Regulated Nuclear-Cytoplasmic Localization of CCAAT/enhancer-binding Protein δ in Osteoblasts*

Insulin-like growth factor I (IGF-I), a 70-amino acid secreted protein, plays a central role in regulating growth and development in mammals and other vertebrates (1, 2). IGF-I promotes the survival, proliferation, and differentiation of many cell types and tissues, including bone, where it enhances osteoblast replication and type I collagen synthesis, among other actions (3, 4). IGF-I is produced by various cells within the skeleton, including osteoblasts (5), and its synthesis is enhanced by systemic and locally produced hormones that regulate skeletal function, such as parathyroid hormone and prostaglandin E2 (PGE2) (5, 6). The increase in IGF-I induced by these hormones may explain their anabolic actions within the skeleton, and IGF-I may serve as a coupling factor to balance the remodeling sequence of resorption and new bone formation (5, 7, 8).

In cultured bone cells, both PGE2 and parathyroid hormone stimulate IGF-I gene and protein expression by a transcriptional mechanism (9–11). These effects on IGF-I gene transcription are mediated by hormone-induced increases in cAMP and subsequent activation of cAMP-dependent protein kinase (PKA) (5, 6, 12). As evidence for this pathway, the major IGF-I promoter can be induced in transient transfection experiments in osteoblasts by a co-transfected catalytic subunit of PKA to the level seen with PGE2 treatment (10). Furthermore, a dominant-interfering mutant regulatory subunit of PKA that does not bind cAMP blocks hormone-activated gene expression (10).

In past studies, we mapped a functional cAMP response element to the 5′-untranslated region of IGF-I exon 1 within a previously footprinted site termed HS3D (10, 13) and showed that this sequence was required for full hormonal responsiveness of the IGF-I promoter in osteoblasts (13). More recently, we identified CCAAT/enhancer-binding protein δ (C/EBPδ) as the critical hormone-regulated transcription factor responsible for PKA-stimulated IGF-I gene transcription through the HS3D sequence (14, 15) and showed that hormones that activate PKA induce binding of C/EBPδ to this site (14).

C/EBPδ belongs to a family of transcriptional regulators that function in tissue differentiation, metabolism, healing, and immune responses (16). Members of the C/EBP family are related structurally, each consisting of an NH2-terminal transactivation region, a central basic DNA-binding domain, and a COOH-terminal dimerization interface termed the leucine zipper segment (16). C/EBPδ proteins share similarities in the latter two domains with a larger group of basic-leucine zipper transcription factors (16, 17). The first C/EBP proteins to be characterized, C/EBPa and C/EBPβ, have key roles in adipocyte differentiation and in gene expression in the liver and other tissues (16, 18–21). C/EBPδ has been implicated in control of adipogenesis and in mediating the acute phase response to inflammatory stimuli (16, 18, 19). In addition, our previous work indicated a regulatory role for this protein in IGF-I gene expression in bone cells (14, 15).

The current experiments were designed to assess mechanisms of activation of C/EBPδ in osteoblasts. We now find that
a PKA-dependent pathway stimulates the rapid nuclear translocation of C/EBPδ in the absence of ongoing protein synthesis. Continual PKA activity is required for nuclear retention of C/EBPδ, because C/EBPδ is quickly removed from the nucleus through an exportin-mediated pathway upon cessation of hormone action. Mutagenesis studies indicate that the basic domain of C/EBPδ is necessary for nuclear localization and that the leucine zipper region permits full nuclear accumulation. In the aggregate, this report defines a pathway for hormone-mediated activation of C/EBPδ through its regulated nuclear import.

**EXPERIMENTAL PROCEDURES**

**Materials—**Timed-pregnant Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Collagenase types 1 and 2 were obtained from Worthington Biochemical Corporation (Lakewood, NJ). Leptomycin B was a gift from Dr. Minoru Yoshida (University of Tokyo, Tokyo, Japan). PGE2, forskolin, and cycloheximide were purchased from Sigma. H-89 and KT 5720 were from Calbiochem (San Diego, CA); [γ-32P]ATP was obtained from PerkinElmer Life Sciences. LY294002 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA), and UO126 was from Promega Corporation (Madison, WI). Recombinant human long lasting IGF-I and analogue, R3IGF-I, was from GenProGroup (Adelaide, Australia), and PDGF-BB was from Life Technologies, Inc. Continual PKA activity is required for nuclear retention of C/EBPδ, according to the manufacturers’ instructions. Experiments were performed as described, and aliquots were stored at -80 °C until use. Western immunoblotting was performed as described previously (15, 25). Immunoreactive proteins were visualized by enhanced chemiluminescence, followed by exposure to x-ray film, or by enhanced chemiluminescence followed by detection and quantitation using Molecular Imager FX imaging system and Quantity One software (Bio-Rad).

**Preparation of Recombinant and in Vitro Translated Proteins—**Preparation of recombinant S-tagged C/EBPδ (S-C/EBPδ) and C/EBPβ in Escherichia coli has been described (15). The 31- amino acid N-terminal S-tag includes a minimal consensus phosphorylation site for PKA (Arg-Gly-Ser). C/EBPδ was translated in vitro using the pET29a/C-activation plasmid and TNT coupled reticulocyte lysate system (Promega Corporation), according to the manufacturer’s instructions.

**PKA Assay—**The purified, recombinant catalytic subunit of PKA was diluted to 10 μg/ml in 100 μg/ml BSA. Recombinant C/EBPδ or C/EBPβ proteins (1 μg each) were mixed on ice with 0.5 μCi of [γ-32P]ATP in assay buffer (100 μM ATP, 10 mM MgCl2, 250 μM BSA, 12.5 mM Tris-Cl, pH 7.5). PKA (10 ng) was added, and the reactions were allowed to proceed for 2 min at 30 °C. The reactions were stopped after being placed on ice by addition of EDTA to 80 mM final concentration. After boiling for 5 min in SDS sample buffer, the samples were separated by SDS-PAGE, and after electrophoresis, gels were stained with Coomassie Brilliant Blue, dried, and exposed to x-ray film for 2 h at ~80 °C with intensifying screens.

**Concentration of Recombinant Plasmids—**Flag-tagged rat C/EBPβ in pCDNA3 (pCDNA3-flag-C/EBPβ) was generated by polymerase chain reaction-mediated mutagenesis. The flag epitope tag (codons underlined) was added to the 5’ end of C/EBPβ in pBluescript-C/EBPβ (15) just downstream of the ATG codon. Three independent, bacterial plasmids containing oligonucleotides: 5′-GGCGATCTGGCCACCATGGCATCTAAGAAGCAGGAGG-3′ (top strand) and 5′-CCATGTGCAGCTTGCCCTCTTA-3′ (bottom strand) were obtained. The amplicons were digested with BamHI and EcoRI and inserted into the corresponding S-tag sites in the cloning vectors. Each vector was transformed into E. coli, and recombinant cells were grown in ampicillin media containing 5% glucose. The plasmid DNA was purified from the bacterial clones using the High Pure Plasmid Purification Kit (Roche). Purified plasmid DNA was then digested with BamHI and EcoRI and inserted into the corresponding sites of pCDNA3 (Invitrogen, Carlsbad, CA) to produce pCDNA3-flag-C/EBPβ. The C/EBPβ depletion plasmids diagrammed in Fig. 8A were prepared using the QuiKChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). C/EBPβΔZip was prepared by introducing a point mutation (C to T) after codon 216, which places a stop codon (bold) just beyond the COOH terminus of the basic region. A KpnI site (underlined) was added to the KO site to aid in identifying the mutant. The oligonucleotides used were as follows: 5′-CCGGCAACATT-AGGGTACCGCATGAGCCAGCAGA-3′ (top strand) and 5′-TCTCGTGCTA-TCCTGGTACTACCTGTTTGCG CG-3′ (bottom strand). For C/EBPβΔZip, the basic region (amino acids 193–216) was deleted in-frame and replaced with a consensus EcoRI site on an unmodified plasmid, containing oligonucleotides 5′-GGCGAGCCCTGATATCCGAGGATGAGC-3′ (top strand) and 5′-CTGATCCTCGGATATTGAGCTGGCAGCGC-3′ (bottom strand). For C/EBPβΔZip, a stop codon (underlined) was added after the stop codon to aid in identifying the mutant. The oligonucleotides used were as follows: 5′-CGCGCCGAACATAGGGTACCGCATGAGCCAGCAGA-3′ (top strand) and 5′-TCTCGTGCTACTCCTGGTACTACCTGTTTGCG CG-3′ (bottom strand). For C/EBPβΔZip, the basic region (amino acids 193–216) was deleted in-frame and replaced with a consensus EcoRI site on an unmodified plasmid, containing oligonucleotides 5′-GGCGAGCCCTGATATCCGAGGATGAGC-3′ (top strand) and 5′-CTGATCCTCGGATATTGAGCTGGCAGCGC-3′ (bottom strand).
the coding region was verified by DNA sequencing.

The EGFP/C/EBPα fusion proteins are diagrammed in Fig. 8A. To generate EGFP containing the basic and leucine zipper regions of C/EBPα (EGFP+BZip), the BZip DNA region of C/EBPα was excised from pcDNAs-Flag-C/EBPαBZip by digestion with XhoI, followed by filling in the overhang with the Klenow fragment of DNA polymerase I, and digestion with BamHI. This DNA fragment was then inserted in-frame into EcoRI (blunted with Klenow) and BamHI-digested pEGFP-C1 (CLONTECH Laboratories, Palo Alto, CA). To produce EGFP with the leucine zipper region of C/EBPα (EGFP+Zip), the EcoRV-EcoRI fragment was excised from pcDNAs-C/EBPα3B and inserted into pEGFP-C1 that had been digested with HindIII (blunted with Klenow) and EcoRI. EGFP containing just the basic segment of C/EBPα (EGFP+B) was prepared as follows. CEBPαBZip was excised from pcDNAs-C/EBPαBZip by digestion with BamHI and EcoRI, and ligated into corresponding sites in the polynucleotide of pEGFP-C1. In this construct there is a stop codon after the basic region of C/EBPα. The portion of C/EBPα 5' to the basic region was then eliminated by mutagenesis using oligonucleotides that overlapped the 3' end of the EGFP coding region (5'-TCCGGACTTGTACAGCTCGTCCATGCCGAGTG-3' (top strand)) and the 5' end of the C/EBPα basic domain (5'-GAGTACCGCCAGGGCCGGCAACATC-3' (bottom strand)). The amplified region was verified by sequencing.

Statistical Analysis—Data are presented as the means ± S.E. Statistical significance was determined using the Student’s t test for paired samples. Results were considered statistically different when p < 0.05.

RESULTS

PGE₂ Stimulates Nuclear Translocation of C/EBPα in Rat Osteoblasts—We previously identified C/EBPα as the key transcription factor mediating cAMP-activated IGF-I gene transcription in primary rat osteoblasts (14, 15). We showed that stimulation of DNA binding of C/EBPα to its critical recognition site in the major IGF-I gene promoter and the subsequent induction of IGF-I gene expression were independent of the new protein synthesis (13). These results indicated that C/EBPα was activated by PGE₂ through post-translational mechanisms in osteoblasts. The current experiments were designed to determine how C/EBPα was regulated in these cells. Fig. 1 shows that incubation of osteoblasts with PGE₂ stimulated the nuclear accumulation of C/EBPα. As seen by immunocytochemistry in Fig. 1A, under control conditions C/EBPα was diffusely distributed within the cell, but after 4 h of incubation with 1 μM PGE₂, the protein was predominantly nuclear. Pre-incubation with cycloheximide at a concentration (2 μM) found previously to block >90% of ongoing protein synthesis in osteoblasts (13) did not prevent accumulation of C/EBPα in nuclei, indicating that pre-existing C/EBPα was translocated to the nucleus in a protein synthesis-independent manner. This interpretation was validated by Western immunoblotting of osteoblast protein extracts (Fig. 1B). In control cells, C/EBPα was detected in cytoplasmic but not nuclear extracts. However, within 2 h of hormone treatment, it was found predominantly among soluble nuclear proteins and was depleted from the cytoplasm. Thus, PGE₂ induces nuclear translocation of C/EBPα in primary cultures of rat osteoblasts.

We next looked at the kinetics of nuclear accumulation of C/EBPα. Fig. 2 shows results of time course studies. Again, under basal conditions C/EBPα was concentrated in <1% of osteoblast nuclei. Within 15 min of PGE₂ treatment, C/EBPα expression was primarily nuclear in 25.5 ± 2.0% of cells. The proportion of cells with predominantly nuclear C/EBPα increased to 64% by 30 min and to ~94% at 1 and 2 h (t₁/₂ of nuclear accumulation = 27.1 min). Upon removal of hormone, C/EBPα rapidly disappeared from nuclei and reaccumulated in the cytoplasm (t₁/₂ = 11.6 min). Only 20.0 ± 2.2% of cells retained prominent nuclear expression by 15 min (the earliest time point examined), and <1% retained prominent expression by 1 h. Thus, in response to PGE₂, C/EBPα was rapidly redistributed from cytoplasm to nucleus and returned to the cytoplasm quickly after termination of the hormonal stimulus.

Nuclear Translocation of C/EBPα Is Dependent on PKA—We next looked at the signaling mechanisms involved in hormonal regulation of the subcellular distribution of C/EBPα. Primary osteoblasts were treated with PGE₂ after pre-incubation with specific protein kinase inhibitors (Fig. 3A). The drugs LY294002 (phosphatidylinositol 3-kinase) or UO126 (MEK1 and 2) did not prevent PGE₂-induced nuclear translocation of C/EBPα and did not alter the primarily cytoplasmic distribution of C/EBPα under control conditions. In contrast, the PKA inhibitors H-89 and KT5720 each prevented the appearance of C/EBPα in nuclei after PGE₂ treatment. To demonstrate that LY294002 and UO126 were effective in osteoblasts, cells were treated with either IGF-I or PDGF in either the absence or the presence of the two inhibitors. As shown in Fig. 3B, pre-incubation with LY294002 inhibited IGF-mediated phosphorylation of the phosphatidylinositol 3-kinase target, Akt, and UO126 prevented phosphorylation of the MEK targets, extracellular signal-regulated kinases 1 and 2, by PDGF. As a further test that PKA was required to stimulate the nuclear translocation of C/EBPα in osteoblasts, cells were co-transfected with expression plasmids for the marker protein, EGFP, and for a modified regulatory subunit of PKA that cannot bind cAMP. This latter protein thus acts to block endogenous enzyme activity (26). Following incubation with forskolin (10 μM for 2 h) to activate adenylate cyclase, the subcellular location of C/EBPα was assessed by immunocytochemistry (Fig. 4). Treatment with forskolin stimulated the nuclear accumulation of C/EBPα in nontransfected cells.
treated with PGE2 for 2 h, washed twice with PBS, and incubated in vehicle (con) for the indicated times. For washout studies, cells were con

d"cylinositol 3-kinase-Akt pathway, and PDGF-BB (0.4 nM) was used for the following concentrations: H-89, 10

The results presented as the mean of two experiments. Inhibitors were used at the concentrations described under “Experimental Procedures,” the MEK-extracellular signal-regulated kinase pathway. Results are presented as the mean of two experiments. Inhibitors were used at the following concentrations: H-89, 10 μM; KT5720, 10 μM; LY294002, 30 μM; and U0126, 10 μM.

but only in 11.2 ± 1.7% of cells transfected with the dominant-interfering regulatory subunit of PKA (p = 0.0013). Thus, PKA activity is required for hormone-regulated nuclear translocation of C/EBPδ.

C/EBPδ Is Not a Direct Substrate for PKA in Vitro—The next series of experiments was designed to determine whether C/EBPδ was phosphorylated by PKA. Other studies have shown that the related transcription factor, C/EBPβ, is a substrate for PKA (27, 28), and inspection of the protein sequence of C/EBPδ revealed several potential PKA phosphorylation sites. To test the hypothesis that C/EBPδ is a substrate for PKA, recombinant C/EBPδ was generated in E. coli, purified, and used in in vitro kinase assays with the purified, recombinant catalytic subunit of PKA. As shown in Fig. 5, under the conditions described under “Experimental Procedures,” the cAMP-regulated transcription factor, CREB, was readily phosphorylated by PKA, whereas a mutant CREB lacking the PKA phosphorylation site at serine residue 133 was not labeled (29). These results demonstrate the specificity of the in vitro kinase assay. C/EBPδ also was not phosphorylated by PKA, but S-C/-EBPδ was labeled, S-C/EBPδ contains a consensus PKA site in the NH2-terminal S-tag. These in vitro experiments show that C/EBPδ does not appear to be a high affinity substrate for PKA.

An Inhibitor of Nuclear Export Does Not Alter the Cytoplasmic Distribution of C/EBPδ in the Absence of Hormone Treatment—The results presented in Figs. 1–4 did not allow us to determine whether C/EBPδ was constitutively cytoplasmic under basal conditions or whether it rapidly shuttled between cytoplasmic and nuclear compartments. To distinguish be-

but did not alter the predominantly cytoplasmic distribution of C/EBPδ in osteoblasts expressing the dominant-interfering PKA regulatory subunit (Fig. 4A, left panels). As shown in Fig. 4B, forskolin treatment induced nuclear translocation of C/EBPδ in 91.7 ± 3.5% of cells transfected with the empty expression plasmid.
between these possibilities, we employed the antibiotic leptomycin B (30, 31), which specifically inhibits chromosome region maintenance 1 (CRM1), the receptor that functions to export proteins from the nucleus (32, 33). Fig. 6 shows that leptomycin B did not alter the subcellular distribution of C/EBPδ under basal conditions and did not influence the ability of PGE₂ to stimulate its nuclear translocation or of H-89 to prevent it. To demonstrate that leptomycin B was effective in osteoblasts, cells were pre-treated with PGE₂ for 2 h, washed with PBS, and then incubated with leptomycin B or with vehicle. Under these experimental conditions, leptomycin B inhibited the exit of C/EBPδ from nuclei (Fig. 7). In vehicle-treated cells, export of C/EBPδ to the cytoplasm was rapid, being nearly complete within 30 min. By contrast, in osteoblasts incubated with leptomycin B, C/EBPδ remained predominantly nuclear for up to 4 h. Based on these results, we conclude that in the absence of hormonal stimulation, C/EBPδ is primarily cytoplasmic and that PKA induces transport of C/EBPδ into the nucleus.

The Basic and Leucine Zipper Domains of C/EBPδ Are Required for Nuclear Targeting in Osteoblasts—We next sought to identify the domains of C/EBPδ that were required for its nuclear localization. The full-length protein contains three major functional segments: an NH₂-terminal transcriptional activation domain, a basic region that mediates DNA binding, and a leucine zipper segment that is responsible for dimerization (16). We generated expression plasmids for full-length and truncated rat C/EBPδ, each containing an NH₂-terminal flag epitope tag to distinguish them from endogenous C/EBPδ (Fig. 8A). Upon transient transfection into primary rat osteoblasts (data not shown) or into the human osteoblast cell line hFOB 1.19, full-length C/EBPδ was found in the nucleus even in the absence of hormone treatment (Fig. 8B, top panel). This precluded us from investigating the regulation of transfected C/EBPδ by PKA. We instead used the various truncation mutants of C/EBPδ to establish the structural requirements for its nuclear localization. A truncation mutant lacking the leucine zipper (C/EBPδΔZip) was primarily nuclear when expressed in hFOB 1.19 cells. In contrast, mutant proteins lacking the basic domain (C/EBPδΔB) or both basic and leucine zipper regions (C/EBPδΔBZip) were predominately cytoplasmic (Fig. 8B, bottom panels). These observations are consistent with results obtained with the related transcription factor, C/EBPβ, which show that the highly conserved basic region contains the nuclear localization sequence (34). In C/EBPδ, however, removal of the leucine zipper led to partial expression in the cytoplasm (Fig. 8B, top, compare the two left panels), indicating that this latter region also contributes to nuclear localization.

These results were confirmed after transfection of EGFP fusion constructs containing different segments of C/EBPδ at their COOH termini (diagrammed in Fig. 8A). EGFP fused to the basic and leucine zipper regions (EGFP+BZip) was exclusively nuclear when expressed in hFOB 1.19 cells. EGFP plus the basic domain (EGFP+B) was predominantly nuclear, and EGFP plus the leucine zipper (EGFP+Zip) was diffusely distributed, as was EGFP (Fig. 8B, lower panels). We interpret these experiments to indicate that a nuclear localization sequence (NLS) resides within the basic region of C/EBPδ but that the leucine zipper contains additional determinants that facilitate full expression within the nucleus.

One NLS Is Sufficient to Translocate a C/EBPδ Dimer into the Nucleus—The hFOB 1.19 cell line does not produce C/EBPδ (assessed by immunocytochemistry and immunoblotting; data not shown). In these cells, transfected C/EBPδΔB was found exclusively in the cytoplasm (Figs. 9, top left panel, and 8B). However, when expressed in primary rat osteoblasts, C/EBPδΔB was concentrated in the nucleus (Fig. 9, top row, third panel from left). These results suggested the possibility that the transfected protein dimerized with endogenous C/EBPδ and used its NLS for translocation into the nucleus. Consistent with this hypothesis, blocking basal PKA activity with H-89 resulted in retention of C/EBPδΔB in the cytoplasm (Fig. 9, top right panel). In confirmation of these results, transfected C/EBPδΔBZip was located in the cytoplasm of both hFOB 1.19 cells and primary rat osteoblasts (Fig. 9, lower panels). C/EBPδΔBZip lacks both the basic and leucine zipper domains and can neither dimerize nor translocate to the nucleus by itself. We interpret these observations to indicate that C/EBPδ forms dimers in the cytoplasm and that one NLS is sufficient for nuclear localization of the dimer when at least basal PKA activity is present in the cells.

DISCUSSION

Our current studies define a mechanism for activation of the transcription factor C/EBPδ in primary rat osteoblasts through its regulated nuclear import by a PKA-mediated pathway. In previous studies, we identified C/EBPδ as the critical transcription factor for induction of IGF-1 gene expression in response to PGE₂ (14, 15). We showed that PGE₂ stimulated PKA in osteoblasts (12) and that PKA induced C/EBPδ to bind to a site termed HS3D located within the major IGF-1 gene promoter, leading to activation of IGF-1 gene transcription (10, 13, 14). This pathway of hormonal stimulation of gene expression also has been shown to be independent of new protein synthesis (13). Using a combination of experimental approaches we now demonstrate that activation of PKA by forskolin or PGE₂ leads to the rapid accumulation of C/EBPδ in osteoblast nuclei. Nuclear translocation was induced within 15 min of hormone treatment (the earliest time point examined), was detected in the majority of osteoblasts by 60 min, and persisted for at least 4 h when cells were continually incubated with PGE₂. Translocation of C/EBPδ into osteoblast nuclei occurred in the presence of the protein synthesis inhibitor cycloheximide, indicating that it was mediated by post-translational mechanisms. Upon removal of hormone, C/EBPδ exited the nucleus rapidly (t½ < 12 min), suggesting that a continuous stimulus was needed to maintain its nuclear localization. Regulated nuclear translocation of C/EBPδ was blocked by the specific PKA inhibitors H-89 and KT5720 and by forced expression of a dominant-interfering regulatory subunit of PKA, indicating that nuclear translocation was stimulated by PKA. Interestingly, C/EBPδ did not appear to be a direct substrate for PKA, because the purified enzyme failed to phosphorylate C/EBPδ in
vitro to a measurable extent. Therefore, activation of C/EBPδ by PKA occurs through an indirect mechanism, perhaps by a PKA-initiated signaling cascade. However, attempts to block this postulated pathway with LY294002 or UO126 were unsuccessful. These latter results may be interpreted to indicate that neither phosphatidylinositol 3-kinase-Akt nor MEK-extracellular signal-regulated kinase pathways control the activity of C/EBPδ in osteoblasts.

This report presents the first example of PKA-dependent nuclear import of C/EBPδ in any cell type, although in a cultured hepatocyte cell line, treatment with tumor necrosis factor-α induced its nuclear accumulation (35). The regulated nuclear import of C/EBPδ defined here appears to resemble the pathway of nuclear translocation of the related transcription factor, C/EBPβ. As shown by several investigators, C/EBPβ resided in the cytoplasm in unstimulated cells and accumulated in the nucleus after treatment with agents that activated PKA with kinetics similar to those observed here for C/EBPδ (28, 36). However, C/EBPδ is a substrate for PKA, and its phosphorylation is required for its nuclear translocation (28). In addition, C/EBPβ can be phosphorylated in vitro by PKA on serines 277 and 299 (27) and in cells on serine 299 (28). An alanine substitution at residue 299 blocked regulated nuclear translocation of C/EBPβ in DKO-1 colon carcinoma cells (28), providing clear evidence for control of nuclear localization by direct phosphorylation. In contrast, we were unable to demonstrate that C/EBPδ was a substrate for PKA in vitro, confirming results of Kageyama et al. (37). C/EBPδ has been shown to become phosphorylated after treatment of HepG2 cells with interleukin-1 (38), and changes in phosphorylation induced by other cytokines have been implicated in its transcriptional activation (38, 39). DNA binding of C/EBPδ to a consensus site also has been shown to be increased ~3-fold after phosphorylation in vitro by casein kinase II (40). No information is available on a role for casein kinase II in modulating the function of C/EBPδ in cells. Other members of the C/EBP family, including C/EBPβ and CHOP, undergo regulated phosphorylation by several different protein kinases. These modifications result in either altered DNA binding (27, 41) or transcriptional activity (28, 42–47). However, in preliminary experiments we were unable to detect an increase in phosphorylated C/EBPδ in primary rat osteoblasts after incubation with PGE2 (data not shown), indicating that this modification may not be part of the mechanism of nuclear translocation induced by PKA in these cells.

Very little is known about the cellular steps controlling nuclear import of members of the C/EBP family of transcription factors. Moreover, no information is available regarding which nuclear import receptors interact with C/EBPδ or which importins are expressed in osteoblasts. We find that C/EBPδ is

**Fig. 6.** Leptomycin B does not interfere with PKA-mediated nuclear translocation of C/EBPδ in rat osteoblasts. Immunocytochemistry for C/EBPδ of primary rat osteoblasts after incubation with vehicle (con), 1 μM PGE2, or 10 μM H-89 in the absence or presence of 10 ng/ml of leptomycin B (lept B). Nuclei stained with Hoechst dye are blue.

**Fig. 7.** Treatment with leptomycin B prevents the exit of C/EBPδ from the nucleus of rat osteoblasts. Immunocytochemistry for C/EBPδ of primary rat osteoblasts after incubation with 1 μM PGE2 for 2 h followed by washes with PBS and addition of vehicle or 10 ng/ml leptomycin B (— lept B or + lept B, respectively) for the times indicated. In the absence of leptomycin B, the t½ for the disappearance of C/EBPδ from the nucleus was 11.8 min, and in the presence of leptomycin B, t½ was >4 h.
In summary, we have shown that stimulation of PKA in primary rat osteoblasts led to the rapid activation of the transcription factor C/EBPδ through its regulated nuclear import. Nuclear targeting required the basic region of C/EBPδ and was enhanced by the presence of the leucine zipper motif. The mechanisms responsible for maintaining C/EBPδ in the nucleus after activation by PKA, or for inducing its nuclear export, remain unknown. Our results suggest that C/EBPδ does not undergo continual shuttling between subcellular compartments under basal conditions or after hormone treatment. Continuous activity of PKA was required to retain C/EBPδ in the nucleus of primary rat osteoblasts, because removal of hormonal stimulus led to rapid redistribution into the cytoplasm (<t₁/₂ < 12 min). The pathways of nuclear export of C/EBPδ involve CRM1, because inhibition of this receptor with leptomycin B (30, 31) caused prolonged retention of C/EBPδ in nuclei after removal of hormone. The segment of C/EBPδ that interacts with CRM1 is not known. The currently recognized consensus sequences for binding to the nuclear export receptor include closely spaced short stretches of leucine residues (51, 52). No typical consensus sequence is found in C/EBPδ. To date, however, no functional studies have been performed to demonstrate direct interactions of C/EBPδ with CRM1.

An intriguing question arising from our current and previous results is whether nuclear localization alone is sufficient for full transcriptional activity of C/EBPδ or whether other modifications of the protein are required. As shown in this report, forced expression of C/EBPδ in osteoblasts resulted in its accumulation in the nucleus and is sufficient to transactivate an IGF-I promoter-reporter gene, although treatment of cells with PGE₂ further increases the level of IGF-I promoter function (14, 15). Thus, potentially more than one mechanism controls the transcriptional response of the IGF-I gene to PKA.

**FIG. 8.** The basic and leucine zipper domains are required for nuclear targeting of C/EBPδ in osteoblasts. A, schematic representations of full-length 268-amino acid C/EBPδ and deletion or truncation mutants and of fusion proteins containing different portions of C/EBPδ joined to the COOH terminus of EGFP. F, flag epitope tag; TA, transactivation domain; B, basic region; Zip, leucine zipper segment. B, immunocytochemistry for the flag epitope tag (upper panels) or immunofluorescence for EGFP (lower panels) after transient transfections of the indicated expression plasmids into fetal human osteoblast cell line hFOB 1.19.

**FIG. 9.** One NLS is sufficient to translocate a C/EBPδ dimer into the nucleus in osteoblasts. Immunocytochemistry for the flag epitope tag of hFOB 1.19 cells (no endogenous C/EBPδ expression) and primary rat osteoblasts (rOB) transfected with the indicated C/EBPδ deletion and truncation mutants and treated with vehicle (-) or 10 μM H-89 for 2 h.
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