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Src Protein and Tyrosine-Phosphorylated Protein Profiles in Marrow Stroma During Osteogenic Stimulation

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Abstract Src protein is essential for the regulation of bone turnover primarily via bone resorption because it is required in osteoclast differentiation and function. We followed temporal changes of Src protein abundance in marrow stromal cells induced to mineralize by dexamethasone (DEX), growth in cold temperature, or both. Given the tyrosine kinase function of Src and its numerous substrates, profiles of phosphotyrosine-containing proteins were followed as well. On day 11 of stimulation, specific alkaline phosphatase (ALP) activity at 30°C decreased under DEX relative to 37°C cultures, in accord with increased cell counts. Mineralization per well under DEX increased by 25% at 37°C, whereas at 30°C it increased by more than threefold regardless of the DEX stimulation. At 30°C, on a per cell basis mineralization increased 2.5 and 3 times with and without DEX, respectively. Cultures at 37°C showed a general drop per cell of many phosphotyrosine-containing proteins on day 3 relative to days 1 and 2 in both DEX-stimulated and nonstimulated cultures; several proteins did recover (recuperate) thereafter. On days 1 and 2, the phosphotyrosine signal was higher in several proteins under DEX stimulation; this trend became inverted after day 3. The changes in abundance per cell of Src protein (pp60src) followed a similar trend, and in addition a truncated Src molecule, p54/52src, was detected as a putative cleavage product presumably representing its carboxy terminus. The pp60src was most abundant, relative to its truncated product, in day 7 nonstimulated cultures, whereas under DEX stimulation the truncated species pp54/52src showed the highest relative abundance on days 7. At 30°C, DEX stimulation accentuated the increase in Src protein on day 3, showed no change on day 7, and returned to increase Src protein on day 10. Potassium ionophorvalinomycin, considered to select against mineralizing osteoprogenitors at 30°C, showed on day 10 in the absence of DEX a relative increase in truncated Src protein compared to both DEX-stimulated and nonstimulated cultures in the absence of valinomycin. On day 7 of DEX stimulation, the presence of valinomycin resulted in low p54/52src. Among phosphotyrosine-containing proteins, a 32–34 kDa band, as yet unidentified, showed the most concordant changes with mineralization induction. P32–34 decreased by DEX on days 2 and 8 and increased by low temperature alone or combined with DEX on day 3. On day 7, p32–34 did not change under DEX, but valinomycin selected cells with less phoshpotyrosine-containing p32–34. Taken together, high Src abundance at the start of osteogenic induction followed by a decrease 1 week later is probably related to energy metabolism-dependent induction of mineralization. This is in temporal accord with the increase in Src truncation and fluctuation in mitochondrial membrane potential (which affects mineralization). The reported binding of amino-terminal Src oligopeptide to p32 ADP/ATP carrier in the mitochondrial inner membrane raises the question of its possible involvement in mitochondria-regulated mineralization. J. Cell. Biochem. 69:316–325, 1998.

Key words: osteoprogenitors; mineralization; marrow stroma; Src; tyrosine kinase dexamethasone

Bone cell mineralization depends on bone extracellular matrix organization; however, induction of mineralization is also regulated by cellular energy metabolism [Lehninger, 1970, Brighton and Hunt, 1978, Shapiro et al., 1982; Wuthier, 1993]. Induction of mineralization is associated with oscillation of mitochondrial membrane potential either affected by dexamethasone (DEX) [Klein et al., 1993c], Krebs cycle inhibitors [Klein et al., 1996a], or prolonged cold exposure [Klein et al., 1996b]. Some tyrosine kinase (TK) inhibitors may under certain concentrations preserve the osteoblastic phenotype while killing nonosteoprogenitor cells [Klein et al., 1997]. The activity of synthetic TK inhibitors (tyrphostins) consists of two effects, one by competing for tyrosine residues, the other by competing for ATP binding sites within the kinase molecule [Levitzki and Gazit, 1995;

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Ullrich and Schlessinger, 1990]. Free ATP in itself may induce cell-mediated mineralization [Hatori et al., 1995]; therefore, extensive short-term inhibition of ATP binding to tyrosine kinase might suffice as an initiator of mineralization. Tyrphostin AG-1478 at nM concentrations specifically inhibits epidermal growth factor (EGF) receptor TK activity, but at higher concentrations it inhibits downstream nonreceptor TK like those of the Src family [Osherov and Levitzki, 1994]. The prototype of this family, pp60src, is known to be essential for bone turnover due to its importance in the maturation and function of osteoclasts [Tanaka et al., 1996], as its absence results in osteopetrosis [Soriano et al., 1991], correctable by normal bone marrow. Bone marrow stroma and Saos2 cell lines in culture are osteogenic rather than osteolytic systems. Increased mineralization under AG-1478 in these systems suggested that Src may be involved in osteoblastic mineralization in addition to its effect on bone resorption. Sigal and Resh [1993] found that Src can bind the ADP/ATP carrier of the mitochondrial inner membrane; it thus becomes relevant to follow changes in Src protein in osteoprogenitor cells during their stimulation to mineralize. We found that a decrease in Src protein starts after 3 days of DEX induction, peaking around day 7, when its truncated species peaked. This was in parallel to the decrease in several phosphotyrosine-containing bands. The results indicate that, while osteoclasts depend on Src, the mineralizing osteoprogenitors may require decreased Src for a limited period, perhaps accompanied by increase in truncated Src species.

MATERIALS AND METHODS

Reagents

ALP kit 104 LL, dexamethasone, ascorbate, β-glycerophosphate, and antiphosphotyrosin antibodies were purchased from Sigma (St. Louis, MO). MAb anti-Src 327 was generated from hybridoma medium [Lipsich, 1983]; Fetal calf serum (FCS) was purchased from Grand Island Biological Company (Grand Island, NY).

Stromal Cell Culture

Bone marrow cell suspension s obtained from femurs and tibiae of female Sabra rats, weighing 60–80 g, were seeded in 25 cm^2 flasks, 10^8 cells/flask. Stromal cells were obtained as described by others [Maniatopoulos et al., 1988] removing the nonadherent hematopoietic cells during the first 10 days of culture. The remaining adherent stromal cells were propagated in the same maintenance medium that consisted of DMEM (Dulbecco's modified Eagle's medium) supplemented with 15% fetal calf serum and antibiotics in a humid, 9% CO_2 atmosphere. For the experimental cultures, stromal cells were removed 2 weeks later by trypsinization and were plated in 96-well microtiter plates, 5,000 cells/well, and grown in osteoprogenitor cell (OPC) stimulation medium. This consisted of maintenance (ordinary) medium containing 10^{-8} M dexamethasone, 50 μg/ml ascorbate, and 10 mM glycerophosphate. The medium (DEX medium) was changed on day 7 and subsequently every 4 days.

Measurement of In Vitro Precipitated Calcium

After 3 weeks in culture, the DEX medium, as opposed to ordinary medium, induces cell-mediated mineralization, which appears as calcium deposits surrounding and covering the foci of cells, detectable by alizarin red staining [Klein et al., 1993a]. On X-ray diffraction, such calcium deposits show a main deflection, characteristic of hydroxyapatite at $2\Theta = 32.8°C$, and on X-ray dispersion analysis mainly calcium and phosphate were seen [Maniatopoulos et al., 1988]. Similar results were obtained in our laboratory using the same system. In this study, the calcium deposits were measured as follows. Plates were washed twice with TBS (Tris-buffered saline) to remove water-soluble calcium and then incubated in 0.5 N HCl overnight. Appropriately diluted samples were measured by the metallochromic method with Arsenazo III as described elsewhere [Klein et al., 1995]. Mineralization was expressed in micrograms of calcium per well or presented as an index of calcium deposits relative to untreated controls.

Quantitative Cell Staining

Cells were stained using the methylene blue (MB) method [Goldman and Bar-Shavit, 1979]. Cells were fixed in 0.5% glutaraldehyde for 30 min, rinsed with distilled water, and air-dried overnight. Borate buffer (0.1 M boric acid brought to pH 8.5 with NaOH), 0.2 ml/well, was added to the cells for 2 min and rinsed with tap water. Cells were then incubated in 0.1 ml of 1% MB in borate buffer for 60 min at room temperature, rinsed with water, and air-dried.

The MB was then eluted from the stained cells by incubation with 0.2 ml of 0.1 N HCl at 37°C for 60 minutes. Optical density (O.D.) of the eluted MB was measured at 620 nm by an optical densitometer. Calibration demonstrated that 1.0 O.D. unit was equivalent to 5×10^4 stromal cells.

Alkaline Phosphatase Activity Assay

Alkaline phosphatase (ALP) activity was measured in situ in microtiter plates. Day 11 of DEX stimulation was selected for cell counts and ALP assay since ALP activity on day 11 showed the best correlation with mineralization [Klein et al., 1993b]. The medium was removed, and cells were washed twice in situ with 0.2 ml TBS (50 mM Tris, 150 mM NaCl, pH 7.6). ALP substrate, pNPP (p-nitrophenyl phosphate) in TBS, 1.33 mg/ml, was dispensed 0.2 ml/well. Plates were placed in the tissue culture incubator for 90 min, and the O.D. of the hydrolyzed pNPP was measured in a optical densitometer at 405 nm. ALP specific activity was expressed as nanomoles/90 min/50,000 cells.

Electrophoresis and Western Blot Development

Stromal cells were seeded, 2×10^5/well, on day (−)3 in six-well plates; on day 0, cultures were stimulated as indicated in the respective experiments. In each plate, three cultures were stimulated and three served as controls. Medium was changed on days 0 and 7, 4 ml per well; plates were harvested for electrophoresis on the indicated days by cell lysis. Prior to cell lysis, cells in one well of each set of three wells (one experimental and one control well in every plate) were trypsinized and counted in a counting chamber. The remaining wells were harvested as follows. Wells, placed on ice, were washed twice with 6 ml of cold phosphate buffered saline (PBS) removing washing solution to dryness. Ice-cold electrophoresis sample buffer (with 7% 2-mercaptoethanol and 3% sodium dodecylsulfate [SDS]), 50 or 100 µl, was added to each well and incubated on ice for 30 min. The lysates were quickly scraped off the plastic with a policeman and transferred to cold tubes, boiled for 5 min, cooled on ice, and stored at −70°C until further use. Samples of lysed cultures representing equal cell numbers were adjusted to equal volumes with plain sample buffer and fractionated by electrophoresis usually on 7–15% polyacrylamid gradient gels. Gels were then electroblotted onto nitrocellulose filters which were blocked with 5% skim milk and 0.2% Tween-20. Filters were first incubated with antiphosphotyrosine antibody, and after washing they were incubated with an anti-mouse IgG antibody conjugated to peroxidase. Filters were exposed to enhanced chemiluminescence (ECL) buffer to generate chemoilluminescence by activated peroxidase conjugates, which was detected by autoradiography.

After radiography, the antiphosphotyrosine antibodies were stripped off the filters, and the above procedure was repeated with a monoclonal anti-Src antibody. Stripping was performed by incubation of the filters in 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS at 50°C for 30 min.

Quantitation of Western Blot Bands

Autoradiographic gel images were analysed on a Power Mac computer using the 1.61 NIH image program. Bands in pairs of adjacent lanes were plotted based on area size and integrated density within peaks, and their pixels/band were analyzed after removal of the background. In blots that were analyzed for tyrosine-phosphorylated proteins, the pixels in each band were expressed as the percent of total pixels in the respective entire lane. The percent band intensity between adjacent lanes was compared considering a difference of more than 40% as a change induced by respective treatments.

RESULTS

Osteogenic Response of Stromal Cells to Low Temperature

Figure 1A shows that specific ALP activity increased under DEX at 37°C more than at 30°C, most of which can be attributed to selection of ALP-expressing cells. Cell counts (Fig. 1B) increased substantially at cold temperature under DEX stimulation. Mineralization (per well) increased under DEX stimulation by 25% at 37°C, whereas at 30°C it was three to four times higher, and its absolute level was similar in the presence or absence of DEX (Fig. 1C). At 30°C there was a discrepancy between ALP activities and mineralization effects of DEX whether calculated per cell (as in Fig. 1C) or per well. It follows that the effect of cold temperature on mineralization is at least partially dissociated from its effect on ALP activity and that increased mineralization at 30°C does not depend exclusively on DEX.

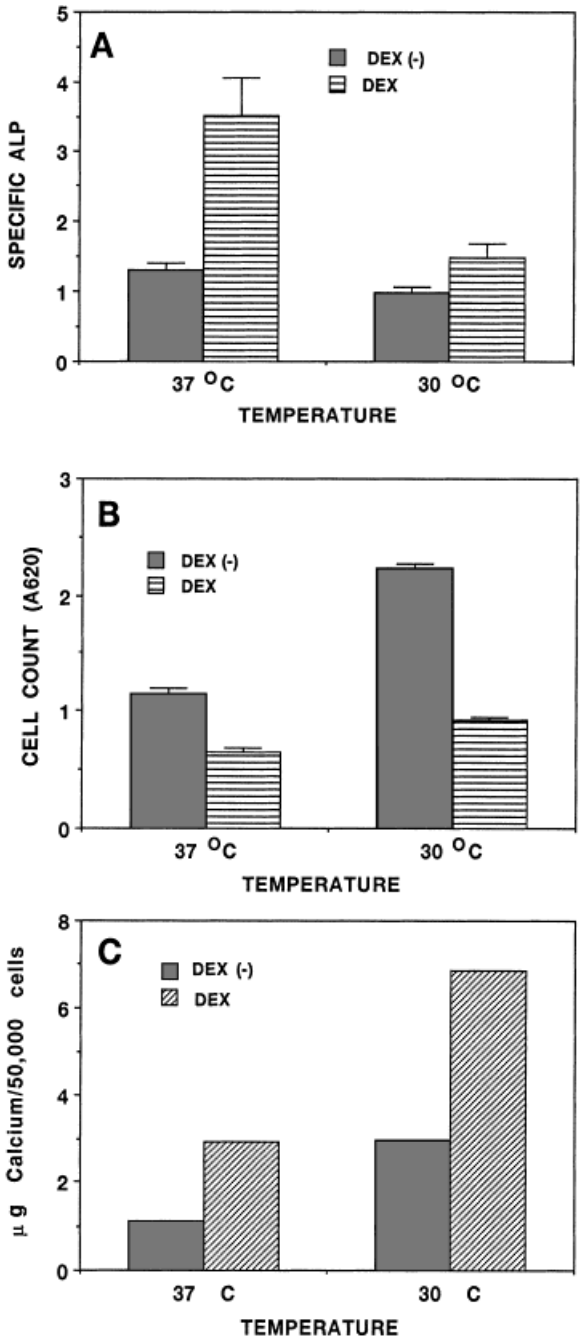

Fig. 1. Osteogenic differentiation in cold temperature in the presence and absence of dexamethasone. Stromal cells seeded on day (−)3 and cultured until day 0 at 37°C, when they were stimulated with either DEX(+) or DEX(−) medium and divided between two incubators, one at 37 and one at 30°C. On day 11 of stimulation, ALP activity was measured at the upper half of the plates (**A**), and cells were quantitatively stained (**B**) at the lower parts of the plates. On day 21 of stimulation, water-insoluble calcium was measured on equally prepared separate plates (**C**). Results are presented as mean ± SE; n = 20 wells per sample.

Profiles of Tyrosine-Phosphorylated Proteins in DEX-Stimulated Stroma

Figure 2 shows a Western blot of stromal cell proteins harvested from cultures on different days of osteogenic stimulation at physiologic temperature. On day 3 there was a general decrease in tyrosine-phosphorylated proteins per cell number (Fig. 2a) followed by a slight selective recovery for some protein bands on day 4, but the bands were still continuously suppressed by DEX. Several tyrosine-phosphorylated bands were prominently suppressed by DEX. For example, DEX strongly decreased a phosphorylated protein (per cell and per total lane phosphorylated tyrosine) of 150 kDa on days 4, 7, and 8 (+DEX/−DEX = 1,606/3,225, 1,542/4,647, and 539/1,485 scanner units per 2×10^5 cells, respectively). DEX suppressed a 66 kDa band on days 4, 7, and 8 (to 45.5%, 40.6%, and 27.7% of untreated cells, respectively). On day 3 there was a general low level of phosphotyrosine-containing proteins regardless of the presence or absence of DEX. DEX also had a differential effect on p38/41 (suppressing phosphorylation to 17.2% of controls on day 2 and increasing it by 90.4%, 43.6%, and 97.1% on days 3, 4, and 8, respectively). DEX suppressed p33 to 23.5% and to 62.3% of the relative phosphorylation level in untreated controls on days 2 and 8 ,respectively.

Figure 2b shows bands of Src protein in the same blot. On day 1, DEX induced a slightly denser and faster running band. This trend reverted starting on days 2 and 3; from day 4, cultures without DEX showed denser and faster running bands relative to those seen in the presence of DEX. This indicates that after day 3 DEX decreases Src protein quantity and its migration rate. On days 2, 3, and 7, the smaller Src bands, $p52/54^{src}$ under DEX stimulation, were more abundant (relative to the major Src band) than that seen in the absence of DEX. From day 3, upon inversion of Src abundance under DEX, its quantity/cell positively correlated with the total phosphotyrosine per cell (see Fig. 6).

Effect of Low Temperature on the Profile of Tyrosine-Phosphorylated Proteins

Figure 3a compares effects of low and normal temperatures on repertoires of tyrosine-phosphorylated proteins from stromal cells on day 3 of osteogenic stimulation. Here electrophoresis

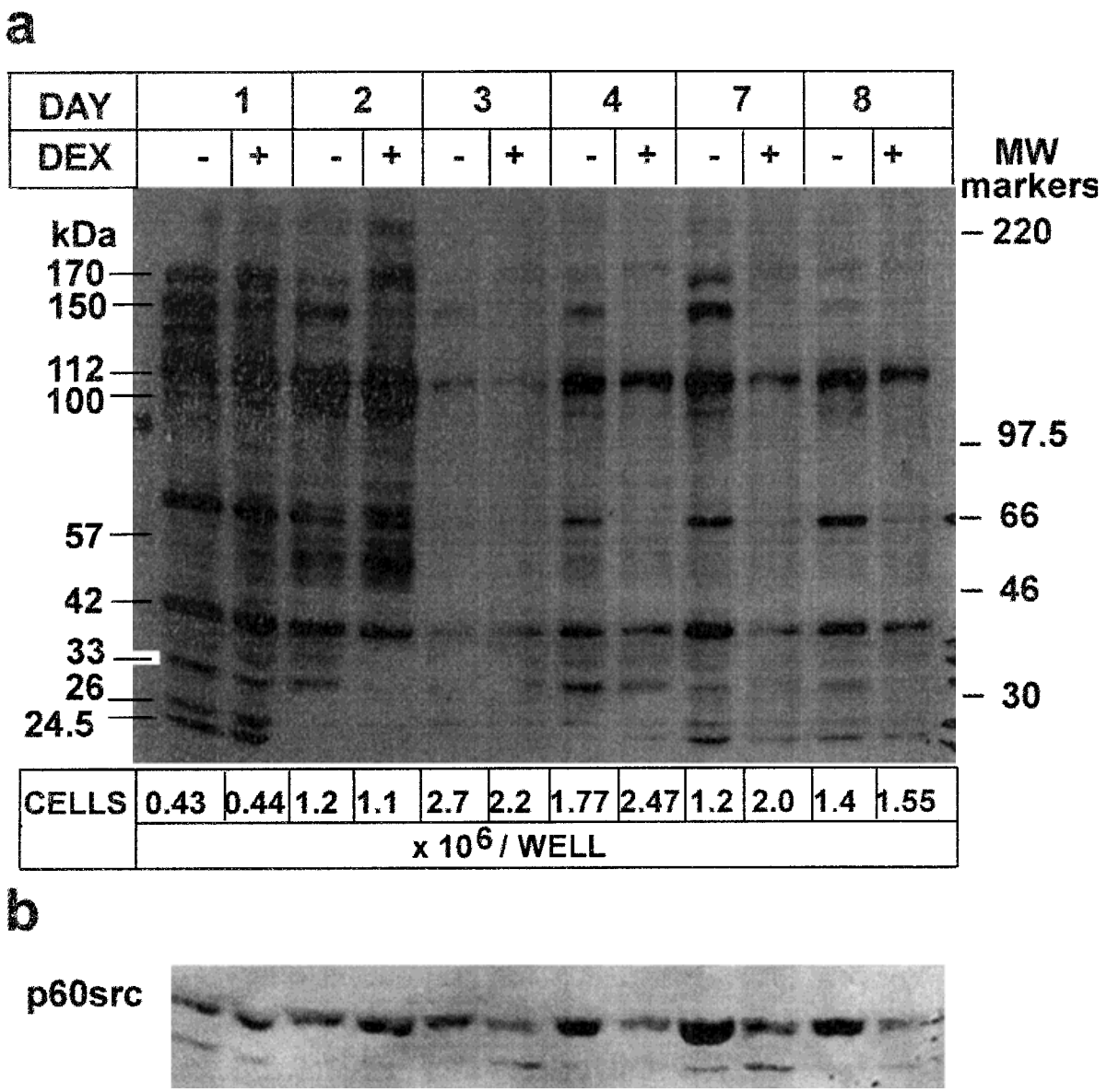

Fig. 2. Profiles of tyrosine-phosphorylated proteins from stromal cell cultures under osteogenic stimulation with and without DEX. Stromal cells seeded in six-well plates, 2×10^5 cells/well, on day (−)3 were stimulated starting from day 0 with or without DEX at 37°C. Each plate contained three stimulated and three nonstimulated wells. On indicated days, cells from one well of each triplicate sample were counted. Cell number/well on harvest day is indicated. Subsequently, each well was exposed to ice-cold electrophoresis sample buffer, 30 min on ice for cell lysis, and then heated in boiling water for 5 min and loaded on a 7% polyacrylamid gel for electrophoresis. After fractionation, proteins were electroblotted onto a 0.45 µm nitrocellulose filter which was exposed to antiphosphotyrosine antibodies and to a peroxidase-conjugated second antibody. The filters were autoradiographically developed (**a**) and subsequently, after removal of these antibodies by excess 2-mercaptoethanol, the filter processing was repeated for anti-SRC antibodies (**b**).

was performed on a 7–15% gradient gel which resolved low molecular sizes better than the 7% gel seen in Figure 2. In the absence of DEX, on day 7 p200 and p38–41 lost more than half of their relative phosphotyrosine signal intensity as compared with day 3, whereas on day 7 p15 showed a signal intensity 2.7 times higher than that of day 3. DEX partially abrogated the decrease in p200 and p38–41 and completely abrogated the increase in p15. On day 3, either DEX stimulation or low temperature increased p49 phosphotyrosine signal and that of its p50 shoulder by 94.6% and 62.3% respectively. Combination of DEX and low temperature further

increased the signal of the p50 shoulder by an additional 16%. The intensity of p38–41 increased by 90.1% only under combination of DEX and low temperature. At 37°C, the intensity of p38–41 decreased from day 3 to day 7 by more than one-half, to 48.9%, and DEX limited its decrease to only 76% of the untreated control. On day 7, DEX increased p38–41 by 35.8%. Phosphotyrosine in p32/33 increased on day 3 by 2.56 and 4.93 times under low temperature and combined treatment with DEX, respectively, whereas on day 7 it was not changed by DEX. Of interest are bands that changed in accord with osteogenic stimuli, (i.e., 30°C, 30°C + DEX, and 37°C + DEX)—for example, p200, p49/50, p32/33 and p15. Of interest is also p38–41, which was prominent only at 30°C + DEX, a condition which, according to Figure 1C, resulted in an enriched mineralizing cell population. The augmented density of bands in low temperature cultures were further accentuated under DEX. Figure 3b shows antibody-detected bands of Src protein on the same blot, similarly to Figure 2b. At 37°C, DEX increased the amount of Src protein/cell on day 3 contrary to the inverted ratio on day 7. On day 3, at 37°C, slightly less Src protein was seen compared to low temperature (1,087 vs. 1,574 scanner units), and in both it was more abundant in the presence (1,301 and 2,308 units, respectively) than in the absence of DEX. These results indicate that low temperature increases the amount of Src protein/cell in stromal cells on day 3 of osteogenic stimulation, a trend amplified by DEX at this time point. On day 3 at low temperature, the increased Src under DEX stimulation was accompanied by slightly less truncated Src protein, 2.64% compared with 5.15% of intact p60c-Src. The Src protein signals in the blot showed a direct correlation with the total phosphotyrosine signals of the respective lanes (Fig. 6).

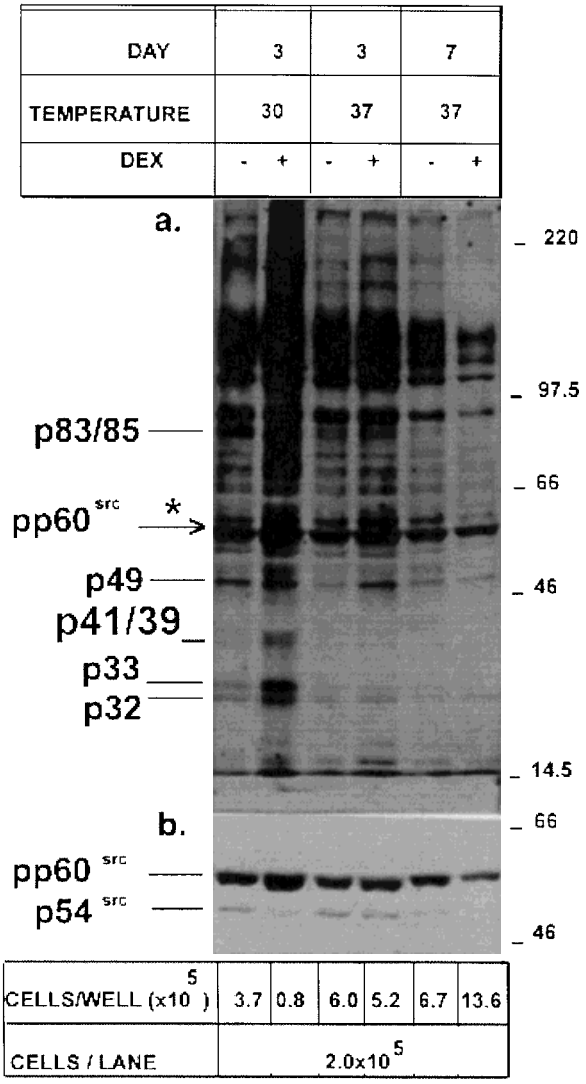

Fig. 3. Differential effect of culture temperature on the tyrosine-phosphorylated protein profile and Src protein. Stromal cells were seeded at the indicated density on day (−)3; on day 0, plates were divided between incubators of 37°C and 30°C, and DEX was added to the stimulation medium for half of each plate. On day (+)3, cell proteins were extracted and run on a 7–15% gradient polyacrylamide gel and electroblotted. Western blots were developed with antiphosphotyrosine antibodies (**a**) and, after stripping, with anti-Src antibodies (**b**). Molecular weight markers are indicated on the right. Selected bands where tyrosine phosphorylation intensity was higher at 30°C than at 37°C in the absence of DEX are indicated on the left. The inferred location of pp60src in panel a is indicated (asterisk) based on the band identified by the anti-Src antibody on the same blot. Cell counts/well upon harvesting are indicated.

Tyrosine-Phosphorylated Proteins and Quantity of Src Protein in Valinomycin-Selected Cells

Figure 4 shows the effect of cold temperature on the repertoire of tyrosine-phosphorylated proteins of stromal cells on day 7 of osteogenic stimulation. Here 10^{-7} M valinomycin was added in part of the cultures since it was shown to select nonmineralizing cells during osteogenic stimulation in the cold, especially when valinomycin was present between days 3 and 7 [Klein et al., 1996b]. It follows that on day 7 valinomycin selected cells with p33/34 containing less phosphotyrosine (55.1% of nonselected cells in the presence and 62.9% in the absence of DEX) and less p83/85 (42.2%) in the presence of DEX. Valinomycin-selected cells also showed

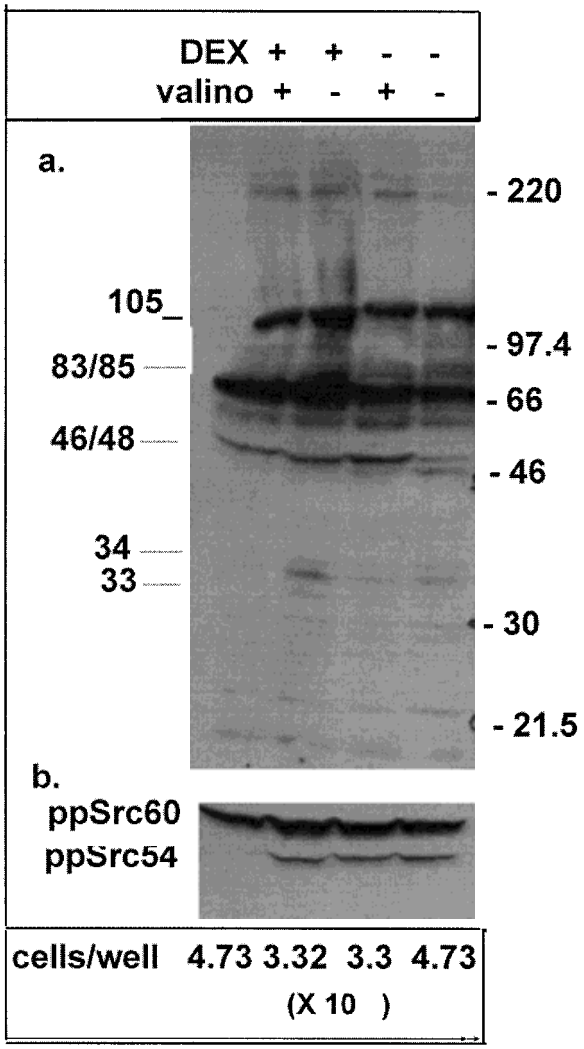

Fig. 4. Phosphotyrosine protein profiles on day 7 of stromal cells cultured at low temperature with or without valinomycin. Stromal cells were seeded on day (−)3 and cultured from day 0 at 30°C with and without 10^{-7} M valinomycin. On day 7, cells were harvested and processed for electrophoresis, blotting, and development (as described in the legend of Fig. 3).

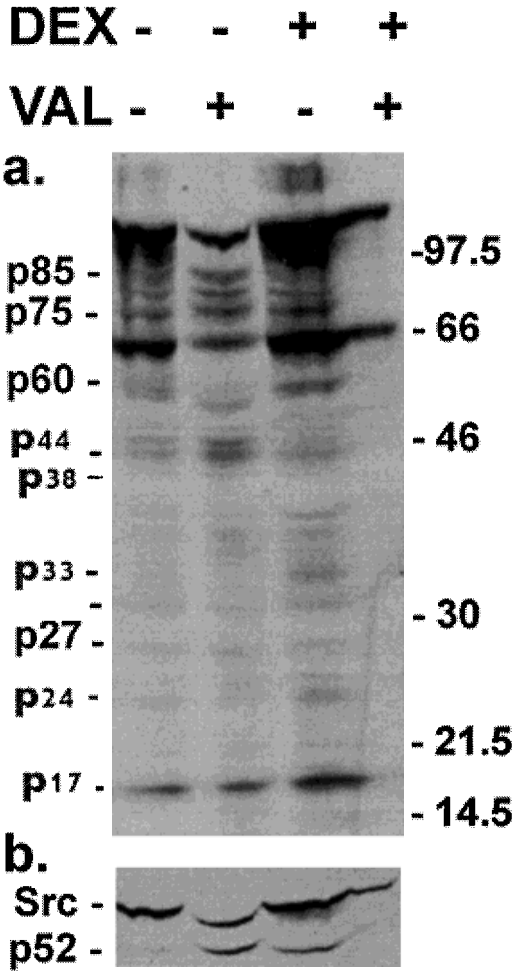

Fig. 5. Phosphotyrosine protein profiles on day 10 of stromal cells cultured in the cold with and without valinomycin. Stromal cells were transferred on day 0 to 30°C, treated, and processed as described in Figures 3 and 4. Media were changed on day 7. The molecular weight of bands showing differential intensity of phosphotyrosine are indicated on the right.

less phosphotyrosine in p110, 31.8% in the presence and 54% in the absence of DEX. In day 7 nonstimulated cultures, abundance of Src (4865 units) and its truncated protein (1,240 units) in valinomycin-selected cells were similar to values in nonselected cells (4,772 and 1,395 units respectively). Contrarily, in DEX-stimulated cells less Src protein (2,127 units) and few truncated Src (87 units) were seen in valinomycin-selected cells. Figure 5a shows that on day 10 valinomycin selected and perhaps helped to induce in stromal cells poorly phosphorylated band profiles mainly in DEX-stimulated cells. These included a decrease in 140–150, 105, 75, 70, 60, whereas in nonstimulated only p140–150 phosphorylation was decreased in valinomycin-selected cells. Other bands like p55, p44, p33/34, p31, p27, p24, p21, and p17 showed increased phosphotyrosine in DEX-stimulated and valinomycin-selected cells. In nonstimulated cells, reciprocally to DEX-stimulated cells, p105, p75 and p70 bands showed increased phosphotyrosine. In day 10 DEX-stimulated cells, Src protein was 3.8 times more abundant than in valinomycin-selected cells, and it was only 5.4 times more than its truncated band vs. 13.5 times in valinomycin-selected cells (Fig. 5b). Contrarily, in the absence of DEX the truncated Src protein was barely detected relative to the pp60Src, opposite to the case on day 7

(Fig. 4b). This corroborates the finding that valinomycin selects nonmineralizing cells in association with more Src protein, which in vivo favors bone resorption, while Src absence favors osteopetrosis. From time-sequence cultures it can be summarized that there is a relative drop in $pp60^{src}$ abundance from day 3 through days 7 and 8 (not shown), with a recovery on day 10. This occurs in stromal cultures under conditions of osteogenic stimulation, like DEX treatment at normal temperature or osteogenic stimulation at cold temperature in the absence of DEX. There is a tendency of Src quantity/cell to decrease in association with a general decrease in tyrosine phosphorylation per cell.

DISCUSSION

At low temperature, DEX did not increase specific ALP activity to the same extent as at 37°C. In the absence of DEX, twice as many cells were found on day 11 at low temperature as in the presence of DEX or at normal temperature with or without DEX. Many of these cells must be ALP-expressing cells, since the specific ALP activity is not sufficiently lower in the absence of DEX to account for the increased cell count. Thus, prolonged exposure to low temperature increases expression and or activity of ALP under these culture conditions. The differential calcium deposition (mineralization) shown in Figure 1C is presented on a per cell basis; when calculated per well at low temperature, mineralization in the presence of DEX was similar to that in its absence. The equal mineralization per well found in the cold DEX-treated and untreated cultures could be in part due to increased pH, but mineralization increases in the cold also in HEPES-buffered medium [Klein et al., 1996b]. Thus, prolonged propagation in the cold increases mineralization beyond the pH effect.

At normal temperature, DEX decreased phosphotyrosine in many of the cellular proteins in association with increased specific ALP activity, suggesting that perhaps also intracellular protein tyrosine phosphatases may increase under DEX at normal temperature. The following three different treatments are associated with osteogenic or mineralization induction in stromal cells: DEX [Bellows et al., 1987; Leboy et al., 1991; Kamalia et al., 1992], low temperature, and nonosteoprogenitor cell selection by valinomycin [Klein et al., 1996b]. In the present study, temporal changes in the phosphotyrosine content of cell proteins were followed under these treatments applied either separately or combined. The abrupt drop in general phosphotyrosine content seen on day 3 cultures, relative to day 4 and regardless of treatment, may reflect decreased proliferation and tyrosine phosphorylation–dependent signaling perhaps coupled with tyrosine phosphatases activity. The most recurring observation regards phosphotyrosine protein p32–34, which under osteogenic conditions decreased towards day 2, increased on days 3 and 4 of osteogenic induction, and decreased again after day 7. This band is yet to be identified and might interact with proteins recently described by Dennet et al. [1997] as inhibitors of cyclin-dependent kinases active in osteoblastic differentiation. Since thyrosine kinase Src protein is absolutely required for osteoclastic function, its presence in the stromal cell population was examined under mineralization induction stimuli. On days 1 and 2 of DEX stimulation, pp60src increased; this trend inverted on day 3 and thereafter, being especially prominent on day 7, when a smaller Src band increased proportionately. This decrease in Src on day 3, lagging 1 day behind the decrease in p33/34, may reflect a diminished enzyme substrate interaction between the $p34^{cdc2}$ complex and Src. In general, the amount of Src protein in each lane directly correlated with total phosphotyrosine of respective lanes in the gels (Fig. 6), but we cannot exclude a correlation which reflects the total amount of cellular protein rather than the level of tyrosine phosphorylation. The anti-Src antibody used in these studies recognizes the truncated Src [Lipsich et al., 1983] missing the amino-terminal fragment required for membrane association [Sigal et al., 1994]. If this contention is still valid, then our Src protein quantitation indicates that at 37°C, from day 3 and especially on days 7 and 8 under DEX stimulation, the proportion of membrane-unattached Src molecules is higher than in the absence of DEX. Given information from time sequence cultures of days 6, 8, and 10 (not shown) and cultures described above, at 30°C DEX decreased pp60src protein on day 6, did not change it on day 7, and increased it on day 8 and 10. Surprisingly, the presence of valinomycin resulted in less pp60src on days 6, 7, and 10; only on day 8 in the absence of DEX was pp60src slightly higher in cultures with valinomycin. It was expected that

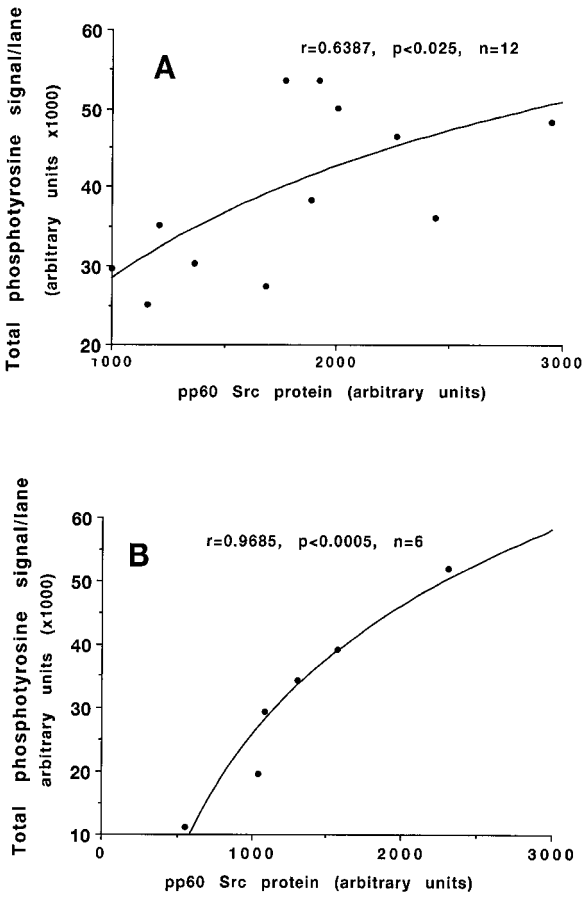

Fig. 6. Correlation between Src protein and total cell phosphotyrosine. ECL signal of pp60^{src} produced by the anti-Src antibody is plotted against the signal produced by the antiphosphotyrosine antibody. Each point represents one lane from Fig. 2 (**A**) and Fig. 3 (**B**).

at cold temperature cells of the osteoprogenitor lineage would react by thermogenesis [Klein et al., 1996b] in association with a mitochondria-aided mineralization initiation process. Although the opposite was found, it is possible that the valinomycin embedded in the plasma membrane antagonized the hydrophobic-dependent plasma-membrane anchorage of pp60^{src} in nonmineralizing cells. Interestingly, in valinomycin cultures the presence of DEX did result in lower pp60^{src}—for example, on days 6, 7, and 10. A possible connection between Src protein and induction of cell-mediated mineralization might be a nonkinase function of Src. Sigal and Resh [1993] have shown that the amino-terminus of Src can bind the mitochondrial ADP/ATP carrier only if presented as a short peptide small enough to cross the outer mitochondrial membrane. This is consistent with cleavage products of Src increasing under DEX on days 7 and 8 at normal temperature and on days 7 and 10 at cold temperature. It also agrees with mitochondrial-membrane potential changes on days 7/8 which accompany changes in mineralization [Klein et al., 1993c, 1996a].

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