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ARTICLES

1,25-Dihydroxyvitamin D_3 Regulates pp60$^{c\text{-}src}$ Activity and Expression of a pp60$^{c\text{-}src}$ Activating Phosphatase

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Abstract The nonreceptor tyrosine kinase, c-src, and the steroid hormone, 1,25-dihydroxyvitamin D_3(1,25$(OH)_2D_3$), are essential to development of the osteoclast phenotype. On the other hand, functional relationships between the activities of c-src and 1,25$(OH)_2D_3$ are as yet unknown. To determine if 1,25$(OH)_2D_3$ modulates c-src in osteoclastogenesis, we tested the steroid's effect on avian marrow-derived osteoclast precursors. We find c-src mRNA and immunoprecipitable c-src protein (pp60$^{c\text{-}src}$) unaltered by 72 h exposure of these cells to 1,25$(OH)_2D_3$ (10^{-11} to 10^{-9} M). Despite no quantitative change in pp60$^{c\text{-}src}$, in vitro kinase assay of the immune complex reveals 1,25$(OH)_2D_3$ dose-dependently accelerates the catalytic activity of pp60$^{c\text{-}src}$, enhancing its autophosphorylation and phosphorylation of exogenous substrate. This observation represents the first documentation, in nontransformed cells, of humoral induction of pp60$^{c\text{-}src}$ kinase. Consistent with the fact pp60$^{c\text{-}src}$ is activated by dephosphorylation of tyrosine 527 (Y527), the phosphotyrosine content of the pp60$^{c\text{-}src}$ immunoprecipitate, measured by immunoblot, is decreased by 1,25$(OH)_2D_3$. Alternatively, mRNA and protein levels of c-src kinase (CSK), which inactivates pp60$^{c\text{-}src}$ by phosphorylating Y527, are not altered by the steroid. In contrast, 1,25$(OH)_2D_3$ enhances mRNA and especially protein levels of avian protein tyrosine phosphatase λ (PTPλ), an enzyme specifically activating pp60$^{c\text{-}src}$ by dephosphorylating Y527 [Fang et al. (1994): J Biol Chem 269:20194–20200]. Thus, treatment of avian osteoclast precursors with 1,25$(OH)_2D_3$ accelerates the catalytic activity of pp60$^{c\text{-}src}$ independent of protein expression. Activation of the kinase may occur via the Y527 dephosphorylating enzyme PTP, expression of which, we show for the first time, is regulated. J. Cell. Biochem. 67:432–438, 1997.

Key words: pp60$^{c\text{-}src}$; 1,25-dihydroxyvitamin D_3; phosphatase; c-src kinase; tyrosine kinase

pp60$^{c\text{-}src}$, the product of the c-src protooncogene, is a widely expressed nonreceptor tyrosine kinase. Activation of the pp60$^{c\text{-}src}$ catalytic domain prompts phosphorylation of multiple target proteins, including those participating in signal transduction and cytoskeletal organization [reviewed in Brickell, 1992].

Despite its relative ubiquity, a rate-limiting biological role for pp60$^{c\text{-}src}$ has until recently escaped definition. In 1991, Soriano et al. [1991] documented platelets and neural tissue in mice in which the c-src gene had been deleted by homologous recombination function normally. This observation was surprising, as the protooncogene is particularly abundant in these two tissues [Ross et al., 1988; Golden and Brugge, 1989] and platelet aggregation activates the kinase [Clark et al., 1994]. Equally unexpected was the fact c-src−/− mice develop osteopetrosis [Soriano et al., 1991], a disease of dysfunctional osteoclasts incapable of resorbing bone, thus yielding a sclerotic skeleton. Further studies documented the resorptive defect of c-src−/−osteoclasts reflects failure of polarization, an event essential to the resorptive process [Boyce et al., 1992]. These observations document that a biological event, namely osteoclast polarization and subsequent bone resorption, is absolutely dependent on pp60$^{c\text{-}src}$ expression. Thus, regulation of the protooncogene's expression and activity in osteoclasts and their precursors is an issue of importance.

Abbreviations: BMMs, avian bone marrow macrophages precursors; CSK, c-src kinase; PTPλ, avian protein tyrosine phosphatase λ; Y527, tyrosine 527; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3.

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The osteoclast is a physiological polykaryon and the principal if not exclusive resorptive cell of bone. It is of hematopoietic origin [Coccia et al., 1980] and a member of the monocyte/macrophage family [Udagawa et al., 1990]. In fact, when appropriately cultured, macrophages or their precursors differentiate into osteoclast-like cells with the capacity to resorb bone [Udagawa et al., 1990]. Furthermore, while pp60$^{c\text{-}src}$ is minimally present in macrophage precursors, the protooncogene is progressively and specifically expressed as the cells assume the osteoclast phenotype [Tanaka et al., 1992].

$1,25(OH)_2D_3$ is the biologically active form of vitamin D. While this secosteroid is classically viewed as a calcium-regulating hormone, its biological repertoire reflects the fact that it prompts differentiation of a number of native and transformed cells, the former including osteoclast precursors [Clohisy et al., 1987]. In fact, commitment of macrophage progenitors to osteoclast differentiation is absolutely dependent on the presence of $1,25(OH)_2D_3$ [Takahashi et al., 1988].

The observation that pp60$^{c\text{-}src}$ and $1,25(OH)_2D_3$ are both essential to the resorptive process raises the possibility that the steroid regulates the protooncogene. To this end we find that, in marrow-derived avian osteoclast precursors, $1,25(OH)_2D_3$ accelerates pp60$^{c\text{-}src}$ kinase-specific activity without impacting expression. pp60$^{c\text{-}src}$ activation by the steroid is characterized by autophosphorylation and phosphorylation of exogenous substrate. The fact that the phosphotyrosine content of $1,25(OH)_2D_3$-activated pp60$^{c\text{-}src}$ declines indicates steroid exposure prompts dephosphorylation of the regulatory tyrosine kinase residue, Y527 [Thomas et al., 1991; McFarland et al., 1993]. This conclusion is supported by the observation that $1,25(OH)_2D_3$ enhances steady-state mRNA and protein levels of the avian pp60$^{c\text{-}src}$-specific protein, tyrosine phosphatase PTPλ.

RESULTS

$1,25(OH)_2D_3$ Amplifies pp60$^{c\text{-}src}$ Specific Activity

Our first experiments were aimed at determining if total pp60$^{c\text{-}src}$ content and kinase activity are modulated by $1,25(OH)_2D_3$. Accordingly, avian bone marrow macrophage precursors (BMMs), isolated in homogeneity, were cultured for 3 days with various concentrations of $1,25(OH)_2D_3$ and lysed in the presence of kinase and phosphatase inhibitors, and the lysate was immunoprecipitated with excess anti-pp60$^{c\text{-}src}$ mAb 327. Half the immunoprecipitate was subjected to in vitro kinase assay, a technique involving resuspension in buffer containing [$^{32}P_\gamma$]ATP and p62, a pp60$^{c\text{-}src}$-specific substrate, followed by SDS-PAGE autoradiography. The other half of the pp60$^{c\text{-}src}$ immunoprecipitate was probed, by immunoblot, for pp60$^{c\text{-}src}$ content.

$1,25(OH)_2D_3$ dose-dependently promotes pp60$^{c\text{-}src}$ autophosphorylation and phosphorylation of exogenous substrate, p62 (Fig. 1). Enhanced phosphorylation is detectable at picomolar concentrations of the steroid reflecting those normally circulating. These data indicate $1,25(OH)_2D_3$ amplifies total pp60$^{c\text{-}src}$ kinase activity, a phenomenon due either to increased expression of the c-src protein or induction of specific activity. As seen in Figure 2, however, pp60$^{c\text{-}src}$ content is unaltered in marrow macrophages in which $1,25(OH)_2D_3$ has accelerated pp60$^{c\text{-}src}$ kinase activity. Thus, $1,25(OH)_2D_3$ enhances pp60$^{c\text{-}src}$ specific activity without changing expression of the protooncogene.

$1,25(OH)_2D_3$ Decreases pp60$^{c\text{-}src}$ Phosphotyrosine Content

The activity of pp60$^{c\text{-}src}$ is modulated by its state of Y phosphorylation [reviewed in Cooper and Howell, 1993], which we next explored. The protooncogene was immunoprecipitated from cells treated with $1,25(OH)_2D_3$ for 3 days, and

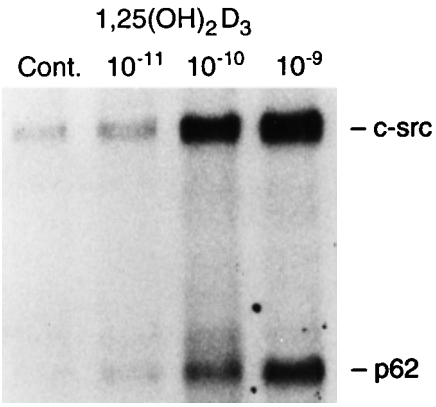

Fig. 1. $1,25(OH)_2D_3$ increases autophosphorylation of pp60$^{c\text{-}src}$ and phosphorylation of exogenous substrate. BMMs were cultured for 72 h ± 10^{-9} M $1,25(OH)_2D_3$. Lysates from equal numbers of treated and control cells were immunoprecipitated with excess anti-c-src mAb 327 and total complexes bound to GammaBind Plus beads. P62, a pp60$^{c\text{-}src}$-specific substrate, and [$^{32}P_\gamma$]-ATP were added. After 3 min the samples were subjected to SDS-PAGE and autoradiography.

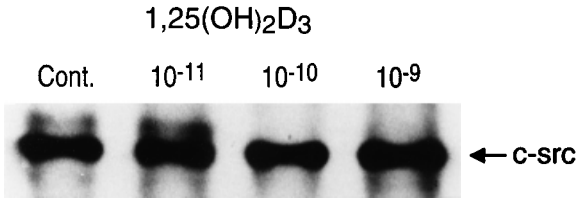

Fig. 2. Treatment of avian marrow macrophages with $1,25(OH)_2D_3$ fails to alter the level of $pp60^{c\text{-}src}$. BMMs were treated with various concentrations of $1,25(OH)_2D_3$ for 3 days. Equal numbers of cells were washed and lysed, and lysates were immunoprecipitated with excess anti-c-src mAb antibody 327. Immunoblot of aliquots of immunoprecipitate normalized for protein was performed with the same antibody.

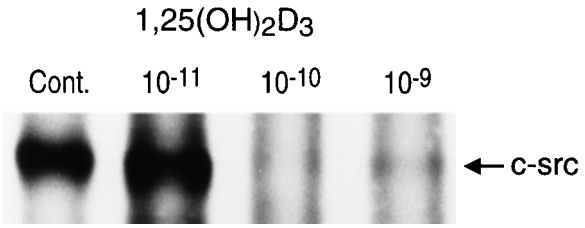

Fig. 3. $1,25(OH)_2D_3$ decreases the phosphotyrosine content of $pp60^{c\text{-}src}$ in a dose-dependent manner. Avian BMMs were cultured for 72 h ± varying concentrations of $1,25(OH)_2D_3$. Lysates were immunoprecipitated with excess anti-c-src mAb 327 and the immune complexes subjected to immunblot analysis using the antiphosphotyrosine mAb antibody, 4G10.

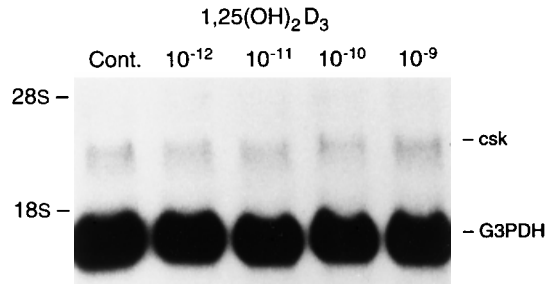

Fig. 4. $1,25(OH)_2D_3$ fails to alter mRNA levels of the $pp60^{c\text{-}src}$ kinase, csk. Avian BMMs were treated with various concentrations of $1,25(OH)_2D_3$ for 3 days. RNA was isolated and subjected to Northern analysis using a full-length avian csk cDNA. Loading was normalized to G3PDH mRNA.

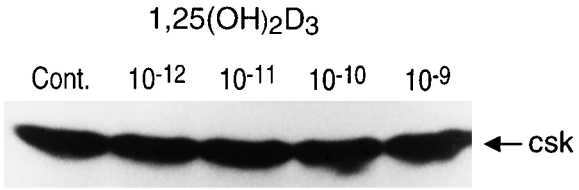

Fig. 5. $1,25(OH)_2D_3$ fails to alter protein levels of the $pp60^{c\text{-}src}$ kinase, csk. BMMs were cultured for 72 h ± varying concentrations of $1,25(OH)_2D_3$. Lysates from equal numbers of cells were immunoprecipitated with excess anti-csk MAP and immunoprecipitate subjected to immunoblot using an anti-csk polyclonal antibody.

the phosphotyrosine content was determined by immunoblot using an anti-phosphotyrosine antibody. As seen Figure 3, $1,25(OH)_2D_3$ dose-dependently decreases $pp60^{c\text{-}src}$ phosphotyrosine content, again exerting its effect in the picomolar range. In this regard, the steroid's capacity to diminish $pp60^{c\text{-}src}$ phosphotyrosine reciprocally reflects its impact on kinase activity (compare Figs. 1 and 3).

$1,25(OH)_2D_3$ Fails to Alter CSK Expression

With the knowledge that $pp60^{c\text{-}src}$ is hypophosphorylated in $1,25(OH)_2D_3$-treated cells, we turned to the mechanism by which the steroid induces Y dephosphorylation and thus activates the kinase. The state of Y527 phosphorylation is regulated by specific phosphatases and kinases, particularly c-src kinase (CSK) [Thomas et al., 1991; Okada and Nakagawa, 1989], and we asked if $pp60^{c\text{-}src}$ Y hypophosphorylation reflects inhibited CSK expression. Thus, RNA extracted from cells treated with $1,25(OH)_2D_3$ for 3 days was subjected to Northern analysis with a full-length murine CSK cDNA (provided by Dr. Akira Imamoto, Fred Hutchinson Cancer Research Center, Seattle, WA). As seen in Figure 4, $1,25(OH)_2D_3$ fails to impact expression of mRNA of this src family–specific kinase. Most importantly, CSK protein, measured by immunoblot in the same cells, mirrors mRNA levels (Fig. 5). Thus, $1,25(OH)_2D_3$ activation of $pp60^{c\text{-}src}$ is not mediated via CSK expression.

$1,25(OH)_2D_3$ Induces Avian PTPλ Expression

Protein tyrosine phosphatase lambda (PTPλ) is an avian tyrosine phosphatase which selectively dephosphorylates $pp60^{c\text{-}src}$ Y527, thereby activating the enzyme [Fang et al., 1994]. To determine if $1,25(OH)_2D_3$ enhances PTPλ expression, a possible means by which the steroid might activate $pp60^{c\text{-}src}$, we treated BMMs with $1,25(OH)_2D_3$ for 72 h. PTPλ mRNA was measured by Northern analysis using a full-length cDNA (a gift of Dr. H. Hanafusa, Rockefeller University, New York, NY) and PTPλ protein by immunoblot with a polyclonal antibody (a gift of Dr. H. Hanafusa, Rockefeller University, New York, NY). As seen in Figures 6 and 7, respectively, steroid-exposed cells specifically express increased amounts of PTPλ mRNA and protein.

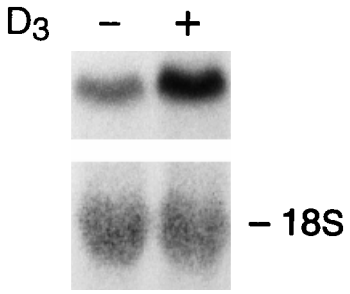

Fig. 6. 1,25(OH)$_2$D$_3$ increases mRNA levels of PTPλ. BMMs were treated with 1,25(OH)$_2$D$_3$ (10^{-8} M) for >72 h. RNA was isolated and subjected to Northern analysis using a full-length avian PTPλ cDNA. Loading was normalized to 18S RNA.

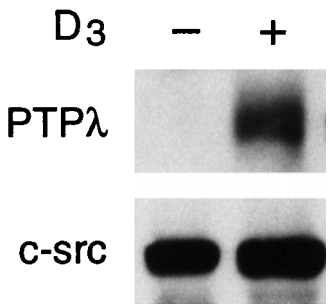

Fig. 7. 1,25(OH)$_2$D$_3$ increases PTPλ protein levels. BMMs were cultured for 72 h ± 1,25(OH)$_2$D$_3$ (10^{-8} M). Lysates from equal numbers of cells were immunoprecipitated and the immune complexes subjected to immunoblot using a polyclonal antibody to PTPλ. Note that levels of pp60$^{c\text{-}src}$ protein are unchanged in the same lysate by treatment with 1,25(OH)$_2$D$_3$.

DISCUSSION

In 1991, Soriano et al. [1991] made the surprising observation that deletion of the c-src gene induces osteopetrosis as the unique phenotypic abnormality. Shortly thereafter, Boyce and coworkers [1992] showed the c-src–deleted osteopetrotic mouse capable of generating osteoclasts which are, however, dysfunctional due to their inability to polarize. This defect is most evident in failure of c-src−/− osteoclasts to form their resorptive organelle, namely the ruffled membrane. While the mechanism by which pp60$^{c\text{-}src}$ prompts ruffled membrane formation is unknown, the protooncogene itself polarizes to this complex infolding of plasmalemma [Tanaka et al., 1992], an event probably involving transport of pp60$^{c\text{-}src}$, and other ruffled membrane residing proteins, along the microtubular network in osteoclasts contacted with appropriate extracellular matrix [Abu-Amer et al., 1997]. The fact that pp60$^{c\text{-}src}$ also locates in integrin-bearing focal adhesion complexes [Schaller et al., 1994] raises the possibility the protooncogene is essential to transmission of matrix-derived signals prompting the resorptive phenotype.

Because the appearance of pp60$^{c\text{-}src}$ in macrophages is temporally associated with osteoclastogenesis [Tanaka et al., 1992], an event necessitating 1,25(OH)$_2$D$_3$, the aim of our study was to determine if the steroid alters pp60$^{c\text{-}src}$ activation in osteoclast precursors. To this end, we turned to a model of generated avian osteoclast-like cells [Alvarez et al., 1991]. In this circumstance, avian marrow macrophage precursors are maintained in culture, during which time they acquire many features of osteoclasts, including multinucleation, tartrate-resistant acid phosphatase expression, and the capacity to pit bone. These precursor cells are isolated in homogeneity and large numbers and as such lend themselves to meaningful biochemical analysis.

We find a dramatic increase in pp60$^{c\text{-}src}$ kinase activity in avian osteoclast precursors exposed to physiological levels of 1,25(OH)$_2$D$_3$, which, in these cells, induce osteoclast-associated markers such as the $\alpha_v\beta_3$ integrin [Mimura et al., 1994]. Accelerated pp60$^{c\text{-}src}$ kinase activity is unaccompanied by alterations of protooncogene expression, documenting the steroid impacts specific activity. Steroid induction of pp60$^{c\text{-}src}$ kinase activity is detectable only after 72 h, suggesting 1,25(OH)$_2$D$_3$-mediated cell maturation, involving sequential gene expression, may be required.

A variety of cytokines, including colony stimulating factor-1 [Courtneidge et al., 1993], platelet-derived growth factor [Gould and Hunter, 1988; Weernink and Rijksen, 1995], epidermal growth factor [Weernink and Rijksen, 1995], and endothelin, activate pp60$^{c\text{-}src}$ [Simonson and Herman, 1993]. Induction of pp60$^{c\text{-}src}$ specific activity by a steroid is unique and particularly impressive as the magnitude of change supersedes that induced by other agonists. Most importantly, we document for the first time direct humoral induction of pp60$^{c\text{-}src}$ activity in nontransformed cells, particularly those in which the protooncogene is functionally significant.

Interestingly, those pp60$^{c\text{-}src}$ agonists described to date exert their effect within minutes [Courtneidge et al., 1993; Gould and Hunter, 1988; Weernink and Rijksen, 1995; Simonson and Herman, 1993], generally in a rapidly transient nature indicating that the phenomenon is nongenomic. 1,25(OH)$_2$D$_3$, on the other hand, is

a classical steroid hormone which activates its intracellular receptor to serve as a transcription factor [Darwish and DeLuca, 1993]. Thus, while $1,25(OH)_2D_3$ has nongenomic properties [Norman et al., 1992], the steroid operates primarily by prompting transcription. This mechanism of action is in keeping with the 3 days of exposure to $1,25(OH)_2D_3$ required to consistently induce $pp60^{c\text{-}src}$ specific activity and consistent with a trans event mediated by the vitamin D receptor.

The kinetic activity of $pp60^{c\text{-}src}$ is governed primarily by the state of phosphorylation of Y527 [Cooper and Howell, 1993]. When phosphorylated, the amino acid residue associates with the molecule's own SH2 region, inactivating the catalytic domain. Thus, activated $pp60^{c\text{-}src}$ is typically Y-hypophosphorylated relative to the inactive enzyme. Our observation that the steroid prompts $pp60^{c\text{-}src}$ Y hypophosphorylation at a time the enzyme is active indicates the predominant effect of $1,25(OH)_2D_3$ involves Y527 dephosphorylation.

Decreased phosphotyrosine content of the protooncogene, when derived from vitamin D–treated cells, and induction of $pp60^{c\text{-}src}$ autophosphorylation by $1,25(OH)_2D_3$, measured by in vitro kinase assay, may seem contradictory. These events, however, represent two disparate phenomena. Specifically, $pp60^{c\text{-}src}$ phosphotyrosine content, as determined by immunoblot, reflects the in vivo phosphorylation state of Y527, the kinase-regulating residue. In contrast, in vitro kinase assay is a postlysis measure of $pp60^{c\text{-}src}$ enzymatic activity in which the autophosphorylation target, Y416 [Kmiecik et al., 1988], serves as a convenient marker.

With the knowledge that $pp60^{c\text{-}src}$ is hypophosphorylated in $1,25(OH)_2D_3$-treated cells, we turned to the mechanism by which the steroid induces tyrosine dephosphorylation and thus activates the kinase. The state of Y527 phosphorylation is regulated by specific kinases and phosphatases. The best characterized $pp60^{c\text{-}src}$ Y527 kinase is CSK [Thomas et al., 1991; Okada and Nakagawa, 1989]. This enzyme specifically inactivates src family kinases by phosphorylating their carboxyl terminal Y residues [Cooper et al., 1986]. In fact, cells derived from mice in which the CSK gene is deleted contain enhanced $pp60^{c\text{-}src}$ specific activity [Nada et al., 1993]. On the other hand, despite the steroid's induction of $pp60^{c\text{-}src}$ activity, $1,25(OH)_2D_3$ fails to alter expression of CSK mRNA or protein.

Attention has recently turned to the possibility that, in contrast to the negative effect of CSK-mediated phosphorylation, src family kinases may be activated by protein tyrosine phosphatase (PTP)–mediated dephosphorylation of regulatory Y residues [Woodford-Thomas and Thomas, 1993]. In fact, overexpression of the receptor-like PTPα dephosphorylates Y527, thereby enhancing $pp60^{c\text{-}src}$ specific activity three- to sixfold while concomitantly inducing transformation and tumorigenesis [Zheng et al., 1992]. Similarly, the transmembrane PTP, CD45, dephosphorylates the negative regulatory site of the src family members, lck and fyn, an event essential to T cell activation [McFarland et al., 1993; Sieh et al., 1993].

Hanafusa's laboratory recently demonstrated avian PTPλ, a protein homologous to CD45, selectively dephosphorylates $pp60^{c\text{-}src}$ Y527, thereby activating the enzyme [Fang et al., 1994]. We therefore asked if $1,25(OH)_2D_3$ enhances PTPλ expression, a possible means whereby the steroid may activate $pp60^{c\text{-}src}$. In fact, $1,25(OH)_2D_3$ induces PTPλ mRNA and protein in avian osteoclast precursors. This observation, which represents initial documentation of PTP regulation by a physiologically relevant molecule, suggests that induction of the dephosphorylating enzyme may represent the mechanism of steroid-mediated c-src activation. Thus, $1,25(OH)_2D_3$ may promote bone resorption, in part, by activating through specific PTP induction, $pp60^{c\text{-}src}$, a protein critical to osteoclast polarization.

MATERIALS AND METHODS

Reagents

Monoclonal antibody (mAb) [Hall et al., 1994; Lipsich et al., 1983], directed against the c-src protein, was a gift from Dr. Joan Brugge. p62, a substrate for $pp60^{c\text{-}src}$ [Richard et al., 1995], was produced in our laboratory. Monoclonal antiphosphotyrosine antibody (4G10) was purchased from UBI (Lake Placid, NY). Antiavian PTPλ polyclonal antibody was a gift of Dr. H. Hanafusa (Rockefeller University, New York, NY). CSK mAb was a gift from Dr. Andre Veillette (McGill University, Montreal, Quebec). $1,25(OH)_2D_3$ was a gift of Dr. Milan Uskokovic (Hofmann La-Roche, Inc., Nutley, NJ). Digitonin with high solubility in water was purchased from Wako Chemicals (Richmond, VA). The ECL kit was obtained from Amersham Co. (Arlington Heights, IL). All other chemicals were ob-

tained from Sigma Chemical Company (St. Louis, MO).

Cell Culture

Avian bone marrow macrophages (BMMS) which differentiate into osteoclasts were isolated from calcium-deprived laying hens [Alvarez et al., 1991]. The chicken whole bone marrow preparation was layered on a Ficoll-Hypaque gradient, and the cells at the gradient interface were collected and incubated in α-MEM, supplemented with 5% fetal bovine and 5% chicken charcoal-stripped serum (Gibco, BRL, Gaithersburg, MD) at 39°C in 5% CO_2. After 24 h in culture, nonadherent cells were collected and resuspended in fresh medium at 5 × 10^6 cells/ml. Cells were then maintained in the same α-MEM indicated above and plated according to each experimental condition.

Immunoprecipitation (IP)

Adherent cells were scraped from the dish in the presence of digitonin lysis buffer (1% digitonin, 10 mM triethanolamine, 150 mM NaCl, 10 mM iodoacetoamide, 1 mM EDTA, 10 µg/ml aprotinin, 1 mM AEBSF, pH 7.8) [Katagiri et al., 1993] and incubated at 37°C with gentle rocking for 30 min. The cells were then passed through a 25 gauge needle and spun at 10,000 rpm for 10 min in a microfuge. Lysates were precleared with excess of protein A-Sepharose (Sigma Chemical Company) and Protein G-Sepharose (Pharmacia, Piscataway, NJ). Cleared lysates were incubated with various antibodies followed by protein A or G beads, as indicated, and the beads containing immune complexes were washed extensively with lysis buffer.

Immunoblotting

Immunoprecipitated proteins were boiled in the presence of SDS-sample buffer (0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 10% glycerol, 0.05% (w/v) bromophenol blue, distilled water) for 5 min and subjected to electrophoresis on 7.5–10% SDS-PAGE [Laemmli, 1970]. Proteins were transferred to nitrocellulose membranes using a semidry blotter (Bio-Rad, Richmond, CA) and incubated in blocking solution (10% skim milk prepared in PBS containing 0.05% Tween-20) to reduce nonspecific binding. Membranes were washed with PBS/Tween buffer and exposed to primary antibodies, washed again four times, and incubated with secondary goat antimouse HRP-conjugated antibody. Membranes were washed extensively, and an ECL detection assay was performed following manufacturer's directions.

In Vitro Kinase Assay

The method described by Clark and Brugge [1993] was followed, with slight modification. Beads containing immunoprecipitated pp60$^{c\text{-}src}$ were washed with kinase lysis buffer (1.5% NP-40, 150 mM NaCl, 25 mM Tris, pH 8.0, 25 mM NaF, 100 µM $NaVO_3$) and then preincubated with 15 µl of kinase reaction buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 100 µM $NaVO_3$) for 10 min at 22°C. The kinase reaction was started by addition of 5 µCi of [^{32}P]ATP (6,000 Ci/mmol) (Amersham Co.), 5 µM ATP, 1 µg enolase (all final concentrations). The reaction was terminated after 2 min by the addition of an equal volume of 2× SDS-sample buffer and heating at 65°C for 5 min. Samples were subjected to electrophoresis on SDS-PAGE gel, dried, and exposed to film.

Isolation and Analysis of mRNA

Total cellular RNA was isolated from osteoclast precursors using RNAzol (Tel-Test, Inc., Friarswood, TX). Equal amounts of RNA (8–10 µg) were treated with formaldehyde, separated on 0.8% agarose gels, and blotted to nitrocellulose with a vacuum blotter. Northern analysis was performed [Medhora et al., 1993] using full-length CSK and PTPλ cDNAs labeled by the random primer method (Boehringer-Mannheim, Indianapolis, IN).

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