Distinct Roles of p130Cas and c-Cbl in Adhesion-Induced or Macrophage Colony-Stimulating Factor-Mediated Signaling Pathways in Prefusion Osteoclasts

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Both p130Cas and c-Cbl have been reported to play critical roles in osteoclast function as downstream targets of c-Src kinase. The purpose of this study was to examine adhesion- and macrophage colony-stimulating factor (M-CSF)-induced tyrosine phosphorylation of these two molecules in prefusion osteoclasts (pOCs) derived from either Src+/− or Src−/− mice and to directly compare the roles of p130Cas and c-Cbl in osteoclast function. Cell attachment of normal pOCs to vitronectin induces tyrosine phosphorylation of p130Cas and, to a much lesser extent, of c-Cbl. Treatment with M-CSF results in further tyrosine phosphorylation of both p130Cas and c-Cbl, suggesting cooperation between α, β integrin and the M-CSF receptor, c-Fms, in osteoclasts. However, M-CSF induces tyrosine phosphorylation of c-Cbl, but not p130Cas, in pOCs in suspension, confirming the role of c-Cbl as a downstream effecter of c-Fms. This observation also suggests that M-CSF-mediated p130Cas phosphorylation requires ligand engagement of α, β integrin. In Src-deficient pOCs plated on vitronectin, although M-CSF highly induces Cbl phosphorylation, it does not affect p130Cas phosphorylation. These results suggest that in osteoclasts 1) tyrosine phosphorylation of p130Cas depends on α, β integrin-mediated cell adhesion, even in the presence of M-CSF; 2) on the other hand, c-Cbl phosphorylation is predominantly activated by M-CSF and is independent of cell adhesion; 3) lastly, although c-Src is essential for both adhesion- and M-CSF-mediated phosphorylation of p130Cas, it is clearly not required for c-Cbl phosphorylation in M-CSF-treated pOCs. Taken together, p130Cas and c-Cbl play distinct roles in the signal transduction pathways that mediate cytoskeletal organization in osteoclasts.

Osteoclasts are highly differentiated, bone-resorbing cells of hemopoietic origin. The nonreceptor tyrosine kinase c-Src has been shown to be essential for osteoclast function, as target disruption of c-Src in mice caused osteopetrosis (1). Activation of c-Src in osteoclasts is dependent upon ligand engagement of α, β integrin (2), which is also important for osteoclast function. c-Src kinase has been shown to mediate the tyrosine phosphorylation of p130Cas and c-Cbl in osteoclasts (3, 4), suggesting that both molecules are downstream targets of c-Src in osteoclasts and might play important roles in bone resorption. Several lines of evidence have demonstrated that c-Cbl is involved in modulating the cytoskeleton and cell motility of osteoclasts (5) and also in recruiting the ubiquitin-conjugating enzyme UbcH7 to receptor tyrosine kinase such as macrophage colony-stimulating factor (M-CSF) receptor (6, 7) and nonreceptor tyrosine kinase c-Src, leading to the ubiquitination and degradation of these molecules in macrophages and fibroblasts (8). p130Cas has also been reported to play a critical role in cytoskeletal reorganization such as the formation of podosomal actin rings in osteoclasts (3, 9, 10). Although both c-Cbl and p130Cas have been suggested to mediate the Src-dependent signaling pathways in osteoclasts, a functional relationship among these molecules in these pathways has not been directly compared in the same cell system. This study examines the effects of adhesion and M-CSF on tyrosine phosphorylation of p130Cas and c-Cbl in osteoclasts, using Src+/− and Src−/− prefusion osteoclasts (pOCs) derived from the in vitro coculture system. We show that tyrosine phosphorylation of p130Cas depends on α, β integrin-mediated cell adhesion and that c-Src is essential for both adhesion- and M-CSF-induced phosphorylation of this molecule.

Materials and Methods

Antibodies and other reagents

Vitronectin (Vn) was obtained from Life Technologies, Inc. (Grand Island, NY). Antibodies specific to c-Cbl (mAb 17) and p130Cas (mAb 21) were purchased from Transduction Laboratories, Inc. (Lexington, KY); phospho-tyrosine (4G10) and c-Fms were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY); c-Src (mAb327) was purchased from Oncogene Research Products (Cambridge, MA). 1α,25-Dihydroxyvitamin D3 [1α,25(OH)2D3] and collagenase were obtained from Wako Chemicals (Dallas, TX), and dispase was purchased from Roche (Indianapolis, IN). Murine M-CSF was obtained from R&D Systems (Minneapolis, MN).

Animals

BALB/c mice were obtained from Taconic Farms (Germantown, NY). Heterozygote Src+/− mice obtained from The Jackson Laboratory (Bar Harbor, ME) were mated in our laboratory, and Src−/− mice were phenotypically distinguished from their Src+/− siblings by lack of tooth eruption. All animals were cared for and housed under conditions approved by the institutional animal care and use committee guide.

Abbreviations: Cas, Crk-associated substrate; 1α,25(OH)2D3, 1α,25-dihydroxyvitamin D3, M-CSF, macrophage colony-stimulating factor; pOC, prefusion osteoclast; Vn, vitronectin.
**Cell cultures**

pOCs were prepared as described previously with slight modifications (11). Briefly, spleen cells isolated from 2- to 5-wk-old Src−/− or their normal littermates were cocultured with osteoblastic MB1.8 cells for 5–6 days in the presence of 10 nM 1α,25(OH)2D3. pOCs were released from dishes with 10 mM EDTA after removing MB1.8 cells with collagenase-dispase. The purity of this pOC preparation is about 90%, which is equivalent to that reported previously (88–95%) (11) and high enough for biochemical analysis (2, 9, 10).

**Cell adhesion and M-CSF treatment**

After isolation, pOCs (8 × 10^5 cells/condition) were washed twice with serum-free aMEM containing 0.1% BSA (Sigma-Aldrich Corp., St. Louis, MO) and allowed to attach to polystyrene dishes coated with Vn (20 μg/ml) or kept in suspension. As both cell spreading of pOCs and tyrosine phosphorylation of p130Cas after cell adhesion peak at 30–60 min after plating (2), pOCs were plated on Vn-coated dishes for 60 min and then treated with M-CSF (2.5 nM) for the indicated periods, and an equal volume of 2× TNE lysis buffer [20 mM Tris (pH7.5), 300 mM NaCl, 2 mM EDTA, 2% Nonidet P-40, 2 mM Na3VO4, 20 mM NaF, 20 μg/ml leupeptin, 1 μg/ml aprotinin, and 2 mM phenylmethylsulfonylfluoride] was added to the plates. Clarified lysates were subjected to immunoprecipitation and immunoblotting.

**Immunoprecipitation and immunoblotting**

Immunoprecipitation and immunoblotting were performed as previously described (2). Briefly, lysates were precipitated with anti-p130Cas, c-Cbl, c-Src, or c-Fms antibodies (2 μg) for 2 h at 4°C, followed by protein G-Sepharose for 1 h at 4°C. After washing with lysis buffer (four times), proteins were separated on an 8% SDS-PAGE and blotted onto Immobilon-P membrane (Millipore Corp., Bedford, MA). After blocking with 100 mM NaCl, 10 mM Tris, 0.1% Tween 20, and 2% BSA, the membrane was incubated with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies, and detected with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Arlington Heights, IL).

**Results and Discussion**

We first examined the effects of cell adhesion and M-CSF on tyrosine phosphorylation of p130Cas and c-Cbl in normal pOCs. To confirm our previous data (2, 10), adhesion of pOCs to Vn-coated dishes induced tyrosine phosphorylation of p130Cas in a time-dependent manner (Fig. 1, upper panels, lanes 1–5). M-CSF induced further tyrosine phosphorylation of p130Cas (Fig. 1, upper panels, lane 7); however, when cells were kept in suspension, phosphorylation of p130Cas by this cytokine was barely detected (lanes 6), suggesting that M-CSF-mediated phosphorylation of p130Cas might depend upon ligand engagement of αβ3 integrin. As reported previously (5, 12), cell adhesion induced c-Cbl tyrosine phosphorylation (Fig. 1, lower panels, lanes 1–5); however, this phosphorylation was much weaker than that of p130Cas. On the other hand, c-Cbl phosphorylation was induced more markedly by M-CSF than by cell attachment to Vn (Fig. 1, lower panels). Moreover, c-Cbl was tyrosine-phosphorylated by this cytokine even when cells were kept in suspension, suggesting that c-Cbl phosphorylation might be dominantly regulated by M-CSF, rather than by cell adhesion, in osteoclasts.

The slight phosphorylation of c-Cbl by adherence is in contradiction to the results reported by Sanjav et al. (5), who found that integrin ligation induces strongly phosphorylation via c-Src. The difference between our results and theirs might be due to the osteoclast cell types used. We use purified prefusion osteoclasts under serum-free conditions, whereas they used already spread multinucleated osteoclasts in which various intracellular signals might be evoked in the processes of cell fusion, spreading, and migration.

The involvement of c-Src in αβ3-integrin-mediated phosphorylation of p130Cas and c-Cbl has been reported (2, 5) and is also confirmed in Fig. 3 (lanes 1 and 2). Therefore, we examined the involvement of Src kinase in M-CSF-induced tyrosine phosphorylation of p130Cas and c-Cbl. We first evaluated whether M-CSF activated tyrosine phosphorylation of its receptor, Fms, even in Src-deficient cells. As shown in Fig. 2, M-CSF induced c-Fms phosphorylation in the absence or presence of c-Src. Interestingly, we also observed that M-CSF binding resulting in a fast kinetic (0.5 min) of c-Fms phosphorylation, which was followed by down-regulation of the receptor (Fig. 2).

We then examined the effects of M-CSF on tyrosine phosphorylation of p130Cas and c-Cbl in Src-deficient cells. As shown in Fig. 3, in Src−/− pOCs, p130Cas was not tyrosine-phosphorylated by M-CSF (upper panels, lanes 2–6), whereas this cytokine markedly evoked c-Cbl phosphorylation even...
in the absence of c-Src (middle panels, lanes 2–6). These data suggest that c-Src is essential for both adhesion- and M-CSF-induced phosphorylation of p130Cas whereas it is not necessary for c-Cbl phosphorylation in the presence of the cytokine. A previous study (13) found that M-CSF did not induce tyrosine phosphorylation of c-Cbl in Src-deficient adherent multinucleated osteoclasts. The present study used purified prefusion osteoclasts under serum-free condition, in which M-CSF-mediated signaling could be enhanced.

In summary, we show that 1) tyrosine phosphorylation of p130Cas depends on ligand engagement of αvβ3 integrin, even in the presence of M-CSF. c-Src is essential for both adhesion- and M-CSF-induced phosphorylation of this molecule. 2) On the other hand, c-Cbl phosphorylation is predominantly activated by M-CSF, but not by αvβ3 integrin, as this cytokine induces phosphorylation of c-Cbl in pOCs in suspension. Moreover, c-Src is not essential for c-Cbl phosphorylation by M-CSF in osteoclasts. Taken together, p130Cas and c-Cbl play distinct roles in the signal transduction pathways in osteoclasts (Fig. 4). Recently, TNF receptor-associated factor 6, a downstream molecule of receptor activator of nuclear factor κB ligand and IL-1, has been reported to associate with c-Src (9, 14). The molecular complex, including c-Src, TNF receptor-associated factor 6, protein-tyrosine kinase 2, p130Cas, and c-Cbl, might be involved in various functions, including cytoskeletal reorganization, protein ubiquitination, cell migration, and apoptosis.

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