Cbl-mediated Degradation of Lyn and Fyn Induced by Constitutive Fibroblast Growth Factor Receptor-2 Activation Supports Osteoblast Differentiation

Received for publication, March 4, 2004, and in revised form, June 2, 2004
Published, JBC Papers in Press, June 9, 2004, DOI 10.1074/jbc.M402469200

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Fibroblast growth factors (FGFs) play an important regulatory role in skeletal development and bone formation. However, the FGF signaling mechanisms controlling osteoblast function are poorly understood. Here, we identified a role for the Src family members Lyn and Fyn in osteoblast differentiation promoted by constitutive activation of FGF receptor-2 (FGFR2). We show that the overactive FGFR2 S252W mutation induced decreased Src family kinase tyrosine phosphorylation and activity associated with decreased Lyn and Fyn protein expression in human osteoblasts. Pharmacological stimulation of Src family kinases or transfection with Lyn or Fyn vectors repressed alkaline phosphatase (ALP) up-regulation induced by overactive FGFR2. Inhibition of proteasome activity restored normal Lyn and Fyn expression and ALP activity in FGFR2 mutant osteoblasts. Immunoprecipitation studies showed that Lyn, Fyn, and FGFR2 interacted with the ubiquitin ligase c-Cbl and ubiquitin. Transfection with c-Cbl in which the RING finger was disrupted or with c-Cbl with a point mutation that abolishes the binding ability of the Cbl phosphotyrosine-binding domain restored Src kinase activity and Lyn, Fyn, and FGFR2 levels and reduced ALP expression in mutant osteoblasts. Thus, constitutive FGFR2 activation induces c-Cbl-dependent Lyn and Fyn proteasome degradation, resulting in reduced Lyn and Fyn kinase activity, increased ALP expression, and FGFR2 down-regulation. This reveals a constitutive Cbl-mediated negative feedback mechanism controlling Lyn, Fyn, and FGFR2 degradation in response to overactive FGFR2 and indicates a role for Cbl-dependent down-regulation of Lyn and Fyn in osteoblast differentiation induced by constitutive FGFR2 activation.

Fibroblast growth factors (FGFs)§ belong to a family of polypeptides that regulate cell proliferation, differentiation, and survival in many cell types (1). These biological effects are mediated by ligand interaction with high affinity cell-surface receptors (FGFRs). Ligand binding induces dimerization of the receptor, resulting in activation of intrinsic tyrosine kinase and of multiple downstream signaling processes that contribute to the regulation of cell growth and differentiation in several tissues (2). In bone, FGFs play essential roles in skeletal development and postnatal osteogenesis (3, 4) through interactions with FGFR1–3, which are differentially expressed during development (5). The importance of FGF signaling in bone formation is exemplified by the finding that several mutations in FGFRs induce premature ossification of cranial sutures (craniosynostosis) in humans (6, 7). Most FGFR mutations result in ligand-independent dimerization, phosphorylation, and constitutive receptor activation (5–7). Gain-of-function mutations in FGFR2 were found to induce changes in osteoblast proliferation, differentiation, and survival in mice and humans (8–11). In human osteoblasts, we have shown that single missense point mutations (S252W and P253R) located in the linker region between the second and third extracellular immunoglobulin domains of FGFR2 activate the expression of early and late osteoblast differentiation genes, including alkaline phosphatase (ALP), type I collagen (COL1A1), and osteocalcin in vitro and in vivo (11, 12), a phenotype that is mediated in part through protein kinase C activation (13, 14). The role of other FGF signaling pathways in the osteoblast phenotype induced by overactive FGFR2 mutations remains unknown.

The Src family of non-receptor tyrosine kinases mediates a variety of signaling pathways (15). Src family kinases have unique N-terminal and SH2 and SH3 domains that mediate protein/protein interactions and a C-terminal SH1 tyrosine kinase domain (15, 16). Protein-tyrosine kinases of the Src family are involved in the transduction of signals from multiple transmembrane tyrosine kinase receptors, including epidermal growth factor (EGF) (16), platelet-derived growth factor (PDGF) (17), and FGF (18–21). Activation of these receptors causes phosphorylation of Src family kinases and increases their intrinsic activity. Src family kinase activity is also controlled by binding of multiple proteins to SH2 or SH3 domains. The proto-oncogene c-cbl has been shown to associate with the SH3 domain in some Src family kinases and thereby may control their kinase activity (22). C-Cbl can interact with several members of the Src kinase family, including c-Src, Lyn, Yes, and Syk (23–31). This interaction leads to ubiquitination and degradation of Src proteins by the proteasome, showing that c-Cbl is a negative regulator of the Src family of non-receptor tyrosine kinases (31–33). c-Cbl also acts as a ubiquitin-protein isopeptide ligase for receptor tyrosine kinases such as EGF, PDGF, and FGF receptors, resulting in their down-regulation after ligand binding (34–37). The molec-
ular mechanisms by which c-Cbl exerts this negative regulation involve c-Cbl recruitment and polyubiquitination of activated receptors (38, 39). The important domains of c-Cbl involved in the negative regulation of EGF and PDGF receptors were found to be the phosphotyrosine-binding (PTB) domain, which is involved in c-Cbl binding to the activated receptor, and the RING finger, which directs recruitment of the ubiquitin system (40–42). FGFR can recruit Cbl by another indirect mechanism involving the docking protein FRS2α (37). Although intrinsic tyrosine kinase activity is inhibited by poptargeted FGFR mutations is associated with increased FGFR2 down-regulation (43), it is unknown if c-Cbl plays a role in FGFR2 degradation induced by overactive FGFR2 mutations.

Given that c-Src was found to be a negative regulator of osteoblast differentiation in mice (44), we hypothesized that constitutive activation of FGFR2 induced by FGFR2 mutations in human osteoblasts may result in alterations in recruitment and degradation of Src family kinases, which may contribute to premature osteoblast differentiation. In this work, we show that the activating FGFR2 S252W mutation enhances c-Cbl-dependent down-regulation of Lyn and Fyn and subsequent inhibition of their kinase activity. This effect is biologically relevant in that it reduces the alkaline phosphatase up-regulation induced by the activating FGFR2 mutation in human osteoblasts. Moreover, we show that, in addition to Lyn and Fyn down-regulation, the activating FGFR2 mutation induces c-Cbl-dependent ubiquitination of FGFR2. This study reveals a common Cbl-dependent mechanism controlling Lyn, Fyn, and FGFR2 down-regulation in response to overactive FGFR2 and indicates a role for Cbl-dependent down-regulation of Lyn and Fyn in the increased osteoblast differentiation induced by constitutive FGFR2 activation.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—Normal and FGFR2 mutant human calvaria cells obtained from Apert and control fetuses were immortalized by transfection with the origin-defective large T antigen of the SV40 oncogene as described previously and called Apert and control cells (11). Apert mutant cells display increased expression of osteoblast marker genes such as ALP, COL1A1, and osteocalcin and increased in vitro osteogenic capacity compared with normal control cells, a phenotype that is similar to the pathological features observed in fetal Apert calvaria (12). The control cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 292 mg/liter glutamine, 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Stimulation of Src Kinase Activity—Src kinase activation is an early event shown to be involved in activation of signaling pathways induced by the G protein-coupled lysophosphatidic acid (LPA) receptor (47). LPA receptor activation is coupled to several heterotrimeric G proteins such as G12, which activate Src tyrosine kinases (48). One mechanism involves LPA-induced tyrosine phosphorylation of Pyk2 and c-Src activation (49). To determine the role of the decreased Src family kinase activity in the osteoblast phenotype induced by FGFR2 activation, confluent control and Apert osteoblasts were treated with LPA (10 μM; Sigma) or the selective α1-adrenergic agonist UK 14,304 (10 μM; Sigma), which can activate Src family kinases (49, 50). Apert and control cells were treated for 24 h, and then total Src kinase activity was determined as described below. After 24 h of treatment, ALP activity, an early marker of osteoblast differentiation that is constitutively increased in FGFR2 human mutant osteoblasts compared with control cells (11–14), was determined as described (11).

Inhibition of Proteasome Activity—Lactacystin is a specific proteasome inhibitor that acts by binding covalently to the active-site N-terminal threonine residue in certain proteasome β-subunits (51). To determine whether Lyn, Fyn, and FGFR2 are degraded by the proteasome in osteoblasts, FGFR2 mutant cells were treated with lactacystin (10 μM; Calbiochem) for 24 h, and Lyn, Fyn, and FGFR2 protein levels were determined by Western blot analysis as described below.

In Vitro Kinase Assay—Total Src activity was determined using an assay kit (Upstate Biotechnology, Inc. and Euromedex, Mundolsheim, France) designed to measure the phosphotransferase activity of all Src kinases. The assay kit is based on measurement of phosphotransferase by Src family kinases of a specific substrate peptide (KVEKIGETGYVYK) using the transfer of the γ-phosphate of [5-32P]ATP. Proteins (10 μg) were diluted in reaction buffer (100 mM Tris-HCl (pH 7.2), 125 mM MgCl2, 25 mM MnCl2, 2 mM EGTA, 250 μM sodium orthovanadate, and 2 mM dithiothreitol) with 10 μM of manganese/ATP mixture (1 mM/100 μl) and incubated for 10 min at 30 °C with agitation. Proteins were precipitated with 20 μl of 40% trichloroacetic acid at room temperature for 5 min, and 25 μl of the final solution were spotted onto phosphocellulose P-81 paper squares, which were washed five times with 0.75% phosphoric acid. After 3 min in aceton, the assay squares were transferred to a scintillation vial containing 5 ml of scintillation mixture and measured in a scintillation counter.

Transient Transfection—All cells were plated at 2500 cells/cm² the day before transfection. The cells were cotransfected with the plasmid (2.5 μg/3-cm dish) and pSV-β-galactosidase control vector (50 ng of β-galactosidase; Promega) in Dulbecco’s modified Eagle’s medium with 1% fetal calf serum. Cells were incubated with empty vector (pcDNA3) or Lyn, Fyn, 70Z-Cbl, or Cbl G50E vector and Exgen (Euromedex) according to the manufacturer’s instructions. Efficiency of transfection was controlled by determination of β-galactosidase activity (β-galactosidase reporter gene assay; Roche Applied Science). The number of β-galactosidase-positive cells was counted 24 h post-transfection.

Reverse Transcription (RT)-PCR Analysis—The levels of lyn, fry, COL1A1, and COL5A1 mRNAs were analyzed by reverse transcription using random primers. Total cellular RNA from each sample were reverse-transcribed, and the cDNA samples were then divided and amplified by PCR using specific primers for ALP, COL1A1, lyn, lyn, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences for lyn were 5′-TTGGAAGTGTAACCTCTCCG-3′ (sense) and 5′-TGCCTGGTATAGAAGTCAACTCG-3′ (antisense), and those for lyn were 5′-CATCACCAGGATACGGC-3′ (sense) and 5′-TCTCCCGCTGTATGC-3′ (antisense). The primers for ALP, COL1A1, and GAPDH were as described (14). Southern blot analysis was performed by running aliquots of amplified cDNA blots on 6–15% SDS-polyacrylamide gradient gels. Electrophoresed proteins were transferred onto polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences). Autoradiographic signals were quantified using a scanner densitometer (Transydine General Corp., Ann Arbor, MI), and the signal for each gene was related to that of GAPDH.

Immunoprecipitation and Western Blot Analysis—Cell proteins were extracted in lysis buffer consisting of 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mM calyculin A, 50 mM microcystin LR, and 2 mM NaVO3 (45, 46). Lysates were clarified by centrifugation at 12,000 × g for 30 min at 4 °C, and protein content of the supernatants was determined using the DC protein assay (Bio-Rad). For Western blot analysis, equal aliquots (80 μg) of protein lysates were resolved on 6–15% SDS-polyacrylamide gradient gels. Electrophoresed proteins were transferred onto polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences). The membranes were then reacted with either anti-phospho-Src antibody (Upstate Biotechnology, Inc.), which recognizes all Src family members phosphorylated at Tyr416, or with anti-Lyn or anti-Fyn antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); or with anti-actin antibody (1:200; Sigma) and then incubated for 1 h with the appropriate affinity-purified anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.), probed with peroxidase-coupled specific secondary antibodies, and visualized using enhanced chemiluminescence detection kits (Amersham Biosciences). The signals for each band were measured by scanning densitometry and corrected for actin.

For immunoprecipitation analysis, equal aliquots (300 μg) of protein lysates were immunoprecipitated using 2.5 μg of specific antibody (anti-FGFR2, anti-Cbl, anti-Lyn, anti-Fyn, or anti-ubiquitin; Santa Cruz Biotechnology) and incubated overnight at 4 °C in a rotating device. Aliquots of 20 μl of protein lysates (80 μg) were added and incubated for 1 h at 4 °C. Immunoprecipitates were then collected by centrifugation at 1200 × g for 3 min, and the pellets were washed four times with lysis buffer and resuspended in 50 μl of running buffer. Aliquots were then subjected to electrophoresis as described above, and membranes were reacted with anti-Lyn, anti-Fyn, anti-Cbl, or anti-ubiquitin, or with anti-FGFR2 antibodies. Membranes were probed with peroxidase-coupled specific secondary antibodies and visualized by enhanced chemiluminescence.

For analysis of the kinase activity of specific Src family members, we used the Sam68 protein, which functions as a substrate for Src tyrosine
kinases. Sam68 phosphorylation by Src family members reflects tyrosine kinase activity. Equal amounts of lysate proteins were immunoprecipitated for 18 h at 4 °C with the following antibodies (2 μg/ml): monoclonal anti-Src60 (Upstate Biotechnology, Inc.), monoclonal anti-Fyn, polyclonal anti-Yes (Upstate Biotechnology, Inc.), polyclonal anti-Fgr (Upstate Biotechnology, Inc.), or polyclonal anti-Lyn (BD Biosciences). Sepharose beads (20–30 μl) were added during the last hour of incubation. Immunoprecipitates were washed three times with kinase reaction buffer containing 50 mM Tris (pH 7.4), 25 mM β-glycerophosphate, 20 mM MgCl2, and 1.0 mM dithiothreitol and incubated with 10 μM [32P]ATP (50 μCi/ml) and 2.5 μg of Sam68 (p68) tyrosine kinase substrate (Santa Cruz Biotechnology) for 30 min at 30 °C. The reaction was stopped by addition of 2× buffer, and the products were resolved by SDS-PAGE. The incorporation of [32P]phosphate was visualized by autoradiography.

Data Analysis—Experiments were repeated two to four times, and the results presented are representative of all experiments. The results are expressed as the mean ± S.E. and were analyzed using the statistical package super-ANOVA (Macintosh, Abacus Concepts, Inc., Berkeley, CA). Differences between mean values were evaluated with a minimal significance of p < 0.05.

RESULTS

The Overactive FGFR2 S252W Mutation Reduces Src Kinase Activity in Osteoblasts—To assess whether activation of FGFR2 induced by the gain-of-function S252W mutation alters total Src activity in mutant osteoblasts, we assessed the phosphorylation state of Src family kinases by Western blot analysis using a substrate that is phosphorylated by all Src family members. As shown in Fig. 1A, we observed a significant decrease in phosphorylation of Src family kinases at the tyrosine corresponding to Try416 in cells bearing the activating S252W mutation compared with control cells (Fig. 1A). The kinase assay confirmed that total Src kinase activity was reduced by ~2-fold in mutant osteoblasts compared with control cells (Fig. 1B). Consistently, the in vitro kinase assay performed in FGFR2 mutant osteoblasts revealed decreased kinase activity of several Src kinase family members. Notably, the kinase activity of Lyn and Fyn was markedly decreased in mutant cells compared with control cells (Fig. 1C). Western blot analysis was then performed to investigate whether the observed decreased Lyn and Fyn kinase activity resulted from a decreased amount of Lyn and Fyn proteins. As shown in Fig. 1D, the amount of Lyn and Fyn proteins was markedly reduced in FGFR2 mutant osteoblasts compared with control cells. In contrast, RT-PCR analysis showed a similar amount of lyn or fyn transcripts in FGFR2 mutant cells compared with control cells (Fig. 1E). These results show that the overactive FGFR2 S252W mutation reduces the amount of Lyn and Fyn, resulting in decreased Lyn and Fyn kinase activity, which contributes to the decreased total Src kinase activity in mutant osteoblasts.

Both Pharmacological Activation of Src Kinases and Overexpression of Lyn and Fyn Reduce ALP Up-regulation Induced by Overactive FGFR2—To determine whether the reduced Src family kinase activity may contribute to the premature osteoblast differentiation induced by the overactive FGFR2 mutation, we examined the effect of LPA, a known activator of Src kinase family members (48, 49), on Src kinase activity and expression of ALP, which is predictive of osteoblast differentiation. As shown in Fig. 2A, total Src kinase activity was de-
increased in FGFR2 mutant cells, and treatment with 10 μM LPA for 24 h restored this activity measured in a kinase assay. Similarly, treatment of FGFR2 mutant cells with UK 14,304 significantly increased total Src kinase activity, although to a lesser degree compared with LPA at the same concentration (Fig. 2A). Similar effects were found after 48 h of treatment (data not shown). As expected, ALP activity was increased in FGFR2 mutant cells compared with control cells, reflecting the effect of the overactive FGFR2 mutation (11–14). Treatment with LPA or UK 14,304 at concentrations that increased total Src kinase activity (Fig. 2A) led to reduced ALP activity in mutant cells (Fig. 2B). To confirm this finding, we determined the effects of LPA on ALP mRNA expression. ALP mRNA levels were increased in FGFR2 mutant cells compared with control cells, confirming our previous results (11–14). Treatment with LPA reduced ALP mRNA up-regulation in mutant cells (Fig. 2, C and D). Similar results were found after stimulation with bryostatin (data not shown), which has been shown to activate Fyn kinase (52). These results indicate that pharmacological activation of Src family kinases abolishes the ALP up-regulation induced by FGFR2 activation.

Because LPA may stimulate signaling pathways other than Src family kinases, including mitogen-activated protein kinase (53), we wished to determine more specifically the role of Lyn and Fyn by overexpressing these kinases in mutant cells. Transient transfection of mutant osteoblasts with Lyn or Fyn vectors resulted in increased Lyn and Fyn protein levels as determined by Western blot analysis (Fig. 3, A and B). Transfection with Lyn or Fyn markedly reduced ALP mRNA in mutant osteoblasts (Fig. 3, A and B). Consistently, transfection with Lyn or Fyn reduced ALP activity induced by the overactive FGFR2 mutation (Fig. 3C). Transfection with Lyn also reduced COLIA1 mRNA levels in mutant cells (Fig. 3A). In contrast, transfection with Fyn did not correct the up-regulation of COLIA1 transcripts induced by the overactive FGFR2 mutation (Fig. 3B). These results indicate that the reduced amount of Lyn and Fyn proteins in mutant cells contributes to ALP up-regulation induced by the FGFR2 mutation and that the reduction of Lyn (but not Fyn) is involved in COLIA1 up-regulation induced by the overactive FGFR2 mutation.

Proteasome Inhibition Reduces Lyn and Fyn Degradation and ALP Up-regulation Induced by Overactive FGFR2—Because Src family kinases can be controlled by proteasome degradation, we considered the possibility that the decreased amount of Lyn and Fyn induced by the overactive FGFR2 mutation may result from increased proteasome degradation. To test this hypothesis, FGFR2 mutant cells were treated with lactacystin, a specific proteasome inhibitor, and Lyn and Fyn levels were determined by Western blot analysis. Inhibition of proteasome activity with lactacystin (10 μM) resulted in increased Lyn and Fyn protein levels in mutant osteoblasts (Fig. 4A). Consistent with this finding, treatment with lactacystin greatly increased total Src kinase activity in FGFR2 mutant cells (Fig. 4B). Biochemical analysis showed that inhibition of proteasome activity with lactacystin markedly inhibited the increased ALP activity induced by the activating FGFR2 mutation (Fig. 4C). In contrast, lactacystin treatment had no effect on ALP mRNA levels in mutant cells as determined by RT-PCR analysis (data not shown). These results indicate that the reduced Lyn and Fyn protein levels induced by the overactive FGFR2 S252W mutation result in part from their increased degradation by the proteasome and that this effect contributes to the ALP up-regulation induced by the overactive FGFR2 mutation in osteoblasts.

We previously reported that the FGFR2 S252W mutation results in down-regulation of FGFR2 protein levels in mutant osteoblasts (12). Given that tyrosine kinase receptors can be degraded by ubiquitination and proteasome degradation (34–37), we examined whether the down-regulation of FGFR2 in mutant cells may be related to proteasome degradation. Consistent with our previous data, immunoprecipitation analysis showed decreased FGFR2 protein levels in mutant cells compared with control cells (Fig. 4D). Inhibition of proteasome degradation with lactacystin (10 μM) increased FGFR2 protein levels in mutant cells, although the levels remained lower than in control cells (Fig. 4D). These results indicate that down-regulation of FGFR2 in mutant osteoblasts results, at least in part, from increased degradation by the proteasome.

c-Cbl Interacts with Lyn, Fyn, and FGFR2 in Mutant Osteoblasts—c-Cbl is an adaptors protein that was found to interact with active Src family members and to mediate their ubiquitination. We therefore asked whether c-Cbl may interact with Lyn and Fyn in mutant osteoblasts to mediate ubiquitination of these proteins induced by the overactive FGFR2 mutation. As
shown in Fig. 5, c-Cbl co-immunoprecipitated with Lyn and Fyn in FGFR2 mutant osteoblasts and in control cells, indicating physical interactions between these proteins (Fig. 5, A and B). The amount of c-Cbl associated with Lyn or Fyn was lower in mutant cells compared with control cells (Fig. 5, A and B), most likely as a result of the decreased amount of Lyn and Fyn proteins in mutant osteoblasts (Fig. 1D). Furthermore, immunoprecipitation studies showed that Lyn and Fyn in control and mutant cells were associated with ubiquitin (Fig. 5, A and B), indicating that these two proteins are targeted to proteasome degradation once associated with c-Cbl. The high amount of ubiquitin associated with Lyn and Fyn in mutant cells suggests that these proteins are more degraded than in control cells. These results support the finding that the total Src kinase activity in mutant osteoblasts is decreased, at least in part, as the consequence of increased proteasome degradation of Lyn and Fyn.

**c-Cbl-mediated Lyn and Fyn Degradation in FGFR2 Mutant Osteoblasts Involves the RING Domain of c-Cbl**—The RING finger of c-Cbl is known to be important for Src protein ubiquitination. To determine whether c-Cbl-mediated Lyn and Fyn ubiquitination in FGFR2 mutant osteoblasts is dependent on the RING domain of c-Cbl, mutant cells were transfected with 70Z-Cbl, in which the RING finger is disrupted (30), and changes in total Src kinase activity and in Lyn and Fyn protein levels were determined. As shown in Fig. 6A, transfection of FGFR2 mutant osteoblasts with 70Z-Cbl increased total Src kinase activity to the level in control cells. Transfection with 70Z-Cbl resulted in increased Lyn and Fyn protein levels in mutant cells as revealed by immunoprecipitation analysis (Fig. 6B), reflecting the inhibitory effect of 70Z-Cbl on proteasome degradation of Lyn and Fyn. This indicates that the increased c-Cbl-dependent Lyn and Fyn degradation and subsequent reduction in total Src kinase activity induced by the FGFR2 mutation in osteoblasts require the presence of the RING finger domain of c-Cbl. The PTB domain in c-Cbl may also be another domain controlling ubiquitination of Src proteins induced by FGFR2 activation. We therefore examined the role of the PTB domain in c-Cbl-mediated Lyn and Fyn degradation in FGFR2 mutant osteoblasts. Transfection of FGFR2 mutant cells with the Cbl G306E mutant, in which a point mutation abolishes the ability of the Cbl PTB domain to bind Src (30), increased total Src kinase activity (Fig. 6A). Consistently, the c-Cbl G306E mutant increased Lyn protein levels in FGFR2 mutant cells as revealed by immunoprecipitation analysis (Fig. 6B). Interestingly, the c-Cbl G306E mutant had no effect on Fyn protein levels, which remained as low as in pcDNA-transfected mutant cells (Fig. 6A). This suggests that c-Cbl-dependent ubiquitination of Lyn (but not Fyn) by the activating FGFR2 S252W mutation may be dependent on interaction with the PTB domain of c-Cbl.

**Dominant-negative c-Cbl Inhibits ALP Activity Induced by FGFR2 Activation**—To assess the functional implication of c-Cbl in the up-regulation of ALP expression induced by the FGFR2 mutation, mutant cells were transfected with the 70Z-Cbl or c-Cbl G306E mutant, and ALP activity was determined. As shown in Fig. 6C, transfection with 70Z-Cbl and, to a lesser extent, the c-Cbl G306E mutant decreased ALP activity induced by the FGFR2 mutation (Fig. 6C). These results, together with our findings shown in Fig. 6 (A and B), indicate that ALP up-regulation induced by the overactive FGFR2 S252W mutation results, at least in part, from Lyn and Fyn down-regulation mediated by c-Cbl interaction via the RING domain and possibly the PTB domain.

The RING and PTB Domains of c-Cbl Are Involved in the Increased FGFR2 Degradation Induced by the FGFR2 Mutation—c-Cbl was previously found to associate with and to me-
iate ubiquitination of EGF and PDGF tyrosine kinase receptors (40–42). Here, we assessed whether c-Cbl associates with constitutively activated FGFR2 and is involved in FGFR2 degradation in mutant osteoblasts. As shown in Fig. 7A, FGFR2 co-immunoprecipitated with c-Cbl in mutant and control osteoblasts. Less FGFR2 was associated with c-Cbl in mutant cells compared with control cells because of the lower amount of FGFR2 in mutant cells (Fig. 4D). Furthermore, immunoprecipitation studies showed that Lyn and Fyn were associated with FGFR2 in mutant and control cells (Fig. 7A). These data indicate that FGFR2 interacts with Lyn and Fyn and with c-Cbl, which can then mediate proteasome degradation of these three molecules in osteoblasts. The RING finger and PTB domains of c-Cbl were previously found to be required for ubiquitination of tyrosine kinase receptors. For example, the 70Z-Cbl mutant is unable to induce the ubiquitination of the EGF receptor (30, 42) and a point mutation (G306E) that inactivates the PTB domain leads to inhibition of the ligand-induced ubiquitination of the PDGF receptor (54, 55). We used these c-Cbl mutants as dominant-negative vectors to assess the role of c-Cbl-interacting domains in FGFR2 degradation induced by the overactive FGFR2 mutation. As shown in Fig. 7B, transfection with the 70Z-Cbl mutant led to increased FGFR2 levels in mutant cells compared with the control vector as revealed by immunoprecipitation analysis. Transfection with the c-Cbl G306E mutant also led to increased FGFR2 levels, albeit to a lesser extent than with the 70Z-Cbl mutant (Fig. 7B). Thus, the RING domain and, to a lesser degree, the PTB domain of c-Cbl appear to be involved in the increased c-Cbl-dependent FGFR2 degradation induced by the overactive FGFR2 mutation. This provides a mechanism by which constitutive FGFR2 activation in osteoblasts induces c-Cbl-dependent recruitment of FGFR2, Lyn, and Fyn, leading to their proteasome degradation, decreased kinase activity, and hence increased osteoblast differentiation.

**DISCUSSION**

In this study, we examined the role of specific Src kinase family members in the premature osteoblast differentiation induced by constitutive FGFR2 activation in osteoblasts. We have shown here that FGFR2 activation induced by the overactive FGFR2 S252W mutation induced c-Cbl-mediated Lyn and Fyn down-regulation, resulting in decreased Lyn and Fyn protein levels and activity and increased expression of early markers of osteoblast differentiation. Interestingly, not all Src family kinases were down-regulated to the same extent by the FGFR2 mutation, as mostly Lyn and Fyn proteins were decreased in FGFR2 mutant cells compared with control cells. The down-regulation of these proteins was sufficient to inhibit total Src kinase activity in mutant cells. We suggest that Lyn and Fyn down-regulation induced by the overactive FGFR2 mutation results from proteasome degradation because inhibition of proteasome activity by lactacystin prevented both Lyn and Fyn degradation and increased total Src kinase activity in FGFR2 mutant osteoblasts. The mechanisms of protein degra-
Data are the mean ± S.E. of six values. A, p < 0.05 versus control cells; b, p < 0.05 versus pcDNA-transfected mutant cells.

FIG. 6. Implication of the RING and PTB domains of c-Cbl in Lyn and Fyn degradation in FGFR2 mutant osteoblasts. A, FGFR2 mutant osteoblastic cells were transfected with 70Z-Cbl, c-Cbl G306E, or the empty vector (pcDNA). After 24 h, total Src kinase activity was determined in cell lysates as described under “Experimental Procedures.” B, cell lysates were immunoprecipitated (IP) with Lyn or Fyn and blotted with anti-Lyn and anti-Fyn antibodies. C, after 24 h, ALP activity was determined biochemically. PNP, paranitrophenol. Data are the mean ± S.E. of six values. a, p < 0.05 versus control cells; b, p < 0.05 versus pcDNA-transfected mutant cells.

FIG. 7. The RING and PTB domains of c-Cbl are involved in FGFR2 degradation induced by the overactive FGFR2 S252W mutation. A, cell lysates from control and FGFR2 mutant osteoblastic cells were immunoprecipitated (IP) with FGFR2, Lyn, or Fyn; resolved by SDS-PAGE; and blotted with anti-Cbl or anti-FGFR2 antibody. B, FGFR2 mutant osteoblastic cells were transfected with 70Z-Cbl, c-Cbl G306E, or pcDNA. After 24 h, cell lysates were immunoprecipitated and blotted with anti-FGFR2 antibody.

finger domain (30). We also examined the potential role of the PTB domain in c-Cbl-dependent Lyn and Fyn degradation induced by the overactive FGFR2 mutation. We found that the c-Cbl G306E mutant, which inactivates the PTB domain of c-Cbl, increased total Src activity in FGFR2 mutant cells compared with control cells, suggesting that the PTB domain of c-Cbl is involved in ubiquitination of Src family members in our cellular system, in which constitutive FGFR2 activation positively influenced Lyn and Fyn ubiquitination. Because the c-Cbl G306E mutation corrected Lyn (but not Fyn) protein expression in FGFR2 mutant cells, the PTB domain of c-Cbl does not appear to be involved in c-Cbl-mediated down-regulation of Fyn in FGFR2 mutant cells. Src proteins are known to bind to Cbl through the SH2 or SH3 domain (59), and recent data indicate that Fyn can interact through binding of the SH3 domain to the Cbl proline-rich region (24). The proline-rich region in the C-terminal half of Cbl may thus be necessary for Cbl to interact with Fyn and to induce its degradation induced by the overactive FGFR2 mutation. Thus, multiple domains may be involved in the molecular mechanisms responsible for Lyn/c-Cbl and Fyn/c-Cbl interactions, resulting in their degradation in FGFR2 mutant osteoblasts.

We had previously shown that constitutive activation of FGFR2 by the FGFR2 S252W mutation induces FGFR downregulation in mutant osteoblasts in vitro and in vivo (12). However, the molecular mechanism of FGFR2 down-regulation induced by the activating mutation was unidentified. After ligand binding, FGFR is known to be down-regulated by internalization and degradation, which occurs in part through FGFR ubiquitination (37). Cbl was recently found to control FGFR1 degradation after ligand activation by an indirect mechanism involving Grb2, which functions as a link between the docking protein FRS2α and Cbl (37). In the present system, we found that FGFR2 interacted with both Cbl and ubiquitin in osteoblasts. Inhibiting proteasome activity resulted in increased FGFR2 levels in mutant cells, indicating that the overactive FGFR2 mutation induces Cbl binding to the receptor, resulting in FGFR2 ubiquitination and proteasome degradation. This provides a Cbl-dependent mechanism by which
FGFR2 is down-regulated in response to constitutive activation of FGFR2. Following ligand binding, activated EGF and PDGF receptors have been shown to be down-regulated through Cbl recruitment and binding to its SH2-like domain (38, 39). Recent data also indicate that Cbl is required for ligand-induced degradation of EGF receptors (60). In contrast, no association was found between FGFR3 and Cbl during FGFR3 ubiquitination induced by overactive FGFR3 G380R and K650E mutants (61). Thus, our data provide novel evidence that FGFR2 is down-regulated by proteasome degradation via Cbl recruitment and association with FGFR2 in response to ligand-independent constitutive activation of the receptor by the FGFR2 S252W mutation. The RING finger of c-Cbl is known to play an essential role in ubiquitin ligation and mediates desensitization of tyrosine kinase receptors (38, 40, 42). Consistent with this, the expression of 70Z-Cbl was found to increase PDGF (54) and EGF (42) receptor kinase activity. In addition, the PTB domain in the N-terminal half of Cbl binds to phosphorylated EGF and PDGF receptors and acts as a negative regulator of these receptor tyrosine kinases (32, 42, 55). Our finding that FGFR2 down-regulation was abolished by both 70Z-Cbl and c-Cbl G306E mutants indicates that the Cbl-dependent FGFR2 degradation induced by the overactive FGFR2 S252W mutation involves the RING and PTB domains of Cbl. These data imply that Cbl plays a major role not only in the ubiquitination of Lyn and Fyn, but also in FGFR2 down-regulation induced by the overactive FGFR2 mutation in osteoblasts. The molecular mechanisms for this tripartite molecular interaction likely involve constitutive FGFR2-dependent phosphorylation of Src family kinases, leading to phosphorylation of c-Cbl and activation of the ubiquitin ligase activity of c-Cbl, resulting in ubiquitination and proteasome degradation of Lyn, Fyn, and FGFR2. This model is consistent with the finding that Src family kinase-dependent signaling mechanisms; it enhances Lyn and Fyn degradation via the proteasome, resulting in reduced kinase activity and increased ALP expression, and induces c-Cbl-dependent FGFR2 down-regulation in mutant osteoblasts. This reveals a common Cbl-mediated negative feedback mechanism controlling both Lyn and Fyn degradation in response to constitutive FGFR2 activation and provides additional insight into the molecular mechanisms by which constitutive FGFR2 activation promotes osteoblast differentiation in humans.

In conclusion, our data indicate that c-Cbl recruitment induced by constitutive FGFR2 activation induces two mechanisms; it enhances Lyn and Fyn degradation via the proteasome, resulting in reduced kinase activity and increased ALP expression, and induces c-Cbl-dependent FGFR2 down-regulation in mutant osteoblasts. These findings provide additional insight into the molecular mechanisms by which constitutive FGFR2 activation promotes osteoblast differentiation in humans.

Acknowledgments—We thank Drs. A. Sanjay and R. Baron (Department of Cell Biology, Yale University School of Medicine, New Haven, CT) for the gift of cbl mutant plasmids, Dr. P. Auburger (INSERM U526, Nice, France) for the Lyn and Fyn plasmids, and Drs. W. Horne (Department of Cell Biology, Yale University School of Medicine) and E. Lepelantine for critical review of the manuscript.

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Cbl-mediated Degradation of Lyn and Fyn Induced by Constitutive Fibroblast Growth Factor Receptor-2 Activation Supports Osteoblast Differentiation

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J. Biol. Chem. 2004, 279:36259-36267.
doi: 10.1074/jbc.M402469200 originally published online June 9, 2004

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