Osteoclasts Express High Levels of pp60$^{c\text{-}src}$ in Association with Intracellular Membranes

W. C. Horne, L. Neff, D. Chatterjee, A. Lomri, J. B. Levy, and R. Baron

Departments of Cell Biology and Orthopaedics, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract. Deletion of the c-*src* gene in transgenic mice by homologous recombination leads to osteopetrosis, a skeletal defect characterized by markedly deficient bone resorption (Soriano, P., C. Montgomery, R. Geske, and A. Bradley. 1991. *Cell.* 64:693–702), demonstrating a critical functional role of pp60$^{c\text{-}src}$ in osteoclast activity. Since decreased bone resorption could result from a defect either within the osteoclast or within other cells present in its environment, indirectly affecting osteoclast functions, we determined which cell(s) in bone expressed high levels of pp60$^{c\text{-}src}$ Measuring pp60$^{c\text{-}src}$ protein and kinase activities in osteoclasts and immunolocalizing pp60$^{c\text{-}src}$ in bone, we find that expression of pp60$^{c\text{-}src}$ is nearly as high in osteoclasts as in brain and platelets. In contrast, other bone cells contain only very low levels of the protein. In addition, expression of the c-*src* gene product increases when bone marrow cells are induced to express an osteoclast-like phenotype by 1,25-dihydroxyvitamin D_3, further suggesting that high expression of pp60$^{c\text{-}src}$ is part of the osteoclast phenotype. Three other *src*-like kinases, c-*fyn*, c-*yes*, and c-*lyn*, are also expressed in osteoclasts at ratios to pp60$^{c\text{-}src}$ similar to what is found in platelets. These *src*-related proteins do not, however, compensate for the absence of pp60$^{c\text{-}src}$ in the *src*$^-$ mice, thereby suggesting that pp60$^{c\text{-}src}$ may have a specific function in osteoclasts. Although further work is necessary to elucidate what the critical role of pp60$^{c\text{-}src}$ in osteoclasts is, our observation that the protein is associated mostly with the membranes of intracellular organelles suggests the possibility that this role might be at least in part related to the targeting or fusion of membrane vesicles.

THE c-*src* protooncogene is highly conserved throughout evolution and widely expressed (9, 14). While the level of expression is low in most cells, some cell types, particularly neurons (10) and blood platelets (20, 47), express the gene product (pp60$^{c\text{-}src}$) and the c-*src* kinase activity at high levels. Although the physiological role of pp60$^{c\text{-}src}$ is not fully understood (9, 14, 35), it is known that pp60$^{c\text{-}src}$ and the other members of the *src* family, which share highly conserved sequences both within and outside the kinase catalytic domain, play important roles in signal transduction mechanisms that contribute to the regulation of cell growth and development (6, 9). They can be activated by various transmembrane tyrosine-kinase receptors, such as the PDGF receptor (28), and in turn, phosphorylate various substrate proteins on tyrosine residues, including the ras GTPase activating protein (9, 15), one of the subunits of the IP_3-kinase (p85) (17, 23) and two proteins with relative molecular masses of 80 kD and 85 kD, which colocalize with F-actin in peripheral extensions of normal cells and in matrix attachment structures (podosomes) in v-*src*-transformed cells (50).

To better understand the physiological role of pp60$^{c\text{-}src}$, Soriano et al. (41) have performed the targeted disruption of its encoding gene by homologous recombination in mouse embryos. Surprisingly, cell proliferation and other basal functions did not seem to be altered in the mutant c-*src*$^-$ mice and no obvious phenotypic or functional abnormalities were noticed in brain and other neuronal tissues or in platelets, possibly due to the presence in these cells of other *src*-like tyrosine kinases, e.g., c-*fyn* and/or c-*yes*, which might impart a degree of functional redundancy with pp60$^{c\text{-}src}$ (41). Unexpectedly, striking skeletal abnormalities with a phenotype of osteopetrosis were observed in the recombinant mice. These included a failure of the incisors to erupt, a slower growth, shorter and abnormally shaped long bones, and a decreased bone marrow cavity. All these changes are characteristic of impaired osteoclast activity (33). The presence of osteoclasts in apparently normal numbers in the c-*src*$^-$ animals suggested that the defect was with the function rather than the differentiation of the cells. These data therefore suggested a critical role of normal c-*src* expression in osteoclast function, and the lack of other phenotypic changes in these mutant mice suggested that the requirement for normal c-*src* expression in bone was more stringent than in other tissues. The failure of osteoclasts of c-*src*$^-$ mice to resorb bone at a normal rate could, however, be due either to alterations within the osteoclasts or to alterations in other cells, which would be necessary for the complete differentiation or activation of the osteoclast (43). This study was consequently undertaken to determine the cellular and subcellu-

lar distribution of pp60$^{c\text{-}src}$ in bone. Our results indicate that pp60$^{c\text{-}src}$ is expressed at high levels in osteoclasts, where it is associated predominantly with intracellular organelles, but not in other bone cells. Osteoclasts were also found to express c-*fyn*, c-*lyn*, and c-*yes*. Culturing bone marrow cells with 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$),[1] which induces differentiation into osteoclast-like cells (4, 5), resulted in increased expression of pp60$^{c\text{-}src}$. These results, taken together with the observation of Soriano et al. (41), suggest that pp60$^{c\text{-}src}$ is part of the osteoclast phenotype and may perform a function associated with intracellular vesicles in the osteoclast that is essential for normal bone resorption.

Materials and Methods

Cell and Tissue Preparation

All buffers used for cell isolation and lysis contained protease inhibitors (0.1 mM PMSF, 1 μM pepstatin, and 1 μM leupeptin). Osteoclasts were isolated from adult laying hens fed a calcium-deficient diet for 14 d (51). Tibias and femurs were quickly dissected and placed in ice-cold phosphate-buffered saline containing 0.1% bovine serum albumin (PBS-BSA). All subsequent steps were performed on ice. After splitting the bone longitudinally, the medullary bone was removed and rinsed with PBS-BSA to remove bone marrow cells, and then minced, and the cells were dissociated from the bone fragments by shaking. The resulting suspension was filtered through 100-μm nylon mesh to separate the cells from the bone fragments. The bone fragments were resuspended in PBS-BSA and the process was repeated four times. Pooled filtrates were centrifuged at 150 *g* for 5 min. The cell pellet was resuspended in 0.25% NaCl (10 ml per hen) and kept on ice for 7 min with mild vortexing every 90 s to lyse erythrocytes. After adding an equal volume of 1.6% NaCl, the final cell suspension was passed through a 50-μm nylon mesh to remove debris and the cells were recovered by centrifugation at 150 *g* for 5 min. The pelleted cells were resuspended in PBS-BSA (15 ml/hen; sample OC1) and layered over 30 ml of 70% heat-inactivated fetal bovine serum, 30% αMEM and kept on ice for 80 min to permit the osteoclasts to sediment at unit gravity. The top 20 ml was removed to a clean tube (sample OC2S) and the remaining 20 ml was centrifuged at 150 *g* for 5 min. The partially purified osteoclast pellet was then resuspended in PBS-BSA (15 ml/hen; OC2P), layered over 30 ml of 100% FBS (heat-inactivated), and kept on ice for an additional 40 min. The top 25 ml was aspirated, and the remaining 15 ml in the tube was centrifuged as before, generating an osteoclast preparation (OC3) that was used immediately or kept frozen at −70°C for future use. On average, the starting cell suspension contained approximately one osteoclast/500–1,000 cells. After the first unit gravity sedimentation, the purity was 1 osteoclast to 100–150 other cells. In the final osteoclast preparation the purity was 1 osteoclast to 5–10 contaminating cells. Since unit gravity sedimentation favored the selection of the much larger osteoclasts, we estimate that ~90% of the protein in these preparations was derived from osteoclasts.

Whole chicken brain was removed and minced in ice-cold PBS-BSA. The minced tissue was homogenized in a Dounce homogenizer with a loose-fitting pestle in 5 vol of ice-cold RIPA buffer (29) or PBS containing 0.5% SDS, and insoluble material was removed by centrifugation for 15 min at 27,000 *g*.

Platelets were isolated from chicken and human blood anticoagulated with 1:10 vol of 3.8% sodium citrate. All procedures were performed at room temperature to minimize platelet activation and subsequent aggregation during centrifugation. The blood was centrifuged for 10 min at 190 *g*, and the platelet-rich plasma was removed to a clean tube. The platelets were washed in PBS containing 5 mM EGTA and the final pellet was lysed in either RIPA buffer or PBS containing 0.5% SDS.

Bone Marrow Cell Cultures

Bone marrow cells were isolated from the tibias and femurs of white leghorn chickens (SPAFAS, Norwich, CT) that had been fed a calcium-deficient diet (Ralston Purina, Richmond, IN) for 1–2 wk after hatching in αMEM containing 2% heat-inactivated chicken serum (Sigma Chemical Co., St. Louis, MO), 8% heat-inactivated FBS (Sigma Chemical Co.), 50 UI/ml penicillin, and 50 μg/ml streptomycin. Mononuclear cells were fractionated on Ficoll-Paque (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) by sedimentation to the medium–Ficoll interface. To deplete these preparations of the most mature stromal and myelomonocytic cells, isolated cells were plated for 16 h at 5×10^6 cells/ml in the same medium used in the isolation procedure (3). Nonadherent cells were recovered after 16 h of culture, replated in 150-mm tissue culture plates at a density of 6×10^6 cells/ml (8×10^5 cells/cm^2), and cultured for 2–6 d. Cells were grown in the presence or absence of 10^{-8} M exogenous $1,25(OH)_2D_3$ (provided by Dr. Milan Uskokovic, Hoffman-LaRoche, Nutley, NJ).

Western Blots

Western blotting (29, 46) was performed using alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (Promega Corp., Madison, WI), except when the amount of pp60$^{c\text{-}src}$ was to be quantitated, in which case biotinylated mAb 327 was used as the primary antibody and [^{125}I]streptavidin (20–40 mCi/mg; Amersham Corp., Arlington Heights, IL) was used to detect the bands; mAb 327 was biotinylated using the Pierce biotinylation kit (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions.

Immunoprecipitation and Kinase Assay

Kinase activity was measured by a method derived from those of Levy and Brugge (29) and Kypta et al. (28). Samples were lysed in RIPA buffer. Equal amounts of protein (200–400 μg) were incubated with antibody on ice for 45 min, and the immune complexes were precipitated with protein A–agarose (for rabbit polyclonal antibodies) or protein G–agarose (for mouse mAbs). The beads were washed twice with RIPA buffer and once with 100 mM NaCl, 10 mM Tris, and 5 mM $MgCl_2$, pH 7.0. In some cases, immunoprecipitated pp60$^{c\text{-}src}$ was split into two equal portions, one to be used for assaying kinase activity and the other for Western blot to quantitate the amount of pp60$^{c\text{-}src}$ present. Kinase activity was measured by incubating the beads with 5 mg acid-denatured rabbit muscle enolase and 100 mM [γ-^{32}P]ATP (5 μCi/sample) in 20 mM Hepes (pH 7.5) and 10 mM $MnCl_2$ for 15 min on ice. The samples were subjected to SDS-PAGE on 8% gels, which were stained, dried, and autoradiographed.

Immunocytochemistry

Immunoperoxidase. White leghorn chickens (SPAFAS) that had been fed a calcium-deficient diet (Ralston Purina) for 1 wk after hatching, and 4-d-old Wistar rat pups were perfused, via the heart or the femoral artery, respectively, with PBS for 1 min, followed by paraformaldehyde (2%), lysine (0.75 M), sodium periodate (0.01 M) (PLP) for 5 min. The proximal tibias were dissected out and slices were cut out of the primary spongiosa area under the growth plate. The slices were fixed in PLP for an additional 4 h at 4°C and then washed in PBS containing 10% DMSO as a cryoprotectant. The tissue was subsequently quick-frozen and 40-μm sections were prepared on a cryostat (Bright Instr. Co., Ltd., Huntingdon, UK) using tungsten carbide knives. Sections were incubated overnight at 4°C in mAb 327, a monoclonal antibody against chicken pp60$^{c\text{-}src}$ (Oncogene Science, Inc., Manhasset, NY) diluted in PBS + 0.1% BSA.

After washing (2 h) in PBS + 0.1% BSA, the sections were incubated with Fab fragments of peroxidase-labeled goat anti-mouse IgG (Biosys, France). Sections were incubated with the secondary antibodies at a dilution of 1:100 in PBS + 0.1% BSA for 2 h at 20°C. After washing, the sections were reacted in DAB (1 mg/ml in 0.05 M Tris buffer, pH 7.4; Polysciences Inc., Warrington, PA) in the presence of 0.1% H_2O_2 and post-fixed in ferrocyanide-reduced OsO_4. After embedding in Epon (Polybed 812; Polysciences Inc.), 1-μm-thick sections were cut with a glass knife and counterstained with methylene blue-azure II for identification of areas of interest. Selected areas were then sectioned with a diamond knife and stained with lead citrate. Grids were viewed on a JEOL-CX 100 electron microscope (JEOL U.S.A. Inc., Peabody, MA).

Immunofluorescence Labeling. For immunofluorescence staining of bone tissue, white leghorn chickens maintained on a calcium-deficient diet for 1 wk after hatching were perfused with PLP as described above. The proximal tibias were dissected out and slices were cut out of the primary spongiosa area under the growth plate. The slices were fixed in PLP for an additional 4 h at 4°C, washed in PBS for 1 h, and infiltrated overnight with 30% sucrose in PBS. The tissue was subsequently quick-frozen, and 6-μm sections were prepared on a Bright cryostat using tungsten carbide knives

1. *Abbreviations used in this paper*: $1,25(OH)_2D_3$, 1,25 dihydroxyvitamin D_3; PLP, paraformaldehyde, lysine, sodium periodate.

and transferred to slides. For immunofluorescence staining of isolated osteoclasts, osteoclasts were isolated from 4-d-old Wistar rat pups as described elsewhere (42) and cultured overnight on glass coverslips or slices of cortical bovine bone. The cells were fixed in 3.7% formaldehyde in PBS for 10 min at room temperature and washed with PBS. Further processing of tissue sections and isolated cells was identical. All subsequent incubations were performed at room temperature with PBS containing 0.05% saponin and 5% normal goat serum. The samples were incubated for 1 h with PBS-saponin-normal goat serum to block nonspecific binding, then for 1 h with mAb 327 diluted 1:25. The samples were washed with PBS-saponin and incubated with rhodamine-conjugated goat anti-mouse IgG (Boehringer Mannheim Corp., Indianapolis, IN) for 1 h in the dark. After washing with PBS, the samples were mounted in FluorSave (Calbiochem Corp., La Jolla, CA). Microscopy was performed using a confocal microscope (model MRC 600; Bio-Rad Laboratories, Richmond, CA) with a krypton laser.

Immunogold Labeling. Immunogold labeling was performed by the method of Tokuyasu (45), with some modifications. White leghorn chickens maintained on a calcium-deficient diet for 1 wk after hatching were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 5 min. The proximal tibias were dissected out and the growth plate was removed and fixed by immersion in 4% paraformaldehyde plus 0.1% glutaraldehyde for 4 h at 4°C. The fixed tissue was washed in PBS and infiltrated overnight in 2.3 M sucrose plus 15% polyvinylpyrolidone. Sections were cut on an ultracryotome (Reichert Scientific Instruments, Buffalo, NY). Fixed sections on grids were transferred to PBS containing 1% BSA for 30 min. All subsequent incubations were performed at room temperature. Grids were incubated with mAb 327 diluted 1:25 in PBS + 1% BSA for 1 h, and then washed and incubated with a 1:50 dilution of anti-mouse IgG–colloidal gold complexes (10 nm; Amersham Corp.) for 15 min. After washing in PBS, the grids were counterstained with uranyl acetate and embedded in methyl cellulose. Grids were viewed on a JEOL-CX100 electron microscope (JEOL U.S.A. Inc.).

Subcellular Fractionation

Osteoclasts were resuspended in 5 vol of ice-cold HARM's buffer (10 mM triethanol amine, 10 mM acetic acid, 1 mM EDTA, pH 7.4) containing 0.25 M sucrose and protease inhibitors and passed 8–10 times through a 25-gauge needle. The homogenate was centrifuged at 1,000 *g* for 10 min and the supernatant was centrifuged at 10,000 *g* for 10 min to pellet mitochondria. The pellet was washed once in HARM's buffer. The 10,000 *g* supernatant was centrifuged at 100,000 *g* for 90 min in a rotor (model SW41; Beckman Instruments, Inc., Fullerton, CA) to sediment microsomal membranes. The supernatant was retained and the pellet was washed by being resuspended in HARM's buffer without sucrose and centrifuged at 100,000 *g* for 90 min.

Northern Blots

Total RNA from the bone marrow cells cultured with or without 1,25-$(OH)_2D_3$ for 6 d was isolated using the guanidium isothiocyanate method (7). Poly (A+) mRNA was purified from bone marrow total RNA using the PolyATract mRNA isolation system (Promega Corp.). Formamide-denatured poly (A+) mRNA was electrophoresed on 1% formaldehyde/agarose gels and transferred to nylon membranes (Hybond N^+; Amersham Corp.). Membranes were prehybridized for 30 min at 65°C in rapid hybridization buffer (Amersham Corp.) and hybridized in the same buffer with a gel-purified 1.65-kb cDNA probe encoding the chicken c-*src* (30) for 2 h at 65°C. The probe was labeled with [α-^{32}P]dCTP by nick translation (39). Membranes were washed twice under high stringency conditions in 2× SSC/0.1% SDS for 15 min each at room temperature, and once each in 1× SSC/0.1% SDS and 0.1× SSC/0.1% SDS for 30 min at 65°C. Membranes were exposed to x-ray film at −70°C using an intensifying screen.

Protein Assay

Protein concentrations were determined with the Micro BCA protein assay reagent (Pierce Chemical Co.).

Antibodies

mAb 327, which recognizes chicken pp60$^{c\text{-}src}$ (32), was obtained from J. Brugge and Oncogene Science Inc. A second mAb against pp60$^{c\text{-}src}$, LA074, raised against residues 2–17 of pp60$^{v\text{-}src}$, was obtained from Quality Biologics Inc. (Camden, NJ). Polyclonal antibodies against the *src*-related kinases were obtained from Drs. J. Bolen (c-*blk*, c-*fgr*, c-*fyn*, c-*hck*, c-*lck*, and c-*lyn*) (25), J. Brugge (c-*yes*), S. Courtneidge (c-*yes*) (28), and R. Perlmutter (c-*fyn*) (8).

Results

Immunoblotting and src Kinase Activity

Osteoclasts were isolated from calcium-deficient hens and the proteins were immunoblotted with mAb 327. The results demonstrated that the characteristic c-*src* 60-kD band became progressively enriched during purification of the osteoclasts (Fig. 1 *A*). pp60$^{c\text{-}src}$ was detectable only at much lower levels in the contaminating cells, which are mostly derived from bone and bone marrow but also contain osteoclasts, albeit at low numbers (Fig. 1 *B*). The amount of pp60$^{c\text{-}src}$ in the most pure preparations of osteoclasts was greater than the level of the protein in homogenates of whole chicken brain, a tissue known to be rich in pp60$^{c\text{-}src}$ (10), but less than the level in purified human platelets (Fig. 1 *B*).

To determine on a more quantitative basis the level of pp60$^{c\text{-}src}$ activity in osteoclasts relative to other cell types, tyrosine kinase activity was assayed using enolase as a substrate. The results demonstrated (Fig. 1 *C*) that the level of pp60$^{c\text{-}src}$ kinase activity present in osteoclasts was comparable to the activity in brain and about one-quarter of the level in platelets ($27 \pm 20\%$, $n = 4$). For comparison, it has been reported that pp60$^{c\text{-}src}$ kinase activity in tissues such as muscle, spleen, thymus, and bone marrow are only 3–5% of the activity in platelets, while activity in normal fibroblasts is <1% of that in platelets (19, 20). Furthermore, the assay showed that the pp60$^{c\text{-}src}$ kinase activity present in the initial unfractionated bone cell preparations sedimented almost entirely with the osteoclast fraction and was barely detectable in bone marrow and other bone mononuclear cells, confirming that the pp60$^{c\text{-}src}$ in the initial bone cell preparations is almost exclusively in the osteoclasts.

Immunocytochemistry

Further evidence of specific high expression of pp60$^{c\text{-}src}$ in osteoclasts was obtained in immunocytochemical analysis of normal neonatal rat and chicken bone. When immunoperoxidase staining with mAb 327 was performed, the osteoclasts were very strikingly labeled, in contrast to other bone cells (i.e., osteoclasts, osteocytes, cells lining the bone surfaces, and bone marrow cells) in which the amount of pp60$^{c\text{-}src}$ was below the level of detection (Fig. 2, *A* and *C*). Among osteoclasts, pp60$^{c\text{-}src}$ appeared to be expressed at different levels, with some cells showing more intense staining than others (not shown), suggesting that the amounts of pp60$^{c\text{-}src}$ in osteoclasts might differ, depending on the functional state of the cell. The high expression of pp60$^{c\text{-}src}$ in osteoclasts relative to other bone cells was confirmed by immunofluorescence staining of bone tissue (Fig. 2 *B*).

While it is known that pp60$^{c\text{-}src}$ is bound to the cytoplasmic surface of membranes via a myristic acid group at the NH_2 terminus of the protein (6), examination of the sections at the EM level, although often suggesting enrichment close to the plasma membrane, failed to demonstrate restriction to a specific plasma membrane domain or to specific intracellular membranes. On the contrary, the labeling appeared to be distributed throughout the cytosol (Figs. 2 *C* and 3 *A*), including the ruffled border area (Fig. 3 *C*), sug-

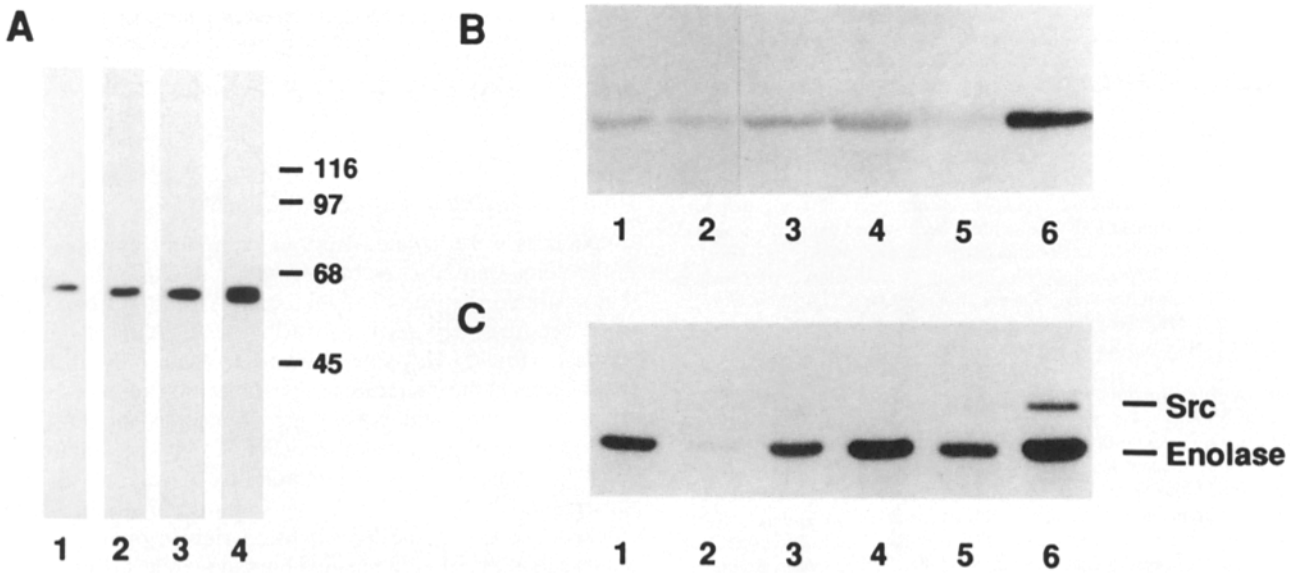


Figure 1. Avian osteoclasts are enriched in pp60$^{c\text{-}src}$. Osteoclasts were isolated from calcium-deprived hens as described in Materials and Methods. (*A*) Preparations of increasing relative abundance of osteoclasts were solubilized in 0.5% SDS. Equal amounts of protein (100 μg) were subjected to SDS-PAGE and transferred to nitrocellulose and the pp60$^{c\text{-}src}$ was detected by immunoblotting with mAb 327, demonstrating that pp60$^{c\text{-}src}$ copurifies with osteoclasts. Lane *1*, 1 osteoclast (OC), 1,000 other cells; lane *2*, 1 OC, 200 other cells; lane *3*, 1 OC, 50 other cells; lane *4*, 1 OC, 5 other cells. Positions of molecular mass standards are indicated in kilodaltons. (*B*) Samples that were taken at different stages of the osteoclast purification, described in Materials and Methods, as well as chicken brain and human platelets, were solubilized in 0.5% SDS. Equal amounts of protein (100 μg) were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with mAb 327. The results demonstrate that nearly all of the pp60$^{c\text{-}src}$ sediments with the osteoclasts. Lane *1*, unfractionated bone cells (1 OC, 500 other cells); lane *2*, unsedimented cells after the first unit gravity sedimentation (<1 OC, 2,000 other cells); lane *3*, sedimented cells from the first unit gravity sedimentation (1 OC, 100 other cells); lane *4*, final osteoclast preparation (1 OC, 5 other cells); lane *5*, chicken brain; lane *6*, human platelets. (*C*) *Src* tyrosine kinase activity. Samples taken at different stages of the osteoclast purification, as well as chicken brain and human platelets, were assayed for *src* tyrosine kinase activity. The cells were lysed with RIPA buffer, equal amounts of protein (250 μg) were immunoprecipitated with mAb 327, and the immunoprecipitates were assayed for tyrosine kinase activity by measuring ^{32}P incorporation into enolase as described in Materials and Methods. The results demonstrate that the *src* kinase activity sediments with the osteoclasts, and that the level of *src* kinase activity in osteoclasts is comparable to levels in brain and platelets. Lane *1*, unfractionated bone cells (1 OC, 300 other cells); lane *2*, unsedimented cells after the first unit gravity sedimentation (<1 OC, 2,000 other cells); lane *3*, sedimented cells from the first unit gravity sedimentation (1 OC, 100 other cells); lane *4*, final osteoclast preparation (1 OC, 7 other cells); lane *5*, chicken brain; lane *6*, human platelets.

gesting that c-*src* might be associated with multiple membranes within the osteoclast, or even, as unlikely as it may seem, soluble in the cytosol. A similarly diffuse pattern was also observed by immunofluorescence of frozen sections (Fig. 2 *B*). Since this diffuse pattern could also result from the procedures we used, we then used immunogold and confocal immunofluorescence techniques to localize pp60$^{c\text{-}src}$ in osteoclasts. Osteoclast pp60$^{c\text{-}src}$ was only weakly labeled by immunogold, possibly due to limited access to the epitopes, but the results strongly suggested an association with membranes, particularly intracellular membranes (Fig. 3 *B*).

To further analyze the distribution of pp60$^{c\text{-}src}$ in osteoclasts, isolated cells were studied by confocal microscopy. For this purpose, isolated rat osteoclasts were allowed to adhere to glass coverslips or to slices of bovine cortical bone, fixed, and stained with either mAb 327 or LA074. With both antibodies, intracellular staining was apparent and was distributed throughout the cells in optical sections taken at various levels from the top of the cell to the level of attachment to the substrate (Fig. 4). A clearly punctate pattern of staining was seen in osteoclasts on glass (Fig. 4, *A*, *C–E*), while the pattern in cells on bone was more diffuse (Fig. 4 *B*). Although an accumulation of stained particles was sometimes observed along the cytoplasmic face of the plasma membrane (Fig. 4 *C*), staining of the plasma membrane and lamellipodia was low or undetectable relative to the intracellular punctate structures. Most often, the pp60$^{c\text{-}src}$-containing structures were distributed unevenly through the cell interior, usually well separated from the plasma membrane.

Subcellular Fractionation

To further establish the association of pp60$^{c\text{-}src}$ with osteoclast membranes, we then performed cell fractionation and examined the level of the protein by immunoblot analysis. We examined membrane and cytosol fractions prepared from purified osteoclasts, as described in Materials and Methods, for pp60$^{c\text{-}src}$. As shown in Fig. 5, little of the pp60$^{c\text{-}src}$ was present in the cytosol (lane *3*). Of the two membrane fractions, the 100,000-*g* pellet (lane *2*), enriched in plasma membrane, golgi, and endosomal markers, contained more pp60$^{c\text{-}src}$ than the 10,000-*g* pellet (lane *1*), which was enriched in mitochondrial markers. These data confirmed that pp60$^{c\text{-}src}$ is membrane associated in osteoclasts, as in other cell types (1, 6, 11, 16, 22, 24, 31, 36–38).

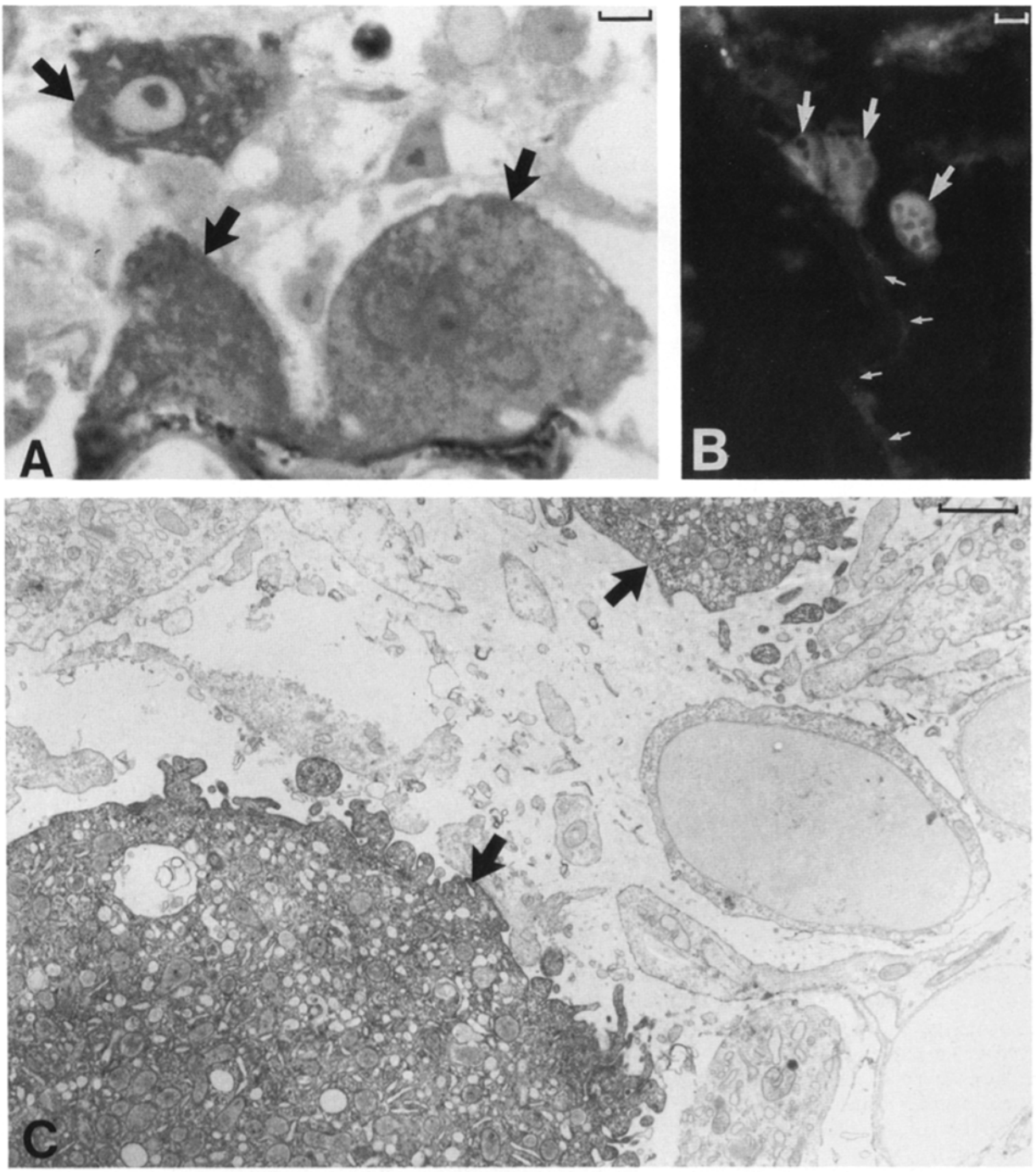


Figure 2. pp60$^{c\text{-}src}$ is highly enriched in osteoclasts but not in other bone cells. Elevated expression of pp60 in osteoclasts (*large arrows*, *A–C*) but not other nearby cells (*small arrows*, *B*) in chick growth plate, demonstrated by immunoperoxidase staining (*A* and *C*) and immunofluorescence of frozen sections (*B*) with mAb 327. With both techniques, pp60$^{c\text{-}src}$ was found to be distributed throughout the cytoplasm without apparent polarity (see also Fig. 3). Bars: (*A*) 5 μm; (*B*) 20 μm; (*C*) 2 μm.

Induction of c-src during Bone Marrow Differentiation

Since it appeared that high levels of c-*src* expression constituted part of the phenotype of mature osteoclasts, we then investigated whether expression of the c-*src* gene was specifically induced during the differentiation of this cell type. Bone marrow cells from calcium-deprived chicks were cultured for 2–6 d in the presence or absence of 10^{-8} M exogenous $1,25(OH)_2D_3$. We have previously demonstrated that, in this culture system, $1,25(OH)_2D_3$ induces the expression of several osteoclast markers (carbonic anhydrase II, vitronectin receptor, tartrate-resistant acid phosphatase, and Na^+/K^+ ATPase) (5). In the cells cultured with $1,25(OH)_2D_3$, the amount of pp60$^{c\text{-}src}$ (Fig. 6) and *src* kinase activity (not shown) at day 4 was about two-fold greater than the level in untreated cells. However, the amount of pp60$^{c\text{-}src}$ also increased over time in control cells, albeit at a slower rate than in the $1,25(OH)_2D_3$-treated cells, confirming a

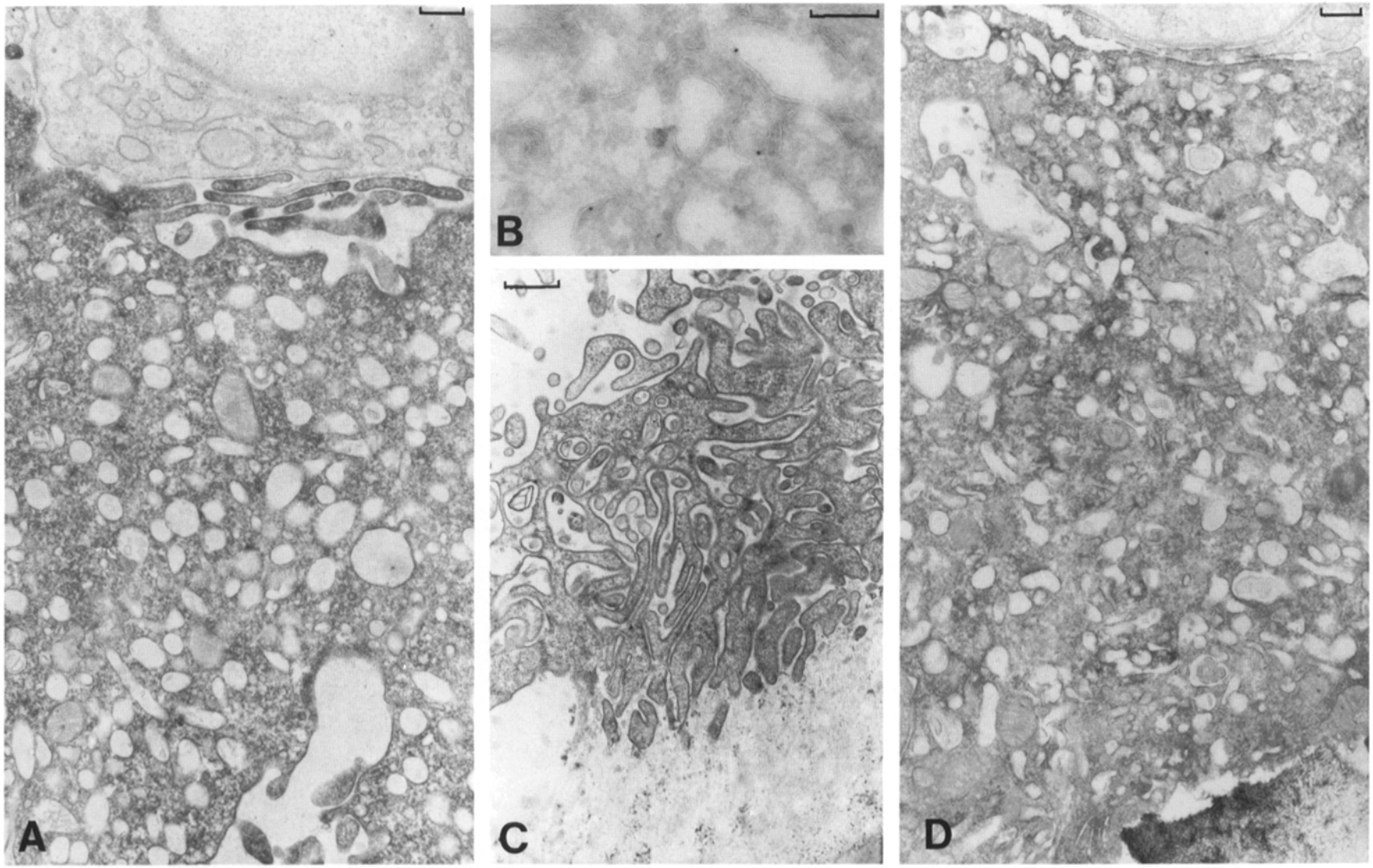


Figure 3. Widespread membrane-associated distribution of pp60$^{c\text{-}src}$ and c-*yes* in osteoclasts. (*A*, *C*) Immunoperoxidase staining of chick bone with mAb 327, showing the distribution of pp60$^{c\text{-}src}$ throughout the cytoplasmic compartment of osteoclasts, near the basolateral membrane (*A*, *top*), and the ruffled border region (*C*). A cell that is not an osteoclast is visible at the top of *A*, with undetectable levels of pp60$^{c\text{-}src}$ expression. (*B*) The intracellular localization of pp60$^{c\text{-}src}$ was confirmed by immunogold labeling of osteoclasts with mAb 327, demonstrating the association of pp60$^{c\text{-}src}$ with the limiting membranes of intracellular organelles. (*D*) Immunoperoxidase staining of chick bone with anti-c-*yes*, demonstrating a localization throughout the cell, similar to that of pp60$^{c\text{-}src}$. (*Bottom*, bone matrix; *top*, basolateral domain.) Bars: (*A*, *C*, and *D*) 0.5 μm; (*B*) 0.2 μm.

previous report of increased expression of pp60$^{c\text{-}src}$ and *src* kinase activity during marrow cell differentiation in the presence of serum (18). When c-*src* mRNA was examined in cells cultured for 6 d with or without $1,25(OH)_2D_3$, the levels were only slightly higher (20–80%) in the $1,25(OH)_2D_3$-treated cells than in control cells (not shown).

Src-related Tyrosine Kinases

Finally, we also screened for other members of the *src* family that share some functional properties of pp60$^{c\text{-}src}$ (6), in order to determine whether the apparent sensitivity of osteoclasts to mutation of the c-*src* gene could possibly be explained by their failure to express such related tyrosine kinases. Tyrosine kinase assays were performed on osteoclast lysates using antibodies in the immunoprecipitation step that are directed against unique sequences of individual *src*-related kinases (25, 28, 32). In addition to pp60$^{c\text{-}src}$ kinase activity, c-*fyn*, c-*lyn*, and c-*yes* kinase activity was detected (Fig. 7). While the kinase activities of the other members of the family, c-*blk*, c-*fgr*, c-*hck*, and c-*lck*, were essentially absent, we cannot rule out the possibility that one or more are present at undetectable levels. The levels of activity of c-*fyn*, c-*lyn*, and c-*yes* in osteoclasts were 1–5% of the pp60$^{c\text{-}src}$ kinase activity, similar to the relative activities of these kinases in human platelets. In immunocytochemical studies, osteoclasts were strongly stained by antibodies to c-*yes*, with an intracellular distribution similar to that seen for pp60$^{c\text{-}src}$ stained with mAb 327 (Fig. 3 *D*). On the other hand, only weak staining was seen with antibodies to c-*fyn* and c-*lyn*.

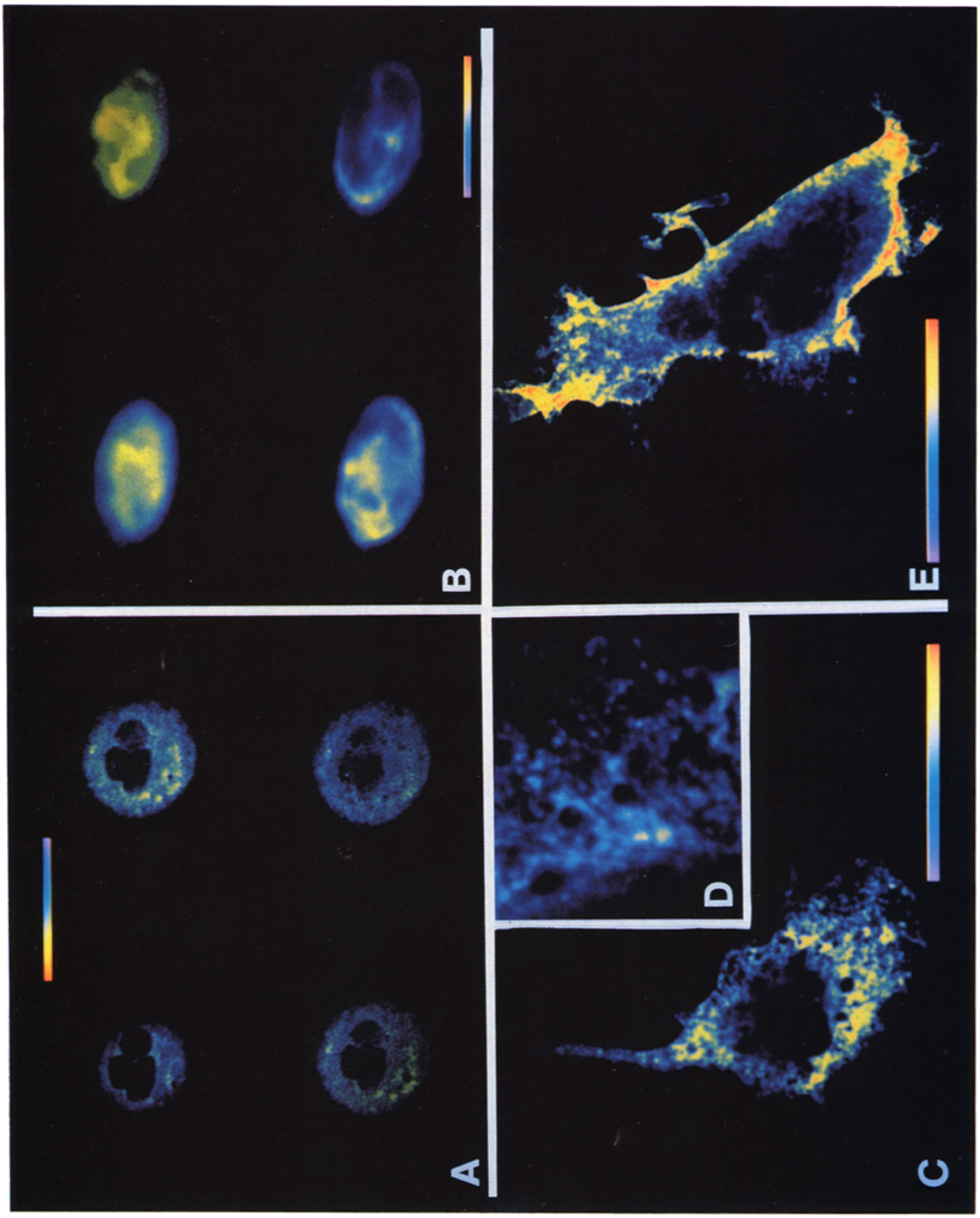
Discussion

The report by Soriano et al. (41) that the *src*$^-$ mouse is osteopetrotic was a dramatic demonstration that pp60$^{c\text{-}src}$ plays a key role in osteoclast function. The aim of this study was to identify which bone cells expressed pp60$^{c\text{-}src}$, and with what subcellular distribution, as a first step in analyzing the requirement for this protein in bone resorption. Although our results do not address the issue of pp60$^{c\text{-}src}$'s functional role in bone resorption, they indicate that the protein is specifically expressed at high levels in the osteoclast, as shown by immunocytochemistry, immunoblotting, and *src* kinase activity. Induction of differentiation of bone marrow mononuclear cells toward an osteoclast-like phenotype by treatment with $1,25(OH)_2D_3$ (4, 5, 33) led to higher levels of pp60$^{c\text{-}src}$ than in untreated cells, further suggesting that osteoclasts are characterized by elevated c-*src* expression. These results clearly demonstrate a direct link between the osteoclast and c-*src* expression, and strongly suggest, but by no means prove, that the osteoclast may be the site of the specific defect in c-*src*$^-$ mice that leads to impaired osteoclast function and osteopetrosis. This possibility is further supported by the ability of Soriano, Mundy, and colleagues to reverse the osteopetrotic condition of the *src*$^-$ mice by transplanting hematopoietic tissues (liver or bone marrow) from normal mice (Mundy, G., and P. Soriano, personal communication).

While this initial study did not attempt to identify specific functions of pp60$^{c\text{-}src}$ in osteoclastic bone resorption, some possibilities are suggested by considering the immunolocalization results and the known characteristics of the protein in other cells. As in other cells, the protein is virtually entirely membrane bound. Most striking is the fact that, although we cannot exclude the presence of lower levels of pp60$^{c\text{-}src}$ at the plasma membrane, the higher concentration of the protein is found at the limiting membrane of intracellular vesicles. This is in contrast to the situation in platelets, where the majority of the pp60$^{c\text{-}src}$ is associated with the plasma membrane and the membrane of the surface-connected canalicular system (16). Both confocal and electron microscopy suggest that these vesicles are not distributed in any polarized fashion within the osteoclast. Interestingly, this pattern of staining is similar to what has been observed in cells derived from MDCK cells (48, 49), COS cells (26), and NIH 3T3 cells (12), which overexpress pp60$^{c\text{-}src}$ after transfection. In the case of the overexpressing NIH cells, pp60$^{c\text{-}src}$ colocalized in part with vesicular components of the endocytic pathway (12). Although the possibility was raised that this distribution resulted from overexpression of the protein, our results in normal cells, both in situ and on glass, suggest that the association of pp60$^{c\text{-}src}$ with vesicles found in the perinuclear region may be functionally significant. The association of pp60$^{c\text{-}src}$ with secretory granules and vesicles has also been reported in platelets (37), chromaffin cells (22, 36), neurons (1, 24), and PC12 cells (31), further suggesting that the protein may contribute to vesicle targeting or membrane fusion. In the osteoclast, targeted delivery of intracellular vesicles to the apical surface of the cell is required to secrete enzymes into the resorption compartment and to insert the proton pump and other transporters involved in acid secretion at the apical ruffled border membrane (2). Although the apparent association of pp60$^{c\text{-}src}$ with intracellular vesicles in the osteoclast is consistent with this possible function, further work will be required to clarify this issue.

It has also been suggested that pp60$^{c\text{-}src}$ may be involved in integrin-related attachment and signal transduction mechanisms, another potential role that is of particular interest with regard to osteoclasts. In platelets, inhibiting the binding of the integrin GP IIb-IIIa to fibrinogen blocks agonist-induced tyrosine kinase activity (21), while in epidermal carcinoma cells, antibody-mediated clustering of integrin $\alpha_5\beta_1$ activates tyrosine kinases (27). In osteoclasts, integrins, specifically $\alpha_v\beta_3$ (the vitronectin receptor), are involved in bone resorption (13), probably mediating the attachment of the osteoclast to the bone matrix (52) and participating in the sealing-off of the extracellular bone resorbing compartment. Although pp60$^{v\text{-}src}$ is known to associate with focal points of adhesion in v-*src*-transformed fibroblasts (34, 40), pp60$^{c\text{-}src}$ never appears in focal adhesion plaques or rosettes (podosomes) in NIH cells that overexpress pp60$^{c\text{-}src}$ (12). In agreement with these results, our confocal microscopic results fail to show a specific association of pp60$^{c\text{-}src}$ with adhesion structures. Integrin binding to bone matrix might nevertheless involve pp60$^{c\text{-}src}$ and provide a signal leading to the development of cell polarity and the formation of the ruffled border at the site of bone resorption. Consistent with such a possible involvement of protein tyrosine kinases in integrin function in osteoclasts, preliminary studies in our laboratory indeed suggest that a rapid, transient wave of tyrosine phosphorylation is triggered in isolated osteoclasts upon exposure to RGD-containing peptides that bind to integrin.

Redundancies in the functional associations of the several


A
B
C
D
E

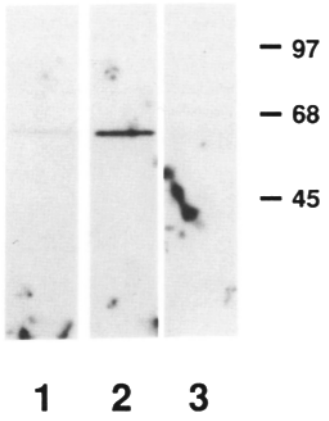


Figure 5. Osteoclast pp60$^{c\text{-}src}$ is membrane associated. Purified osteoclasts (1 OC, 6 other cells) were homogenized and the homogenates were fractionated by differential centrifugation as described in Materials and Methods. Proteins were solubilized in RIPA buffer and equal amounts of protein (100 μg) from the membrane and cytosol fractions were subjected to immunoprecipitation with mAb 327. The immunoprecipitates were run on SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblot with biotinylated mAb 327 and [^{125}I]streptavidin. The 100,000-*g* pellet is mostly highly enriched in pp60$^{c\text{-}src}$, while the protein is not detected in the 100,000-*g* supernatant. Lane *1*, 10,000-*g* pellet; lane *2*, 100,000-*g* pellet; lane *3*, 100,000-*g* supernatant.

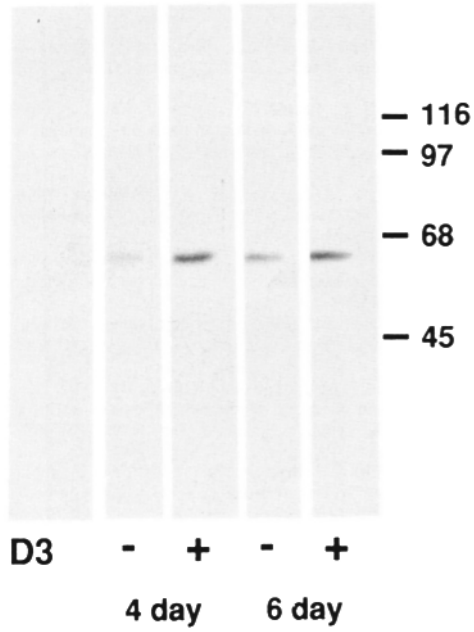


Figure 6. Increased pp60$^{c\text{-}src}$ expression during osteoclast differentiation. Chick marrow mononuclear cells were cultured for 4 and 6 d in the presence (+) or absence (−) of 10^{-8} M 1,25(OH)$_2$D$_3$. The cells were harvested by gentle scraping and lysed in 0.5% SDS. Equal amounts of protein were subjected to SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblot with mAb 327. Molecular weight markers are indicated in kilodaltons. The amount of pp60$^{c\text{-}src}$ is increased when cells are cultured in the presence of 1,25(OH)$_2$D$_3$.

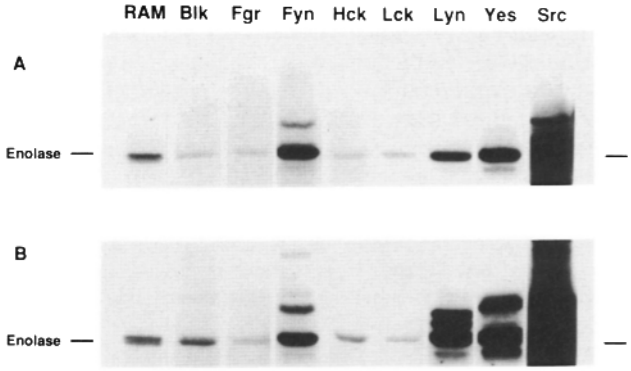


Figure 7. Osteoclasts express other members of the *src* family. Equal amounts of protein (100 μg for *src*, 200 μg for the other kinases) from lysates of chicken osteoclasts (*A*) and human platelets (*B*) solubilized with RIPA buffer were immunoprecipitated with antibodies against individual members of the *src* family or with rabbit anti-mouse IgG (*RAM*), and the immunoprecipitated material was assayed for tyrosine kinase activity as in Fig. 1 *C*.

members of the *src* family present in platelets and neurons (23, 25, 44) could potentially explain the absence of gross alteration of platelet and nervous system function in the c-*src*$^-$ transgenic mice (41). The apparent specificity of the bone resorption defect in the c-*src*$^-$ mice implied either that bone cells lack other *src*-like proteins that could fill the functional role of pp60$^{c\text{-}src}$, or that the protein performs some function that is specific to both pp60$^{c\text{-}src}$ and bone cells. To address this issue, we tested osteoclast preparations for the presence of other members of the *src* family that are known to share certain functional properties with pp60$^{c\text{-}src}$ (28). The presence of three other members of the *src* family, c-*fyn*, c-*lyn*, and c-*yes*, in osteoclasts, and at levels comparable to the levels in platelets or brain, clearly indicates that the resorption defect in the *src*$^-$ mouse cannot be explained simply by the absence of other *src*-like proteins. The presence in osteoclasts of these members of the *src* family also demonstrates that the scope of the contribution of nonreceptor tyrosine kinases to osteoclast function extends beyond pp60$^{c\text{-}src}$, notwithstanding the dramatic effect of the deletion of the c-*src* gene on bone resorption. The degree of functional redundancy of these enzymes in the osteoclast, as well as in other cell types, remains to be determined, and functions that are unique to pp60$^{c\text{-}src}$ are likely points at which the deletion of the c-*src* gene has its deleterious effect on bone resorption. Further support for a specific requirement for pp60$^{c\text{-}src}$ in bone resorption and remodeling is provided by the fact that *fyn*$^-$ and *yes*$^-$ mice generated by the Soriano laboratory are not osteopetrotic (Soriano, P., personal communication).

In summary, pp60$^{c\text{-}src}$ is expressed at high levels in os-

Figure 4. Intracellular vesicles in osteoclasts are highly enriched in pp60$^{c\text{-}src}$. Confocal immunofluorescence localization of pp60$^{c\text{-}src}$ in isolated rat osteoclasts on glass (*A*, *C*, *D*, *E*, stained with LA074) and bone (*B*, stained with mAb 327). In *A* and *B*, 0.6-μm optical sections were taken progressively from near the top of the cell (*upper left*) to the level of attachment to the substratum (*lower right*), showing that the punctate (*A*) or more diffuse (*B*) staining of pp60$^{c\text{-}src}$ is distributed throughout the cell, without apparent polarity. In the cells on glass (*A*, *C–E*), the staining pattern is distinctly punctate, often surrounding small circular unstained regions. In *C* and *D*, the predominant localization is toward the center of the cell (*left*, in *D*). The images are pseudocolor projections; bars indicate the relative intensity of staining, with low intensity indicated by blue and purple and high intensity indicated by yellow and red. Bars: (*A*) 30 μm; (*B*) 40 μm; (*C*) 22 μm; (*E*) 35 μm. For *D*, the bar in *C* is 10 μm.

teoclasts, where it is predominantly associated with intracellular membranes. Although further work will be required to determine the role of pp60$^{c\text{-}src}$ in osteoclasts, these results demonstrate a direct link between the osteoclast and c-*src* expression, and may help explain the fact that targeted disruption of this protooncogene leads to impaired function and osteopetrosis.

We thank Dr. Joan Brugge for helpful discussion and suggestions, as well as for providing mAb 327 and a polyclonal antibody against c-*yes*. We also thank Dr. Joseph Bolen for providing antibodies against c-*blk*, c-*fgr*, c-*fyn*, c-*hck*, c-*lck*, and c-*lyn*, Dr. Sara Courtneidge for providing an antibody against c-*yes*, and Dr. Roger Perlmutter for providing an antibody against c-*fyn*. We are grateful to Mr. Phillipe Male for his assistance with the confocal microscopy, and Dr. Stephen Warren for critically reviewing the manuscript.

This research was supported by NIH grant DE04724 (R. Baron) and American Cancer Society Institutional Research grant IN31-33 (W. C. Horne). D. C. Chatterjee was the recipient of a Swebelius Cancer Research Award and A. Lomri of an Arthritis Foundation Postdoctoral Fellowship.

Received for publication 3 July 1992 and in revised form 18 August 1992.

References

1. Barnekow, A., R. Jahn, and M. Schartl. 1990. Synaptophysin: a substrate for the protein tyrosine kinase pp60$^{c\text{-}src}$ in intact synaptic vesicles. *Oncogene.* 5:1019–1024.
2. Baron, R., M. Chakraborty, D. Chatterjee, W. Horne, A. Lomri, and J.-H. Ravesloot. 1992. Biology of the osteoclast. *In* Physiology and Pharmacology of Bone. G. R. Mundy and T. J. Martin, editors. Springer Publishing Company, New York. In press.
3. Beug, H., A. von Kirchbach, G. Doderlein, J. F. Conscience, and T. Graf. 1979. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. *Cell.* 18:375–390.
4. Billecocq, A., J. Emanuel, S. Jamsa-Kellokumpu, B. Prallet, R. Levenson, and R. Baron. 1990. 1,25(OH)2D3 induces the concomitant expression of the vitronectin receptor, sodium pumps and carbonic anhydrase II in avian bone marrow cells. *In* Calcium Regulation and Bone Metabolism. D. V. Cohn, F. H. Glorieux, and T. J. Martin, editors. Elsevier Science Publishers B. V., Amsterdam. 152–156.
5. Billecocq, A., J. Rettig-Emanuel, R. Levenson, and R. Baron. 1990. 1a,25-Dihydroxyvitamin D_3 regulates the expression of carbonic anhydrase II in nonerythroid avian bone marrow cells. *Proc. Natl. Acad. Sci. USA.* 87:6470–6474.
6. Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff. 1991. Oncogenes and signal transduction. *Cell.* 64:281–302.
7. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.
8. Cooke, M. P., K. M. Abraham, K. A. Forbush, and R. M. Perlmutter. 1991. Regulation of T cell receptor signalling by a src family protein-tyrosine kinase (p59^{fyn}). *Cell.* 65:281–291.
9. Cooper, J. A. 1989. The *src* family of protein-tyrosine kinases. *In* Peptides and Protein Phosphorylation. B. Kemp and P. F. Alewood, editors. CRC Press, Inc., Boca Raton, FL. 85–113.
10. Cotton, P. C., and J. S. Brugge. 1983. Neural tissues express high levels of the cellular src gene product pp60$^{c\text{-}src}$. *Mol. Cell. Biol.* 3:1157–1162.
11. Courtneidge, S. A., A. D. Levinson, and J. M. Bishop. 1980. The protein encoded by the transforming gene of avian sarcoma virus (pp60^{src}) and a homologous protein in normal cells (pp60$^{proto\text{-}src}$) are associated with the plasma membrane. *Proc. Natl. Acad. Sci. USA.* 77:3783–3787.
12. David-Pfeuty, T., and Y. Nouvian-Dooghe. 1990. Immunolocalization of the cellular src protein in interphase and mitotic c-src overexpressor cells. *J. Cell Biol.* 111:3097–3116.
13. Davies, J., J. Warwick, N. Totty, R. Philp, M. Helfrich, and M. Horton. 1989. The osteoclast functional antigen, implicated in the regulation of bone resorption, is biochemically related to the vitronectin receptor. *J. Cell Biol.* 109:1817–1826.
14. Eiseman, E., and J. B. Bolen. 1990. src-related tyrosine protein kinases as signaling components in hematopoietic cells. *Cancer Cells (Cold Spring Harbor).* 2:303–310.
15. Ellis, C., M. Moran, F. McCormick, and T. Pawson. 1990. Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. *Nature (Lond.).* 343:377–381.
16. Ferrell, J. E., Jr., J. A. Noble, G. S. Martin, Y. V. Jacques, and D. F. Bainton. 1990. Intracellular localization of pp60$^{c\text{-}src}$ in human platelets. *Oncogene.* 5:1033–1036.
17. Fukui, Y., S. Kornbluth, S. M. Jong, L. H. Wang, and H. Hanafusa. 1988. Phosphatidylinositol kinase type I activity associates with various oncogene products. *Oncogene Res.* 4:283–292.
18. Gee, C. E., J. Griffin, L. Sastre, L. J. Miller, T. A. Springer, H. Piwnica-Worms, and T. M. Roberts. 1986. Differentiation of myeloid cells is accompanied by increased levels of pp60$^{c\text{-}src}$ protein and kinase activity. *Proc. Natl. Acad. Sci. USA.* 83:5131–5135.
19. Golden, A., and J. S. Brugge. 1988. The *src* oncogene. *In* The Oncogene Handbook. E. P. Reddy, A. M. Skalka, and T. Curran, editors. Elsevier Science Publishers B.V., Amsterdam. 149–173.
20. Golden, A., S. P. Nemeth, and J. S. Brugge. 1986. Blood platelets express high levels of the pp60$^{c\text{-}src}$-specific tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA.* 83:852–856.
21. Golden, A., J. S. Brugge, and S. J. Shattil. 1990. Role of platelet membrane glycoprotein IIb-IIIa in agonist-induced tyrosine phosphorylation of platelet proteins. *J. Cell Biol.* 111:3117–3127.
22. Grandori, C., and H. Hanafusa. 1988. p60$^{c\text{-}src}$ is complexed with a cellular protein in subcellular compartments involved in exocytosis. *J. Cell Biol.* 107:2125–2135.
23. Gutkind, J. S., P. M. Lacal, and K. C. Robbins. 1990. Thrombin-dependent association of phosphatidylinositol-3 kinase with p60$^{c\text{-}src}$ and p59$^{c\text{-}fyn}$ in human platelets. *Mol. Cell. Biol.* 10:3806–3809.
24. Hirano, A. A., P. Greengard, and R. L. Huganir. 1988. Protein tyrosine kinase activity and its endogenous substrates in rat brain: a subcellular and regional survey. *J. Neurochem.* 50:1447–1455.
25. Huang, M.-M., J. B. Bolen, J. W. Barnwell, S. J. Shattil, and J. S. Brugge. 1991. Membrane glycoprotein IV (CD36) is physically associated with the Fyn, Lyn, and Yes protein-tyrosine kinases in human platelets. *Proc. Natl. Acad. Sci. USA.* 88:7844–7848.
26. Kaplan, J. M., H. E. Varmus, and J. M. Bishop. 1990. The src protein contains multiple domains for specific attachment to membranes. *Mol. Cell. Biol.* 10:1000–1009.
27. Kornberg, L. J., H. S. Earp, C. E. Turner, C. Prockop, and R. L. Juliano. 1991. Signal transduction by integrins: Increased protein tyrosine phosphorylation caused by clustering of β_1 integrins. *Proc. Natl. Acad. Sci. USA.* 88:8392–8396.
28. Kypta, R. M., W. Goldberg, E. T. Ulug, and S. A. Courtneidge. 1990. Association between the PDGF receptor and members of the src family of tyrosine kinases. *Cell.* 62:481–492.
29. Levy, J. B., and J. S. Brugge. 1989. Biological and biochemical properties of the c-src$^+$ gene product overexpressed in chicken embryo fibroblasts. *Mol. Cell. Biol.* 9:3332–3341.
30. Levy, J. B., H. Iba, and H. Hanafusa. 1986. Activation of the transforming potential of p60$^{c\text{-}src}$ by a single amino acid change. *Proc. Natl. Acad. Sci. USA.* 83:4228–4232.
31. Linstedt, A. D., M. L. Vetter, J. M. Bishop, and R. B. Kelly. 1992. Specific association of the proto-oncogene product pp60$^{c\text{-}src}$ with an intracellular organelle, the PC12 synaptic vesicle. *J. Cell Biol.* 117:1077–1084.
32. Lipsich, L. A., A. J. Lewis, and J. S. Brugge. 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma virus. *J. Virol.* 48:352–360.
33. Marks, S. C., Jr. 1984. Congenital osteopetrotic mutations as probes of the origin, structure, and function of osteoclasts. *Clin. Orthop. Relat. Res.* 189:239–263.
34. Nigg, E. A., B. M. Sefton, T. Hunter, G. Walter, and S. J. Singer. 1982. Immunofluorescence localization of the transforming protein of Rous sarcoma virus with antibodies against a synthetic peptide. *Proc. Natl. Acad. Sci. USA.* 79:5322–5326.
35. Parsons, J. T., and M. J. Weber. 1989. Genetics of src: structure and functional organization of a protein tyrosine kinase. *Curr. Top. Microbiol. Immunol.* 147:79–127.
36. Parsons, S. J., and C. E. Creutz. 1986. p60$^{c\text{-}src}$ activity detected in the chromaffin granule membrane. *Biochem. Biophys. Res. Commun.* 134: 736–742.
37. Rendu, F., M. Lebret, S. Danielian, R. Fagard, S. Levy-Toledano, and S. Fischer. 1989. High pp60$^{c\text{-}src}$ level in human platelet dense bodies. *Blood.* 73:1545–1551.
38. Resh, M. D., and R. L. Erikson. 1985. Highly specific antibody to Rous sarcoma virus src gene product recognizes a novel population of pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$ molecules. *J. Cell Biol.* 100:409–417.
39. Rigby, P. W. P., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specificity *in vitro* by nick-translation with DNA polymerase I. *J. Mol. Biol.* 113:237–251.
40. Rohrschneider, L. R. 1980. Adhesion plaques of Rous sarcoma virus-transformed cells contain the src gene product. *Proc. Natl. Acad. Sci. USA.* 77:3514–3518.
41. Soriano, P., C. Montgomery, R. Geske, and A. Bradley. 1991. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell.* 64:693–702.
42. Su, Y., M. Chakraborty, M. Nathanson, and R. Baron. 1992. Differential

effects of the cAMP and PKC pathways on the response of isolated rat osteoclasts to calcitonin. *Endocrinology.* 131:1497–1502.
43. Suda, T., N. Takahashi, and T. J. Martin. 1992. Modulation of osteoclast differentiation. *Endocr. Rev.* 13:66–80.
44. Sudol, M. 1988. Expression of proto-oncogenes in neural tissues. *Brain Res. Rev.* 13:391–403.
45. Tokuyasu, K. T. 1973. A technique for ultramicrotomy of cell suspensions and tissues. *J. Cell Biol.* 57:551–569.
46. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
47. Varshney, G. C., J. Henry, A. Kahn, and F. Phan-Dinh-Tuy. 1986. Tyrosine kinases in normal human blood cells: platelet but not erythrocyte band 3 tyrosine kinase is $p60^{c\text{-}src}$. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 205:97–103.
48. Warren, S. L., and W. J. Nelson. 1987. Nonmitogenic morphoregulatory action of $pp60^{v\text{-}src}$ on multicellular epithelial structures. *Mol. Cell. Biol.* 7:1326–1337.
49. Warren, S. L., L. M. Handel, and W. J. Nelson. 1988. Elevated expression of $pp60^{c\text{-}src}$ alters a selective morphogenetic property of epithelial cells in vitro without a mitogenic effect. *Mol. Cell. Biol.* 8:632–646.
50. Wu, H., A. B. Reynolds, S. B. Kanner, R. R. Vines, and J. T. Parsons. 1991. Identification and characterization of a novel cytoskeleton-associated $pp60^{src}$ substrate. *Mol. Cell. Biol.* 11:5113–5124.
51. Zambonin-Zallone, A., A. Teti, and M. V. Primavera. 1982. Isolated osteoclasts in primary culture: first observations on structure and survival in culture media. *Anat. Embryol.* 165:405–413.
52. Zambonin-Zallone, A., A. Teti, M. Grano, A. Rubinacci, M. Abbadini, M. Gaboli, and P. C. Marchisio. 1989. Immunocytochemical distribution of extracellular matrix receptors in human osteoclasts: a beta 3 integrin is colocalized with vinculin and talin in the podosomes of osteoclastoma giant cells. *Exp. Cell Res.* 182:645–652.