitude of the latex membrane stretching is described as the percent change of length in each dimension. Quantification of phosphorylation of CasSD was scaled with unextended NC-biotinylated CasSD set at 1 and noted below the anti-phospho-Cas blot with SD (n = 4).

(B) Kinase specificity of extension-dependent tyrosine phosphorylation of CasSD. NC-biotinylated CasSD was either extended (100%) or left unextended and then incubated with recombinant c-Src, Csk, Abl1, ZAP-70, or FynT for 2 min at room temperature. Tyrosine phosphorylation of CasSD was analyzed as in (A).

(C) Extension-dependent phosphorylation of CasSD by c-Src measured by two different anti-phospho-Cas antibodies. Samples were prepared as in (A) except for the extent of the latex membrane stretching (40% in the left panel and 100% in the right panel). Equivalent portions of each sample were subjected to SDS-PAGE followed by pCas-165 and pCas-410 immunoblotting and avidin affinity blotting. Quantification of phosphorylation of CasSD was scaled with unextended NC-biotinylated CasSD set at 1 and noted below the anti-phospho-Cas blots with SD (n = 4).

reflection fluorescence (TIRF) or confocal microscope. Further, the stretchable substrate (silicone) had high background fluorescence. Therefore, we looked at αCas1 immunostaining of intact cells during the late phase of spreading on collagen-coated glass coverslips (20 min after plating), when the fast movement of actin cytoskeletons at the periphery is observed (Dubin-Thaler et al., 2004) and the forces required for continuous spreading are generated (Giannone et al., 2004). In RFP-Cas-expressing Cas-deficient fibroblasts, we found that αCas1 staining primarily colocalized with RFP-Cas in the peripheral regions (Figure 5C, top). An anti-phospho-Cas antibody (pCas-165) also exhibited a peripheral staining in the late spreading cells (Figure 5C, bottom), confirming that Cas extension correlated with phosphorylation. These staining patterns did not appear to be artifacts of antibody staining, since αCas3 staining always colocalized with RFP-Cas (Figure 5C, middle). Considering the specificity of αCas1 and αCas3 in immunoblotting (Figure S1C), both αCas1 and αCas3 staining most likely represent the distribution of their Cas epitopes, and not the crossreaction with other cellular protein(s). Indeed, we observed

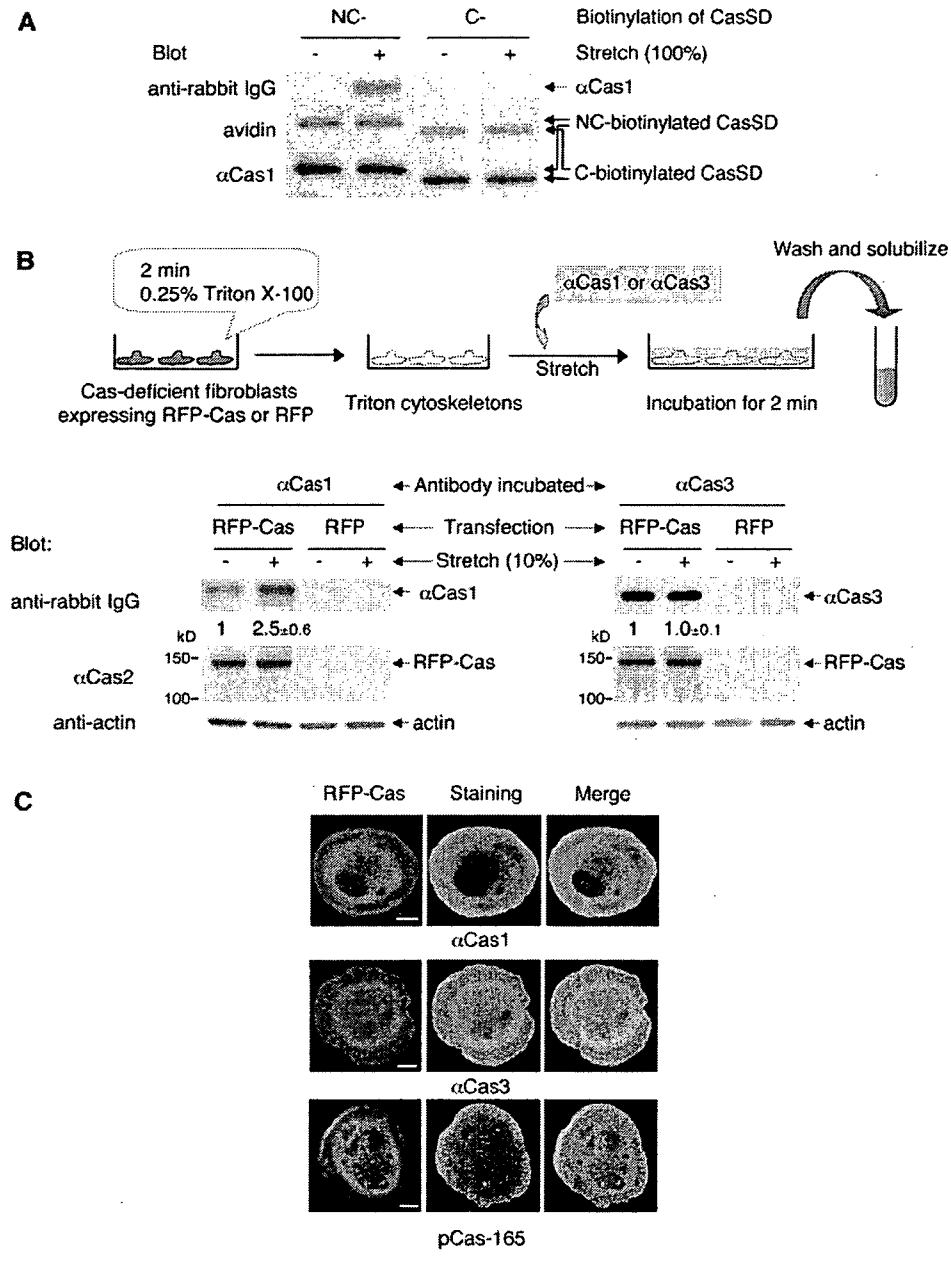

Figure 5. Extension of Cas In Situ and In Vivo

(A) αCas1 recognizes extended CasSD in vitro. NC-biotinylated or C-biotinylated CasSD was either extended (100%) or left unextended in the IPE system. After blocking, CasSD proteins were incubated with αCas1, washed, and solubilized with SDS sample buffer containing 0.12 M DTT. Equivalent portions of each sample were analyzed for quantification of bound αCas1 by anti-rabbit IgG immunoblotting. The amount of NC-biotinylated and C-biotinylated CasSD in each sample was quantified by avidin affinity blotting and αCas1 immunoblotting. Note that the difference in the relative signal intensity between avidin and αCas1 blots is consistent with the molar ratio of biotinylation (NC-biotinylated CasSD:C-biotinylated CasSD ≒ 2:1).

(B) Stretch dependence of αCas1 and αCas3 binding to Cas in Triton cytoskeletons. Triton cytoskeletons were prepared from Cas-deficient fibroblasts transfected with RFP-Cas or RFP alone, either stretched or left unstretched, and incubated with either αCas1 or αCas3 as shown in the diagram. Quantification of bound antibody by anti-rabbit IgG immunoblotting was scaled with unstretched control set at 1 and noted with SD (n = 4).

(C) αCas1 preferentially binds to Cas, where higher traction forces are expected in vivo. Cas-deficient fibroblasts expressing RFP-Cas were plated, fixed after 20 min, and then stained with αCas1, αCas3, or pCas-165. Confocal images are shown for RFP-Cas (left, red channel) and immunostaining (center, green channel) and are merged on the right. Scale bars, 10 μm.

only faint background staining in untransfected Cas-deficient fibroblasts with either αCas1 or αCas3 (data not shown).

These in situ (cytoskeleton stretching) (Figure 5B) and in vivo (intact cell spreading) (Figure 5C) results, together with the observed preference of αCas1 binding for extended CasSD in vitro (Figure 5A), suggest that the in vitro extension of CasSD causes the conformational change of CasSD that is relevant to the force-dependent conformational change of Cas protein in vivo. Therefore, the extension-dependent phosphorylation of CasSD in vitro (Figure 4) appears to be relevant to the force-dependent phosphorylation of Cas in vivo (Figures 1, 2, and 5C).

DISCUSSION

Tyrosine Phosphorylation of Cas Is Involved in Physiological Force Transduction

Cas appears to act as a force transducer in vivo, since the knockdown of Cas expression by siRNA significantly attenuated stretch-dependent Rap1 activity (Figure 2A) and overexpression of wild-type Cas, but not coexpression of the phosphorylation-defective Cas mutant (Cas15YF) enhanced stretch-dependent Rap1 activity (Figure 2B). Phosphorylation by SFK is critical since stretch-dependent Cas phosphorylation was inhibited by the SFK inhibitor CGP77675 (Figure 1A) and attenuated in SYF cells (Figures 1B and 1C). These findings conform to our previous observation of stretch-dependent Cas phosphorylation by SFK in cytoskeletal complexes (Triton cytoskeletons) (Tamada et al., 2004) and indicate that tyrosine phosphorylation of Cas upon cell stretching constitutes a significant pathway for stretch-dependent Rap1 activation in intact cells (Sawada et al., 2001).

Possible Mechanisms for Stretch-Increased Cas Phosphorylation

Although Src was shown to be mechanically activated using genetically engineered reporters (Wang et al., 2005), it is not clear how endogenous c-Src is activated or if it is indirectly activated by force. We observed that Src-dependent phosphorylation of Cas was significantly increased by stretching in c-Src-expressing SYF cells without causing Src kinase activation (Figures 1B and 1C). These findings suggest that activation of the kinase is not primarily responsible for stretch-dependent increase of Cas phosphorylation in vivo. Since the SFK inhibitor greatly attenuated the stretch-dependent increase of Cas phosphorylation (Figure 1A), stretch-dependent alteration of phosphatase activity is also unlikely to be the cause. Further, the tyrosine phosphatase inhibitor sodium orthovanadate did not inhibit stretch-dependent tyrosine phosphorylation of Cas in Triton cytoskeletons (Tamada et al., 2004).

It is unlikely that stretching causes the spatial interaction between the kinase and the substrate, considering the constraints that such a mechanism places on the geometry of the cytoskeleton (Tamada et al., 2004). Since a mechanical modification of the substrate (Cas) was sufficient to increase Cas phosphorylation in vitro, we have focused on the analysis of that possibility.

Extension of Cas by Cellular Force

Both the amino-terminal SH3 and carboxy-terminal Src-binding domains are required for Cas localization at focal adhesions (Nakamoto et al., 1997), where cellular force is expected to be concentrated, and force-dependent signaling involving tyrosine phosphorylation occurs (Geiger and Bershadsky, 2002; Tamada et al., 2004). Since different proteins are known to associate with SH3 and Src-binding domains of Cas (Defilippi et al., 2006), an individual Cas molecule would be anchored to the cytoskeleton-adhesion complex via two distinct sites. In addition, the SH3 domain of Cas binds to FAK (focal adhesion kinase) (Harte et al., 1996). FAK has a FERM (erythrocyte band *4.1*-ezrin-*r*adixin-*m*oesin) domain commonly found in actin-binding proteins (Lee et al., 2004), associates with an actin-binding protein, talin (Chen et al., 1995), and is involved in the dynamic variation in tyrosine phosphorylation within focal adhesions (Ballestrem et al., 2006). Thus, we speculate that the amino-terminal anchor of Cas to the focal adhesion complex is more closely linked to the actin cytoskeleton than the carboxy-terminal anchor and that Cas is subjected to traction forces generated by the actin cytoskeleton (Figure 6).

Although the structures of the SH3 and the serine-rich domains of Cas were reported (Briknarova et al., 2005; Wisniewska et al., 2005), no structural analysis of the substrate domain has been reported, and the structure prediction algorithms (available at the Network Protein Sequence Analysis site) do not give a clear prediction for the structure of Cas substrate domain. We speculate that the intramolecular interactions within the substrate domain constrain its conformation in the absence of traction force and that traction force is required to expose YxxP sites to kinases.

Because our model centers on extension of Cas, the magnitude of force needed for extension is a concern. The force per integrin molecule in the adhesion site was estimated to be on the order of 1 pN (Balaban et al., 2001; Jiang et al., 2003), an order of magnitude below the force needed to reversibly unfold single domains of proteins such as spectrin by AFM (atomic force microscope) (Fisher et al., 1999). However, it has been shown that the protein unfolding force depends exponentially on the loading rate (Carrion-Vazquez et al., 1999), and at a low loading rate, proteins can be unfolded by forces even orders of magnitude below the forces required for unfolding at a high loading rate (Merkel et al., 1999). Further, extension of Cas may not require the force needed to cause "unfolding," i.e., linearization of a mechanically stable distinct structure. Thus, extension of Cas probably can occur by physiological forces at focal contact sites (order of a few pN). Further details of force-dependent extension and phosphorylation of the Cas substrate domain in vitro as well as in vivo are under study.

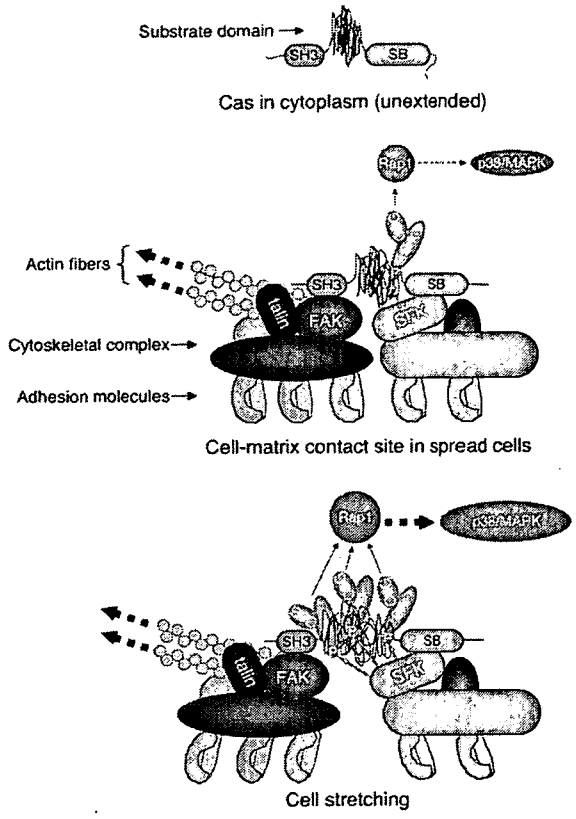

Figure 6. Model of Extension of Cas and Signaling at Cell-Matrix Contact Sites

The top and middle panels represent a Cas molecule with unextended configuration of substrate domain in the cytoplasm and a Cas molecule with moderate extension of substrate domain at the cell-matrix contact site of spread cells, respectively. The bottom panel represents the extension-dependent phosphorylation of the Cas substrate domain by SFK and enhancement of its downstream signaling. SH3 and SB represent the SH3- and the Src-binding domains of Cas, respectively.

Physiological Role of Extension and Phosphorylation of Cas in Force Transduction

Several different experimental approaches indicate that Cas extension plays a role in the direct sensing of traction forces in vivo. In vitro, extension of NC-biotinylated CasSD remarkably enhanced its phosphorylation by exogenous c-Src, FynT, or Abl1 (Figure 4). Extension of CaSD was confirmed by measuring the separation of two halves of YFP linked to the ends of the CasSD (Figure 3B). Stretch-dependent phosphorylation of Cas in cytoskeletons and in intact cells further supports the idea that it is involved in physiological force sensing. Antibody binding to epitopes exposed by extension in regions of higher traction forces shows that Cas is extended in vivo. Although many force-dependent effects are observed within cells, extension of Cas appears to be a primary force-sensing process and not part of a secondary force-response pathway, since extension-dependent phosphorylation of CasSD by active kinases was observed in vitro, where any extraneous biochemical interactions or signaling pathways were completely eliminated. While extracellular matrix proteins also respond to force by unfolding (Oberhauser et al., 2002) and exhibit different functional effects (Zhong et al., 1998), we show here that a cytoplasmic protein, Cas, has a gain of function upon cell stretching in terms of increase in phosphorylation and activation of Crk/C3G-Rap1 signaling.

A much greater percentage extension is required to observe the increase of in vitro phosphorylation of CasSD (Figure 4A, lanes 2–6) than the percentage of cell stretching to observe an increase of in vivo Cas phosphorylation (Figures 1 and 2). In vivo, cytoskeletal filaments will not stretch significantly, and cytoskeletal networks are believed to be strain hardened by cell-generated traction forces; therefore, molecular complexes at "stress-bearing" sites will be greatly extended upon even mild cell stretching. Moreover, cell traction forces will pre-extend the cytoskeleton-bound Cas molecules in spread cells even without stretching (Figure 6, middle). Thus, 10% stretching of intact cells can cause more than 10% extension of "unextended" Cas (Figure 6, top), since traction forces are concentrated at cell-matrix contact sites (Geiger and Bershadsky, 2002). In addition, αCas1 immunostaining shows that Cas is extended in the high-traction force regions of cells where Cas is phosphorylated (Figure 5C).

In other studies, shear stress increases Cas phosphorylation by SFK in vascular endothelial cells (Okuda et al., 1999). Since shear stress is known to modulate the cell contractility (Chien et al., 2005) in which Cas has been shown to play a role (Tang and Tan, 2003), extension of Cas caused by the increased cell contractility might result in shear stress-dependent phosphorylation. Thus, local extension of Cas is likely to be involved in the local response to various types of "mechanical stress" and can possibly account for the versatile function of Cas (Defilippi et al., 2006).

Substrate Priming as a General Mechanism of Cell Signaling

Enhancement of a substrate's susceptibility to phosphorylation by mechanical extension is designated as extension-dependent "substrate priming." The transduction of cell forces into a biochemical signal by mechanical substrate priming could be highly flexible and dynamic. The extent of substrate extension in vivo will depend upon the extent of strain produced locally in the cell, resulting in a graded extent of substrate phosphorylation and, consequently, gradations in the magnitude of downstream signaling events. Substrate priming by mechanical force might be generally involved in kinase signaling, particularly in light of our observation that a number of other cytoskeletal proteins are tyrosine phosphorylated in a stretch-dependent manner (Tamada et al., 2004) and since substrate conformation is a critical determinant in phosphorylation of other SFK substrates (Cooper et al., 1984). Thus, we suggest that substrate priming by localized protein

extension provides a simple mechanism for sensing the level of force on a cell as well as the location at which force is applied.

EXPERIMENTAL PROCEDURES

Antibodies

Polyclonal antibodies against Cas protein (αCas1, αCas2, and αCas3) were described previously (Sakai et al., 1994). The polyclonal anti-phospho-Cas antibodies pCas-165 and pCas-410, a polyclonal anti-phospho-SrcY416 antibody, and a polyclonal anti-phospho-SrcY527 antibody were purchased from Cell Signaling. The polyclonal anti-phospho-Cas antibody αP-Cas460Y (Miyake et al., 2005) was used for in vitro experiments with CasSD. Monoclonal anti-GFP (JL-8) and anti-RFP (anti-DsRed) antibodies were purchased from Clontech and BD Pharmingen, respectively. Monoclonal anti-Src (GD11) and anti-polyHistidine antibodies were purchased from Upstate Biotechnology and Sigma, respectively. Polyclonal anti-actin and anti-Rap1 antibodies were purchased from Santa Cruz Biotech.

Cells and DNA Plasmid Transfection

Human embryonic kidney (HEK) 293 cells, Cas-deficient fibroblasts (Huang et al., 2002), and SYF cells that lack c-Src, c-Yes, and Fyn were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 IU/ml and 100 μg/ml) at 37°C and 5% CO_2. DNA plasmid transfection was performed with Fugene 6 (Roche) according to the manufacturer's protocol. To isolate stably transfected cell lines, pPUR that carried a puromycin-resistant gene (Clontech) was cotransfected, and clones were selected using puromycin (Clontech).

Immunoprecipitation and In Vitro Kinase Assay of Src

To measure the Src kinase activity in SYF cells and stable transfectant cells derived from SYF cells, Src was immunoprecipitated, and an in vitro kinase assay was performed using acid-treated enolase as a substrate. Phosphorylation of enolase was analyzed by anti-phospho-tyrosine immunoblotting. Details of the in vitro kinase assay of immunoprecipitated Src are described in the Supplemental Data section.

RNA Interference Experiments

To decrease the endogenous expression of Cas protein, two different siRNAs, BCAR1-HSS114272 and BCAR1-HSS114273 (Stealth RNAi, Invitrogen), were transfected into HEK293 cells (1×10^5/dish) using Lipofectamine RNAiMAX according to the manufacturer's protocol (180 pmol RNA interference [RNAi] and 9 μl Lipofectamine RNAiMAX/dish) (Invitrogen). Six hours after transfection, culture medium was replaced with fresh DMEM containing 10% FBS. Twenty-four hours after transfection, cells were either stretched or left unstretched and subjected to biochemical analyses.

Quantification of Rap1 Activity

A GST pull-down assay was performed to measure the Rap1 activity using GST-RalGDS-RBD that preferentially bound to Rap1·GTP (Sakakibara et al., 2002). To measure the Rap1 input, equivalent portions of each lysate were directly subjected to SDS-PAGE followed by immunoblotting.

Kinases and Substrates

Recombinant c-Src, FynT, Abl1, Csk, and ZAP-70 were purchased from Invitrogen. Specific kinase activities of c-Src, FynT, Csk, and ZAP-70 were determined by an in vitro kinase assay using poly-Glu/Tyr (4:1) as a substrate (Invitrogen). Abl1 kinase activity was determined by an in vitro kinase assay using Abl1 substrate (Invitrogen). Enolase (rabbit muscle) was purchased from Sigma. Bacterially expressed cdb3 was used as a substrate to measure the ZAP-70 activity.

Preparation of Biotinylated Proteins

Various forms of biotinylated CasSD were prepared using Biotin AviTag technology (Avidity). The Biotin AviTag sequence consists of 15 residues (GLNDIFEAQKIEWHE) and is specifically and efficiently biotinylated by the protein biotin ligase BirA. Biotinylated AviTag-fused proteins were obtained by coexpression with BirA in bacteria (BL21 Star, Invitrogen) cultured in NZCYM medium containing d-biotin (50 μM; Research Organics) at the time of IPTG induction. The molar ratio of biotin to AviTag-fused protein was confirmed to be 2:1 in NC-biotinylated CasSD and NY/CY-NC-biotinylated CasSD, and 1:1 in C-biotinylated CasSD and NY/CY-C-biotinylated CasSD. Details of biotinylated protein preparation are given in the Supplemental Data section.

For chimeric proteins of biotinylated CasSD and YFP components (NY/CY-NC-biotinylated CasSD and NY/CY-C-biotinylated CasSD), yellow fluorescence was observed and estimated in bacteria and in solution by quantitative fluorescence microscopy. Solutions containing NY/CY-NC-biotinylated CasSD or NY/CY-C-biotinylated CasSD were as fluorescent as solutions containing bacterially expressed full-length YFP at the same concentration (0.8 μM).

Plasmids

Plasmids used in this work are described in the Supplemental Data section.

Stretching of Intact Cells

Cells plated on collagen (type I; Sigma-Aldrich)-coated stretchable silicone dishes (Sawada et al., 2001) were either stretched biaxially (and kept stretched) or left unstretched in our cell stretching system (Sawada et al., 2001; Tamada et al., 2004).

Covalent Avidin Coating of Latex Membrane and Preparation of Biotinylated Proteins Specifically Bound to Avidin-Coated Latex Membrane

Avidin (Neutravidin) was covalently immobilized onto the surface of latex membrane by introducing the amine-reactive groups using Friedal-Crafts chemistry, and biotinylated proteins were bound to the immobilized avidin. Details of preparation of biotinylated protein bound to latex membrane are described in the Supplemental Data section.

IPE System

A biotinylated protein-bound latex membrane set in an adjustable tension ring was placed on a lubricated round-shaped glass stage and stretched biaxially and uniformly by pulling down the tension ring (Figure 3A, bottom). Magnitude of the latex membrane stretching was described as percent change of length in each dimension. For example, 100% stretching represented 2-fold expansion in each dimension. To recover the protein for analysis, the protein-bound membrane (Figure 3A, bottom) was incubated with 1× SDS sample buffer containing 0.12 M DTT at 95°C for 5 min. Using amine-reactive, photocleavable biotin analog (NHS-PC-LC-Biotin, PIERCE) (Sawada and Sheetz, 2002), we confirmed that this procedure recovered the majority (>95%) of biotinylated proteins bound to the immobilized avidin.

YFP Amino-Terminal Swapping Assay

NY/CY-NC-biotinylated, NY/CY-C-biotinylated (Figure 3B), or NC-biotinylated CasSD (Figure 3A) bound to avidin-coated latex membrane was prepared as described above. After stretching of latex membrane (100%) or without stretching, biotinylated CasSD proteins were washed two times with 1% BSA and 1% Triton X-100 in PBS and incubated with 2 μM His_6-YFP-N in 1% BSA and 1% Triton X-100 in PBS containing 1 mM DTT for 10 min at room temperature. The bound protein complex on the latex membrane was washed four times with 1% BSA and 1% Triton X-100 in PBS and two times with 1% Triton X-100 in PBS, recovered with 1× SDS sample buffer containing 0.12 M DTT. The samples were subjected to SDS-PAGE followed by anti-polyHistidine immunoblotting and avidin affinity blotting.

In Vitro Extension and Phosphorylation of CasSD

NC-biotinylated or C-biotinylated CasSD bound to avidin-coated latex membrane was prepared as described above. After stretching of latex membrane or without stretching, biotinylated CasSD proteins were washed three times with 0.25% Triton X-100 and 2% BSA in buffer A (20 mM HEPES [pH 7.5], 150 mM NaCl, 4 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 20 μg/ml aprotinin, 0.5 mM EGTA) and three times with 0.1% BSA in buffer A and incubated with recombinant kinases (specific activity of each kinase used: 700 pmol/min phosphate transfer) in 350 μl of kinase reaction buffer (20 mM HEPES [pH 7.5], 0.9 mM ATP, 0.1% BSA, 140 mM NaCl, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 0.5 mM EGTA, 20 μg/ml aprotinin, 1.5 mM DTT, 1.5 mM Na_3VO_4, 0.03% Brij-35) for 2 min at room temperature. After kinase reaction, biotinylated CasSD proteins were washed three times with ice-cold 1% Triton X-100 in PBS containing 1 mM Na_3VO_4, recovered, and solubilized by incubation with 1× SDS sample buffer containing 0.12 M DTT at 95°C for 5 min. Tyrosine phosphorylation of CasSD was determined by anti-phospho-Cas immunoblotting and avidin affinity blotting.

In Vitro Binding of αCas1 to Extended CasSD

NC-biotinylated or C-biotinylated CasSD bound to avidin-coated latex membrane was either extended (100%) or left unextended in PBS containing 1% Triton X-100, 2% BSA, 5% FBS, 1 mM DTT, 20 μg/ml aprotinin, and 0.5 mM EGTA. After 10 min, CasSD proteins on the latex surface were washed three times and incubated for 30 min with PBS containing 2% BSA, 5% FBS, 1 mM DTT, 20 μg/ml aprotinin, and 0.5 mM EGTA to block nonspecific binding and then incubated for 2 min with αCas1 diluted at 1:1200 in the same buffer. After six washes with PBS containing 0.1% Tween-20 and 1 mM DTT, bound proteins were solubilized with 1× SDS sample buffer containing 0.12 M DTT and subjected to SDS-PAGE followed by anti-rabbit IgG or αCas1 immunoblotting and avidin affinity blotting.

Binding of Two Different Anti-Cas Antibodies, αCas1 and αCas3, to Triton Cytoskeletons

Triton cytoskeletons were prepared from Cas-deficient fibroblasts transiently expressing RFP-Cas or RFP alone as described previously (Sawada and Sheetz, 2002). After two washes with buffer A containing 2% BSA and 5% FBS, the buffer was replaced with the buffer A containing 0.5 mM ATP, 2% BSA, 5% FBS, and either αCas1 or αCas3 (1:400 dilution), and Triton cytoskeletons were either stretched or left unstretched. After 2 min of incubation, samples were washed two times with buffer A containing 2% BSA and 5% FBS and four times with buffer A, solubilized with 1× SDS sample buffer containing 20 mM DTT, and subjected to SDS-PAGE followed by anti-rabbit IgG, αCas2, and anti-actin immunoblotting (Figure 5B, upper panel).

Immunofluorescence Staining, Fluorescence Microscopy, and Image Display

Twenty minutes after being plated on collagen (Type-I)-coated coverslips, Cas-deficient fibroblasts expressing RFP-Cas were washed with PBS; fixed with 3.7% formaldehyde in PBS; permeabilized with 0.1% Triton X-100 in PBS; stained using αCas1, αCas3, or pCas-165 as a primary antibody (1:400 dilution for αCas1 and αCas3 and 1:100 dilution for pCas-165) and Alexa Fluor 488 anti-rabbit IgG as a secondary antibody; and then viewed with a confocal microscope (Olympus IX-81 with FV500 system). Image intensity from the green channel (immunofluorescence with αCas1, αCas3, or pCas-165) and the red channel (RFP-Cas) was displayed with the contrast enhanced by setting the highest intensity in each image at the maximum value of the dynamic range and the background (cell-free area) at zero in ImageJ, a free open source Java imaging platform (http://rsb.info.nih.gov/ij/).

Statistical Analysis

Statistical analysis was performed with the paired Student's t test, and $p < 0.05$ was defined as significant.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures and one supplemental figure and can be found with this article online at http://www.cell.com/cgi/content/full/127/5/1015/DC1/.

ACKNOWLEDGMENTS

We thank S. Ohkubo, T. Mandai, M. Cull, M. Galbmillion, and M.L. Bushey for assisting in the construction of the IPE system; P.S. Low, S.K. Hanks, T. Yamamoto, and M. Matsuda for the plasmids; J.M. Fernandez, M. Edidin, T.D. Perez, A. Sakakibara, C.D. Hu, H. Takayanagi, T. Miyazaki, T. Tezuka, M. Saitoh, K. Takeda, H. Ichijo, and K. Nakamura for helpful discussions and consistent support. This work was supported by NIH grant R01 EB001480.

Received: June 9, 2006
Revised: August 20, 2006
Accepted: September 25, 2006
Published: November 30, 2006

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Molecular Mechanism of the Life and Death of the Osteoclast

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ABSTRACT: **The life span of osteoclasts is critically regulated by various cytokines, and therapeutics such as bisphosphonates act directly on osteoclasts and induce apoptosis of the cells. This article will focus on the molecular mechanism of osteoclast apoptosis and summarize the recent advances in this field with an emphasis on the role of intracellular signaling pathways.**

KEYWORDS: **osteoclast; cytokines; apoptosis; Akt; TNF; Rac1; Bim**

THE ROLE OF APOPTOSIS IN BONE HOMEOSTASIS

The homeostasis of the skeletal tissues is maintained by a well-organized regulation of bone formation and bone resorption. This process is called remodeling, in which osteoblasts are mainly involved in the bone formation process and osteoclasts are essential for bone resorption. Osteoclasts are terminally differentiated cells primarily involved in physiological and pathological bone resorption. The life span of osteoclasts is relatively short both *in vitro* and *in vivo*, and once differentiated, they rapidly die in the absence of supporting cells such as osteoblasts or bone marrow stromal cells, or growth factors, such as interleukin (IL)-1, receptor activator of NF-κB ligand (RANKL), and macrophage colony-stimulating factor (M-CSF).[1] Antiresorptive drugs such as estrogen, raloxifene, and bisphosphonates are known to reduce the life span of osteoclasts.[2]

Recent studies have revealed that the rapid cell death of osteoclasts is caused by apoptosis. Apoptosis is a form of programmed cell death that is characterized

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Ann. N.Y. Acad. Sci. 1068: 180–186 (2006).
doi: 10.1196/annals.1346.020

by specific morphological and biochemical properties.[3,4] Morphologically, apoptosis is characterized by a series of structural changes in dying cells: blebbing of the plasma membrane, condensation of the cytoplasm and the nucleus, and cellular fragmentation into membrane apoptotic bodies. Biochemically, apoptosis is characterized by the degradation of chromatin, initially into large fragments of 50–300 kilobases and subsequently into smaller fragments that are monomers and multimers of 200 bases.[4,5] Not only does apoptosis regulate various aspects of the biological activity but also can trigger cancer, autoimmune diseases, and degenerative disorders.

INTRACELLULAR SIGNALING PATHWAYS REGULATING OSTEOCLAST SURVIVAL

Using adenovirus vector-mediated gene transduction system, we found that the activation of extracellular-regulating kinase (Erk) by transducing a constitutively active mutant of Mek1 markedly promoted the survival of osteoclasts.[6] Conversely, inhibiting Erk activation by overexpressing a dominant negative *ras* gene mutant rapidly induced apoptotic cell death of the cells.[6] These results, combined with the fact that anti-apoptotic factors such as RANKL, IL-1, and M-CSF also induce Erk activation in osteoclasts, suggest that the Ras-Erk pathway plays an essential role in osteoclast survival.

Recent studies have revealed the critical role of phosphatidylinositol 3-kinase (PI3K)/Akt pathways in the survival of osteoclasts. Xing *et al.* reported that the transgenic overexpression of Src251, which contains SH2 and SH3 domains of chicken c-Src but lacks the kinase domain, under the control of the tartrate-resistant acid phosphatase (TRAP) promotor-induced osteopetrosis in mice.[7] The mice had a reduced number of osteoclasts, and osteoclasts of Src251 transgenic mice showed apoptotic phenotypes. The activity of Akt in the Src251 transgenic mouse osteoclasts was significantly reduced, and the RANKL-induced Akt activation was impaired in the cells, suggesting that Akt plays a critical role in osteoclast survival, and Akt activation is at least partly mediated by c-Src.[7] Using a mouse osteoclast formation system, Lee *et al.* showed that tumor necrosis factor-α (TNF-α) prolonged the survival of osteoclasts, which was abrogated by either PI3K inhibitors or Mek/Erk inhibitors.[8] They also revealed the involvement of Grb2 and ceramide in TNF-α-induced Erk activation in osteoclasts.[8] A similar effect was observed in IL-1α.[9] Glantschnig *et al.* exhibited the central role of mTOR/S6K pathways in M-CSF- and RANKL-induced osteoclast survival, which was suppressed by rapamycin.[10] We also found that the adenovirus vector-mediated overexpression of constitutively active Akt stimulates osteoclast survival (Fukuda *et al.*, unpublished observation). These results clearly suggest the crucial role of Akt pathways on the osteoclast survival. However, contrary to these previous observations, Sugatani and Ross recently reported that silencing of Akt1

and/or Akt2 by small interfering RNA (siRNA) suppressed osteoclast differentiation but did not affect osteoclast survival.[11] The reason of this discrepancy remains unknown, and further investigation is required to clarify the exact role of PI3K/Akt pathways in the osteoclast survival.

Rac1 is a member of the Rho family small G-proteins and recent studies have revealed that it mediates anti-apoptotic signals in some types of cells. Rac1 is reported to be required for the cytoskeletal organization and bone-resorbing activity of osteoclasts, but its role in the osteoclast survival and function is not fully elucidated yet. To examine the role of Rac1 in the osteoclast survival and function, we examined the effect of dominant negative Rac1 and constitutively active Rac1 expression on osteoclasts.[12] Adenovirus vector-mediated dominant negative Rac1 ($Rac1^{DN}$) expression significantly reduced the pit formation of osteoclasts, and promoted their apoptosis. M-CSF rapidly activated Rac1, consistent with the results reported by Faccio *et al.*,[13] and the pro-survival effect of M-CSF for osteoclasts was abrogated by $Rac1^{DN}$ overexpression. Constitutively active Rac1 enhanced osteoclast survival, which was completely suppressed by PI3K inhibitors, while Mek inhibitors had only partial effects. Using time-lapse video-microscopy, we found that $Rac1^{DN}$ expression reduced the membrane ruffling and spreading of osteoclasts in response to M-CSF. These results strongly suggest that small GTPase Rac1 is critically involved in M-CSF receptor signaling, and mediates survival signaling of osteoclasts primarily by modulating PI3K/Akt pathways. Faccio *et al.* recently demonstrated that a guanine nucleotide exchange factor Vav3 lies upstream of Rac1 activation in osteoclasts, and is involved in the signaling of M-CSF receptor and $\alpha v\beta 3$ integrin.[14]

ROLE OF BCL-2 FAMILY MEMBERS IN OSTEOCLAST APOPTOSIS

The apoptotic process can be divided into two different pathways, the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway.[15] In the death receptor pathway, the activation of a receptor belonging to the TNF receptor family leads to the induction of apoptosis through the activation of aspartate-specific cystein proteases (caspases) 8, and the anti- and pro-apoptotic Bcl-2 family members are critically involved in the intrinsic pathway by regulating cytochrome *c* release from the mitochondrial intermembrane into the cytosol, which leads to the activation of caspase 9.[15] The anti-apoptotic members include mammalian Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1, and *C. elegans* CED-9. So far, more than 20 members of pro-apoptotic Bcl-2 family proteins have been identified in mammals, and they can be further divided into two groups; multi-domain members possessing homology in BH1-3 domains and BH3 domain-only members. Multi-domain members include Bax, Bak, Bok and *D. melanogaster* DEBCL/DROB, and BH3-only members include

mammalian Bad, Bik/Nbk, Bid, Hrk/DP5, Blk, Bim, Noxa and *C. elegans* EGL-1.[16] It was recently reported that BH3-only proteins failed to induce cytochrome *c* release and apoptosis in *bax-/-bak-/-* cells, suggesting that Bax-like proteins mediate death signals from various BH3-only proteins.[17] Bax-like proteins are ubiquitously expressed, while BH3-only members have a more tissue-specific distribution, indicating that the latter may play a role in the tissue/cell-specific regulation of apoptosis.

The activity of BH3-only proteins is strictly regulated to prevent unorganized cell death. The expression of Noxa is induced by p53 or IRF-1, and DP5/Hrk is transcriptionally upregulated in the absence of growth factors or when the cells are exposed to β-amyloid protein. The other mechanism regulating their activity is the posttranslational modification. It is now widely recognized that Bad is phosphorylated at various sites and sequestrated away from Bcl-2 family members by binding to 14-3-3.[18,19] Many kinases including Akt, mitochondrial-anchored protein kinase A, Pak1, Rsk and Raf1, and phosphatase calcineurin have been reported to modify Bad, although the physiological importance of these regulations still remains unclear.

Depolarization of mitochondrial transmembrane potential, chromatin condensation, and cytochrome *c* release from mitochondria into cytoplasm were observed in the apoptotic osteoclasts, implying that cytokine deprivation triggers osteoclast apoptosis through the mitochondrial pathway.

A possible role of anti-apoptotic Bcl-2 family members for osteoclast survival has been reported. Okahashi *et al.*[20] showed that M-CSF treatment increased Bcl-2 mRNA expression in osteoclasts, and we found that adenovirus vector-mediated overexpression of Bcl-xL markedly prolonged survival of the cells *in vitro*.[21] Roodman and co-workers developed a transgenic mouse, in which Bcl-xL together with Simian Virus 40 large T antigen were specifically overexpressed in osteoclasts under the control of the TRAP promotor, and successfully established an immortalized osteoclast precursor cell line from the mice.[22] These results clearly showed that anti-apoptotic Bcl-2 family proteins play important roles in osteoclast survival both *in vitro* and *in vivo*. The mechanism regulating Bcl-2 and Bcl-xL expression in osteoclasts has not been clarified yet, but McGill *et al.* showed that Bcl-2 expression is regulated by Mitf, a transcription factor essential for osteoclast and melanocytes development.[23]

PRO-APOPTOTIC BH3-ONLY PROTEIN BIM IS INVOLVED IN OSTEOCLAST APOPTOSIS

Bim, one of the BH3-only proteins, was first identified as a Bcl-2 interacting protein by screening a λ-phage expression library constructed from a mouse thymic lymphoma.[24] Bim is expressed in hematopoietic, epithelial, neuronal, and germ cells,[25] and alternative splicing generates various Bim isoforms,

including Bim_S, Bim_L, and Bim_{EL}. Bim has been shown to play an essential role in the induction of apoptosis in T lymphocytes, B lymphocytes, and neurons, mainly based on the finding in Bim-deficient animals,[26,27] and its activity is also regulated both transcriptionally and posttranscriptionally.[28] The expression of Bim is downregulated at the transcriptional level by IL-3 signaling through Raf/Erk pathways and/or PI3K/mammalian target of rapamycin (mTOR) pathways in IL-3-dependent Ba/F3 cells.[29] The transcription of Bim is also upregulated in neonatal sympathetic neurons in response to the nerve growth factor deprivation. In healthy cells, Bim_L is bound to LC8, a component of the microtubule-associated dynein motor complex, and is sequestered to this complex in the cytoplasm. Certain apoptosis stimuli induce dissociation of this complex, and allow the translocation of Bim_L-LC8 complex to Bcl-xL on the mitochondrial membrane.[28]

We demonstrated a novel and unique regulation of apoptosis by ubiquitylation-dependent degradation of Bim in osteoclasts.[21] In the presence of M-CSF, Bim is constitutively ubiquitylated and degraded, and cytokine deprivation induced rapid upregulation of Bim due to the reduced level of its ubiquitylation.[21] C-Cbl is a possible candidate of E3 ubiquitin ligase of Bim in osteoclasts because Bim ubiquitylation was reduced in c-Cbl-deficient osteoclasts. Bim-deficient osteoclasts exhibited prolonged survival both *in vitro* and *in vivo*, and however, their bone-resorbing activity was significantly reduced, consistent with the *in vivo* observation that *bim–/–* animals showed mild osteosclerosis.[21] Consistent with our observation, Sugatani and Hruska reported that silencing the *bim* gene by siRNA prolonged the survival of osteoclasts.[11] These results clearly demonstrate that pro-apoptotic protein Bim plays a crucial role in osteoclast apoptosis and activation. Further studies are required to elucidate the mechanism of action and the regulation of Bim in osteoclasts, and the role of Bim in skeletal disorders.

CONCLUSIONS AND PERSPECTIVES

The success of bisphosphonates as the therapeutics of osteoporosis, together with the finding that bisphosphonates act directly on osteoclasts to induce their apoptosis, has attracted a great deal of attention to the molecular mechanism of osteoclast apoptosis, and remarkable progress has been made during the last several years to reveal the signaling pathways involved in the process. Therapeutics targeting these signaling pathways (molecules) will provide a novel treatment for bone diseases.

ACKNOWLEDGMENTS

This work was in part supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Health Science

research grants from the Ministry of Health, Labour and Welfare of Japan to ST.

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Mod Rheumatol (2006) 16:68–74
DOI 10.1007/s10165-006-0460-z

REVIEW ARTICLE

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The role of c-Src kinase in the regulation of osteoclast function

Received: November 21, 2005 / Accepted: January 23, 2006

Abstract The targeted disruption of c-Src impairs osteoclast bone resorbing activity, causing osteopetrosis. Although it has been reported that restoring only the c-Src adaptor function at least partly rescues the skeletal phenotypes, the importance of c-Src kinase activity remains controversial. We here highlight the contributions of the Src adaptor and kinase activities in cytoskeletal organization and osteoclast function using adenovirus vectors containing various mutants of Src or Pyk2. In addition, we describe the importance of c-Src in mitochondria, where it phosphorylates cytochrome *c* oxidase (Cox). Src-induced Cox activity is also required for bone resorbing activity of osteoclasts that require high levels of ATP. Thus, c-Src kinase activity not only on the plasma membrane but also within mitochondria is essential for the regulation of osteoclastic bone resorption.

Key words c-Src · Kinase · Mitochondria · Osteoclast · Pyk2

Introduction

Non-receptor type tyrosine kinase c-Src is a member of a family of nine protein–tyrosine kinases that associate with the cytoplasmic surface of the cellular membrane.[1] Activated mutants of Src are oncogenic, and study of these mutants has implicated c-Src in the control of cell growth and proliferation. However, c-Src is highly expressed in terminally differentiated cells such as platelets and neurons, indicating a physiological role for the protein unrelated to growth control.[2] Soriano et al.[3] reported that targeted disruption of the *c-src* gene in mice induced osteopetrosis, a disorder characterized by decreased bone resorption, without showing any obvious morphological or functional abnormalities in other tissues or cells. They also showed that the osteopetrotic phenotype of *c-src*-disrupted mice is cell-autonomous and occurs in mature osteoclasts.

Osteoclasts are multinucleated, terminally differentiated cells which degrade mineralized matrix during normal and pathological bone turnover.[4] Osteoclastic bone resorption involves the proliferation and homing of the hemopoietic osteoclast progenitors to bone, their differentiation and fusion to form multinucleated cells, and the migration of osteoclasts to and between the resorption sites. Osteoclasts attach to the bone surface and form a tight sealing zone (or "clear zone") enclosing the resorption lacunae, which was frequently compared to a large lysosome. Following the insertion of secretory vesicles, a highly convoluted membrane called the "ruffled border" is formed facing the bone surface.[5] Integrins have been suggested to play a role in osteoclast activity by mediating osteoclast adhesion and regulating the cytoskeletal organization required for both cell migration and formation of sealing zone. v-*src*-transformed cells contain high concentrations of Src as well as other tyrosine phosphorylated proteins in podosome,[6,7] specialized adhesion sites that are structurally and functionally distinct from focal adhesions.[6,8–10] Although many of the same proteins are present in podosomes and focal adhesions, focal adhesions are relatively stable, whereas podosomes are dynamic attachment structures, undergoing assembly and disassembly within minutes.[11–13] Activation of Src may therefore be associated with a shift from stable focal adhesions with actin stress fibers to more dynamic podosome assemblies, possibly regulating cell motility. Indeed, several papers have suggested a role for Src kinase activity in cell spreading and migration.[14–18]

It remains uncertain, however, whether catalytically active Src is required for normal osteoclast function.

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Schwartzberg et al.[19] previously reported that osteoclast-specific expression of kinase-dead Src mutants rescued the $Src^{-/-}$ osteopetrotic phenotype, suggesting that c-Src may act as an adaptor molecule and that c-Src kinase activity may not be critical for bone resorption.[19] In addition, no adverse effects of kinase-dead Src expression in $Src^{+/+}$ and $Src^{+/-}$ animals was observed.[19,20] On the other hand, we have recently reported that down- or up-regulation of c-Src activity modulates osteoclastic bone resorption not only in vitro but also in vivo,[18] leaving the question of the contribution of c-Src kinase activity unsettled.

Here, we show that not only adaptor function but also the kinase activity of c-Src are important in osteoclastic bone resorption, taking advantage of the ability of adenoviral vectors to transduce foreign genes into osteoclasts.[21] In addition, we also found that c-Src in mitochondria regulates osteoclast function via cytochrome c oxidase activity.[22] In this review, we would like to introduce the role of c-Src kinase activity in both plasma membrane and mitochondria.

The structure of c-Src

Src family tyrosine kinases have a common domain organization, with each segment designated as a Src-homology (SH) region. The N-terminal segments includes the SH4 domain, which is a myristoylation and membrane-localization signal, as well as a "unique" domain, which differs among family members. This region is followed in the peptide chain by the SH3 domain, the SH2 domain, the tyrosine kinase (SH1) domain, and a short C-terminal tail, which includes a critical tyrosine residue (Fig. 1A). SH2 and SH3 domains mediate protein–protein interactions in cellular signaling cascades, and are found in many proteins outside the Src family. The SH3 and SH2 domains and the C-terminal tail all have roles in regulating Src kinase activity. It is now clear that phosphorylation of Tyr-527 by a specific kinase, Csk (C-terminal Src family kinase),[23] inhibits Src catalytic activity by creating an intramolecular binding site for the Src SH2 domain. The interaction is believed to result in auto-inhibition by locking the molecule in an inactive state. Displacement of SH2 domains by C-terminal tail dephosphorylation or by competitive binding of optimal SH2/SH3 domain ligands, allows the kinase domain to open, exposing Tyr-416 to phosphorylation. In open state, phosphorylation of Tyr-416 in the activation loop of the kinase domain further upregulates the enzyme (Fig. 1B).[24,25]

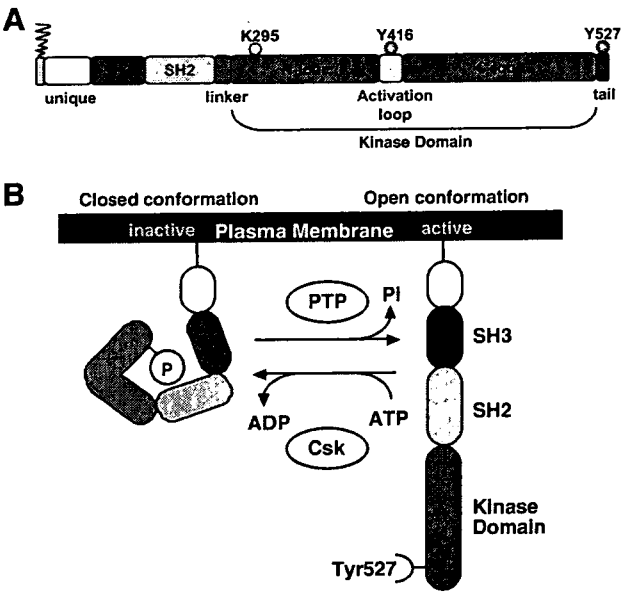

Fig. 1A,B. The structure of c-Src. **A** Schematic illustrations of the c-Src. N-terminal segment includes membrane-localization signal, myristoylation site (also called SH4 domain). The Src homology 3 domain, proline-rich binding site, SH2 domain, which binds to phosphotyrosine, and kinase domain follow in order. There are also a short, C-terminal tail, which includes a critical tyrosine residue. **B** The restrained conformation of c-Src is stabilized by intramolecular interactions among the kinase domain, the SH2/SH3 domains, and the phosphorylated C-terminal tail. Displacement of SH2 and/or SH3 domains, either by C-terminal tail dephosphorylation or by competitive binding of optimal SH2/SH3 domain ligands, allows the kinase domain to open, exposing Tyr-416 to phosphorylation

Pyk2-dependent recruitment of c-Src to the plasma membrane is necessary for bone resorption

Proline-rich tyrosine kinase 2 (Pyk2) has been identified as a major adhesion-dependent tyrosine kinase in osteoclasts, both in vivo and in vitro.[26–28] Pyk2 is a member of the focal adhesion kinase family, highly expressed in cell of the central nervous system and cells of hematopoietic lineage. Pyk2 and FAK share approximately 45% of the overall amino acid identity and have a high degree of sequence conservation surrounding binding site of SH2- and SH3-domain-containing proteins.[29] Ligation of $\alpha_v\beta_3$ integrin either by ligand binding or by antibody-mediated clustering increased Pyk2 tyrosine phosphorylation in osteoclasts. Moreover, in adherent osteoclasts, Pyk2 is tightly associated with c-Src, via its SH2 domain.[17,27] Upon osteoclast adhesion, Pyk2 translocates into the Triton X-100 insoluble cytoskeletal fraction and was concentrated at the cell periphery, co-localizing with F-actin.[27] Furthermore, Sanjay et al.[17] reported that Pyk2 is autophosphorylated upon integrin activation and that Pyk2 autophosphorylation at Y402 is required for the Pyk2/c-Src complex formation via the interaction with the Src SH2 domain. To examine the importance of the binding of Src to Pyk2, we constructed adenovirus vector carrying autophosphorylation site-mutated Pyk2 [$Pyk2^{Y402F}$]. More than 90% of wild-type osteoclast-like cells (OCLs) formed in vitro[30,31] display typical rounded appearances with a clear actin ring formation (Fig. 2A, upper panel). Adenovirus-mediated $Pyk2^{Y402F}$-overexpressed OCLs did not exhibit one large actin ring (Fig. 2A, lower panel) and strongly inhibited the bone-resorbing activity in proportion to $Pyk2^{Y402F}$ expression (Fig. 2B).[21] These data lead us to speculate that the interaction of

Src with Pyk2, mediated by the SrcSH2 domain and the Pyk2 Tyr(P)-402, is required for the normal organization of the osteoclast actin cytoskeleton and for bone resorption. Although the recruitment of Src to autophosphorylated Pyk2 at adhesion sites is a key event in bone resorption, it does not address the question of whether Src kinase activity is required or if the adaptor functions of Src SH2 and SH3 domains are sufficient. To examine whether or not the upregulation of Src kinase activity is sufficient to rescue the decreased pit-forming activity of $Pyk2^{Y402F}$-expressing OCLs, we coinfected OCLs with $AxPyk2^{Y402F}$ and adenovirus carrying kinase-dead C-terminal Src family kinase ($AxCsk^{KD}$), which prevents the phosphorylation of the negative regulatory Tyr-527 and increases Src kinase activity in OCLs.[18] The inhibitory effect of $AxPyk2^{Y402F}$ on osteoclast function was, however, not rescued by co-infection with $AxCsk^{KD}$, leading us to conclude that high Src kinase activity alone is not sufficient to rescue the decreased bone resorbing activity in the $Pyk2^{Y402F}$-expressing OCLs and that the Pyk2-dependent recruitment of Src is necessary for bone resorption.

c-Src kinase activity is required for osteoclast function

To further confirm the importance of Src kinase activity in osteoclasts, we constructed an adenovirus carrying kinase-dead Src (Src^{K295M}). In contrast to the report by Schwartzberg et al.,[19] Src^{K295M}-expression completely disrupted the actin cytoskeleton in osteoclasts and bone-resorbing activity was strongly inhibited in proportion to the expression level of Src^{K295M} protein.[21] These results strongly suggest that it is the presence of the kinase activity of Src in the osteoclast adhesion structures and not just the Src protein that is necessary for actin ring formation and bone resorption. However, the autophosphorylation of Src promotes the adoption of the fully open active conformation,[24,25] and thus, Src^{K295M} might not be an effective adaptor.

To rule out the possibility that the dominant negative effects of Src^{K295M} could be due to a loss of adaptor function, we constructed $AxSrc^{K295M+Y527F}$, which prevents the phosphorylation of the negative regulatory Tyr-527 and promotes the availability of the SH2 and SH3 domains to bind other proteins. The morphology and bone-resorbing activity of $Src^{K295M+Y527F}$-expressing osteoclasts was similar to that of osteoclasts infected with $AxSrc^{K295M}$, further establishing that it is the absence of kinase activity, not the possible failure to bind to Pyk2 and/or other proteins in the adhesion complex, that causes the loss of bone resorbing activity.[21] To further confirm the importance of Src kinase activity, we investigated the functional changes in osteoclasts infected with $AxSrc^{Y416F+Y527F}$. The Y416F mutation reduces kinase activity about 50% by preventing the phosphorylation of a tyrosine on the activation loop of Src.[32,33] Despite its open conformation, $Src^{Y416F+Y527F}$ partially inhibited osteoclast function, consistent with its reduced kinase activity. $AxSrc^{Y416F+Y527F}$-infected osteoclasts exhibited a few small actin rings and still retained partial bone-resorbing activity (about 70% of control).[21] These results lead us to conclude that the localization of catalytically active Src in adhesion structures is required for actin ring formation and bone resorption, at least in vitro.

c-Src kinase activity is required for the efficient rescue of the c-$Src^{-/-}$ osteoclast phenotype

As noted earlier, Schwartzberg et al.[19] previously reported that osteoclast-specific expression of the kinase-dead Src^{K295M} mutant partially restored normal trabecular bone volume and tooth eruption in the $Src^{-/-}$ mice. We then introduced c-Src mutants into c-$Src^{-/-}$ osteoclasts to determine the influence of endogenous c-Src levels in our previous experiments. Under these in vitro experimental conditions, $Src^{-/-}$ OCLs were completely unable to form resorption pits in our assay (Fig. 4). Interestingly, Src^{K295M} and $Src^{K295M+Y527F}$, which did not notably affect the cytoskeletal organization of OCLs, restored (Fig. 3A), albeit extremely minimally (less than 3%), some bone-resorbing activity in $Src^{-/-}$ osteoclasts (Fig. 3B).[21] This minimal effect could be due to the adaptor function of c-Src or to the very low residual kinase activity in these mutants. In contrast, the expression of $Src^{Y416F+Y527F}$, which retains about 50% kinase activity, induced the formation of actin rings and very significantly increased bone-resorbing activity to ~70% of control (Fig. 3).[21] These results clearly show that c-Src kinase activity is indeed required to rescue the disruptive effect of c-Src deficiency on the cytoskeletal organization and bone-resorbing activity of osteoclasts.

The data from our study clearly show that at the level of the individual osteoclast, the kinase activity of Src is indeed required for bone resorption. Most interestingly, although $Src^{-/-}$ OCLs were 100% devoid of resorbing activity, cells reconstituted with Src^{K295M} demonstrated a very minimal (about 3%) but significant ($P < 0.001$) level of pit-forming activity (Fig. 3B), which was still much less than the >70% restoration of pit formation by OCLs reconstituted with $Src^{Y416F+Y527F}$. This minimal restoration of activity by Src^{K295M} suggests a possible explanation of the apparent discrepancy between our results with individual OCLs and the findings of Schwartzberg in animals. It is known that there are severalfold more osteoclasts in $Src^{-/-}$ than in wild type animals, and the degree of osteopetrosis in these animals is moderate.[34] Even a small increase in the activity of the larger number of osteoclasts acting over a longer time than our in vitro bone resorbing assay could induce sufficient additional bone resorption to account for the partial rescue reported by Schwartzberg et al.[19]

c-Src is localized in mitochondria

To define additional candidate proteins implicated in c-Src signaling, we performed a two-hybrid screen using kinase-

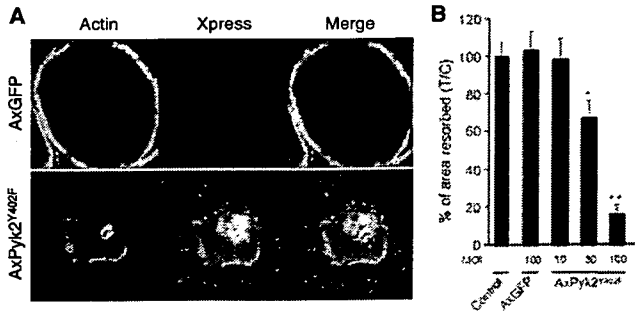

Fig. 2. A Double immunofluorescence staining of F-actin (*red*) and Xpress-tagged Pyk2^{Y402F} (*green*) in osteoclast-like cells (OCLs) infected with AxGFP or AxPyk2^{Y402F}. Adenovirus-mediated Pyk2^{Y402F} overexpression disrupted the one large actin ring formation. **B** Dentine-resorbing activity of OCLs expressing the Pyk2 mutants. OCLs 24 h after the infection at an indicated multiplicity of infection (*MOI*; a measure of titer, which reflects how many viruses infected a cell) were restored by digesting the collagen gel with 0.2% collagenase for 20 min at 37°C and an aliquot of the crude OCL preparation was transferred onto dentine slices (diameter 5 mm) as well as on 96-well culture plates and further cultured for 12 h. The resorbed area on dentine slices was measured using an image analysis system, and the number of TRAP-positive OCLs on culture plates was counted by light microscopy. The bone-resorbing activity of the cells was expressed as the resorbed area per osteoclast. The values are the mean ± SD ($n = 8$; $^*P < 0.05$, $^{**}P < 0.01$ compared with non-infected OCLs)

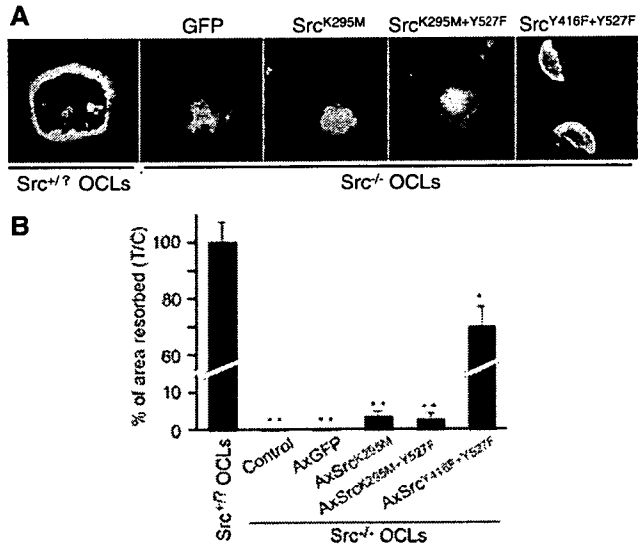

Fig. 3. A F-actin staining in Src$^{-/-}$ OCLs infected with AxGFP, AxSrc^{K295M}, AxSrc$^{K295M+Y527F}$, or AxSrc$^{Y416F+Y527F}$. Although Src^{K295M} and Src$^{K295M+Y527F}$ did not notably affect the cytoskeletal organization of Src$^{-/-}$ OCLs, the expression of Src$^{Y416F+Y527F}$ partly rescued the formation of actin rings. **B** Dentine-resorbing activity of Src$^{-/-}$ OCLs expressing the Src mutants. In contrast to Src^{K295M} and Src$^{K295M+Y527F}$, the expression of Src$^{Y416F+Y527F}$, which retains about 50% kinase activity, very significantly increased bone-resorbing activity. The values are the mean ± SD ($n > 8$; $^*P < 0.05$, $^{**}P < 0.01$ compared with Src$^{+/?}$ OCLs)

dead c-Src as a bait. We screened a mouse brain library and obtained several clones that encode a mitochondrial protein. Mitochondria, the cellular energy plants, generate ATP through oxidative phosphorylation (OXPHOS). OXPHOS, defined as the oxidation of fuel molecules by

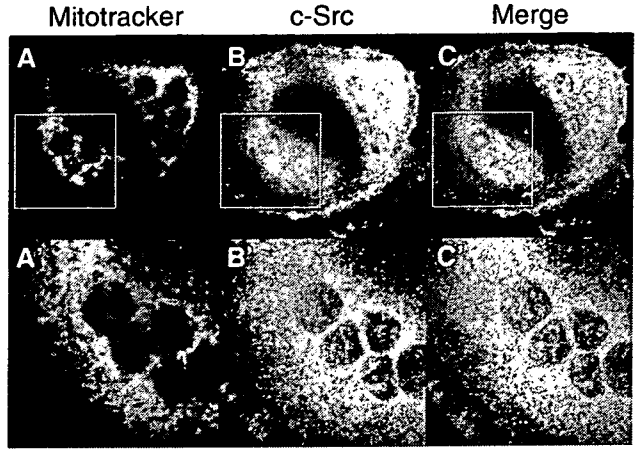

Fig. 4. Mitochondrial localization of c-Src. **A–C** Double immunofluorescence staining of Mitotracker and c-Src in osteoclasts. The localizations of Mitotracker (*red*) and c-Src (*green*) were assessed by confocal microscopy. **A′–C′** Close-up of the boxed region from **A–C**. c-Src staining was detected throughout the cytoplasm as well as at the cell periphery and many of these colocalized with mitochondria in osteoclasts

oxygen and the concomitant transduction of this energy into ATP, is the final process of the complicated biochemical network involved in cellular energy production. The OXPHOS molecular system, which is embedded in the lipid bilayer of the mitochondrial inner membrane, consists of electron acceptors, coenzyme Q and cytochrome c, and five multisubunit protein complexes (complexes I–V). The OXPHOS system comprises about 70 nuclear gene products and 13 mitochondrial gene products.[35,36]

To address whether c-Src might be present in mitochondria, we performed the dual staining of osteoclasts for c-Src and mitochondria using c-Src antibody and Mitotracker. As shown in Fig. 4, the merged image of c-Src (green) and mitochondria (red) revealed that c-Src protein appeared to colocalize with mitochondria. In close inspection of magnified images, c-Src clearly exhibited mitochondrial localization, as evidenced by colocalization with Mitotracker. The immunofluorescent results were also verified by immunoelectron microscopy. c-Src was found to be associated with the inner mitochondrial membrane in Src$^{+/?}$ osteoclasts. A large fraction of the gold particles were close to, or superimposed on, the inner mitochondrial membrane (Fig. 5). In contrast, no mitochondria showed labeling in the c-Src$^{-/-}$ osteoclasts (negative control).

We then examined the tyrosine phosphorylation of mitochondrial proteins from c-Src-overexpressing HEK 293 cells to determine if c-Src functions in mitochondria. Western blots of proteins suspended on two-dimension non-denaturing/denaturing gels with anti-phosphotyrosine suggested that one of the tyrosine-phosphorylated mitochondrial proteins was the cytochrome *c* oxidase (Cox).[22] Cox, which contains 13 subunits, is the terminal oxidase of cell respiration. The three major subunits of Cox are encoded by mitochondrial DNA and form the functional core of the enzyme; this core is surrounded by 10 nuclear-coded small subunits. Cox reduces dioxygen to water with four

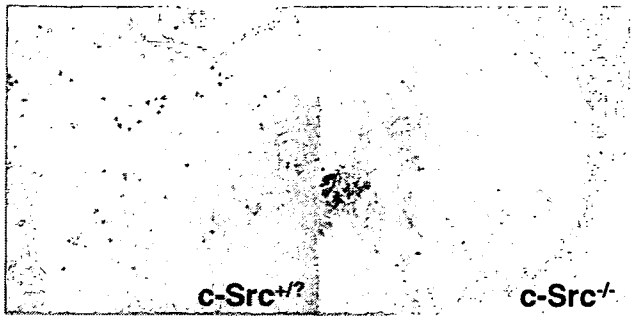

Fig. 5. Immunogold labeling of c-Src in isolated mitochondria from c-Src$^{+/?}$ and c-Src$^{-/-}$ osteoclasts. As predicted from confocal microscopy data, there is strong labeling of mitochondria. Many of gold particles are associated with the inner mitochondrial membrane in c-Src$^{+/?}$ osteoclasts, whereas no labeling was detected in the mitochondria of c-Src$^{-/-}$ osteoclasts

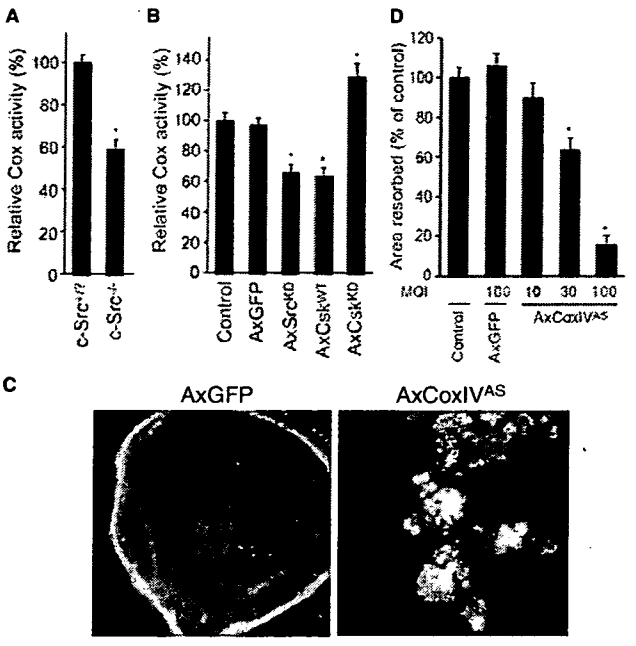

Fig. 6. A Cox activity in c-Src and c-Src$^{-/-}$ OCLs. Cox activity was reduced in c-Src$^{-/-}$ OCLs formed in vitro. The values are mean ± SD (n = 8; *P < 0.01 compared with Src$^{+/?}$ OCLs). **B** Cox activity in OCLs infected with AxGFP, AxSrc^{KD}, AxCsk^{WT}, or AxCsk^{KD} at an MOI of 100. The values are mean ± SD (n = 8; *P < 0.01 compared with uninfected OCLs). **C** Effects of CoxIV antisense (CoxIV^{AS}) on osteoclast morphology. Cocultures infected with AxGFP or AxCoxIV^{AS} at an MOI of 100 were plated on serum-coated glass coverslips for 12 h and then costained for F-actin using rhodamine phalloidin. **D** Dentine-resorbing activity of OCLs expressing CoxIV antisense. An aliquot of the OCL preparation was transferred to dentine and cultured for an additional 12 h. Bone-resorbing activity was progressively and severely inhibited as the MOI of AxCoxIV^{AS} increased. The values are mean ± SD (n = 8; *P < 0.01 compared with uninfected OCLs)

electrons from cytochrome c and four protons taken up from the mitochondrial matrix, without the formation of reactive oxygen species. The energy generated by the passage of electrons down the electron transport chain creates a proton gradient across the membrane that drives ATP synthase to make ATP from ADP.

To examine the functional consequences of the tyrosine phosphorylation of Cox by c-Src, we investigated whether or not c-Src kinase activity affected Cox activity. For this purpose, we used Src family tyrosine kinase-deficient mouse embryonic fibroblasts[37]. Cox activity in Src$^{-/-}$ Yes$^{-/-}$ Fyn$^{-/-}$ cells (SYF) was significantly decreased compared to Yes$^{-/-}$ Fyn$^{-/-}$ cells (Src++). Furthermore, c-Src re-introduction in SYF cells restored Cox activity to the higher level of Src++ cells.[22] These results lead us to speculate that c-Src can promote Cox activity in mouse embryonic fibroblasts. Further support for this conclusion was provided by an experiment showing that Cox activity in the enriched mitochondrial fraction was inhibited by the Src family inhibitor PP2. As in the SYF fibroblasts, Cox activity was reduced in c-Src$^{-/-}$ OCLs formed in vitro (Fig. 6A), providing further evidence that c-Src regulates Cox activity. To test whether modulating c-Src kinase activity in OCLs would affect Cox activity and bone resorption, we used adenovirus vectors containing kinase-dead Src (AxSrc^{K295M}), wild-type C-terminal Src family kinase (AxCsk^{WT}), or kinase-dead Csk (AxCsk^{KD}). Downregulation of c-Src kinase activity by Src^{K295M} or Csk^{WT} overexpression inhibited Cox activity in OCLs, while upregulation of Src kinase activity by AxCsk^{KD} infection induced higher Cox activity (Fig. 6B).[22] Thus, Cox activity is positively correlated with c-Src kinase activity.

Cox activity is correlated with osteoclastic bone resorption

To determine if reducing Cox activity affects the bone-resorbing activity of OCLs, we took advantage of the fact that nuclear-coded Cox subunit IV (CoxIV) is absolutely required for Cox activity.[38] So we constructed an adenovirus vector carrying CoxIV antisense (AxCoxIV^{AS}). The expression of CoxIV in OCLs infected with AxCoxIV^{AS} decreased in a dose-dependent manner. The decreasing levels of CoxIV correlated with decreased Cox activity in a biochemical assay; OCLs infected with AxCoxIV^{AS} showed 45% of basal Cox activity.[22] In addition, there was no significant change in c-Src kinase activity among OCLs infected with AxCoxIV^{AS}.

An actin ring was observed in 85% of the AxGFP-infected OCLs, whereas only 15% of OCLs infected with AxCoxIV^{AS} formed these rings (Fig. 6C). Consistent with the absence of the actin ring, bone-resorbing activity was progressively and severely inhibited by the infection with AxCoxIV^{AS} (Fig. 6D).[22] Interestingly, AxCoxIV^{AS} infection does not affect the survival of OCLs. These results strongly suggest that basal Cox activity is required for maintaining osteoclast morphology and for normal bone resorption. Consistent with these results, treating the cells with the classical Cox inhibitor KCN also decreased bone resorption. Diminished ATP levels by the failure of normal Cox could contribute to the observed loss of bone resorption Indeed, the complex I inhibitor rotenone and the complex

III inhibitor myxothiazol also prevented the bone-resorbing activity of OCLs in a dose-dependent manner, strongly suggesting that the rate of ATP generation by mitochondrial oxidative phosphorylation is critical for osteoclastic bone resorption. Furthermore, the inhibitory effect of $AxCoxIV^{AS}$ on bone resorption was not reversed by Csk^{KD} overexpression, even though c-Src kinase activity was increased in OCLs co-infected with $AxCoxIV^{AS}$ and $AxCsk^{KD}$ as much as it was in OCLs infected with $AxCsk^{KD}$ alone.[22] Taken together, these results indicate that Cox is a signaling effector downstream of c-Src that is necessary for bone resorption. Osteoclasts have a large number of mitochondria and express a high level of c-Src,[5,39,40] and a high level of ATP is required to support acid secretion by the osteoclast v-ATPase, as well as other functions that are required for bone resorption.[5] Thus, decreased Cox activity and the resulting reduction in ATP levels could explain, at least in part, the reduced bone-resorbing activity of $c\text{-}Src^{-/-}$ osteoclasts.

Concluding remarks

c-Src, which is highly conserved throughout evolution and widely expressed, associates with the cytoplasmic surface of cellular membranes. It is generally thought that c-Src's regulation of cell adhesion, movement, and proliferation involves its activity as a plasma membrane-associated molecular switch that links a variety of extracellular cues to specific intracellular signaling pathways. Our study demonstrates that both the formation of a Pyk2/Src complex, presumably in the podosomes, and the kinase activity of Src are required for bone resorption by osteoclasts. Elucidation of the role of Src in osteoclast function requires the identification of the signaling elements whose activities might to be modulated by the recruitment of active Src kinase to Pyk2. Src-catalyzed phosphorylation of Cbl on Tyr-731 is reported to create a binding site for the regulatory p85 subunit of phosphatidylinositol 3-kinase (PI3K),[41,42] and induce its activation and recruitment to the cell membrane. PI3K in turn is involved in a reorganization of the cytoskeleton that results in cell spreading and migration of several cell types including osteoclasts. Expressing the Cbl^{Y731F} mutant in osteoclasts markedly reduced their bone resorbing activity, suggesting that phosphorylation of Cbl-Y731 and the subsequent recruitment and activation of PI3K may be critical signaling events downstream of c-Src in osteoclasts. These results provide further insight into how the attachment of osteoclasts to the bone surface regulates bone resorption and how Src participates in this regulation.

c-Src is required for maintaining the basal Cox activity in mouse embryonic fibroblasts and osteoclasts. Furthermore, basal Cox activity is important for bone-resorbing activity of mature osteoclasts as well as c-Src. On the other hand, the most prominent defects of the SYF cells were reduction of cell proliferation and motility, and it should be noted that these are also ATP-dependent events. We therefore conclude that c-Src in mitochondria regulates Cox activity and that the c-Src/Cox signaling pathway is critical for the bone-resorbing activity of osteoclasts. The downregulation of Cox activity in the absence of c-Src may be involved in the osteopetrotic phenotype of $c\text{-}Src^{-/-}$ mice.

Interestingly, c-Src has also been reported to be present on late endosomes in fibroblasts,[43] synaptic vesicles in PC12 cells,[44] secretory vesicles in chromaffin cells,[45] vesicular structures in osteoclasts,[39,46] and the Golgi apparatus in CHO cells,[47] suggesting that c-Src is involved in multiple intracellular processes as well. In addition, Lyn, another Src family kinase, was found in rat brain mitochondria.[48] More recently, Itoh et al.[49] reported that Dok-4 recruits c-Src in mitochondria as an anchoring molecule and regulates NF-κB activation in endothelial cells. The subcellular fractionation also confirmed the localization of c-Src in all fractions, suggesting that c-Src associates with not only mitochondria but also various intracellular membranes. The identification and characterization of signaling cascades of c-Src on various intracellular membranes present an interesting new avenue for further elucidating Src's role in regulating cell function.

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by invasive synovial hyperplasia. Proliferation of the synovial cells leads to pannus tissue that invades the bare area between cartilage and bone, finally resulting in progressive bone and joint destruction in the affected joints. The ultimate goal of the treatment of RA is to prevent bone and joint destruction and preserve the daily activity of the patients. Recent studies have shown that osteoclasts are involved in the pathogenesis of bone and joint destruction and can be a potent therapeutic target of this disease, and that therapies that inhibit osteoclast formation or function can at least ameliorate the progression of these bone changes. We have previously demonstrated that local injection of Csk virus, which negatively regulates Src family tyrosine kinases, into rat ankle joints with adjuvant arthritis not only ameliorated inflammation but also suppressed bone destruction. There will be no cure for RA until its etiology is elucidated, but suppression of osteoclast activity by regulating various intracellular signaling pathways including c-Src might lead to a novel therapeutic strategy for preventing the joint breakdown associated with RA.

Acknowledgments We would like to thank W.C. Horne and L. Neff for discussion and consistent support.

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