Fluoroaluminate Induces Activation and Association of Src and Pyk2 Tyrosine Kinases in Osteoblastic MC3T3-E1 Cells*

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Fluoride is known to increase bone mass in vivo, probably through stimulation of osteoblast proliferation; however, the mechanisms of fluoroaluminate action in osteoblasts have not yet been elucidated. We have previously shown that in osteoblastic MC3T3-E1 cells, fluoroaluminate stimulates G protein-mediated protein tyrosine phosphorylation (Šuša, M., Standke, G. J. R., Jeschke, M., and Rohner, D. (1997) Biochem. Biophys. Res. Commun. 235, 680–684). Although the Src/Thr kinases Erk1, Erk2, and p70S6K were activated in response to fluoroaluminate, the identity of fluoroaluminate-activated tyrosine kinase(s) remained elusive. In this study, we show that in MC3T3-E1 cells, fluoroaluminate induces a 110-kDa tyrosine-phosphorylated protein that we identify as Pyk2, a cytoplasmic tyrosine kinase related to Fak (focal adhesion kinase). The tyrosine phosphorylation of Pyk2 increased in a dose- and time-dependent manner. The autophosphorylation activity of Pyk2 increased 3-fold and reached its maximum within 10 min of fluoroaluminate treatment. Fluoroaluminate also induced activation of Src and the association of Pyk2 with Src. The phosphorylation of Src-associated Pyk2 increased >20-fold in in vitro kinase assays, suggesting that Pyk2 is phosphorylated by Src. Although MC3T3-E1 cells express much more Fak than Pyk2, Src preferentially associated with Pyk2. In vitro, Pyk2 bound to the Src SH2 domain, suggesting that this interaction mediates the Src-Pyk2 association in cells. These data indicate that osteoblastic cells express Pyk2, which is tyrosine-phosphorylated and activated in response to G protein activation by fluoroaluminate, and that the mechanism of Pyk2 activation most likely involves Src. Thus, Src and Pyk2 are tyrosine kinases involved in G protein-mediated tyrosine phosphorylation in osteoblastic cells and may be important for the osteogenic action of fluoroaluminate.

Fluoride has been known as a bone-forming agent for more than 100 years. Together, the occurrence of osteosclerosis in miners exposed to a mineral consisting of sodium aluminum fluoride (Na3AlF6), the effect of fluoridation of drinking water on bone mass in humans, as well as recent animal studies suggest that fluoride in complex with aluminum is capable of mimicking its anabolic action and could overcome problems of the narrow therapeutic window and side effects.

Little is known about the molecular mechanism of fluoride action in bone at the cellular level. Fluoride in a complex with aluminum (fluoroaluminate, most likely AlF4−) (4) binds to α-subunits of heterotrimeric G proteins in vitro and activates G protein-mediated intracellular signaling pathways (5–8). Numerous reports indicate that in cultures of isolated primary osteoblastic cells as well as osteoblastic cell lines, fluoride and fluoroaluminate can induce proliferation (2, 9–11), although this effect seems to be restricted to a certain population of osteoblastic cells (12). Activation of several enzymes involved in intracellular signal transduction has been implicated in mediating the mitogenic signal of fluoride (including phospholipase C, diacylglycerol kinase, phospholipase D, tyrosine kinases, Erk1, Erk2, and p70S6K) as well as the inhibition of tyrosine phosphatases and adenylate cyclase (2, 13–17).

We (17) and others (2, 18) have recently reported that fluoroaluminate and fluoride increase protein tyrosine phosphorylation in osteoblast-like cells. In our system, fluoroaluminate induced prominent tyrosine phosphorylation of several proteins with apparent molecular masses of ~70, 120, and 130 kDa (17). However, the identity of tyrosine kinases mediating fluoroaluminate-induced tyrosine phosphorylation has remained elusive. In this study, we describe the tyrosine phosphorylation of a previously undetected 110-kDa protein in response to treatment of MC3T3-E1 cells with fluoroaluminate.

The phosphorylation of this protein was weaker than the tyrosine phosphorylation of major proteins that were previously described, and its resolution from the major 120-kDa protein was critical for its detection. Furthermore, we demonstrate that this 110-kDa protein is immunologically indistinguishable from Pyk2 (proline-rich tyrosine kinase 2), a recently described tyrosine kinase shown to be regulated by G protein-coupled receptors in neuronal cells (19, 20). This finding prompted us to investigate the mechanism of Pyk2 activation by fluoroaluminate in osteoblastic MC3T3-E1 cells.

EXPERIMENTAL PROCEDURES

Materials—NaF was purchased from Fluka, and AlCl3, bradykinin, protein A-Sepharose CL-4B, and protein G-Sepharose 4B Fast Flow were from Sigma. Fluoroaluminate solution was prepared immediately before the experiment by mixing stock solutions (500 mM NaF and 10 mM AlCl3) to give a final concentration of 10 mM NaF and 10 μM AlCl3, which has been recommended for formation of fluoroaluminate complexes (4). [32P]ATP (specific activity of 3000 Ci/mmol) was acquired from Amersham Pharmacia Biotech. The SH2 domains immobilized on agarose beads were from Oncogene Science Inc. (PhosphoBindTM SH2 reagents). Antibodies were obtained from the following sources: anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology,
Inc.), anti-Fak monoclonal antibody (mAb)‡ 2A7 (Upstate Biotechnology, Inc. and Transduction Laboratories), goat anti-Fak polyclonal antibody (raised against amino acids 990–1009 of human Pyk2; Santa Cruz Biotechnology), horseradish peroxidase-conjugated secondary antibodies (Cappel), anti-Src monoclonal antibodies LA-074 (Quality Bioproducts) and 05-184 (Upstate Biotechnology, Inc.), and anti-Src polyclonal antibody SRC2 (Santa Cruz Biotechnology).

Cell Culture—The mouse osteoblastic cell line MC3T3-E1 was provided by Dr. J. Caverzasio (University of Geneva, Geneva, Switzerland).

Cells were cultured in α-minimum Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.), penicillin/streptomycin, and 10% fetal calf serum (Life Technologies, Inc.) and 0.5% nonessential amino acids (Life Technologies, Inc.) and 10% fetal calf serum (Life Technologies, Inc.), penicillin/streptomycin, and 10% fetal calf serum (Life Technologies, Inc.). The cells were grown in 10% fetal calf serum (Life Technologies, Inc.) and 10% fetal calf serum (Life Technologies, Inc.). The cells were grown in 10% fetal calf serum (Life Technologies, Inc.). The cells were grown in 10% fetal calf serum (Life Technologies, Inc.). The cells were grown in 10% fetal calf serum (Life Technologies, Inc.).

Immune Complex Kinase Assay—Pyk2, Src, or Fak immune complexes were washed twice before centrifugation, and the supernatant was diluted with 800 µl of Nonidet P-40 lysis buffer and analyzed by Western blotting as described above.

Immunodepletion—The anti-Pyk2 polyclonal antibody was bound to 100 µl of a 1:1 mixture of protein A and protein G-Sepharose (protein A/G-Sepharose) in 500 µl of Nonidet P-40 lysis buffer by incubation at room temperature on a shaker for 1 h. Subsequently, the protein A/G-Sepharose beads were washed three times with 1 ml of lysis buffer to remove unbound antibodies and resuspended in lysis buffer. The beads of cleared cell lysate prepared from fluoroaluminate-stimulated MC3T3-E1 cells (200 µg, 150 µg of total protein) was incubated on ice with 5 µg of Pyk2-specific antibody prebound to protein A/G-Sepharose beads for 1 h. The beads were removed by centrifugation, and the supernatant was incubated with the immobilized antibody again. Equal amounts of protein of the original lysate and of the supernatants after immunodepletion of Pyk2 were analyzed by Western blotting as described above.

Immunoprecipitation—Cleared cell lysates were diluted with Nonidet P-40 lysis buffer to a final protein concentration of 1 mg/ml and incubated with 2 µg of the appropriate antibody for 2–4 h on ice on an orbital shaker. The immune complexes were subsequently captured with 10 µl of protein A- or protein G-Sepharose for 30 min. The beads were washed twice with 1 ml of 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 2 mM EDTA, and 0.5% Nonidet P-40; twice with 1 ml of 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 2 mM EDTA; and once with 30 mM Tris (pH 7.5). For co-immunoprecipitation experiments, the NaCl concentration was 140 mM (nonstringent conditions). The proteins in immune complexes were analyzed either in kinase assays or by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. For reconstitution of denatured proteins from immune complex kinase assays, the immune complexes on protein A-Sepharose were boiled in SDS loading buffer to release immunoprecipitated proteins. Sepharose beads were removed by centrifugation, and the supernatant was diluted with 800 µl of Nonidet P-40 lysis buffer and used for immunoprecipitation as described above.

Immune Complex Kinase Assay—Pyk2, Src, or Fak immune complexes were washed as described above. An additional wash was performed in kinase buffer (30 mM Tris (pH 7.5), 2 mM MgCl₂, and 5 mM MnCl₂). The protein kinase assays were performed in 30 µl of kinase buffer and incubated at 37 °C for 10 min. Immune complexes were washed three times with 1 ml of ice-cold NET (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 2 mM EDTA) to remove free [32P]ATP. The antigens were released from the beads by boiling in SDS loading buffer and subjected to SDS-PAGE. Src immune complexes were separated on longer gels (8 × 9 cm, Mighty Minigel, Amersham Pharmacia Biotech) at 20 mA/gel for a better separation. Gels were dried and exposed on a PhosphorImager screen (Molecular Dynamics, Inc.) for 4–40 h. The bands of interest were quantified with the ImageQuant program (Molecular Dynamics, Inc.) and corrected for background signal.

RESULTS

Fluoroaluminate Induces Tyrosine Phosphorylation of a 110-kDa Protein in Osteoblastic MC3T3-E1 Cells—Previously, we had shown that fluoroaluminate induced the tyrosine phosphorylation of several proteins in osteoblastic MC3T3-E1 cells, which reached a maximum between 30 and 60 min of stimulation (17). Here, we have stimulated serum-depleted cultures with 10 mM NaF and 10 µM AlCl₃ for 20 min. Control cells were left untreated or were treated with 10 µM AlCl₃. In addition to previously observed tyrosine-phosphorylated proteins of 120, 130, and 65–70 kDa, more precise analysis of the Western blots revealed enhanced tyrosine phosphorylation of another 110-kDa protein (Fig. 1A) that is herein referred to as p110. NaF alone induced a qualitatively identical but weaker phosphorysine increase (data not shown). Stimulation of MC3T3-E1 cells with 10 µM AlCl₃ did not result in an alteration of tyrosine-phosphorylated proteins (Fig. 1A).

To examine the kinetics of p110 tyrosine phosphorylation, MC3T3-E1 cells were treated with fluoroaluminate for 1–90 min. Increased p110 tyrosine phosphorylation was evident 2 min after addition of fluoroaluminate to MC3T3-E1 cells and reached a maximum ~15 min after fluoroaluminate addition, remaining high for as long as 40 min (Fig. 1B, upper panel). It is intriguing that the p110 phosphorylation is weak in comparison to phosphorylated Fak, which is responsible for the strong signal detected just above Pyk2. Fak was identified as the band below Pyk2 by immunodepletion of Fak from MC3T3-E1 lysates (data not shown). To investigate whether p110 is involved in receptor-mediated, G protein-coupled signaling in osteoblasts, the ability of bradykinin to stimulate tyrosine phosphorylation of p110 was evaluated. Bradykinin receptors are seven-transmembrane receptors considered to couple to the G class of G proteins and are present on MC3T3-E1 cells (23).

‡ The abbreviations used are: mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol 5-kinase.
Pyk2, a cytoplasmic tyrosine kinase of the Fak family, has recently been shown to participate in signal transduction involving G protein-coupled receptors, such as thrombin and endothelin-1 (data not shown). These experiments indicate that Pyk2 is expressed in osteoblastic cells. Pyk2 and Fak were immunoprecipitated from Nonidet P-40 cell lysates prepared from cultures treated with fluoroaluminate as described for Fig. 1A. Equal amounts of total cellular protein (250 μg) were incubated with 3 μg of either anti-Pyk2 polyclonal antibody or anti-Fak mAb 2A7 for 2 h. Immune complexes were captured by protein A/G-Sepharose, washed extensively, and subjected to kinase autophosphorylation assays as described under “Experimental Procedures.” 

Identification of p110 as Pyk2 tyrosine kinase

Pyk2 protein was detected using a goat polyclonal antibody specifically recognizing Pyk2. Pyk2 and Fak were immunoprecipitated from Nonidet P-40 cell lysates prepared from cultures treated with fluoroaluminate as described for Fig. 1A. Equal amounts of total cellular protein (250 μg) were incubated with 3 μg of either anti-Pyk2 polyclonal antibody or anti-Fak mAb 2A7 for 2 h. Immune complexes were captured by protein A/G-Sepharose, washed extensively, and subjected to kinase autophosphorylation assays as described under “Experimental Procedures.” 

Identification of p110 as Pyk2 Tyrosine Kinase—Pyk2, a cytoplasmic tyrosine kinase of the Fak family, has recently been shown to participate in signal transduction involving G protein-coupled receptors (19, 20). Pyk2 has an apparent molecular mass of 112 kDa (19) and could therefore be a candidate for the 110-kDa tyrosine-phosphorylated protein detected in fluoroaluminate-treated osteoblasts. Pyk2 has been reported to have a restricted tissue distribution (24–26), but no data are available about the presence of the kinase in bone cells. To evaluate whether or not Pyk2 is expressed in osteoblast cells, Pyk2 levels were analyzed by Western blot analysis of extracts from different cell lines and from primary human osteoblast-like cells using an anti-Pyk2 polyclonal antibody that does not cross-react with Fak (see “Experimental Procedures”) (Fig. 2A). An immunoreactive band of the expected molecular mass was detected in MC3T3-E1 cells (Fig. 2A). The signal was stronger in rat pheochromocytoma (PC12) cells, which have been reported to abundantly express Pyk2 (19, 27). Consistent with earlier observations (25), Pyk2 was not detected in NIH3T3 fibroblasts. Low amounts of Pyk2 were present in primary human osteoblasts (Fig. 2A) and primary mouse osteoblasts (data not shown), suggesting that Pyk2 is expressed in osteoblasts in situ.

The protein kinase activity of Pyk2 was demonstrated by immune complex kinase assay (Fig. 2B). Pyk2 and Fak were immunoprecipitated from fluoroaluminate-treated MC3T3-E1 cells. Tyrosine-phosphorylated proteins were detected by Western blotting as described for A.

![Image](https://example.com/image.png)

**Fig. 1.** Fluoroaluminate induces tyrosine phosphorylation of a 110-kDa protein in MC3T3-E1 osteoblasts. A, detection of tyrosine-phosphorylated proteins in fluoroaluminate-treated MC3T3-E1 cells. Preconfluent cultures were serum-starved for 24 h and stimulated by either fluoroaluminate (10 mM NaF and 10 μM AlCl₃) or 10 μM AlCl₃ alone for 20 min or left untreated. Whole cell lysates were prepared as described under “Experimental Procedures.” Equal amounts of protein (250 μg) were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Tyrosine-phosphorylated proteins were detected by anti-phosphotyrosine (upper panel) and bradykinin (lower panel). Serum-starved MC3T3-E1 cells were treated with fluoroaluminate (as described for A) or bradykinin (0.1 μM) for the indicated times. Tyrosine-phosphorylated proteins were detected by Western blotting as described for A.

**Fig. 2.** Identification of p110 as Pyk2 tyrosine kinase. A, expression of Pyk2 in cell lines and primary human osteoblasts (HOB). Cell extracts were prepared from the indicated cell lines and primary cultures, and equal amounts of protein were separated by 10% SDS-PAGE. Pyk2 protein was detected using a goat polyclonal antibody specifically recognizing Pyk2. B, catalytic activity of Pyk2 and Fak in MC3T3-E1 cells. Pyk2 and Fak were immunoprecipitated from Nonidet P-40 cell lysates prepared from cultures treated with fluoroaluminate as described for Fig. 1A. Equal amounts of total cellular protein (250 μg) were incubated with 3 μg of either anti-Pyk2 polyclonal antibody or anti-Fak mAb 2A7 for 2 h. Immune complexes were captured by protein A/G-Sepharose, washed extensively, and subjected to kinase autophosphorylation assays as described under “Experimental Procedures.” 

Supplementary Figure 1

**Supplementary Fig. 1.** Fluoroaluminate induces tyrosine phosphorylation of a 110-kDa protein in MC3T3-E1 osteoblasts. A, detection of tyrosine-phosphorylated proteins in fluoroaluminate-treated MC3T3-E1 cells. Preconfluent cultures were serum-starved for 24 h and stimulated by either fluoroaluminate (10 mM NaF and 10 μM AlCl₃) or 10 μM AlCl₃ alone for 20 min or left untreated. Whole cell lysates were prepared as described under “Experimental Procedures.” Equal amounts of protein (250 μg) were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Tyrosine-phosphorylated proteins were detected by anti-phosphotyrosine (upper panel) and bradykinin (lower panel). Serum-starved MC3T3-E1 cells were treated with fluoroaluminate (as described for A) or bradykinin (0.1 μM) for the indicated times. Tyrosine-phosphorylated proteins were detected by Western blotting as described for A.
Actuation of Pyk2 in Osteoblasts

Pyk2 was immunoprecipitated (IP) from equal amounts of cell extracts (1 mg of protein). The precipitated proteins were resolved by 8% SDS-polyacrylamide gel, suggesting that it might be identical to Pyk2.

Pyk2 Kinase Is Activated in Primary Murine Calvarial Osteoblast-like Cells—The data shown above indicated that Pyk2 is expressed in the MC3T3-E1 cell line and in primary human osteoblast-like cells and that fluoroaluminate can stimulate Pyk2 phosphorylation in MC3T3-E1 cells. To investigate whether fluoroaluminate-induced Pyk2 phosphorylation also occurs in primary cells, we measured Pyk2 phosphorylation in primary cultures of murine calvarial osteoblasts.

Figure 3. Fluoroaluminate induces Pyk2 tyrosine phosphorylation in primary osteoblasts. Primary mouse calvarial osteoblasts were isolated as described under “Experimental Procedures.” Confluent osteoblast cultures were serum-starved for 24 h and stimulated with fluoroaluminate (10 mM NaF and 10 μM AlCl3) for the times indicated. Pyk2 was immunoprecipitated (IP) from equal amounts of cell extracts (1 mg of protein). The precipitated proteins were resolved by 8% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Tyrosine-phosphorylated Pyk2 was detected by Western blotting with antiphosphotyrosine mAb 4G10 (A); total Pyk2 was detected with anti-Pyk2 antibodies (B). Pyk2 is marked by arrowheads.

Pyk2 is marked by arrowheads.

Figure 4. Dose and time dependence of Pyk2 activation and tyrosine phosphorylation by fluoroaluminate in MC3T3-E1 osteoblasts. Cell cultures were treated as described below, and Pyk2 was immunoprecipitated (IP) with Pyk2-specific antibodies. Pyk2 activity or tyrosine phosphorylation in immunoprecipitates was measured. A, dose response of Pyk2 activation. Lysates were prepared from serum-starved MC3T3-E1 cells stimulated with 2, 5, and 10 mM NaF in the presence of 10 μM AlCl3 for 30 min, and Pyk2 kinase activity in immune complexes was measured. B, time course of Pyk2 activation. Lysates were prepared from cells treated with fluoroaluminate (10 mM NaF and 10 μM AlCl3) for 1, 2, 5, 15, 40, and 90 min; Pyk2 kinase activity in immune complexes was measured and quantified using ImageQuant software. Means ± S.D. were calculated from three independent experiments and are expressed as fold induction over untreated controls. C, time course of Pyk2 tyrosine phosphorylation. Lysates were prepared from cells treated with fluoroaluminate (10 mM NaF and 10 μM AlCl3) for the indicated times. Pyk2 tyrosine phosphorylation (upper panel) and Pyk2 amounts (lower panel) in Pyk2 immune complexes were measured by Western blotting with anti-phosphotyrosine and anti-Pyk2 antibodies, respectively.

observed a maximal 2–3-fold increase in Pyk2 kinase activity upon fluoroaluminate treatment of MC3T3-E1 cells (Fig. 4). The dose dependence of this effect was studied by treating MC3T3-E1 cells for 30 min with different concentrations of fluoroaluminate, and Pyk2 activation was analyzed in Pyk2 immune complex kinase assays (Fig. 4A). Maximal autocatalytic activity of Pyk2 was detected with 10 mM NaF (Fig. 4A). The kinetics of the autophosphorylation of Pyk2 were studied by analyzing extracts of cells that had been treated for increasing periods of time with fluoroaluminate (1–90 min). Stimulation of osteoblastic MC3T3-E1 cells with fluoroaluminate induced a rapid elevation of Pyk2 kinase activity (Fig. 4B). The peak of Pyk2 activity was reached after 10-15 min of treatment and declined slowly over 1.5–2 h to basal activity. Elevated Pyk2 kinase activity was also measured with enolase as an exogenous substrate (data not shown). Thus, the data from the immune complex kinase assays suggest that activation of heterotrimeric G proteins by fluoroaluminate induces a moderate, but reproducible increase in Pyk2 kinase activity in MC3T3-E1 cells.
To detect if other protein kinases are present in Pyk2 immune complexes, the gels from Fig. 4 (A and B) were exposed longer, and one weak additional $^{32}$P-phosphorylated protein became visible, which was identical to Src tyrosine kinase (data not shown; see also Fig. 5). It is unlikely that the very small amounts of Src in Pyk2 autophosphorylation reactions account for the strong $^{32}$P incorporation into Pyk2 in the in vitro kinase assay since coprecipitated Src was not highly active (weak autophosphorylation detected only after long exposures), and its kinetics of activation are faster than those of Pyk2 (compare Figs. 4B and 5B). Nevertheless, this result indicated that Src and Pyk2 may physically associate (see below).

Tyrosine phosphorylation of Pyk2 in MC3T3-E1 cells was tested in Pyk2 immunoprecipitates (Fig. 4C, upper panel). Fluoroaluminate-induced Pyk2 phosphorylation increased with time up to 40 min and remained high up to 60 min. The amount of Pyk2 in immune complexes was similar (Fig. 4C, lower panel). This result is in agreement with data in primary osteoblast-like cells (Fig. 3) and with data on p110 phosphorylation in whole cell lysates (Fig. 1B).

Src Kinase Mediates the Tyrosine Phosphorylation of Pyk2 in MC3T3-E1 Cells—The observation that the increase in Pyk2 tyrosine phosphorylation is much more pronounced than the increase in autophosphorylation activity (compare Figs. 1 and 4) suggested that an associated kinase contributed to Pyk2 tyrosine phosphorylation. Src kinase has been demonstrated to associate with Pyk2 (20, 28) and is required for high activation of Erk by Pyk2 in PC12 cells (20). Thus, we studied the association of Pyk2 and Src in MC3T3-E1 cells by Src immune complex kinase assays in fluoroaluminate-treated cells. To avoid possible interference of antibody with Src-Pyk2 complex formation, we used a mAb recognizing N-terminal amino acids of Src (LA-074), a region unlikely to be important in Src-Pyk2 interactions. The immune complexes were washed under non-stringent conditions, subjected to kinase assay, and analyzed by SDS-PAGE and autoradiography.

Autophosphorylation of Src was induced within 1 min, showed a peak of activity 5 min after addition of fluoroaluminate and slowly decreased with time of treatment (band at $\sim$60 kDa) (Fig. 5, A and B). The maximal activation of Src was $\sim$3-fold after 5 min. This increase in Src activity is in agreement with other studies (20, 31). A control blot of the Src immunoprecipitates with anti-Src antibodies showed that a similar amount of enzyme was present in all samples (Fig. 5C). Several other proteins became phosphorylated in Src immune complex kinase assays. These Src-associated proteins are likely to represent either Src substrates or kinases capable of auto-phosphorylation. The strongest incorporation of $^{32}$P into specifically bound proteins was observed in a unknown protein of $\sim$140 kDa (Fig. 5A). Weaker bands were detected at $\sim$70 and 50 kDa; the latter one most likely represents nonspecific phosphorylation of the the antibody heavy chain. Interestingly, a protein of $\sim$110 kDa (p110) was detected in Src immune complex assays in fluoroaluminate-treated MC3T3-E1 cell lysates, but not in untreated controls (Fig. 5A). The intensity of radioactivity incorporated into this protein increased with time (Fig. 5, A and B).

To check whether the p110 protein may represent Pyk2, a portion of the immune complex assays was used to re-immunoprecipitate Pyk2 using specific antibodies. The reprecipitation of Pyk2 from a typical Src immune complex kinase assay showed that Pyk2 was increasingly associated with and/or phosphorylated by Src (Fig. 5D). Quantification of the radioactivity incorporated into Src-associated Pyk2 revealed a $>20$-fold increase after 40 min of fluoroaluminate treatment. This result strongly suggested that the p110 protein in Fig. 5A is identical to Pyk2. Coprecipitation of Pyk2 with Src was also detected with a different anti-Src antibody (mAb 327) (data not shown). Similar experiments using anti-Yes antibodies did not show any association of Pyk2 with Yes, although the Src family member Yes is expressed in MC3T3-E1 cells (data not shown). The presence of Fak (at 125 kDa) in Src immune complexes was not obvious, but could be detected upon reprecipitation with anti-Fak polyclonal antibody (data not shown). Since we also observed increased tyrosine phosphorylation of Fak in response to fluoroaluminate and since Fak and Pyk2 should bind to the SH2 domain in Src (28, 32), it is interesting that the presence of Pyk2 in Src immune complexes is more pronounced. Together, these data show the time-dependent activation of Src kinase and its increased association with Pyk2 and suggest that Pyk2 tyrosine phosphorylation in response to fluoroaluminate is, at least in part, mediated by Src.

Association of in Vitro Phosphorylated as Well as Native Pyk2 with the Src SH2 Domain—Recruitment of Src to Fak and Pyk2 was reported to be mediated by the Src SH2 domain (20, 28, 32). To test whether phosphorylated Pyk2 associated with the SH2 domain of Src, in vitro phosphorylated, immunopurified, denatured, and renatured Pyk2 was incubated with immobilized SH2 domains of Src, phospholipase Cγ, and the p85 subunit of phosphatidylinositol 3-kinase (the p85 PI 3-kinase C-terminal SH2 domain). The binding of $^{32}$P-labeled Pyk2 to individual SH2 domains was analyzed by SDS-PAGE and quantified with a PhosphorImager (Fig. 6A). As a control, Pyk2 was immunoprecipitated under the same conditions. Of three SH2 domains tested, only the Src SH2 domain efficiently bound Pyk2 (Fig. 6A). Thus, purified renatured Pyk2 directly associates with the Src SH2 domain.

To test whether also native Pyk2 can associate with the Src SH2 domain, we performed binding experiments with Pyk2 from cell lysates (Fig. 6B). Here, cell lysates were incubated with the immobilized SH2 domains; in vitro kinase reactions were performed; and associated $^{32}$P-labeled Pyk2 was analyzed by SDS-PAGE after re-immunoprecipitation with specific antibodies. This detection method was necessary because of the low levels of endogenous Pyk2 in MC3T3-E1 cells and the limited sensitivity of the Pyk2-specific antibody, which did not allow direct detection of SH2-associated Pyk2 by Western blotting. $^{32}$P-Labeled Pyk2 was associated with the Src SH2 domain, but not with the phospholipase Cγ and p85 PI 3-kinase SH2 domains (Fig. 6B). This result is in agreement with the selective association of purified renatured Pyk2 with the Src SH2 domain (Fig. 6A). Furthermore, we compared the association of the Src SH2 domain with Pyk2 from control and fluoroaluminate-stimulated lysates. About three times more $^{32}$P-labeled Pyk2 was detected when stimulated lysates were used (Fig. 6B). This result strengthens the notion that in osteoblastic cells, fluoroaluminate induces activation of Pyk2 tyrosine kinase, its association with Src via the Src SH2 domain, and its phosphorylation by Src.

DISCUSSION

Tyrosine phosphorylation is important for G protein-coupled receptor signaling (for a recent review, see Ref. 33). Tyrosine kinases activated by G protein-coupled receptors include the cytoplasmic enzymes Fak and Src family kinases (31, 34, 35), whose substrates are likely to participate in cytokkeletal re-arrangement (e.g. paxillin, p130Cas, and vinculin). The importance of tyrosine phosphorylation for the mitogenic effect of fluoride on osteoblasts was suggested based on the observation that cell proliferation was markedly reduced by tyrosine kinase inhibitors (2, 16). Very limited information is available about the tyrosine kinases participating in fluoride-induced osteoblast signal transduction. Previously, we reported that tyrosine
phosphorylation of several intracellular proteins increases when osteoblastic MC3T3-E1 cells are treated with the G protein activator fluoroaluminate; major bands were observed at 70, 120, and 130 kDa (17). In this study, we described a 110-kDa protein whose tyrosine phosphorylation increased in response to fluoroaluminate. Subsequently, we identified this protein as the tyrosine kinase Pyk2. In addition to fluoroaluminate, the phosphorylation of Pyk2 in MC3T3-E1 cells was also increased upon stimulation of cells with ligands of G protein-coupled receptors, such as bradykinin, thrombin, and endothelin-1, indicating that Pyk2 phosphorylation occurs via a G protein activation mechanism. In agreement with our findings, Pyk2 kinase was originally reported to be activated by intracellular Ca\(^{2+}\) generated by G protein-coupled receptor signaling in neuronal cells (19). This is the first report of Pyk2 expression in osteoblast-like cells and of its regulation by G protein signaling in these cells. Presently, the identity of the G protein regulating Pyk2 activity remains elusive. Taking into account that a common G protein that is activated by bradykinin, thrombin, and endothelin-1 is G\(_{\alpha_q}\), it is possible that G\(_{\alpha_q}\) mediates Pyk2 activation. However, the involvement of G\(_{\alpha_i}\) cannot be excluded since Pyk2 activation is partially sensitive to pertussis toxin (data not shown).

Pyk2 is also known as Cak\(\beta\) (cell adhesion kinase-\(\beta\)) (24) and RAFTK (related adhesion focal tyrosine kinase) (36), and it belongs to the Fak kinase family. Similar to Fak, Pyk2 consists of a kinase domain and large amino- and carboxyl-terminal sequences that contain neither SH2 nor SH3 domains. Both

**Fig. 5.** Time course of fluoroaluminate-induced Src kinase activation and its association with Pyk2 kinase. MC3T3-E1 cells were treated with fluoroaluminate (10 mM NaF and 10 μM AlCl\(_3\)) for the indicated times and lysed, and Src was immunoprecipitated (IP) with monoclonal antibody LA-074. **A**, \(^{32}\)P-containing proteins in Src immune complexes after in vitro kinase reactions. The bands were visualized using a PhosphorImager. The positions of the Src band and of an associated protein (p110) are indicated. B, quantification of results in A with ImageQuant software shown as -fold induction over untreated controls. C, amount of Src in Src immunoprecipitates. Proteins in Src immunoprecipitates were resolved by SDS-PAGE, and Src was visualized by Western blotting with an anti-Src polyclonal antibody. D, Src-associated Pyk2. Proteins from Src immune complexes were released in SDS loading buffer and renatured in lysis buffer, and Pyk2 was reprecipitated with a specific antibody. The amounts of \(^{32}\)P-labeled Pyk2 were quantified with a PhosphorImager, and the results are expressed as -fold increase above untreated control cells. A control for nonspecific binding to protein A-Sepharose was performed without Pyk2 antibodies (last lane).
The binding of Pyk2 to the Src SH2 domain. A, binding of in vitro phosphorylated Pyk2 to the Src SH2 domain. 32P-labeled Pyk2 was generated by autophosphorylation in Pyk2 immune complexes, released from immune complexes by boiling in SDS loading buffer, renatured in SH2 binding buffer, and incubated with agarose-immobilized SH2 domains from Src, phospholipase Cγ (PLCγ), and the p85 subunit of PI 3-kinase (PI3K). SH2-associated Pyk2 was detected after SDS-PAGE and exposure to a PhosphorImager screen (upper panel). As a control, 32P-labeled Pyk2 was re-immunoprecipitated with anti-Pyk2 antibodies (a-Pyk2 Ab). Nonspecific binding of 32P-labeled Pyk2 to protein A-Sepharose was not detected (control). The results were quantified with the ImageQuant program (lower panel). B, binding of native Pyk2 to the Src SH2 domain. Equal amounts of cell lysates (1.5 mg of protein) from fluoroaluminate-treated (+) or unstimulated (−) MC3T3-E1 cells were incubated with agarose-immobilized SH2 domains from Src, phospholipase Cγ, and the p85 subunit of PI 3-kinase. The associated kinases were allowed to autophosphorylate in the presence of [32P]ATP and were dissociated from the SH2-agarose by boiling in SDS loading buffer. The samples were diluted in lysis buffer, and Pyk2 was re-immunoprecipitated with specific antibodies, subjected to SDS-PAGE, and quantified with a PhosphorImager.

proteins also seem to share some functional characteristics, such as the downstream targets paxillin and p130Cas (26, 30, 37, 38) and the ability to associate with Src family kinases (20, 32, 39). On the other hand, Pyk2 and Fak clearly show different roles of Pyk2 and Fak, it is interesting that preferential association of Src with Pyk2, but not with Fak, was observed in fluoroaluminate-stimulated cells. Although Fak also becomes tyrosine-phosphorylated upon fluoroaluminate treatment of MC3T3-E1 cells, we failed to detect a significant increase in Fak autophosphorylation activity under the same conditions (data not shown). In addition, Fak was not increased in Src immune complexes of fluoroaluminate-treated cells. This observation is in agreement with the unaffected Fac kinase activity since autophosphorylation of Fak at tyrosine 397 is required for Src binding (32). This interesting differential behavior of Fak and Pyk2 could be due to different modes of activation of the two related kinases.

Both Fak and Pyk2 are potently activated by integrins, receptors that mediate cell adhesion to extracellular matrix proteins (26, 30, 38, 41, 45). In addition, recent reports indicate that stimulation of the platelet-derived growth factor receptor can lead to Fak activation (42, 43). However, there are no reports of Pyk2 phosphorylation mediated by receptor tyrosine kinases, such as the receptors for platelet-derived growth factor or epidermal growth factor. Moreover, we did not observe a significant increase in Pyk2 tyrosine phosphorylation upon treatment of MC3T3-E1 cells with platelet-derived growth factor, epidermal growth factor, or insulin (data not shown). One possible difference between Pyk2 and Fak could be that Pyk2 is more responsive to activation by G protein-coupled receptors, whereas Fak might be more responsive to activation by receptor tyrosine kinases. Consistent with this proposal is the observation that Fak phosphorylation and activity were not changed with increasing intracellular Ca2+ levels, which strongly activated Pyk2 (19), and that Pyk2, but not Fak, associated with paxillin upon stimulation of epithelial cells with angiotensin II (44). Future studies will determine which conditions account for the selective activation of one or the other of the two related kinases.

Our data indicate that fluoroaluminate transduces a signal to a G protein, which, in turn, activates (directly or indirectly) Src and Pyk2 kinases and causes their association. The kinetics of Src activation are slightly faster than those of Pyk2 activation, phosphorylation, and association with Src, suggesting that Src activation occurs first and therefore may be a trigger for Pyk2 activation. Autophosphorylation of tyrosine 402 in Pyk2 has been shown to be a prerequisite for association with Src (20). This is consistent with our data showing that there is a substantial Pyk2 basal activity in unstimulated cells. This basal Pyk2 activity may come from continuous integrin stimulation via adherence of cells in culture to plastic or in vivo to the extracellular matrix. Thus, Pyk2 activation may result from a combination of basal integrin signaling and activated G protein/Src-mediated signaling.

What is the role of Pyk2 in osteoblast-like cells? Tyrosine phosphorylation has been proposed to be required for osteoblast proliferation in response to fluoroaluminate (2). Among the tyrosine kinases activated in fluoroaluminate-treated MC3T3-E1 osteoblasts, Pyk2 is the first enzyme that shows basal activity in unstimulated cells. This basal activity may come from continuous integrin stimulation, as has been suggested for Pyk2 kinases in other cell types. Pyk2 has been suggested to mediate Erk (19, 20) and c-Jun N-terminal protein kinase activation (27, 29). Erk activation is generally recognized as a signal in mitogenesis and, in rare cases, in differentiation (47). Presently, there is not much information on the role of c-Jun N-terminal protein kinase in osteoblasts. Generation of Pyk2-deficient mice and analysis of their bones and bone cells in vitro should be a useful approach to address some of these questions.
Activation of Pyk2 in Osteoblasts

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