Identification of a Novel 135-kDa Grb2-binding Protein in Osteoclasts*

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The tyrosine kinase receptor for macrophage colony-stimulating factor and the non-receptor tyrosine kinase c-Src play critical roles in osteoclast differentiation and function. Since the ubiquitously expressed adaptor protein Grb2 plays an important role in several tyrosine kinase signal transduction pathways, we used a filter binding assay to identify osteoclast proteins that bind to Grb2. In osteoclasts, there were three major Grb2-binding proteins, two of which, mSos and c-Cbl (p120), have been previously identified as Grb2-binding proteins in many cell types. The third protein, p135, had a restricted pattern of expression and was present at high levels in authentic osteoclasts and osteoclast-like cells formed in an in vitro co-culture system. In addition to binding Grb2 in the filter binding assay, p135 was isolated in complexes with endogenous Grb2 from osteoclast cell extracts. The association of p135 and Grb2 was dependent on an intact Src homology 3 domain and furthermore, was shown to preferentially interact with the N-terminal Src homology 3 domain of Grb2, which is similar to the interaction of mSos and Grb2 in other cell types. p135 was not recognized by antibodies against several known Grb2-binding proteins and thus may be a novel Grb2-binding protein.

Signaling events mediated by tyrosine kinases play an important role in regulating cell growth, differentiation, and transformation. Evidence that the action of certain tyrosine kinases is also critical in controlling the cellular process of bone resorption is supported by the finding that deletion of the gene encoding the non-receptor tyrosine kinase c-Src induces osteopetrosis in mice, a disease characterized by a marked decrease in bone resorption (1). The osteoclast, a terminally differentiated cell solely responsible for bone resorption, expresses high levels of the c-Src protein and other Src family members (2, 3) and has been identified as the defective cell in the c-Src-deficient mice (4, 5). Similarly, defective M-CSF3 leads to osteopetrosis by impairing osteoclast differentiation (6, 7), and the tyrosine kinase receptor for M-CSF has been shown to be highly expressed in osteoclasts (8, 9). Even though several tyrosine kinases have been identified, little is known about the downstream targets involved in tyrosine kinase signaling pathways in the osteoclast.

In other cell types, one protein that mediates the interaction of tyrosine kinases with their targets is the ubiquitously expressed growth factor receptor binding protein 2 (Grb2). Grb2 is an adaptor protein that lacks catalytic activities and consists of one Src homology (SH) 2 domain flanked by two SH3 domains (10). The SH2 domain of Grb2 facilitates binding to specific phosphorylated tyrosine residues such as the phosphorylated sites found in ligand-activated growth factor receptors, while the SH3 domains of Grb2 interact with proline-rich sequences found in a variety of cytosolic proteins. Among these, the best characterized is son-of-sevenless (Sos), a regulator of activated tyrosine kinase receptors to the Ras pathway. Another Grb2-binding protein is Shc, whose interaction with the SH2 domain of Grb2 depends upon the tyrosine phosphorylation of Shc by activated growth factor receptors or by oncogenic tyrosine kinases such as v-src and v-fps (18, 19). The association of Shc with the Grb2-Sos complex aids in transmitting signals from activated tyrosine kinase receptors to the Ras pathway. Another Grb2-binding protein is Sps, whose interaction with the SH2 domain of Grb2 depends upon the tyrosine phosphorylation of Shc by activated growth factor receptors or by oncogenic tyrosine kinases such as v-src and v-fps (18, 19). The association of Shc with the Grb2-Sos complex provides another means through which the Ras pathway can be activated (15). Aside from its role in the Ras pathway, Grb2 may also be involved in regulating different cytoskeletal changes (20, 21) and has been implicated in regulating the process of endocytosis, based on evidence of in vitro binding to the GTPase dynamin (22–24).

Because of the importance of Grb2 in tyrosine kinase-related signaling in other cell types, we examined the potential role of Grb2 in osteoclast signaling. Utilizing an in vitro filter binding assay, successfully used elsewhere to identify cell-specific Grb2-binding proteins (25), we identified proteins in osteoclast lysates that are capable of binding to Grb2, including a novel 135-kDa protein that is highly enriched in osteoclasts and in osteoclast-like cells (OCLs) generated in a murine co-culture system. This protein binds to Grb2 in an in vitro assay and physically associates with Grb2 in osteoclast lysates.

MATERIALS AND METHODS

Antibodies—The polyclonal antibody against the C-terminal domain of Grb2, the anti-c-Cbl antibody, anti-mSos, and the monoclonal antibody against GST were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

COC + D3, coculture in the presence of vitamin D3; TL, total lysate; PAGE, polyacrylamide gel electrophoresis; RIP, radioimmune precipitation buffer; SH2, Src homology 2; SH3, Src homology 3; 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; GST, glutathione S-transferase; BSA, bovine serum albumin.
Grb2-associated Protein in Osteoclasts

**Tissue Extracts and Cell Culture**—Extracts were prepared from the different tissues and osteoclasts. RIPA buffer (10 mM Tris-HCl, pH 7.2, 158 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS supplemented with inhibitors including 1 μg/ml leupeptin, aprotinin, and pepstatin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 1 mM Na3VO4) using a Barmann mixer (Bar- rington, IL). The resulting extracts were centrifuged at 16,000 × g for 20 min and the supernatants were collected.

Membrane fractions were isolated by enzymatic digestion using 0.1% collagenase and 0.2% dispase. The resulting cells were cultured in a minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum and passed twice before lysis.

Peritoneal macrophages were isolated from CD-1 mice that were injected intraperitoneally with thioglycollate as described previously (26). After injection, the cells were isolated from the peritoneal cavity and cultured as described (27).

OCLs were obtained in the murine co-culture system by culturing neonatal calvarial osteoblasts (2 × 10^6 cells/10-cm plate) with bone marrow cells isolated after flushing the marrow from mouse femurs and tibias (30 × 10^6 cells/plate) in Dulbecco's modified Eagle's medium containing 1% calf serum and 10% dialyzed fetal bovine serum until reaching confluence.

HER 14 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated calf serum. At 70% confluence, cells were seeded for 16 h. Dulbecco's modified Eagle's medium containing 1% calf serum and stimulated for 20 min with recombinant human EGF (275 ng/ml) prepared in RIPA lysis buffer. After 10 min incubation with protein A-Sepharose (Sigma), immune complexes were washed twice with RIPA buffer containing the protease and phosphatase inhibitors described above for RIPA.

**Grb2-binding proteins in Osteoclasts**—To detect Grb2-binding proteins utilizing far Western blotting techniques, filters containing immunoprecipitated material, proteins binding to GST-Grb2 fusion protein, or proteins from total cell lysates were first probed with diazoylated GST-Grb2 fusion protein (1 μg/ml in TBST (50 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Triton X-100) containing 3% BSA and 1 mM dithiothreitol) for 2 h at room temperature and then were reacted with TBST. To test proteins binding to GST-Grb2, we utilized anti-GST monoclonal antibodies (1:1000 dilution in TBST, 3% BSA) according to the Western blotting procedures described above followed by visualization of GST-Grb2-binding proteins by ECL.

**Membrane Localization**—After 5 days in the presence of 1,25(OH)2D3, live cells were washed twice with 1-methionine and 1-cysteine-free Dulbecco's modified Eagle's medium (Life Technologies, Inc.) and labeled for 12 h in this medium containing 10% dialyzed fetal bovine serum and 125 μCi/ml [35S]methionine (10 μCi/ml, >37 Tbq/mmol; Amersham Corp.). OCLs were purified from contaminating osteoblasts as described above, rapidly washed twice with ice-cold phosphate-buffered saline, and lysed immediately in RIPA buffer.

**Competition Experiments**—GSTVPPVPPPPRPFRPGK (SOS) Grb2-binding peptide (provided by Dr. J. Brugge, ARIAD Pharmaceuticals, MA) was used to examine the inhibition of Grb2-interaction with Grb2-binding proteins in OCL lysates. Equal amounts of lysate from unlabelled or [35S] metabolically labelled OCLs were first incubated with GST beads (1 μg) for 2 h at 4°C, and then the precleared lysates were incubated with GST-Grb2 beads (1 μg) alone or in the presence of increasing concentrations of SOS peptide for 4 h at 4°C. In addition, [35S]-labelled lysates were bound to beads containing the N-terminal SH3 domain of Grb2 (GST-SH3), the entire Grb2 protein (GST-Grb2), or the C-terminal SH3 domain of Grb2 (GST-CSH3). The beads were washed, and the Grb2-binding proteins were eluted and separated on SDS-PAGE. Antibodies against c-Cbl and mSos were used in Western blot analysis to detect the respective proteins. In [35S]-labeled lysates, the binding of Grb2 to p135, c-Cbl, and mSos in the presence or the absence of SOS peptide was detected by fluorography.

**Subcellular Fractionation**—Nuclear, membrane, and cytosolic fractions of OCLs were obtained using the method previously described (24). The nuclear pellet was washed twice with a hypotonic buffer to remove contaminating cytosolic proteins, and the nuclear proteins were extracted with 420 mM NaCl and 20% glycerol. The supernatants containing membrane and cytosolic fractions were adjusted to 150 mM NaCl, centrifuged at 200,000 × g for 90 min at 4°C, and the resulting supernatant (cytosolic fraction) and the pellet (membrane fraction) were treated with 1% SDS to solubilize the proteins. In some assays, cells were homogenized and fractionated without detergent present, as described previously (2).

**RESULTS**

**GST-Grb2 Fusion Protein Binds to a 135-kDa Protein That Is Highly Expressed in Osteoclasts**—To compare the pattern of Grb2-binding proteins in osteoclasts relative to other cell types, cellular lysates were probed with a GST fusion protein containing the entire coding sequence of Grb2. In the majority of cells and tissues examined (Fig. 1), Grb2 binds to a similar set of major proteins including the previously identified mSos (14, 16) as well as a ubiquitously expressed 120-kDa protein (see below). However, this assay also detected a 135-kDa Grb2-binding protein (p135) that is highly enriched in OCLs as well as in ROCs relative to other tissue and cell types (Fig. 1, A and B).

Since osteoclasts are derived from cells of the monocyte/macrophage lineage, we then determined whether the 135 kDa Grb2-binding protein was expressed in a macrophage cell line (P388D1) and in primary peritoneal macrophages (pMΦ). Fig. 1C shows that, in contrast with OCL lysates, p35 was barely detected in either of the two macrophage cell lysates. In addition, we found that expression of p135 in the co-culture system is dependent on the presence of 1,25(OH)2D3, which induces
the differentiation of hematopoietic progenitor cells to osteoclasts, thereby further suggesting that this protein is associated with cells of the osteoclast lineage rather than with other hematopoietic cell lineages (Fig. 1C, compare COC1D3 to COC2D3). Similarly, p135 was not detected in primary osteoblasts, one of the bone cell types that could remain at low levels in the co-culture system even after purification of the OCLs by EDTA. In an attempt to determine the subcellular localization of p135, we separated lysates prepared from OCLs into nuclear, membrane, and cytosolic fractions and detected p135 by far Western blotting with the GST-Grb2 fusion protein. The purity of the cellular fractions was tested by using specific markers for each fraction (data not shown). As shown in Fig. 2, p135 appeared to be enriched in the membrane fraction but was also detected in the cytosol.

**Grb2 and p135 Associate in Vivo**—Since the far Western blotting assay is based upon in vitro association of Grb2 with other proteins, we then determined whether a complex of Grb2 and p135 exists in OCLs in vivo. For this purpose, OCL lysates were immunoprecipitated with normal rabbit serum (NRS) or Grb2-specific antibody, and detection of the p135-binding protein in the immune complexes was performed utilizing the GST-Grb2 fusion protein in the filter binding assay. Fig. 3A demonstrates that p135 is present in the complex formed with Grb2-specific antibody but not with normal rabbit serum. In addition, both p120 (see below) and mSos were found to be associated with Grb2 in OCL lysates. Fig. 3B confirms that a large amount of Grb2 was immunoprecipitated from OCL lysates with the Grb2-specific antibody and not with NRS. These results indicate that, in addition to the binding of p135 to Grb2 in a filter binding assay, complexes of p135 and Grb2 are constitutively present in vivo in OCLs.

**p120 Is Composed of c-Cbl Whereas p135 Is a Novel Grb2-binding Protein in Osteoclasts**—The 120-kDa protein c-Cbl is known to bind to the SH3 domain of Grb2 in human hematopoietic cells (33–35), and we have found that it is expressed in OCLs. We therefore determined whether the p120 or p135 Grb2-binding proteins were related to c-Cbl. c-Cbl was identified as one of the proteins present in OCL lysates that was able to bind to GST-Grb2 agarose beads but not to GST beads alone (Fig. 4A). We then performed immunoprecipitation analysis from OCL lysates with NRS and α-c-Cbl antibody, and we blotted the immunoprecipitated material and supernatant that remained after α-c-Cbl immunoprecipitation with α-c-Cbl antibodies in Western analysis (Fig. 4B) and the GST-Grb2 fusion protein utilizing far Western blotting procedures (Fig. 4C). As shown in Fig. 4B, the vast majority of c-Cbl was immunoprecipitated from OCL lysates with α-c-Cbl antibody (α-Cbl IP) even though a small amount of c-Cbl remained in the supernatant after immunoprecipitation (α-Cbl-Stap). The GST-Grb2 fusion protein was able to bind to a protein immunoprecipitated

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**Fig. 1. Identification of p135, a novel Grb2-binding protein in ROC and OCLs.** A, B, and C, equal amounts of protein from tissue extracts or cell lysates were separated by SDS-PAGE on 8% gels, transferred to nitrocellulose membrane, and probed with dialyzed GST-Grb2 fusion protein. C, co-cultures of bone marrow cells and osteoblasts were grown in the presence (COC + D3) or absence (COC – D3) of 1,25(OH)2D3 for 8 days and then treated with EDTA. PM, peritoneal macrophages; P388D1, macrophage cell line; OB, osteoblasts; Lung Fb, lung fibroblasts. The position of p135 is indicated by the arrow.

**Fig. 2. p135 is enriched in the OCL membrane fraction.** OCL lysates were fractionated into membrane (M), cytosolic (C), and nuclear (N) fractions. Equal amounts of protein from the starting material (SM) and the three different fractions were assayed for the presence of Grb2-binding proteins as described in Fig. 1. The positions of p135 and mSos are indicated by the arrows.

**Fig. 3. Grb2 and p135 associate in vivo in OCLs.** OCL lysates were immunoprecipitated with NRS and anti-Grb2 antibody (Grb2). Immune complexes were either resolved on 8% SDS-PAGE and probed with the GST-Grb2 fusion protein (A) or on 12% gels and blotted with Grb2 antibodies (B). TL represents total cell lysate prepared from OCLs. The arrows indicate the position of mSos, p135, p120, and Grb2. IP, immunoprecipitate.
with c-Cbl-specific antibodies (Fig. 4C, α-Cbl-IP) that co-migrated with the p120 Grb2-binding protein in total cell lysates detected by far Western blotting (Fig. 4C, TL). In contrast, analysis in the Grb2 filter binding assay revealed that p135 could not be detected in the c-Cbl immune complex (Fig. 4C, α-Cbl-IP) and remained in the supernatant subsequent to immunoprecipitation with c-Cbl antibodies (Fig. 4C, α-Cbl-Sup). Thus, although p120 Grb2-binding protein is mostly comprised of c-Cbl, p135 is not immunologically related to this protooncogene.

We then used antibodies against several proteins with apparent molecular masses of approximately 135 kDa involved in signaling processes, including pp130, Jak1, Jak2, Tyk2, the Src substrate p120, the Abi SH3-binding protein 3BP1, c-Abi itself, and the Crk SH3-binding protein C3G, to probe the immunoprecipitated material and the supernatants remaining after immunoprecipitation with the GST-Grb2 fusion protein. None of these antibodies could immunoprecipitate the p135 Grb2-binding protein, leading us to the conclusion that p135 is an as yet unidentified Grb2-binding protein (data not shown).

The Interaction between p135 and Grb2 Requires an Intact SH3 Domain of Grb2—Having demonstrated that Grb2 associates with p135 in osteoclasts and OCLs, we then used Grb2 fusion proteins containing mutations in the SH2 and SH3 domains of Grb2 to determine the region of Grb2 that was responsible for this interaction. As a positive control for this experiment, cell lysates prepared from a cell line overexpressing the EGFR (HER14) were used in the assay and confirmed that wild-type Grb2 binds to mSos in unstimulated and stimulated cells as well as to the activated EGFR in EGF-stimulated cells (Fig. 5A). As previously reported (14, 36), substitution of Pro by Leu at position 49 in the N-terminal SH3 domain of Grb2 dramatically reduced the association of Grb2 with c-Cbl (Fig. 5A). The position of p135, the EGFR, and mSos are indicated by the arrows.

containing the N-terminal SH3 domain of Grb2 alone whereas binding of these three proteins to a fusion protein containing the Grb2 C-terminal SH3 domain was markedly diminished (Fig. 6A). Cumulatively our data suggest that the association of p135 with Grb2, at least in vitro, requires the presence of an intact N-terminal SH3 domain of Grb2. Interestingly, SH3 sequences from other adaptor molecules such as Crk failed to bind to p135 (data not shown), suggesting that the association of p135 with the SH3 domain of Grb2 is relatively specific among adaptors.

Based on this SH3-dependent interaction, a proline-rich peptide (GYPVPPPVPPRRCOGK) corresponding to the highest affinity binding sequence in Sos (37) was used in competition experiments to compare the binding of p135 to mSos and c-Cbl with Grb2. The peptide was added in increasing concentrations to 35S-labeled OCL lysates incubated with GST-Grb2 fusion protein. Association of all three Grb2-binding proteins including mSos, c-Cbl, and p135 with Grb2 was inhibited with the SOS peptide over a similar concentration range (Fig. 6B). Inhibition of both mSos and c-Cbl binding to Grb2 by the SOS peptide was further confirmed in Western blot analysis utilizing the respective antibodies (Fig. 6C). Therefore, competition experiments utilizing this peptide further confirm the importance of SH3-dependent association of Grb2 with p135 and suggest that the three Grb2-binding proteins may be binding to Grb2 with similar affinities.

M-CSF Does Not Affect the Binding of p135 with Grb2 in OCLs but Induces the Association of Grb2 with a Tyrosine-phosphorylated p85 Protein—Since post-translational modifications such as tyrosine phosphorylation have been shown to affect various protein interactions, we examined the effect of M-CSF, an agent that induces the tyrosine phosphorylation signaling pathway in OCLs, on the association of p135 with Grb2. As shown in Fig. 7A, M-CSF induces the tyrosine phosphorylation of a subset of GST-Grb2-binding proteins, including p56Shc, an unidentified p85 protein, and c-Cbl. In other cell types, M-CSF treatment leads to the tyrosine phosphorylation of Shc and its subsequent association with Grb2, a result that we have confirmed in OCLs (Fig. 7A and data not shown). p85, like Shc, is tyrosine-phosphorylated in response to M-CSF treatment and only associates with GST-Grb2 in M-CSF-treated cells as observed in both the anti-phosphotyrosine blot (Fig. 7A) and the far Western blot (Fig. 7C) of GST-Grb2-binding proteins in lysates prepared from untreated and treated OCLs. Even though M-CSF treatment induces the tyrosine phosphorylation of c-Cbl (Fig. 7A), the association of

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* K. Insogna, personal communication.
c-Cbl and Grb2 is similar in both untreated and treated cells (Fig. 7B). In contrast, both p135 and mSos are not tyrosine-phosphorylated in response to M-CSF (Fig. 7A), and their binding to Grb2, as shown in Fig. 7C, is not affected by treatment of cells with this agent. Furthermore, the results described above were confirmed in vivo by demonstrating that the level of p135, c-Cbl, and mSos in the complex formed with Grb2 utilizing Grb2-specific antibodies does not change upon treatment of OCLs with M-CSF, whereas p85 association with Grb2 is only observed in M-CSF-treated cultures (Fig. 7D).

**DISCUSSION**

Grb2 is a ubiquitously expressed adaptor protein that in many cell types links signaling events initiated by tyrosine kinases to downstream pathways. Based on the importance of specific tyrosine kinases such as c-Src in the maintenance of normal osteoclastic bone resorption (5), and the M-CSF receptor in the differentiation of precursor cells to mature osteoclasts (38) and in functions of the mature cell (39), we sought to identify Grb2-binding proteins that would provide information concerning tyrosine kinase signaling pathways in these cells. In ROCs and OCLs, three major Grb2-binding proteins were detected. Two of the three proteins, mSos and c-Cbl, have been identified as Grb2-binding proteins in other cell types. mSos is the guanine nucleotide exchange factor for p21ras and is the Grb2-dependent connection between tyrosine kinases and the Ras signaling pathway (11–17). The ubiquitously expressed 120-kDa Grb2-binding protein is at least in part c-Cbl, a protooncogene product recently shown to associate with Grb2 but whose function remains unknown (34, 35). In contrast, the third major Grb2-binding protein, p135, was found to be highly expressed in authentic rabbit osteoclasts and in OCLs formed in a co-culture system, relative to other cells of the monocyte-macrophage lineage, other bone cells such as osteoblasts, and various other cell types. In the co-culture system, p135 expression was dependent upon the addition of 1,25(OH)2D3, further establishing the association of p135 expression with the osteoclast phenotype (28).

Subcellular fractionation experiments demonstrated that p135 is present predominantly in the membrane fraction of OCLs. Interestingly, mSos also appeared to be present in the membrane fraction in OCLs when in most other cell types mSos is generally found in the cytosol and translocates to the membrane only after formation of the mSos-Grb2 complex upon cellular activation. The fact that mSos is present at substantial...
levels in membrane fractions in OCLs suggests that these cells may be somewhat activated under our culture conditions. However, we cannot exclude the possibility that, in contrast to other cell types, mSos and p135 are associated with membranes in OCLs even under unstimulated conditions.

Utilizing GST-Grb2 fusion proteins containing single inactivating point mutations in either the SH2 (S90N) or N-terminal SH3 (P49L) domain and GST fusion proteins that express the individual N-terminal or C-terminal SH3 domains, we were able to determine that, as for mSos and c-Cbl (14, 33, 35), the association between Grb2 and p135 was dependent on an intact N-terminal SH3 domain of Grb2. In addition, a proline-rich SOS peptide effectively inhibited the association of p135, mSos, and c-Cbl with Grb2, thereby suggesting that like mSos and c-Cbl, the SH3 domain of Grb2 is interacting with proline-rich sequences in p135, a possibility that can only be confirmed after identification and/or cloning of p135. After identifying and cloning p135, a detailed quantitative analysis of the binding affinities of p135, mSos, and c-Cbl must await expression and purification of the three Grb2-binding proteins.

In our experiments, mSos, c-Cbl, and p135 are constitutively associated with Grb2 in this SH3-dependent manner under basal cellular conditions. We then determined whether treatment of OCLs with an agent such as M-CSF would affect binding of p135 with Grb2. Similar to mSos, p135 is not tyrosine-phosphorylated in response to M-CSF treatment, and the association of p135 with Grb2 is not altered in treated cells at least as detected in in vitro filter binding assays and in the Grb2 immune complex in vivo. In agreement with reports in macrophage cell lines (40), c-Cbl is tyrosine-phosphorylated in M-CSF-treated OCLs, even though this post-translational event does not affect the binding of c-Cbl with Grb2. Therefore, SH3-dependent interactions of the binding proteins, mSos, c-Cbl, and p135, with Grb2 do not appear to be altered upon treatment of OCLs with M-CSF. In contrast, M-CSF induces the tyrosine phosphorylation of p56Lck and an unidentified p85 protein, and association of the two proteins with Grb2 can only be detected in M-CSF-treated OCLs, thereby suggesting that the interaction of p56Lck and p85 with Grb2 may be mediated via the Grb2 SH2 domain, which concurs with reports of the nature of the Shc-Grb2 association in other cell types (19).

Despite our attempts to positively identify p135, this protein did not appear to be related to any other known signaling proteins of similar molecular weights. Because p135 migrates very close to c-Cbl on SDS-PAGE, we also explored the possibility that p135 may be related to c-Cbl. Utilizing an antibody directed against the c-Cbl protein, we were not able to detect p135 in the c-Cbl immune complex by far Western blotting, and p135 could be detected in the supernatant that remained after immunoprecipitation with c-Cbl antibody. These results imply that p135 is not immunologically related to c-Cbl, at least as determined with this particular antibody. Furthermore, the possibility that p135 is a proteolytic degradation product of mSos is highly unlikely based on the fact that mSos is ubiquitously expressed, and we would therefore expect to see p135 or other degradation products of mSos in other cell types. Therefore, our data strongly suggest that the OCL-enriched p135 is a novel Grb2-binding protein that, as shown for mSos and c-Cbl (16, 34), associates with Grb2 in vivo.

Several studies have demonstrated that the major role of the Grb2 adapter molecule is to couple activated receptor tyrosine kinases to the Ras signaling pathway. No direct interaction between Grb2 and non-receptor tyrosine kinases such as c-Src have been demonstrated, even though in both v-src-transformed fibroblasts and platelet-derived growth factor-stimulated cells, two cell types in which the Src tyrosine kinase is activated, the adaptor protein Shc becomes tyrosine-phosphorylated and associates with the SH2 domain of Grb2, thereby enabling the Shc-Grb2-mSos complex to transduce signals from Src to the Ras pathway. Activation of the Ras pathway is known to have important functional consequences on cell proliferation and differentiation, thereby indicating that Grb2 has a role in regulating these two cellular processes. Aside from its link to the Ras pathway, there is increasing evidence that Grb2 is involved in processes related to the cytoskeleton such as actin organization (20) and membrane ruffling (21). Furthermore, in neurons, Grb2 has been shown to bind in vitro via its SH3 domain to synapsin I and the GTPase dynamin, two proteins that play an important role in the regulation of synaptic vesicle function (41-43). In non-neuronal cells, dynamin is involved in clathrin-mediated endocytosis (44, 45), and in vitro binding to Grb2 stimulates its GTPase activity (29, 24). Therefore, the function of Grb2 in the osteoclast, a terminally differentiated cell, although possibly related to proliferation or differentiation of precursors, may also involve processes critical to the functioning of the mature cell such as those associated with the cytoskeleton, endocytosis, cell motility, and/or vesicular trafficking. Identification of p135 after microsequence analysis and cloning will provide a clearer understanding of the role of the Grb2-p135 complex in osteoclast biology.

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