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CBL-GRB2 Interaction in Myeloid Immunoreceptor Tyrosine Activation Motif Signaling

Rae Kil Park,*† Wade T. Kyono,* Yenbou Liu,* and Donald L. Durden2‡

In this study, we provide the first evidence for role of the CBL adapter protein interaction in FcγRI receptor signal transduction. We study the FcγRI receptor, an immunoreceptor tyrosine activation motif (ITAM)-linked signaling pathway, using IFN-γ-differentiated U937 myeloid cells, termed U937IF cells. CBL is constitutively associated with both GRB2 and the ITAM-containing receptor subunit, FcγRIy of FcγRI, providing direct evidence that CBL functions in myeloid ITAM signaling. FcγRI cross-linking of U937IF cells induces the tyrosine phosphorylation of CBL that is associated with an altered CBL-GRB2 interaction. Both GRB2-SH3 and SH2 domains bind CBL in resting cell lysates; upon FcγRI stimulation, phosphorylated CBL binds exclusively to the GRB2-SH2 domain. Glutathione-S-transferase fusion protein data demonstrate that the constitutive interaction of CBL with GRB2 and CRKL is mediated via two discrete regions of the CBL C terminus. The proximal C terminus (residues 461–670) binds to GRB2 constitutively, and under conditions of receptor activation binds to the tyrosine-phosphorylated SHC adapter molecule. The distal C terminus of CBL (residues 671–906) binds the CRKL adapter protein. The data demonstrate that the CBL-GRB2 and GRB2-SOS protein complexes are distinct and mutually exclusive in U937IF cells, supporting a model by which the CBL-GRB2 and GRB2-SOS complexes function in separate pathways for myeloid FcγRI signaling. The Journal of Immunology, 1998, 160: 5018–5027.

The cbl gene, originally described as the transforming gene of the Caenorhabditis elegans, which is a putative negative regulator of RAS in the LET-23 pathway (epidermal growth factor receptor subunit), is closely related to the SLI-1 gene product recently cloned in C. elegans, which is a putative negative regulator of RAS in the LET-23 pathway (epidermal growth factor receptor subunit). The data demonstrate that the constitutive interaction of CBL with GRB2 and CRKL is mediated via two discrete regions of the CBL C terminus. The proximal C terminus (residues 461–670) binds to GRB2 constitutively, and under conditions of receptor activation binds to the tyrosine-phosphorylated SHC adapter molecule. The distal C terminus of CBL (residues 671–906) binds the CRKL adapter protein. The data demonstrate that the CBL-GRB2 and GRB2-SOS protein complexes are distinct and mutually exclusive in U937IF cells, supporting a model by which the CBL-GRB2 and GRB2-SOS complexes function in separate pathways for myeloid FcγRI signaling. The Journal of Immunology, 1998, 160: 5018–5027.

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Materials and Methods

Antibodies

The FcγRIα-specific cross-linking Abs were generously provided by Medarex (West Lebanon, NH). The mAb 197 and mAb 32.2 are specific for adapter proteins (e.g., GRB2, CRK, CRKL, NCK) that regulate the guanine nucleotide exchange factors, “son of sevenless” (SOS) and C3G in mammalian cells following the activation of the TCR (12–14). Marcilla et al. reported that stimulation of multiple FcγRI classes (FcγRI, FcγRII, and FcγRIII) in HL-60 cells with IgG/anti-IgG complexes induces the tyrosine phosphorylation of CBL (9). Stimulation with these immune complexes results in the activation of FcγIRI, FcγRIIA, and FcγRIII receptors, making it more difficult to interpret these results. Matsuo et al. and Tanaka et al. subsequently implicated CBL in FcγRII/III signaling in macrophages and THP-1 cells, respectively (14, 15). The function of CBL, tyrosine phosphorylation and/or the interaction of CBL with adapter proteins as it relates to specific signaling through the FcγRI receptor in myeloid cells and the regulation of RAS have not been thoroughly studied.

We investigated the role of the CBL adapter protein interaction following specific cross-linking of the FcγRI receptor in myeloid signaling. CBL is bound to the FcγRIy subunit of the FcγRI receptor in myeloid cells, providing direct evidence that CBL is involved in ITAM signaling. Our data demonstrate that CBL binds in vitro to GRB2 and CRKL molecules via different domains of the CBL C terminus. CBL is tyrosine phosphorylated after FcγRI cross-linking, and this phosphorylation is associated with an altered CBL-GRB2 interaction. At the same time, the GRB2-SH2 domain inducibly binds to SHC after FcγRI stimulation. These events are associated with the conversion of GDP to GTP (unpublished observation). Taken together, our data support a model by which the CBL-GRB2 interaction may modulate the interaction between GRB2 and SOS in FcγRI signaling in myeloid cells.

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the FcyRIα subunit; mAb 32.2 is a F(ab′)2 fragment of IgG. The cross-linking Ab was a rabbit anti-mouse F(ab′)2 fragment purchased from Organon Teknika (West Chester, PA). Anti-CBL Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine, anti-SHC Abs, and anti-CRKL antisera were purchased from Upstate Biotechnology (Lake Placid, NY), and the anti-GRB2 mAb (G16720) was obtained from Transduction Laboratories (Lexington, KY). GRB2 immunoprecipitations were performed with polyclonal anti-GRB2 (C-231) against residues 195–217 of human GRB2 molecule from Santa Cruz Biotechnology. The anti-γ subunit (FcyRIγ) antisera 5927 was prepared in our laboratory, as described (16, 17), and the 4D8 anti-γ mAb was generously provided by J. Kochan (Hoffman-La Roche, Nutley, NJ) (18). Preimmune immunoprecipitations were performed with an equal amount of purified rabbit IgG.

**Differentiation and stimulation of U937 cells**

U937 cells were maintained in RPMI 1640 with 10% FCS and differentiated with 250 U/ml human rIFN-γ (obtained from Genentech, San Francisco, CA) for 4 days (termed U937IF cells). U937IF cells were cultured at a concentration of 5 × 10^6 cells/ml, and the medium was replenished with fresh IFN-γ (250 U/ml) every 2 days, as described (16, 19). At the time of performing cross-linking experiments, the U937IF cells are 48 h from the addition of fresh IFN-γ. Flow-cytometric analysis of U937IF cells demonstrated the expression of the FcyRI and FcyRII receptors on these cells (data not shown). For stimulation of FcyRI receptors on U937IF cells, cells were washed twice in cold HBSS and adjusted to a concentration of 4 × 10^6 cells/ml. 0.5 ml aliquots were incubated on ice for 30 min with anti-FcyRI Abs (0.25 μg/sample). Cross-linking Abs used in Figure 1 were the 32.2 (F(ab′)2 fragment) and 197 (whole IgG) anti-FcyRI mAbs. Experiments shown in Figures 2, 3, and 6 and were conducted with the 32.2 (F(ab′)2 fragment), and Figures 4 and 5 were performed with 197 Ab cross-linking, as described (19). In both 32.2 and 197 cross-linking experiments, we added 10 μg/ml rabbit anti-mouse (F(ab′)2 fragment) Ab at 37°C for different times. Stimulated cells were cooled rapidly with cold HBSS and centrifuged at 500 × g for 5 min in a cold centrifuge. The cell pellet was lysed with 800 μl of Triton X-100 extraction buffer (EB buffer) on ice for 30 min or resuspended in 25 μl of 1× sample buffer per 1 × 10^6 cells for whole cell lysates.

**Immunoprecipitation**

Cell lysates were prepared in a lysis buffer (EB) containing 1% Triton X-100, pH 7.6, 50 mM NaCl, 1% BSA, 1 mM PMSF, 1% aprotinin, 5 mM EDTA, 50 mM NaF, 0.1% 2-ME, 5 μM phenylarsine oxide, and 100 μM vanadate. Lysates were cleared by centrifugation at 15,000 × g for 45 min at 4°C. For precipitation of specific protein, we added 3 to 10 μl of the appropriate Ab to clarified cell lysates. After incubation on ice for 2 h, 100 μl of a 10% suspension of Formalin-fixed *Staphylococcus aureus* was added to the immunoprecipitate (IP) and incubated on ice for 1 h. The absorbed immune complexes were washed three times in EB buffer and resuspended with 25 μl of 1× sample buffer. After boiling at 98°C for 5 min, samples were resolved by SDS-PAGE.

**Electrophoresis and immunoblotting**

Immunoprecipitates were resolved on 10 or 15% acrylamide, 0.193% of bisacrylamide gels by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (1 μAh/cm²) using a dry transfer system (Eilard, Seattle, WA), as described (16). The blot was incubated with blocking solution (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5% powered milk) for 1 h at room temperature and then incubated with specific anti-phosphotyrosine, anti-SHC, anti-GRB2, or anti-CRKL Ab, for 2 h at room temperature with continuous agitation. After three washes in rinse solution (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl), the membranes were incubated at room temperature for 1 h with secondary Ab conjugated with horseradish peroxidase for enhanced chemoluminescence (ECL; Amersham, Arlington Heights, IL) or conjugated with alkaline phosphatase for colorimetric development. To reprobe the membrane, we stripped membrane with 0.1 M glycine, pH 2.5, at room temperature for 30 min and then reblotted with primary Ab.

**GST fusion protein experiments**

The C terminus of CBL was defined as the domain containing 11 PXXP motifs, as previously described (1, 2). We prepared GST fusion constructs representing the entire C terminus of the CBL molecule (residues 461–906) or the proximal C terminus (residues 461–670) or the distal C terminus (residues 671–906). We subcloned these cDNAs into the pGEX using a two-staged PCR reaction initially to clone into the pBS and subsequently into pGEX2T vector for expression in *Escherichia coli*. Preparation of GST-GRB2 fusion constructs were as previously described by Lioubin et al. (20). We performed DNA sequence analysis to confirm the identity and fidelity of the N-terminal GST-GRB2-SH3, C-terminal GRB2-SH3 domain, GRB2-SH2 domain, and CBL C-terminal fusion constructs. GST fusion proteins were affinity purified from cell lysates of *E. coli* DH5α by adsorption to glutathione Sepharose beads. Sepharose-bound GST fusion proteins were washed several times and stored −80°C. Sepharose-bound GST fusion proteins were added to lysates (EB lysis conditions) of resting or FcyRI-stimulated U937IF cells, incubated for 1 h at 4 degrees C. Breads were then washed with EB buffer without vortexing, and bound proteins were eluted with 2× SDS sample buffer at 95°C, and resolved by SDS-PAGE. The glutathione Sepharose-bound GST fusion protein (10 μg) was confirmed by Bradford protein assay and by performing SDS-PAGE on an aliquot of the Sepharose-bound GST fusion proteins eluted from the beads. Equivalent amounts of each GST fusion protein were used in each experimental group confirmed by Coomassie blue staining of the protein gels after transfer of protein.

**Results**

**CBL is tyrosine phosphorylated and associates with phosphorylated proteins following FcyRI cross-linking**

Our results demonstrate that CBL is tyrosine phosphorylated in resting U937IF cells (Fig. 1A, lane 2; Fig. 2A, lane 2; and Fig. 5A,
CBL tyrosine phosphorylation is tightly controlled in U937IF following FcγRI stimulation (Fig. 2, lanes 4–8). The tyrosine phosphorylation of CBL (Fig. 2, lane 6) demonstrates the CBL mobility shift in FcγRI-stimulated cells (Fig. 2, lanes 3 to 6). From these data, we conclude that the mobility shift in CBL is unrelated to its induced tyrosine phosphorylation state in myeloid cells.

We then explored in more detail the kinetics of CBL phosphorylation following FcγRI stimulation (Fig. 2A). The tyrosine phosphorylation of CBL is tightly controlled in U937IF following FcγRI stimulation (Fig. 2A, lanes 4–8). PMA stimulation of U937IF cells induced a mobility shift in CBL similar to that induced by FcγRI stimulation, in the absence of tyrosine phosphorylation (Fig. 2, A and B, lane 3). PMA stimulation induced the dephosphorylation of CBL coincident with a pronounced mobility shift. In contrast, FcγRI stimulation of U937IF cells induces a rapid tyrosine phosphorylation of CBL (Fig. 2A, lanes 2–8) with complete tyrosine dephosphorylation observed 15 min after receptor activation (Fig. 2A, lane 6). The CBL mobility shift is maximal at 15 min and disappears 30 to 60 min following FcγRI stimulation (Fig. 2B, lanes 6–8).

CBL is bound to FcγRIγ subunit in U937IF cells

Anti-CBL immunoprecipitations performed on U937IF cell lysates were probed with anti-γ subunit antisera (Fig. 2C). We detected the presence of the FcγRIγ subunit, an ITAM-containing receptor subunit, in CBL immunoprecipitates (Fig. 2C, lanes 2–8). FcγRIγ subunit protein is detected readily in CBL immunoprecipitates from resting, PMA-, and FcγRI-stimulated cells (Fig. 2C, lanes 1–3). This indicates that FcγRIγ subunit protein is associated with CBL under conditions of FcγRI stimulation.
2–8) (16, 18, 23, 24). We previously reported that FcγRI stimulation induces a mobility shift on SDS-PAGE in the FcγRIγ subunit, forming γ₀ and γ₁ bands (16). Phosphoamino acid analysis demonstrated that this γ₀/γ₁ pattern is due to the FcγRI-induced tyrosine and serine/threonine phosphorylation of the γ₁ protein (16, 17). In particular, the γ₁ isoform is predominantly serine phosphorylated upon FcγRI stimulation in U937IF cells (16). The characteristic γ₀ and γ₁ bands of FcγRIγ on SDS-PAGE previously reported to occur after PMA and FcγRI stimulation were clearly observed in the CBL IPs (Fig. 2C, lanes 3 and 4). The pattern of γ₀ and γ₁ observed to coimmunoprecipitate with CBL in both resting and FcγRI-stimulated U937IF cells (Fig. 2C, lanes 2–7) is similar to the pattern observed in our anti-γ subunit IPs performed on FcγRI-stimulated cells, as previously described (16). Importantly, the detection of the coimmunoprecipitating FcγRIγ protein shown in Figure 2C, lanes 2 to 8, is blocked completely by preincubation of the immunoblotting antisera (5927) with a FcγRIγ-specific peptide (NQETYETLKHEKPPQ) (Fig. 3A, lane 2), confirming the identity of the coprecipitating γ subunit protein (16). The decreased quantity of FcγRIγ bound to CBL at 60 min after receptor stimulation shown in Figure 2C is not a consistent finding in all experiments performed (Fig. 2C, lane 8). We did not detect CBL or FcγRIγ in preimmune immunoprecipitations performed on the same lysates (Fig. 2, A and C, lane 1). These IPs showed strong background signal when probed with goat anti-rabbit secondary (Fig. 1B, lane 1; Fig. 2B, lanes 1 and 9); however, at multiple exposure times using ECL we did not observe a CBL-specific band in these blots nor did we observe the coprecipitation of FcγRIγ (Fig. 2C, lane 1).

Similar results were obtained when we performed anti-γ subunit immunoprecipitations using the 4D8 anti-γ mAb and probed these blots for the CBL protein (Fig. 4A, lanes 1–3). We previously reported that the 4D8 anti-γ subunit Ab coimmunoprecipitates SYK and FcγRIγ (24). We used a series of FcγRIγ-specific peptides to determine the binding specificity of the 4D8 mAb (Fig. 3, A and B). We discovered that the 4D8 mAb binds to a defined peptide within the FcγRIγ protein (Fig. 3, A and B) (SDGVYTLGLSTR) and that this peptide blocks the immunoprecipitation of γ by 4D8 and not IP by 5927 antisera (Fig. 3A, compare lane 7 to 3). Using this information, we designed a separate set of experiments to determine the specificity of the FcγRIγ-CBL interaction in U937IF cells (Fig. 4C). Immunoprecipitation performed with 4D8...
immunoblot performed on anti-FcγRI

Anti-FcγRI immunoprecipitation performed on U937IF cells after FcγRI stimulation. We incubated the lysates with different peptides (Table 3) to assess the specificity of the FcγRI immunoprecipitates. Lanes are as described in Fig. 2A. C. Specificity of FcγRIγ-cbl interaction. C represents separate experiment performed. An anti-cbl immunoblot was performed on anti-FcγRIγ subunit immunoprecipitates with 4D8 Ab and anti-cbl immunoprecipitates. Lanes are as described in Fig. 2A. C.

CBL protein (Fig. 4C, lane 5). The reasons for this result are unclear and under active investigation. The addition of peptide (NQETYETLHKEKPPQ) in lane 3 was observed to increase the coprecipitation of CBL and FcγRIγ (Fig. 4C, lane 3). The mechanism for this augmented CBL-γ coprecipitation in vitro is unclear. The preimmune lane in Figure 4C shows high background at the exposure used to demonstrate both CBL-specific bands in lanes 1 to 6; at earlier exposures we observe no CBL-specific bands in these preimmune IPs using the ECL system. The reciprocal IP of CBL and FcγRIγ (Figs. 2 and 4), combined with data from the peptide block experiments (Figs. 3 and 4), provide convincing evidence that CBL and FcγRIγ form a complex in vivo in myeloid cells.

**The CBL-GRB2 interaction is modulated during FcγRI stimulation**

Immunoprecipitation of CBL from resting and FcγRI-stimulated cells (Fig. 5, A and B) confirmed the tyrosine phosphorylation of CBL and the constitutive CBL-GRB2 association (Fig. 5C). In this and other experiments, we observe a small decrease in the amount of GRB2 protein bound to CBL under conditions of FcγRI stimulation at 1 and 5 min following receptor cross-linking (Fig. 5C, compare lanes 2–4; Fig. 6, lanes 1 and 2). The negative immunoblot in Figure 5, lane 5, occurs as result of short exposure time used to see mobility shift in CBL; at longer exposure, the positive control lysates show distinct CBL bands. We were interested in defining the modules of GRB2 (SH3 and SH2 domains) that associate with CBL under conditions of rest vs FcγRI stimulation (Fig. 5, A–D). GST fusion protein constructs representing the N- and C-terminal GRB2-SH3 domains and the GRB2-SH2 domain were used to characterize the in vitro binding of CBL to GRB2 during FcγRI stimulation of U937IF cells (Fig. 5D). Both the C-terminal GRB2-SH3 domain and the GRB2-SH2 domain bind CBL in resting cell lysates (Fig. 5D). Upon FcγRI stimulation, the C-terminal GRB2-SH3 domain no longer binds CBL present in the U937IF cell lysates. The tyrosine-phosphorylated CBL remained exclusively bound to the GRB2-SH2 domain in the FcγRI-stimulated myeloid cells (Fig. 5D). In these experiments, the N-terminal GRB2-SH3 domain was noted to bind a small quantity of the SOS molecule, but did not bind detectable levels of CBL in resting or stimulated cell lysates (Fig. 5D). Lane 5 of Figure 5, A and B, represents U937IF cell lysates from 1 × 10^6 cell equivalents loaded per lane in the CBL IP (lanes 1–4).

**CBL-GRB2 and SOS-GRB2 protein complexes are distinct in U937IF cells**

Based on the data shown in Figure 5D, we postulate that CBL tyrosine phosphorylation modulates the CBL-GRB2 interaction in vivo and that the physical interaction between CBL and GRB2 may regulate the capacity of GRB2 to bind SOS. We then asked whether the CBL-GRB2 protein-protein complexes are distinct from GRB2-SOS complexes in myeloid cells (Fig. 6). CBL immunoprecipitates were noted to contain GRB2, but no detectable SOS (Fig. 6, lanes 1 and 2). In contrast, GRB2 IPs contain SOS and minimal CBL (Fig. 6, lanes 5 and 6), and SOS IPs contain GRB2, but no CBL (Fig. 6, lanes 3 and 4). The quantity of GRB2 bound to CBL decreases following FcγRI stimulation (10–15% change) (Fig. 5C, lanes 3 and 4; Fig. 6, lanes 1 and 2), and CBL IPs contain several-fold greater amounts of GRB2 as compared with amount of CBL detected in GRB2 IP, suggesting that most of GRB2 in the cell is not bound to CBL. Although the CBL and SOS IP brought down similar amounts of GRB2, we were unable to
detect evidence of SOS and CBL in the same protein-protein complex (Fig. 6, compare lanes 1 and 2 with 3 and 4). The lack of detection of CBL coimmunoprecipitating with the anti-GRB2 antisera (Fig. 6, lanes 5 and 6) could be explained by the immunoreactivity of the polyclonal anti-GRB2 antisera used to IP GRB2 (C-231 binds to residues 197–217 in the C terminus of GRB2, the region mediating the GRB2-CBL interaction shown in Fig. 5D).

**Differential binding of CBL C terminus to GRB2 and CRKL adapter molecules**

Previous reports suggested that CBL can bind to CRKL and GRB2 in hemopoietic cells (25). To define the region of CBL-mediating constitutive binding of these adapter proteins in myeloid cell lysates, we divided the C terminus of CBL into two proline-rich
subdomains and prepared GST bacterial fusion protein constructs of each. Data from our GST-CBL pull-down experiments are shown in Figure 7. Our GST fusion constructs consisted of the entire C terminus of CBL protein (residues 461–906), the proximal C terminus (residues 461–620, containing a PPVPPR consensus), and the distal C terminus (residues 621–906, containing 2 YXX-PXXP motifs). These fusion proteins were purified using glutathione-Sepharose beads and used to characterize binding to the GRB2 and CRKL adapter proteins in U937IF cell lysates prepared from resting or FcγRI-stimulated cells. Equivalent amounts of GST (10 μg) or GST fusion proteins are incubated with cell lysates prepared from resting or FcγRI-stimulated cells. Bound proteins are resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with specific antisera in Western blot analysis (immunoblot Ab shown on left border of Fig. 7). Figure 7 shows the immunoblot analysis for CBL, SHC, CRKL, and GRB2 of proteins that bind to GST vs GST-CBL fusion constructs under conditions of rest or FcγRI stimulation. We demonstrate that the proximal CBL C terminus, CBL (PC), binds the GRB2 molecule (Fig. 7, lanes 5 and 6), whereas the more distal CBL C terminus, CBL (DC), selectively binds to CRKL (Fig. 7, lanes 7 and 8). The GST protein alone did not bring down CBL, SHC, GB2, or CRKL (Fig. 7, lanes 1 and 2), and CBL (PC) did not bind CRKL; the CBL (DC) did not bind GRB2. Interestingly, the presence of CRKL is required for the C terminus of CBL to bring down the endogenous CBL molecule in these pull-down experiments, suggesting that CRKL may serve as the molecular bridge between GST-CBL (DC) and endogenous CBL by virtue of its capacity to bind the CBL (DC) via CRKL-SH3 and endogenous CBL via the CRKL-SH2 domain. In contrast, the interaction of CBL with SHC only occurs following FcγRI stimulation and is mediated via the GB2 binding region of CBL (PC) region (Fig. 7, lanes 4 and 6). The SHC binding noted in Figure 7 correlates well with the kinetics of tyrosine phosphorylation of SHC and the interaction of tyrosine-phosphorylated SHC with the GRB2-SH2 domain (21) (data not shown). Lane 9 is a whole cell lysate of U937IF cells stimulated with FcγRI cross-linking used to test the integrity of the immunoblots.

**Discussion**

The identification and characterization of substrates for protein tyrosine kinases activated by ITAM-linked receptors will enhance our understanding of the signaling events that occur following engagement of these receptors. Evidence from the study of hematopoietic cells supports a role for CBL as a substrate for nonreceptor protein tyrosine kinases in FcγRI, B cell receptor, TCR, and integrin receptor signaling (8–11, 14, 26–28). Ota and Samelson reported in rat basophilic leukemia cells evidence that CBL N terminus may antagonize the activation of SYK kinase in the FcγRI signaling pathway (27). Matsuo et al. reported that cross-linking FcγRI receptor in THP-1 cells induces the tyrosine phosphorylation of CBL, and Tanaka et al. implicated CBL in murine FcγRI/FcγRII signaling in macrophages (14, 15). There are no reports of CBL tyrosine phosphorylation following FcγRI stimulation. In this work, we provide the first experiments implicating CBL and the CBL-GRB2 interaction in FcγRI signaling. CBL is tyrosine phosphorylated in resting IFN-γ-differentiated U937 cells and undergoes increased tyrosine phosphorylation (10-fold increase)
following FcγRI stimulation (Figs. 1A, 2A, and 5A). In other experiments, we observe that other GRB2-binding proteins, SLP-76, VAV, and SHC, are only tyrosine phosphorylated upon FcγRI stimulation (21) (unpublished observation), suggesting a specific role for the basal level of CBL tyrosine phosphorylation observed in myeloid cells. This basal level of CBL phosphorylation is also observed in primary cultures of human bone marrow-derived macrophages and non-IFN-differentiated THP-1 myeloid cells (data not shown). We have also performed similar biochemical experiments in IFN-starved U937 and THP-1 cells and in primary bone marrow-derived human macrophages with similar results, suggesting that the signaling events reported in this work are not the immediate consequence of IFN stimulation. Our data demonstrate that PMA induces a mobility shift in CBL under conditions in which CBL is dephosphorylated (Fig. 2, A and B, lane 3). This mobility shift is reversed 30 to 60 min following FcγRI stimulation (Fig. 2B, lanes 7 and 8). Other experiments show that potato acid phosphatase treatment of CBL IPs results in a dephosphorylation of CBL and a concomitant loss of the mobility shift, and that the omission of the tyrosine phosphatase inhibitors, phenylarsine oxide (PAO) and vanadate, from the EB lysis buffer results in tyrosine dephosphorylation of CBL with no alteration in the CBL mobility shift (unpublished observation). These data are consistent with the data of Liu et al. demonstrating that phosphorylation of two serine residues, S619 and S629, in the CBL C terminus serves as binding site for the 14-3-3 protein in T cells (26). We conclude that FcγRI receptor aggregation results in the tyrosine phosphorylation of CBL in myeloid cells. Additional analysis will be required to prove that CBL is a substrate for serine/threonine kinases activated by FcγRI cross-linking.

We sought additional lines of evidence for role for CBL in FcγRI signaling. We surmised that if CBL is directly involved in FcγRI signaling, the FcγRI receptor complex would contain significant amounts of CBL protein. Our data demonstrate that tyrosine-phosphorylated CBL is constitutively bound to FcγRI subunit in resting and stimulated U937IF cells (Fig. 2, A and C, lanes 2–8). The FcγRIγ-CBL interaction is confirmed using anti-γ and anti-CBL immunoprecipitations and peptides to block the immunoprecipitation of γ (4D8 Ab) (Figs. 3 and 4) and the detection of γ by S927 antisera in anti-CBL IPs (Figs. 2–4). We used a series of γ-chain-specific peptides to determine the binding site for the anti-FcγRIγ mAb (4D8) originally described by Schoeneich et al. (Fig. 3) (18), and then used the 4D8-specific peptide to confirm the specificity of the γ-CBL coprecipitation in U937IF cells (Figs. 3 and 4). In several systems, the ITAM-containing receptor subunit binds constitutively to the nonreceptor protein tyrosine kinase SYK (29–31), suggesting that the constitutive CBL-γ subunit interaction may be mediated by a multimeric protein complex containing the FcγRIγ subunit, SYK and CBL. The constitutive association of CBL with FcγRIγ in IFN-prime U937 cells is consistent with the data from our laboratory showing that SYK binds to the FcγRIγ subunit in a constitutive manner (22). Constitutive binding of SYK to the ITAM motif occurs in both platelets and B cells (31, 32). The data of Iwashima et al. demonstrate that the tyrosine phosphorylation of SYK or ZAP-70 is not required for their association with ITAM receptor subunits (30). The aggregation of the FcγRI receptor complex activates SYK kinase activity, which may result in the tyrosine phosphorylation of CBL (22, 33). Lupher et al. reported that CBL contains a PTB motif capable of an inducible binding to the tyrosine-phosphorylated ZAP-70 kinase in activated T cells (34). These data along with the report of Fournel et al., demonstrating that ZAP-70 and SYK can bind to CBL and that CBL is a substrate for SYK in COS cells, support a potential interaction between SYK and CBL in FcγRI signaling (35). Ota and Samelson reported that CBL interacts with the SYK kinase, alters SYK-γITAM signaling, and regulates γITAM function in myeloid cells (27). From these combined data, we conclude that CBL is associated with FcγRIγ subunit and that the CBL-GRB2 interaction functions in FcγRI signaling in myeloid cells. Our preliminary experiment and the results of Ota et al. (27) suggest that the CBL-FcγRIγ interaction is not direct and that an indirect FcγRIγ-SYK-CBL interaction exists in myeloid FcγRI signaling.

The GRB2-SOS interaction is a critical event in the activation of RAS in many cell types (36–38). Previous studies from our laboratory implicated SHC, GRB2, RAF-1, and MAP kinase in FcγRI signaling, suggesting a role for RAS in this signaling pathway (21). In hemopoietic cells, four major GRB2-binding proteins undergo rapid tyrosine phosphorylation upon ITAM stimulation: 1) CBL, 2) SLP-76, 3) LNK, and 4) VAV (33, 39–42). The role of these complex adapter proteins in ITAM signaling and the molecular consequences of their tyrosine phosphorylation and binding to GRB2 or other adapter proteins (CRK, CRKL, NCK, SHC) remain to be determined. Buday et al. reported upon T cell activation, CBL rapidly dissociates from GRB2 and binds to CRKL (25). This work demonstrated that the capacity of CBL to bind to the N- and C-terminal GRB2 SH3 domains in vitro is strongly reduced in activated T cells. We performed experiments with GST fusion proteins representing different modular domains of the GRB2 molecule to test the hypothesis that phosphorylation of CBL could alter its interaction with GRB2 following FcγRI stimulation in myeloid cells (Fig. 5D). We observed at a very early time point following FcγRI stimulation, a qualitative change in the interaction between the domains of the GRB2 molecule and CBL in vitro (Fig. 5D). In U937IF cells, CBL is constitutively bound to GRB2 (Fig. 5C). Our in vitro data demonstrate that this interaction is mediated via the combined GRB2-SH3 and GRB2-SH2 domains (Fig. 5D). The GST-GRB2 fusion constructs used in these experiments bind to a free pool of CBL not already complexed to GRB2 in the U937IF lysates. Importantly, this pool of cellular CBL that binds to GRB2 in our in vitro experiments would not be the same species of CBL bound to the GRB2-SH3 and SH2 domains in vivo. Our results therefore reflect an altered potential for interaction between CBL and GRB2 under conditions in which free CBL in the lysate becomes tyrosine phosphorylated. Tyrosine phosphorylation of CBL is associated with the decrease in GRB2-SH3-CBL interaction, leaving CBL bound to the GRB2-SH2 domain (Fig. 5D). Tyrosine-phosphorylated CBL could be conformationally altered such that it will not bind to the GRB2-SH3 domain. Alternatively, CBL tyrosine phosphorylation may result in the binding of CBL to another molecule, thereby preventing the CBL-GRB2-SH3 interaction. The data reported by Buday et al. in T cells demonstrate that upon TCR activation, CBL is tyrosine phosphorylated and the GRB2-SH2-CBL interaction is reduced dramatically at the same time that the CBL-CRKL complex is augmented (25). In our experiments, the CBL-GRB2 interaction is qualitatively altered (Fig. 5D) with mild reduction in the total quantity of CBL-GRB2 binding (Fig. 5C, lane 3; Fig. 6, lanes 1 and 2).

Other laboratories have observed the binding of CBL to GRB2-SH2 domain, but the mechanism and importance of this binding are not understood (5). Experiments performed in C. elegans support a model by which the slt-1 homologue of CBL modulates signaling events through its direct interaction with the GRB2 molecule (3). The SLT-1 protein contains a conserved consensus GRB2-SH2 binding motif (YXNX) and directly binds the GRB2 homologue in C. elegans. In contrast, mammalian CBL does not contain a direct binding site for the GRB2-SH2 domain and most likely interacts via another phosphoprotein in our system.
CBL, GRB2, and SOS in myeloid cells. It is of course possible that different lysis buffer conditions will support the detection of trimolecular complex between CBL, GRB2, and SOS in U937IF cells. It is also plausible that the CBL-GRB2 interaction could function in a parallel pathway for the activation of RAS via a nucleotide exchange factor other than SOS, or that the CBL-GRB2-SOS trimolecular complex is unstable and difficult to detect in FcγRII signaling. These possibilities are currently under exploitation in our laboratory to understand the role of CBL and CBL-GRB2 interaction in the regulation of RAS in myeloid cells.

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