Journal of Cellular Biochemistry 71:116–126 (1998)

Opposing Effects of Cyclosporin A and Tyrphostin AG-1478 Indicate a Role for Src Protein in the Cellular Control of Mineralization

Jaqueline Stekelenburg,[1] Benjamin Y. Klein,[1,2]* Hannah Ben-Bassat,[1] and Nathan Rojansky[3]

[1]Laboratory of Experimental Surgery, Hadassah Medical Center, Jerusalem, Israel
[2]Department of Orthopedic Surgery, Hadassah Medical Center, Jerusalem, Israel
[3]Department of Obstetrics and Gynecology, Hadassah Medical Center, Jerusalem, Israel

Abstract Cyclosporin A (CsA) induces osteoporosis but not through direct activation of osteoclasts. CsA also inhibits cell-mediated mineralization in marrow stromal cell culture, whereas the tyrphostin AG-1478 increases mineralization. These antagonistic effects on mineralization were used to discern molecules that underwent phosphorylation changes in association with their opposing effects on mineralization. In parallel, quantitative changes in Src protein were followed. Multiple dexamethasone (DEX)-stimulated stromal cell cultures were grown with and without a mineralization-inhibiting dose (0.1 µM) of CsA and were harvested on different days of DEX stimulation. Immunoblots of gel-fractionated cell extracts showed that the most noticeable changes in tyrosine phosphorylated proteins (TPP) were seen on day 8 of DEX stimulation. At least 15 TPP bands, mostly smaller than 53 kDa, were more prominent in CsA-treated cultures on day 8. Under CsA, Src protein quantity decreased on day 8, but its cleavage product (52/54 kDa) was sixfold more abundant then on day 7. Day 8 was chosen to test the effect of AG-1478 on the CsA-induced TPP changes. Dimethyl sulfoxide (DMSO) alone, the solvent of AG-1478, increased mineralization in CsA-treated versus CsA-untreated cultures and slightly decreased Src and its cleavage product. AG-1478 at 5 µM, in CsA cultures increased the specific alkaline phosphatase activity threefold, with a slight change in mineralization relative to controls grown with DMSO alone. This was accompanied by decreased intensity of several TPP bands smaller than 36 kDa. In contrast, treatment with 50 µM of AG-1478 increased the intensity of TPP bands at the same molecular size range. This high AG-1478 dose decreased cell counts selecting mineralizing cells. The results indicate that increased Src protein cleavage product on day 8 by CsA is associated with mineralization inhibition, which is opposed by DMSO and 50-µM AG-1478, thus antagonizing the effect of CsA on mineralization. Direct or indirect interaction between Src and TPP, antagonistically affected by CsA and AG-1478, is likely to underlay cellular control of mineralization. Changes in p19 and p29 intensity showed association with mineralization that was reflected by a significant direct and inverse correlation, respectively, with calcium precipitation per cell. J. Cell. Biochem. 71:116–126, 1998.

Key words: alkaline phosphatase; osteogenic induction; pp60Src; tyrosine phosphorylation

Cyclosporin A (CsA), an inhibitor of the phosphatase calcineurin, inhibits cell-mediated mineralization in dexamethasone (DEX)-stimulated marrow stroma [Klein et al., 1997b]. In contrast, some tyrosine kinase (TK) inhibitors (such as AG-1478) may under certain concentrations preserve the osteoblastic phenotype by preferentially killing nonosteoprogenitor cells [Klein et al., 1997a]. Tyrphostin AG-1478 at nanomolar concentrations specifically inhibits EGF receptor TK activity, but at higher concentrations it inhibits downstream nonreceptor TK inhibitors like those of the Src family [Osherov and Lewitzki, 1994]. The prototype of this family, pp60src, has been shown to be required for bone remodeling because it is indispensable for maturation and function of osteoclasts [Tanaka et al., 1996]. Absence of pp60Src results in osteopetrosis [Soriano et al., 1991] that is correctable by normal bone marrow. Bone marrow stroma and Saos2 cell lines in culture are osteogenic rather than osteolytic systems. Therefore, increased mineralization under AG-1478 in these systems suggested that Src may be involved in osteoblastic mineralization in addition to its osteoclastic effect on bone resorption. In the present study, the two drugs with oppos-

*Correspondence to: Benjamin Klein, Laboratory of Experimental Surgery, Hadassah Medical Center, P.O. Box 12000 Ein-Kerem, Jerusalem, Israel.

Received 11 May 1998; Accepted 12 May 1998

ing effects on cell-mediated mineralization were used to discern thyrosine phosphorylated proteins (TPP) involved in mineralization in relation to changes in pp60Src content. We found that Src protein progressively decreased up to day 8 of osteogenic induction by DEX accompanied by diminishment of its truncated band. This decrease occurred in parallel to the decrease in several phosphotyrosine-containing bands, usually smaller than 53 kDa. CsA, added on day 3, antagonized the decrease in TPP (most prominently) on day 8, also antagonizing the decrease in the truncated Src protein p52Src. The tyrphostin AG-1478 added 48 h before day 8 in CsA-treated cultures differentially affected the abundance of TPP. The results also indicate that a 19- and a 29-kDa TPP correlated with pp60Src association with cell-mediated mineralization.

MATERIALS AND METHODS

Reagents

The alkaline phosphatase (ALP) kit 104 LL, DEX, ascorbate, (β-glycerophosphate and anti-phosphotyrosin antibodies were purchased from Sigma (St. Louis, MO). Monoclonal antibody anti-Src 327 was generated from hybridoma medium [Lipsich et al., 1983]. Fetal calf serum was purchased from Grand Island Biological Company (Grand Island, NY).

Stromal Cell Culture

Bone marrow cell suspensions obtained from femurs and tibiae of female Sabra rats, weighing 60–80 g, were seeded in 25-cm^2 flasks at 10^8 cells per flask. Stromal cells were obtained as described elsewhere [Maniatopoulos et al., 1988] by 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 Dulbecco 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 at 5,000 cells per well and grown in osteoprogenitor cell (OPC) stimulation medium. This medium consisted of maintenance (ordinary) medium containing 10^{-8} M DEX [Bellows et al., 1987; Leboy et al., 1991], 50 μg/ml ascorbate, and 10 mM glycerophosphate. The medium (DEX medium) was changed on day 7 and every 4 days thereafter.

Measurement of In Vitro Precipitated Calcium

After 3 weeks in culture, the DEX medium, as opposed to ordinary medium, induces cell-mediated mineralization that appears as calcium deposits surrounding and covering foci of cells and is detectable by alizarin red staining [Klein et al., 1993a]. On X-ray diffraction, such calcium deposits show a main deflection characteristic of hydroxyapatite at $2\Theta = 32.8°$; on X-ray dispersion analysis, mainly calcium and phosphate were seen [Maniatopoulos et al., 1988]. Similar results were obtained in our laboratory when using the same system. In the present study, the calcium deposits were measured as follows. Plates were washed twice with Tris buffered saline (TBS) 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 the deposits found in untreated controls.

Quantitative Cell Staining

Cells were stained by 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 min. 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.

ALP Activity Assay

ALP activity was measured in situ in microtiter plates. Day 11 of DEX stimulation was selected for cell counts and ALP assay because 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). The ALP sub-

strate, *p*-nitrophenyl phosphate (pNPP) in 1.33 mg/ml TBS, was dispensed at 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 per 90 min per 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 respective experiments. In each plate, three cultures were stimulated and three served as controls. Medium was changed on days 0 and 7, 4 ml/well, and plates were harvested for electrophoresis on indicated days by cell lysis. Prior to cell lysis, cells in one 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 and aspirated 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 that were blocked with 5% skim milk and 0.2% Tween 20. Filters were incubated with anti-phosphotyrosine antibody, and after washing they were incubated with a anti-mouse IgG antibody conjugated to peroxidase. Filters were exposed to ECL buffer to generate chemoilluminesence by activated peroxidase conjugates that were detected by autoradiography.

After radiography, the anti-phosphotyrosine antibodies were stripped off the filters and the 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.

Analysis of Phosphorylated Bands

Autoradigraphic films were scanned, and images were loaded into a Power Mac computer as TIFF IBM files. Pairs of gel lanes representing phosphorylated protein bands of treated and control cultures were plotted in parallel by using the NIH image software. The profiles of these bands, consisting of integrated band area and density, were measured after background subtraction and individual peak definition. The percentage for peaks of each band was calculated relative to the number of total bands of each lane. Indices for percentage values of parallel (experimental vs. control) bands were considered to have changed in phosphorylation only when the larger peak superseded the smaller one at least by 40%.

Statistics

Differences in cell counts, ALP activity, and mineralization in cell cultures were analyzed by Student's t-test by using the SPSS program.

RESULTS

Effect of CsA on TPP

Figure 1A shows the pattern of TPPs in marrow stroma cultured with and without CsA. CsA was added on day 3 of OPC stimulation with DEX, and samples were harvested on different days as indicated. From day 5 a relative decrease in phosphorylated tyrosine is evident (lane 3), particularly in bands smaller than 44 kDa. The reduction in tyrosine phosphorylated bands in the medium to low-molecular-weight bands peaked on day 8 (lane 7) and recovered on day 10 of DEX stimulation (lane 9). CsA resisted this reduction in phosphorylated band intensity of medium to low molecular size especially on days 5 and 8. On day 5, relative to controls and to other days, a tyrosine phosphorylated band of 41 kDa was differentially more abundant (lane 4). The most prominent increase in tyrosine phosphorylated bands induced by CsA was found on day 8, including bands of 42/43, 38, 36, 28, 27, 26, 23, 20, and 19 kDa (lane 8). Therefore, day 8 was chosen to test the effect of TPP inhibition (by tyrphostin AG-1478) on phosphorylated bands induced by CsA and on osteogenic stimulation in culture.

Before focusing on day 8 tyrosin phosphorylation profiles, Src protein was quantitatively analyzed on the same Western blots. Figure 1B

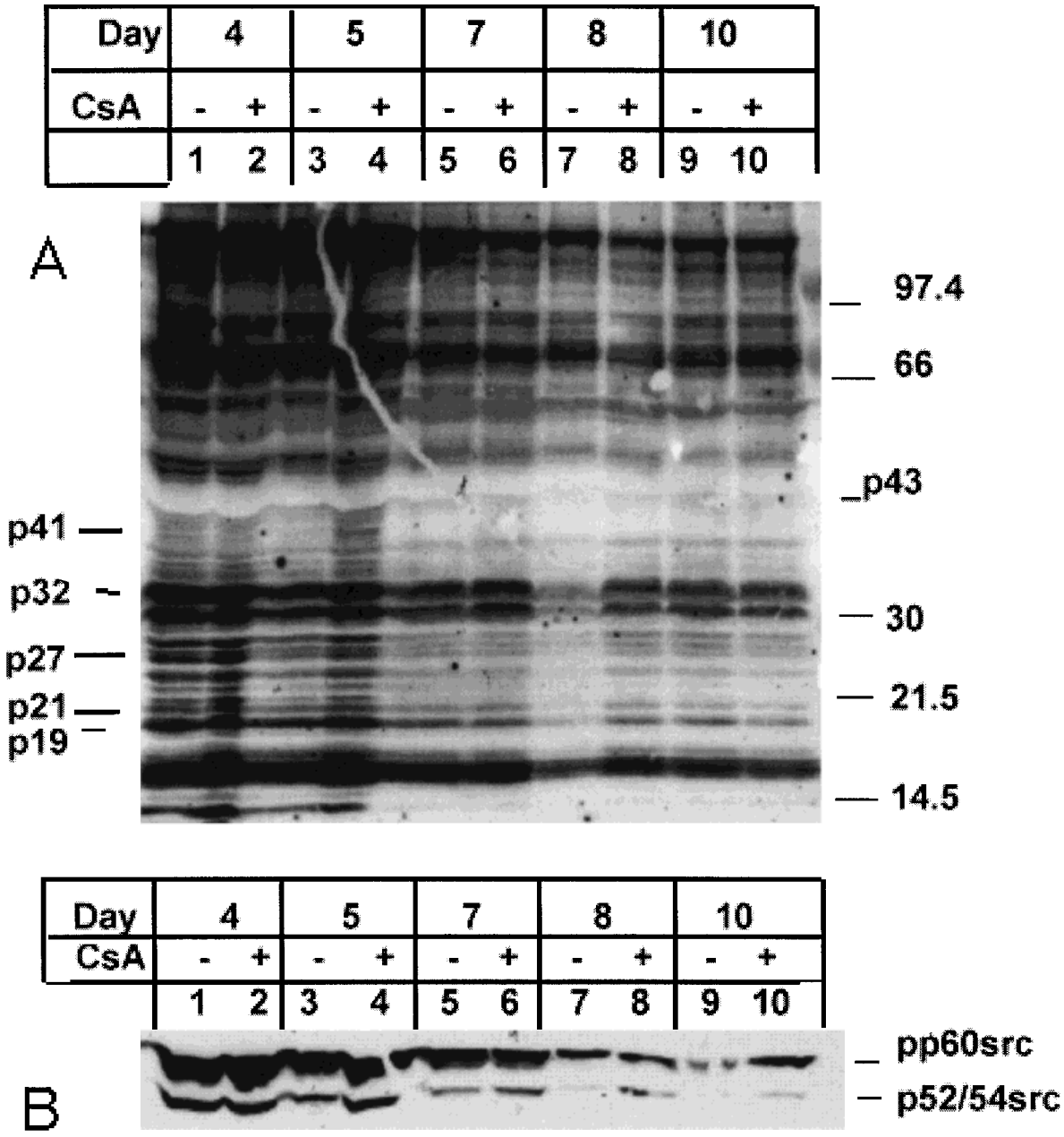


Fig. 1. The effect of cyclosporin A (CsA) on the repertoire of tyrosine phosphorylated proteins. Marrow stromal cell cultures, stimulated with dexamethasone medium on day 0, were exposed form day 3 to a mineralization inhibiting dose (0.1 μM) of CsA. On indicated days, different cultures were harvested, each with its own control (without CsA). Samples, representing 4 × 10^5 cells each, were fractionated on a 7–15% sodium dodecylsulfate polyacrylamide gel. **A:** The electroblotted filters were developed after exposure to anti-phosphotyrosine antibodies (PT-66). **B:** Stripped filters were developed again after exposure to monoclonal anti-Src antibody 327. Scanned autoradiography image was computer analyzed for relative changes of paired lanes. Coomassie blue-stained gels of postblotting remnant proteins were done to ensure equal distribution between paired lanes (not shown). Molecular weight markers are indicated on the right; relative changes in phosphorylated bands (A) or protein quantity (B) are presented.

shows the amount of Src protein in cells cultured with and without CsA on indicated days of DEX stimulation. The monoclonal anti-Src antibody recognized two bands, the pp60Src and its proteolytic cleavage fragment of 52/54 kDa, as indicated by Lipsich et al. [1983]. Figure 1 shows that on day 8 the 52/54-kDa Src-protein band increased in CsA-treated cultures. In contrast, on day 10 the 52/54-kDa Src cleavage product proportionally diminished,

whereas the pp60Src band augmented at least sixfold compared with controls.

Effect of AG-1478 on Tyrosine Phosphorylated Profiles in CsA-Treated Cultures

Figure 2A shows day 8 patterns of TPP of cells cultured with CsA, which were inhibited with different concentrations of tyrphostin AG-1478; each is compared with its uninhibited control exposed to the tyrphostin carrier (dimethyl sulfoxide; DMSO). The tyrphostin was added to the cultures 48 h before sample harvesting, a period too short for inducing cell death but long enough for inhibiting newly synthesized, or recycled, phosphate-free tyrosine sites, to enable inhibition of phosphoryla-

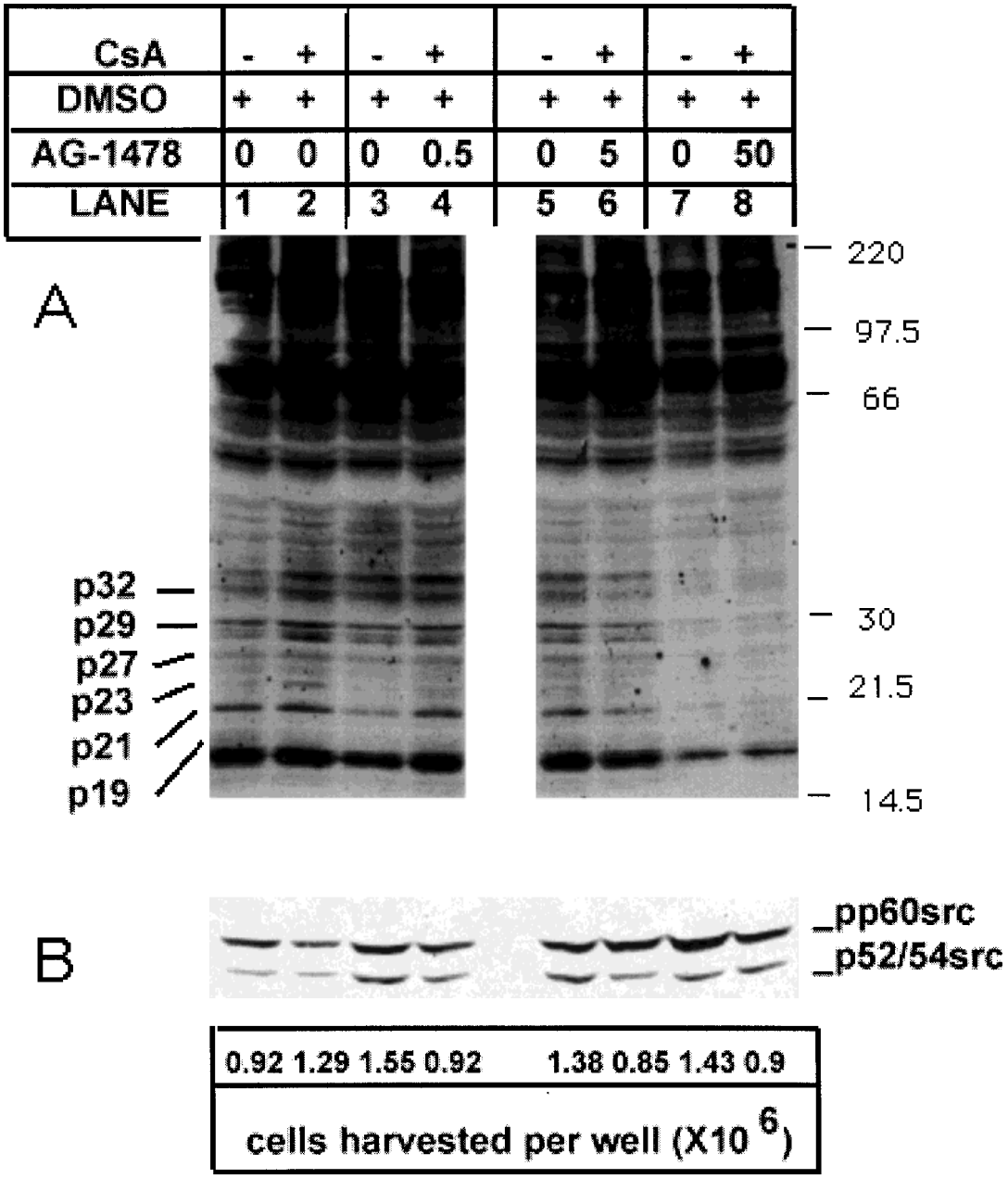


Fig. 2. Resisting and assisting effects of tyrphostin AG-1478 on cyclosporin A (CsA)-induced phosphorylated bands. Stromal cells cultured as described in Figure 1 were all harvested on day 8 after 48 h of exposure to indicated doses of AG-1478. For each plate, separate controls without CsA but with the AG-1478 solvent (dimethyl sulfoxide) alone were used. **A:** Molecular weight markers are in roman type. Several phosphorylated bands of those undergoing changes of more than 40%, relative to paired controls, are in bold type. **B:** Cell counts of representative wells are shown below Src quantities. Samples were adjusted to 4×10^5 cells/lane.

tion. Figure 2 shows that on day 8 CsA (with DMSO carrier) generally increased tyrosine phosphorylated bands of medium and low molecular weight, including bands of 44, 42, 41, 36, 34, 30, and 23 kDa, which increased at least 40% above those of untreated controls (compare lane 2 with 1). In the presence of low AG-1478 dose (0.5 μM, lane 4) CsA still increased TPP while decreasing the 42-kDa band. The intermediate concentration of 5 μM (lane 6) resulted in reduction of the 36-, 34-, 31-, 29-, 27-, 23-, and 21-kDa bands. The high dose (50 μM AG-1478, lane 8) resulted in only slightly higher tyrosine phosphorylation for the 36-, 32-, 30-, 27-, 21-, and 19-kDa bands, whereas p29 was lower (see plots of lanes 7 and 8 in Fig. 7A).

Figure 2B shows changes in intensity of Src protein bands in cultures grown with CsA and treated with different tyrphostin concentration. These Src signals were derived from the Western blot prepared by striping the anti-phosphotyrosine antibodies from the blot shown in Figure 2A and developed de novo with anti-Src antibody as described in Figure 1.

CsA slightly decreased the amount of the pp60src protein on day 8 (lane 2 vs. 1), similarly to day 8 cultures in Figure 1B (lane 8 vs. 7). The only difference between these two couples of lanes is that in Figure 2 the presence of DMSO resulted in equal levels of truncated Src, whereas the absence of DMSO and presence of CsA resulted in higher truncated Src. The CsA effect of augmenting TPP was antagonized by 0.5 and 5 μM AG-1478 but less with 50 μM AG-1478. Calculated indices of Src relative to Src quantities in DEX control cultures (treated with the DMSO carrier) show that only the 5 μM AG-1478 increased the pp60src band (1.27 vs. 0.687 of the control index). The indices between pairs of lanes under 0.5 μM and 50 μM AG-1478 were practically unchanged, i.e., 0.702 and 0.655 respectively. The 52/54 kDa (Src fragment) was slightly decreased by CsA, and this effect was slightly enhanced by 0.5 and 5 μM AG-1478 but reversed by 50 μM AG-1478. In the absence of DMSO, CsA on day 8 increased the amount of the 52/54-kDa cleavage of Src protein at least sixfold (Fig. 1B), which did not occur in the presence of DMSO (Fig. 2B).

Interference of AG-1478 With the CsA Effect on Stromal Cell Osteogenic Differentiation

To determine whether AG-1478 antagonizes the effects of CsA on osteogenic activity, DEX-stimulated stromal cells in the presence of CsA were exposed to different doses of the tyrphostin AG-1478 by using protocols similar to those described for the Western blot experiments. Cell counts and ALP activity were measured on day 11 of DEX stimulation and mineralization was measured on day 21. Figure 3B shows that 0.5 μM AG-1478 slightly but significantly increased the cell count relative to that of controls, 5 μM did not change the cell count, and the 50-μM concentration decreased the cell count by 30%. Figure 3A shows that 0.5 μM AG-1478 added on day 6 to cultures grown in the presence of CsA did not change specific ALP activity, whereas 5 μM increased it threefold and 50 μM only slightly increased specific ALP activity. Thus, the results shown in Figure 3A,B indicate that 5 μM AG-1478 increased ALP activity per se, whereas 50 μM AG-1478 increased it by selection of ALP-expressing cells. Figure 3C shows the effects of AG-1478 on the mineralization relative to cultures grown only with CsA and DMSO. The increased mineralization seen in cultures treated with AG-1478 at 0.5 and 5 μM was not statistically significant, whereas 50 μM AG-1478 increased mineralization significantly ($P = 0.044$) relative to that of untreated controls. On the per-cell basis, the increased mineralization in cultures treated with 50 μM AG-1478 also indicates that selection of cells associated with mineralization occurred. In conclusion, the low AG-1478 dose did not show a differential effect on ALP activity or mineralization in cultures grown with CsA but only a slight increase in cell counts. The intermediate dose increased specific ALP activity but not mineralization, whereas the high dose had a slight effect on specific ALP activity; however, it increased mineralization per cell.

Tyrosine Phosphorylation Correlation With Src

Tyrphostin-treated and -untreated pairs of lanes were subjected to image analysis of autoradiographed bands to compare parallel bands. Arbitrary units of bands from Figure 2 of each molecular size were plotted against the pp60src signal quantity of the respective lane. The TPP bands with a correlation coefficient significantly different from zero are shown in Figure 4. These are p21, p27, p30, and p32, which are at the lower molecular size range. To examine whether the TPP relate to Src in accord with their molecular size, the regression between molecular weight and the correlation coefficient

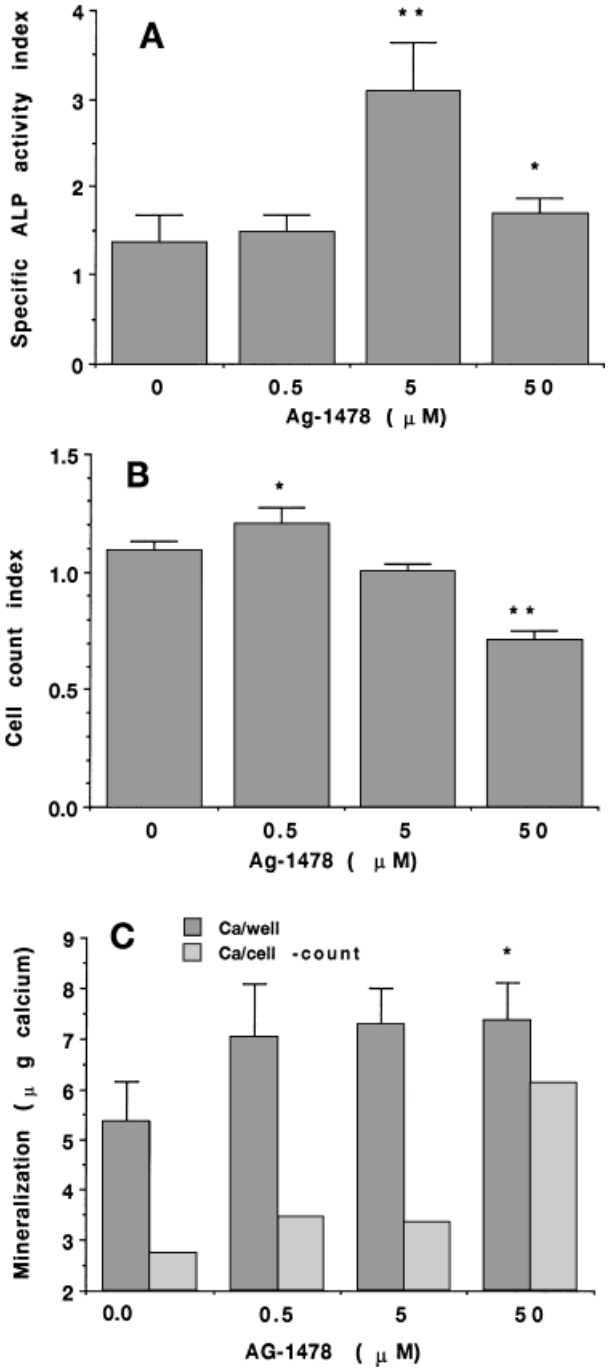


Fig. 3. Effect of AG-1478 on osteogenic stimulation of cultured marrow stromal cells. Cells were prepared for dexamethasone stimulation (n = 20 wells) on day 0 and exposed to 0.1 μM cyclosporin A on day 3 and to indicated concentrations of AG-1478 on day 6, similar to the conditions described in Figure 2. Medium was changed on days 3, 7, and 9 for the (**A**) alkaline phosphatase (ALP) assay and for (**B**) cell counts performed on day 11 and on day 14 for the (**C**) mineralization assay performed on day 21. A: Specific ALP activity is expressed as an index of AG-1478-treated samples relative to dimethyl sulfoxide carrier controls, $*P < 0.005$, $**P < 0.001$. B: For cell counts, $*P = 0.02$, $**P < 0.001$. C: Mineralization is expressed as mean ± SEM micrograms of calcium per well and as calcium precipitates per 50,000 cells, $*P = 0.044$.

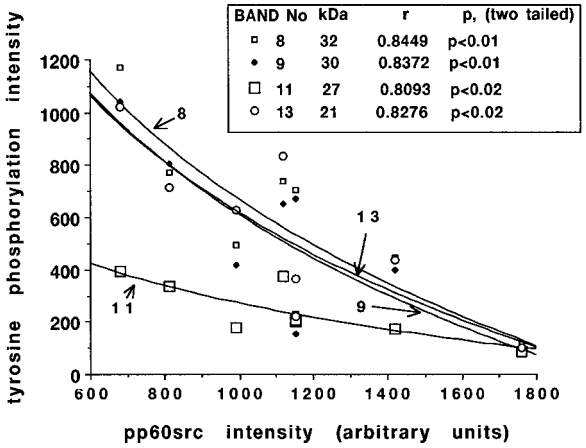


Fig. 4. Correlation between tyrosine phosphorylated proteins and pp60Src protein. Intensities of phosphorylated bands from 53 kDa and below were derived from the image analysis shown in Figure 2 and plotted against respective intensities of pp60Src. Logarithmic regression is shown for values of four of 15 molecular sizes that presented significant correlation with Src.

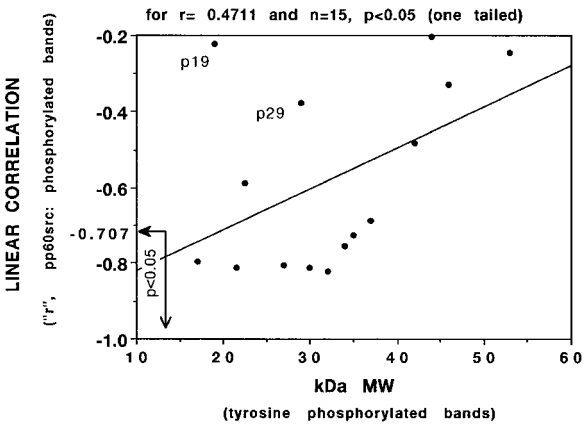


Fig. 5. Dependence of tyrosine phosphorylated proteins (TPP) on pp60Src for molecular size. Linear regression analysis is shown for the correlation coefficient of pp60Src-associated TPP versus molecular size. The arrow on the Y axis indicates point from which r becomes significantly associated with Src. The two TPP bands, p19 and p29, that deviate substantially from the regression line are indicated.

(between TPP and Src) was computed. Figure 5 shows that there is an association between molecular size of TPP and their correlation with Src protein intensity, even in the presence of outlayers p19 and p29. Figure 6 shows that p19 and p29 are in direct and inverse correlation with mineralization, respectively. The reciprocal quantity of these two bands under 50 μM tyrphostin is shown in Figure 7A by comparing the density plots in the absence (lane 7) and presence (lane 8) of AG-1478. The drug results in more abundant p19 and less p29. The quanti-

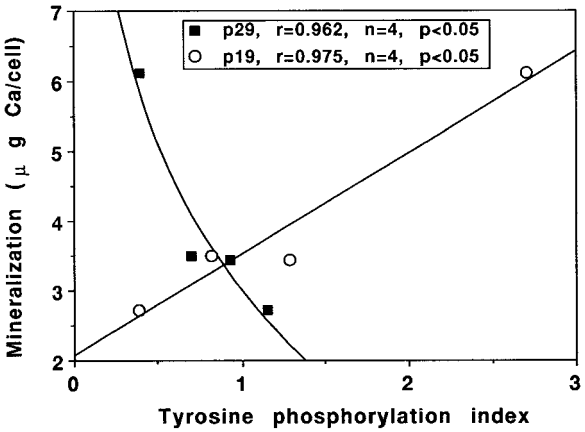


Fig. 6. Dependence of mineralization per cell on the change in intensity of individual tyrosine phosphorylated protein (TPP) bands. Regression was computed between mineralization per cell and the change in TPP band intensity under different doses of AG-1478. The direct linear correlation for p19 and the inverse exponential correlation for p29 are presented as the only significant correlations for 15 other bands.

fied bands of all the lanes of Figure 2 are listed in Figure 7B.

DISCUSSION

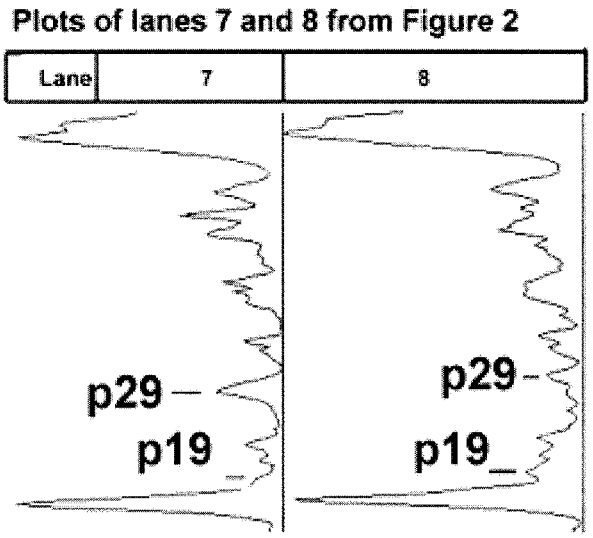
In a previous study [Klein et al., 1997b], DEX-stimulated stromal cell cultures were shown to undergo a decrease in cell-mediated mineralization in the presence of 0.1 μM CsA. Because CsA inhibits phosphatase activity of calcineurin, one of its direct effects should be a delay in protein dephosphorylation, i.e., increase in the phosphorylated molecules of some proteins. In the present study, we detected unidentified macromolecules that underwent tyrosine phosphorylation changes (mainly increases) by CsA during osteogenic induction in cultured marrow stroma. Subsequently, we chose to continue this study focusing on day 8 for two reasons. First, day 8 cultures exhibited the most prominent changes in tyrosine phosphorylation, similar to the effect on day 8, of a CsA analogue (SDZ 220–380; not shown). Second, day 8 ± 1 has been found to coincide with a temporary surge in mitochondrial retention of rhodamine 123 [Klein et al., 1993c]. Rhodamine retention marks either mitochondrial mass per cell or inner membrane potential per mitochondrial mass. The magnitude of the surge in rhodamine retention near day 8 may predict the extent of mineralization as measured on day 21 [Klein et al., 1996].

Influence of the mineralization inhibitor CsA on TPP was tested on day 8 by using tyrphostin AG-1478; this agent inhibits EGF receptor TK at submicromolar concentrations. At the micromolar range, AG-1478 influences the signal transduction pathway downstream to membrane receptors; for example, in fibroblasts it inhibits the TK activity of Src protein [Osherov and Levitzki, 1994]. We found that the tyrphostin carrier DMSO itself increased mineralization. This required discerning those osteogenic effects of the tyrphostin, which superseded osteogenic effects of its DMSO carrier. Table I, which summarizes the results of the study, underscores the patterns of TPP and marks phenotypes of stromal cell populations differentiating into osteogenic cells. The results of TK inhibition with a high dose of AG-1478 indicate that increase in the presence of phosphorylated bands 36, 32, 30, 27, 21, and 19 kDa is associated with decreased mineralization. In contrast, decrease in these bands is associated with increased ALP activity with less mineralization. This pattern seen under the intermediate tyrphostin dose may reflect a delay or block in osteogenic differntiation.

Src, the product of the c-src proto-oncogene, has been implicated in the regulation of bone differentiation [Soriano et al., 1991]; Src may be important for osteoclastic function [Yoneda et al., 1993; Hall et al., 1994; Tanaka et al., 1996]. The parameters tested by our culture system of marrow stroma are not known to reflect osteoclastic effects in DEX-stimulated cultures. Thus, Src may also play a role in osteoblast function. The relative amounts of Src protein versus its cleavage product suggest that increased Src cleavage on day 8 may be associated with a decrease in mineralization level. This was particularly prominent in DEX-stimulated stroma cultured in the presence of 0.1 μM CsA, a concentration shown to inhibit mineralization [Klein et al., 1997b].

Use of DMSO, especially when carrying 50 μM tyrphostin, combined with CsA has greatly diminished the surge in Src cleavage product and concomitantly increased mineralization and/or osteogenic differntiation. The surge in the full-length Src in day 10 cells cultured with CsA further indicates that abundance of Src protein may have a negative effect on the osteoblastic phenotype in addition to its well-known positive effect in osteoclasts. In general, tyrosine phosphorylation of the bands below 53 kDa showed inverse correlation with the amount of pp60Src; four of those with significant correlation are shown in Figure 4.

A



B Quantification of bands from Figure 2

	Src		Molecular size of phosphorylated bands (in kDa)														
Lane	p60	p52	53	44	42	41	36	34	33	32	30	29	27	23	21	19	17
1	992	215	2606	229	167	376	249	662	933	495	419	341	179	172	628	350	2863
2	682	205	3459	731	679	966	1005	1783	2036	1171	1043	698	392	497	1023	243	3140
3	1154	595	3511	818	1631	1557	849	1747	1980	706	674	497	206	112	364	159	2397
4	811	306	3445	715	795	1563	618	1545	1860	774	804	453	338	351	716	202	2759
5	1118	584	2973	392	271	489	388	1136	1846	741	651	546	376	440	835	389	2791
6	1420	295	3185	390	193	362	199	580	508	445	397	335	173	219	438	234	2458
7	1758	406	3091	458	437	467	195	233	294	113	86	453	85	178	99	152	1724
8	1153	420	3090	706	280	639	369	355	364	230	155	206	201	217	219	475	1838

Fig. 7. Comparative quantitation of tyrosine phosphorylated protein and Src bands on day 8, with and without tyrphostin treatment. **A:** Parallel plots of controls (**lane 7**) and 50 μM AG-1478 (**lane 8**). **B:** List of band size for each lane obtained from Figure 2. **Lanes 2, 4, 6, and 8** represent treatment with 0, 0.5, 5 and 50 μM AG-1478, respectively, and **lanes 1, 3, 5, and 7** represent treatment with the respective dimethyl sulfoxide carrier.

The correlation coefficient in this figure suggested that the association of pp60Src with the presence of phosphorylated bands is related to the molecular size of these bands. First, the prominent changes in phosphorylated bands appeared in molecules below 53 kDa. Second, plotting the r values (of correlation between phosphorylation and pp60Src levels) against molecular weights of phosphorylated bands (Fig. 5) clearly demonstrates association with molecular size: the smaller the phosphorylated band size, the larger the quantity per cell of pp60Src.

The band of p19 is an outlayer in this correlation, which raises the possibility that if the effect of p19 on mineralization is connected with pp60Src it is likely to be unrelated to the kinase activity of pp60Src. When phosphorylation changes in all 15 bands below 53 kDa are plotted against respective values of mineralization per cell, only two bands showed significant correlation (Fig. 6). Band p19 showed direct linear correlation with mineralization, and p29 showed a logarithmic inverse correlation with mineralization. These two bands may rep-

TABLE I. Summary of Experimental Results for Day 8 of Stimulation With Dexamethasone*

Index parameters	Increase or decrease	Tyrosine phosphorylated protein (kDa)	ALP index	Cell count index	Mineral (μg calcium/ cell count)	pp60src index	p52/54 Src index
CsA/control	↑	43, 42, 38, 36, 28, 27, 26, 23, 20, 19	n.d.	Increase[a]		n.c.	6
	↓	85, 76			Decrease[a]		
CsA + DMSO/ control + DMSO	↑	44, 42, 41, 36, 34, 30, 23	n.c.	n.c.	2.7	0.687	0.953
	↓	19					
0.5 μM AG-1478[b]	↑	27, 23	n.c.	1.15	3.45	0.7	0.511
	↓	42					
5 μM AG-1478[b]	↑		3	n.c.	3.49	1.27	0.505
	↓	36, 34, 33, 29, 27, 23, 21					
50 mM AG-1478[b]	↑	36, 32, 30, 27, 21, 19	n.c.[c]	0.675	6.125	0.655	n.c.
	↓	29					

*CsA, cyclosporin A; DMSO, dimethyl sulfoxide; DEX, dexamethasone; ALP, alkaline phosphatase; n.d., not done; n.c., not changed; ↑, increased index; ↓, decreased index.
[a]Data from Klein et al. [1997b].
[b]Index of CsA (days 3–11) + AG-1478 vs. DEX control + DMSO.
[c]Increased specific ALP was indirect by selection of ALP-expressing cells.

resent part of a ubiquination ladder of a protein processed by proteosomes.

The specific mechanism by which Src affects osteogenic differentiation regarding the osteoblastic phenotype is not clear. There are numerous hypotheses to follow experimentally. One attractive possibility is to identify kinase and protein binding functions that are directed to interfere with energy metabolism at the mitochondrial level. This suggestion is based on the potential of short N-terminal src peptides to gain access to the inner mitochondrial membrane and to bind the mitochondrial ADP/ATP carrier [Sigal and Resh, 1993]. Some c-Src family substrates are located in the mitochondria [see, e.g., Suzuki et al., 1997], and their function and/or accessibility to this organelle may depend on the catabolic steady state of Src proteins. Recent experiments in our laboratory suggest that the N-terminus of the c-Src protein belongs to the differential segment (6–8 kDa) between the two c-Src bands (not shown), for which the biochemical significance is yet to be determined.

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