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Ephrin-A1 Induces c-Cbl Phosphorylation and EphA Receptor Down-Regulation in T Cells

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Eph receptor tyrosine kinases are expressed by T lineage cells, and stimulation with their ligands, the ephrins, has recently been shown to modulate T cell behavior. We show that ephrin-A1 stimulation of Jurkat T cells induces tyrosine phosphorylation of EphA3 receptors and cytoplasmic proteins, including the c-cbl proto-oncogene. Cbl phosphorylation was also observed in peripheral blood T cells. In contrast, stimulation of Jurkat cells with the EphB receptor ligand ephrin-B1 does not cause Cbl phosphorylation. EphA activation also induced Cbl association with Crk-L and Crk-II adapters, but not the related Grb2 protein. Induction of Cbl phosphorylation upon EphA activation appeared to be dependent upon Src family kinase activity, as Cbl phosphorylation was selectively abrogated by the Src family inhibitor 4-amino-5(4-chlorophenyl)-7-(tert-butyl)pyrazolo[3,4-d]pyrimidine, while EphA phosphorylation was unimpaired. Ephrin-A1 stimulation of Jurkat cells was also found to cause down-regulation of endogenous EphA3 receptors from the cell surface and their degradation. In accordance with the role of Cbl as a negative regulator of receptor tyrosine kinases, overexpression of wild-type Cbl, but not its 70-Z mutant, was found to down-regulate EphA receptor expression. Receptor down-regulation could also be inhibited by blockade of Src family kinase activity. Our findings show that EphA receptors can actively signal in T cells, and that Cbl performs multiple roles in this signaling pathway, functioning to transduce signals from the receptors as well as regulating activated EphA receptor expression. The Journal of Immunology, 2003, 170: 6024–6032.

Immunology and Allergy, Department of Pediatrics, Infection, Immunity, Injury, and Repair Program, Research Institute, Hospital for Sick Children, and University of Toronto, Toronto, Canada

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3 Address correspondence and reprint requests to Dr. Chaim M. Roifman, Division of Immunology and Allergy, Department of Pediatrics, Infection, Immunity, Injury, and Repair Program, Research Institute of Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8. E-mail address: croifman@sickkids.on.ca

4 Abbreviations used in this paper: RTK, receptor tyrosine kinase; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; PBL-T, peripheral blood T cells; PDGF, platelet-derived growth factor; PI3-kinase, phosphoinositide 3-kinase; PP2, 4-amino-5(4-chlorophenyl)-7-(tert-butyl)pyrazolo[3,4-d]pyrimidine.

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Inducible Cbl phosphorylation was also observed in peripheral blood T cells, which selectively express EphA1 and EphA4 receptors. EphA receptor activation also found to alter Cbl interactions with the signal transduction adapters Crk-L and Crk-II, but not the related Grb2 protein. Induction of Cbl phosphorylation upon ephrin-A stimulation, but not Eph receptor activation, was found to be specifically abrogated by the Src family inhibitor 4-amino-5-(4-chlorophenyl)-7-[(tert-butyl)pyrazolo[3,4-d]pyrimidine (PP2), suggesting that Src family kinases may play a role in RTK-mediated activation of Cbl.

c-Cbl is a member of a family of related scaffold proteins (sl-1, d-Cbl, c-Cbl, Cbl-b, and Cbl-3) (19, 20) and undergoes tyrosine phosphorylation in response to activation of a number of receptors, including the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) tyrosine kinase receptors (21, 22). Upon activation, c-Cbl can associate with a variety of signaling molecules, including the tyrosine kinases Zap-70, Syk, and Src family kinases; the adapter proteins Crk, Shc, and Grb2; and effectors such as p85 PI3-kinase and Vav (19, 20, 23, 24). The function of Cbl in these complexes remains ambiguous, but it is suggested to act as a scaffold, serving to juxtapose these disparate signaling molecules.

Cbl can also induce physical down-regulation of activated EGF, PDGF, and CSF receptors from the plasma membrane through induction of receptor ubiquitination, targeting them for internalization (19, 25–27). This activity is derived from its Ring finger domain, an E3 ubiquitin-ligase (28, 29). This appears to form a negative feedback loop regulating the intensity and duration of receptor signaling. In accordance with the known properties of Cbl as a regulator of RTKs, we show here that cell surface expression of endogenous EphA receptor decreases following ephrin-A1 stimulation of Jurkat cells, and proteasome-dependent degradation of the EphA3 receptor is observed. EphA4 receptor expression could also be down-regulated in transfected cells by overexpression of wild-type Cbl, but not of a ubiquitin-ligase inactive 70-Z Cbl protein.

Materials and Methods

Abs and recombinant proteins

Monoclonal anti-phosphotyrosine was obtained from Upstate Biotechnology (Lake Placid, NY). Abs to Cbl, Crk-L, Crk-II, Grb2, Myc, and Fyn were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ephrin-human Fc chimeric fusion proteins were purchased from R&D Systems (Minneapolis, MN). Purified human IgG was purchased from Serotec (Oxford, U.K.).

Cell lines and isolation of T lymphocytes

The mature T cell line Jurkat was grown in complete RPMI-10 medium. PBL were purified from fresh peripheral blood by centrifugation on Ficoll and were cultured with PHA and IL-2 for 3 days in complete RPMI-10 medium before transfer to RPMI-10 with IL-2. Suspension cells were collected after 6–8 days of culture. The resultant T cell lymphocytes are typically >95% CD3⁺.

RT-PCR analysis

Total RNA was purified using RNAeasy (Qiagen, Valencia, CA). First-strand cDNA was made with random primer and Superscript II (Invitrogen, San Diego, CA). The following primer pairs were used for analysis of Eph receptor expression, creating PCR products each of ~400 bp: EphA1, gaagcttcggaactgctg and ctcgactactctgaactgcaag; EphA2, cttgaccc cgctgtgctct and gaagcttcggaacctgactggt; EphA3, cttgaccc gcgctctcgaagtcgag and cgcggagctcgaactcaac and gaagg caagcttgctcgcgctgc; and EphA7, gaagcttcggaactgactgcaag and cacccacttagcgaactctctctctcagc; PCRs were performed with the cycle conditions 94°C for 30 s, 60°C for 30 s, and 68°C for 45 s for 35 cycles.

T cell stimulation

Soluble ephrin-Fc fusion proteins (dimerized) were precomplexed by incubation on ice with F(ab)₂ goat anti-human Fc (Pierce, Rockford, IL) to form soluble oligomers. F(ab)₂ goat anti-human Fc was used as a control where necessary. Irrelevant Fc-fusion protein or purified human IgG were used as specificity controls.

Pull-down assays, immunoprecipitation, and Western blotting and kinase assays

For immunoprecipitation, cells were resuspended in ice-cold 1% Triton lysis buffer consisting of 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 1 mM sodium orthovanadate. Abs and protein G-Sepharose were added to cleared lysates and incubated at 4°C overnight. For ligand pull-down assays, cells were incubated on ice for 30 min with the ephrin-Fc ligands before transfer to 37°C for the indicated times. Triton X-100 was replaced with 1% IGA-230, and ligand was precipitated by addition of protein G-Sepharose beads. Samples were separated by SDS-PAGE and transferred to nitrocellulose membrane, and immunoblotting Abs were added in PBS, 0.1% Tween 20. Bound Abs were detected using HRP-conjugated Abs and chemiluminescent reagents. All experiments were repeated at least three times.

Transfection

293T cells were transiently transfected with Myc-tagged EphA4 (0.2 µg), Cbl, and 70-Z Cbl (2 µg each) cDNA in pcDNA3 vector as indicated using Lipofectamine (Invitrogen). The amounts of transfected DNA were equalized with empty pcDNA3. Cells were harvested after 48 h.

Eph receptor down-regulation assays

Jurkat cells (5 × 10⁶) in serum-free RPMI were incubated at 37°C with a saturating concentration of ephrin-A1-Fc (10 µg/ml) or control IgG protein for 30 min. Cells were stripped of bound ligand by acid wash with RPMI (pH 3.0) for 30 s and washed three times in ice-cold complete RPMI-10 before resuspension in cold PBS. Cells were stained with 10 µg/ml ephrin-A1-Fc for 1 h on ice, washed three times in PBS, and incubated on ice with PE-labeled anti-Fc. Bound ligand was detected by flow cytometry. To further analyze changes in cells expressing the highest EphA levels, the 25% most intensely ephrin-A1-staining cells were gated and analyzed in parallel to the total population. In some experiments cells were preincubated with the Src family inhibitor PP2 (5 µM) or the inactive analog PP3 at 37°C.

Results

Ephrin-A stimulates EphA receptor activation and tyrosine phosphorylation in T cells

RT-PCR analysis of Jurkat T cells demonstrated the presence of mRNA for EphA1, EphA2, and EphA3 receptors (Fig. 1A), while analysis of purified peripheral blood T cells (PBL-T) revealed preferential expression of EphA1 and EphA4. Incubation of both Jurkat cells and PBL-T on ice with soluble ephrin-A-Fc chimeric protein, followed by staining with FITC-labeled anti-Fc to detect bound ligand, revealed strong staining with ephrin-A1 to -A5 upon flow cytometric analysis (not shown), demonstrating the expression of functional EphA receptors at the cell surface.

To examine EphA receptor signaling. Jurkat cells were stimulated with oligomeric ephrin-A1 or ephrin-A3 for 15 min at 37°C. This resulted in the appearance of a strongly phosphorylated band of ~125 kDa in anti-phosphotyrosine blots of total cell lysates and minor bands of 100 and 65 kDa (Fig. 1B). Control stimulations with IgG did not induce tyrosine phosphorylation (not shown). Members of the ephrin-A subclass demonstrate highly degenerate binding to EphA receptors (1, 2), and therefore, the similarity of responses to ephrin-A1 and ephrin-A3 was not unexpected. The dominant phosphorylated 125-kDa band appeared to represent ligand-induced EphA receptor phosphorylation, being of approximately the expected m.w. to be a member of the EphA subfamily (1, 2). The p125 phosphorylation was induced rapidly, was maximal after 10–15 min, and declined to a near basal level by 60 min (Fig. 1B, right panel). In the absence of Abs capable of immunoprecipitating many EphA receptors, it is difficult to examine endogenous receptor activation. However, we used the previously demonstrated ability of ephrin-A ligands to pull-down EphA receptors under permissive lysis conditions (18). Cells were incu-
125-kDa phosphoprotein was recognized by Abs to EphA3 (Fig. 2B). EphA1 was not detected in ephrin-A1 pull-downs despite attempts to Western blot with several different Abs to the receptor. However, as Ephs may act as heteroreceptor complexes, we cannot rule out the possibility that EphA1 is present, but undetectable due to the limited sensitivity of the assay. In accordance with the degeneracy of ephrin-A binding to EphA receptors, the 125-kDa EphA3 receptor could be specifically precipitated by all the ephrin-A ligands examined (not shown). All the EphA receptors are reported to be catalytically active tyrosine kinases (2), and in vitro kinase assays performed with ephrin-A pull-downs revealed strong phosphorylation of the 125-kDa protein consistent with autophosphorylation (Fig. 2C).

**Ephrin-A stimulation induces c-Cbl phosphorylation**

There were several candidates for the 100-kDa phosphorylated protein observed in ephrin-A1-stimulated total cell lysates. One of them, Cas, lies downstream of EphA receptors in NIH-3T3 cells (30). Although Cas undergoes ephrin-A1-inducible tyrosine phosphorylation in 3T3 cells, Cas phosphorylation was not detected upon ephrin-A1 stimulation of Jurkat (not shown). A second candidate was the proto-oncogene c-cbl. Many receptor tyrosine kinases, such as the EGF and PDGF receptors, activate c-Cbl through phosphorylation (22). Immunoprecipitation of Cbl revealed a rapid induction of tyrosine phosphorylation upon ephrin-A1 stimulation (Fig. 3A). Cbl phosphorylation could be detected within 30 s (not shown), peaking at levels 2- to 3-fold higher than basal at 10 min. Similar levels of Cbl phosphorylation (1.5- to 2.5-fold increase) could also be induced by ephrin-A2 and ephrin-A3 (Fig. 3B). Cbl phosphorylation was not, however, altered in response to stimulation with ephrin-B1 (Fig. 3C), although Jurkat express mRNA for multiple EphB receptors (EphB1–4 and 6, not shown).

To determine whether ephrin-A1-induced Cbl phosphorylation was restricted to cell lines or could also occur in primary T cells, we examined whether ephrin-A1 could stimulate Cbl phosphorylation in PBL-T. RT-PCR analysis of EphA receptors in PBL-T revealed preferential expression of EphA1 and EphA4, rather than the EphA1 and EphA3 observed in the mature T cell line Jurkat (Fig. 1A). However, ephrin-A1 stimulation similarly resulted in c-Cbl phosphorylation (Fig. 3D). Induction of Cbl phosphorylation was lower in primary T cells (consistently a 1.5-fold increase by densitometry) than in Jurkat cells, but this was probably due to a
lower level of EphA receptor expression on primary T cells (as assessed by flow cytometry, not shown). Whether EphA1, commonly expressed by Jurkat and PBL-T, is alone responsible for activating Cbl, or whether many EphA receptors induce Cbl phosphorylation remains to be determined.

**Src family kinase inhibitor, PP2, blocks EphA-mediated Cbl phosphorylation, but not EphA activation**

Cbl phosphorylation in hemopoietic cells often appears to result from activation of soluble cytoplasmic kinases, most particularly members of the Src family (31–33). We therefore examined whether Src kinases might play a role in EphA receptor-induced Cbl phosphorylation.

Interaction between the T cell Src family kinase Fyn and Cbl is well described in response to Ag receptor and cytokine stimuli. Fyn has been shown to bind Cbl both through its SH3 domain and in a phosphorylation-dependent manner via its SH2 domain. Anti-phosphotyrosine blotting of Fyn immunoprecipitates from ephrin-A1-stimulated Jurkat cells revealed increased association of a 100-kDa Cbl-like phosphoprotein (Fig. 4A), although reblotting with anti-Cbl was unable to unambiguously confirm the identity of the protein as Cbl. Fyn catalytic activity was also found to increase upon ephrin-A1 stimulation (not shown), suggesting that Fyn could indeed play a role in phosphorylation of proteins downstream of the activated EphA receptor. A role for Src family kinases in Cbl activation was further suggested when pretreatment of Jurkat T cells with the Src family-specific kinase inhibitor PP2, but not its inactive analog PP3, was found to inhibit ephrin-A1-induced Cbl phosphorylation (Fig. 4B). PP2 is reported to be a highly specific inhibitor of Src family members (34), and EphA phosphorylation, resulting predominantly from autophosphorylation, was unaltered in its presence (Fig. 4B, right panel), confirming the specificity of PP2. This inhibition of Cbl phosphorylation was specific to PP2, as neither PP3, the Syk family inhibitor piceatannol, nor the MAP kinase kinase inhibitor PD 98059 affected the induction of Cbl phosphorylation (Fig. 4C), suggesting that Src family kinases are indeed involved in ephrin-A1-induced Cbl phosphorylation.

**Ephrin-A1 stimulation regulates the role of c-Cbl in signal transduction**

There are at least two distinct aspects to c-Cbl function in T cells, one being a central role in connecting downstream signaling pathways (19, 23). Cbl appears to act as a scaffold for the assembly of a large complex of signal transduction proteins. We therefore examined the influence of ephrin-A1 stimulation on a number of well-described Cbl interactions. One of these is with the adapter protein Grb2 (35–38). Grb2 was immunoprecipitated from ephrin-A1-stimulated Jurkat cells, and the association of tyrosine-phosphorylated proteins was examined by Western blotting. Ephrin-A1 stimulation resulted in increased association of a 100-kDa phosphoprotein in Crk-L immunoprecipitates (Fig. 5A, left panel), which was confirmed as Cbl by Western blotting with anti-Cbl Abs (Fig. 5A, right panel). While Cbl association with Grb2 increased 2- to 2.5-fold, it was not present in control immunoprecipitation with irrelevant IgG (not shown). The correlation between Cbl/Grb2 interaction and Cbl phosphorylation is in accordance with binding mediated through the recognition of phosphorylated Cbl tyrosine residues by an SH2 domain of Grb2-L as previously described (35, 36). Tyrosine phosphorylation of Grb2-L itself (42 kDa) could not be detected. Jurkat cells also express the closely related Grk-II gene product, and ephrin-A1 induced similar binding of pp100 to Grk-II (Fig. 5B; a similar 2-fold increase, confirmed as Cbl by direct blotting with anti-Cbl, not shown). Cbl also undergoes a well-characterized interaction with the adapter protein Grb2 in response to certain stimuli (37, 39, 40). However, while basal association of Grb2 with a 100-kDa phosphorylated protein was evident, no increase in the phosphorylation of this protein was observed upon ephrin-A1 stimulation (Fig. 5C), suggesting that the Grb2-Cbl complex may not participate in EphA receptor signaling. In addition to modulating Cbl association with the Grb2 proteins, ephrin-A1 stimulation was found to alter interaction with p85 PI3-kinase and Vav (not shown), suggesting that EphA receptor activation may regulate many transduction pathways through Cbl.

**FIGURE 3.** Ephrin-A stimulation induces Cbl phosphorylation. **A**, Jurkat cells (10⁷) were stimulated with 1 μg/ml ephrin-A1-Fc for the indicated times. Cbl was immunoprecipitated from cell lysates in 1% Triton and blotted with anti-phosphotyrosine. Loading was controlled by reblotting with anti-Cbl. **B**, Cells were stimulated with ephrin-A1 to -A3 as indicated and analyzed as described in A. **C**, Jurkat cells were stimulated with ephrin-B1 for 10 min, and Cbl phosphorylation was examined as described in A. All experiments were repeated at least three times. **D**, PBL-T (5 × 10⁶) from two unrelated individuals were stimulated with 1 μg/ml ephrin-A1 for the indicated times, and Cbl phosphorylation was examined as described in A. Matching lysates were blotted with anti-Cbl to control protein loading.

![Image](https://example.com/image.png)
EphA receptors undergo down-regulation from the cell surface upon ephrin-A1 stimulation

c-Cbl has a second important function in addition to a role in signal transduction. Cbl is known to be responsible for the physical down-regulation of receptor tyrosine kinases from the cell surface, including the EGF, PDGF, and CSF receptors (27, 41, 42). This would appear to form part of a negative feedback loop regulating the intensity and duration of receptor signaling. The down-regulation of receptors requires receptor kinase activity (27) and may be dependent upon phosphorylation of Cbl (25), suggesting that the EphA receptors could be subject to Cbl-mediated down-regulation.

We were unable to coimmunoprecipitate EphA receptor and Cbl from Jurkat cells, either because of the absence of an effective immunoprecipitating Ab to EphA3 or because of a relatively low level of receptor expression. However, a constitutive complex could be isolated by coexpression of EphA receptor and Cbl in 293T cells (Fig. 6A). Furthermore, overexpression of wild-type Cbl, but not the 70-Z Cbl mutant, was found to decrease EphA expression, although they bound the receptor equally well. 70-Z Cbl has a functional extended SH2 domain, allowing it to bind activated receptors, but contains a deletion in its ubiquitin ligase ring finger domain, rendering it unable to induce RTK ubiquitination and, thus, down-regulation (26, 43). 70-Z Cbl did not decrease EphA expression, but, rather, elevated it, further suggesting that activated EphA receptors undergo degradation as the result of interaction with wild-type Cbl.

To assess the potential for endogenous EphA receptor down-regulation in Jurkat T cells, we analyzed cell surface expression of EphA receptors by ligand binding assay. Jurkat cells were incubated with a saturating concentration of ephrin-A1-Fc (10 μg/ml) or control IgG protein for 30 min at 37°C to induce receptor down-regulation. Cells were then stripped of bound ligand by acid wash (RPMM, pH 3.0) and stained with ephrin-A1-Fc ligand for 1 h on ice to detect EphA receptor expression. Bound ligand was detected by a secondary incubation on ice with PE-labeled anti-Fc and subsequent flow cytometric analysis. EphA receptor expression was consistently decreased following incubation at 37°C with ephrin-A1, but not with control protein (Fig. 6B). This was most notable when the cells initially displaying the highest levels of EphA receptor expression were examined separately (~25% of total), where an ~80% drop in EphA receptor surface expression was seen (Fig. 6B, right panel). Down-regulation of EphA receptors reached a plateau after ~40 min (Fig. 6C), roughly paralleling the disappearance of phosphorylated EphA receptor from total cell lysates (Fig. 1C). Control experiments using incubation with ephrin-A1 on ice did not show altered EphA receptor expression (not shown). Interestingly, preincubation with the Src family inhibitor PP2, but not PP3, was also found to block ephrin-A1-induced EphA
FIGURE 6. EphA receptor cell surface expression is down-regulated following ephrin-A1 stimulation. A, 293T cells were transiently transfected with Myc-tagged EphA4, Cbl, and 70-Z Cbl cDNA in the pcDNA3 vector. EphA4 was immunoprecipitated with anti-Myc, and samples were blotted with anti-Cbl and anti-Myc as indicated. Matching lysates were blotted with anti-Cbl to determine the expression of transfected Cbl proteins and anti-Giα to control protein loading. One representative experiment of four is shown. B, Jurkat cells (5 x 10⁶) were incubated with 10 μg/ml ephrin-A1-Fc or control IgG protein for 30 min at 37°C. Cells were then stripped of bound ligand by acid wash before resuspension in cold PBS for staining. Cells were stained on ice with 10 μg/ml ephrin-A1-Fc and incubated with PE-labeled anti-Fc, and bound ligand was detected by flow cytometry. The average of three independent experiments is shown with SEs. Right panel, Cells initially displaying the highest levels of EphA receptor expression were examined separately (25% of total) and demonstrated an ~80% drop in EphA expression. C, Cells were treated with 10 μg/ml ephrin-A1-Fc or control IgG protein for the indicated periods of time at 37°C, and receptor expression was analyzed as described in B. The average of three experiments is shown. D, Jurkat cells were pretreated with the Src family inhibitor PP2 or inactive PP3 for 37°C for 30 min before addition of 10 μg/ml ephrin-A1 or control IgG for an additional 30 min. Receptor expression was analyzed as described in B. E, Jurkat cells were stimulated with ephrin-A1 or control IgG as indicated for up to 60 min at 37°C. Cells were lysed, 1 μg of ephrin-A1-Fc was added to each stimulated sample, and EphA3 receptor precipitated by Fc ligand pull-down. Receptor expression was revealed by blotting with anti-EphA3. Specificity was controlled by control immunoprecipitation with IgG. Lower panel, Sample loading was controlled by blotting matching lysates with anti-Giα. F, Cells were pretreated with the proteasomal inhibitor MG-132 (50 μM) for 30 min before stimulation with ephrin-A1 at 37°C. EphA3 expression was then examined by pull-down and blotting with anti-EphA3 and was controlled by blotting matching lysates with anti-Erk2. One representative experiment of three is shown.
down-regulation (Fig. 6D), suggestive of a requirement for Src kinase activity similar to that for induction of Cbl phosphorylation.

The coexpression of EphA4 and Cbl in HEK-293 cells was observed to decrease receptor protein expression. Similarly, the level of EphA3 protein expression in Jurkat cells was found to decrease upon stimulation with ephrin-A1, but not control protein (Fig. 6E), suggesting that the EphA3 receptor was not only removed from the cell surface, but subsequently degraded. Indeed, pretreatment with the proteasomal inhibitor MG-132 prevented ligand-induced disappearance of EphA3 (Fig. 6F).

**Discussion**

Our finding that EphA activation induces Cbl phosphorylation brings these receptors into a larger group of tyrosine kinase receptors demonstrating the ability to regulate and be regulated by Cbl. These observations also add to our understanding of EphA receptor signaling and function in cells of the immune system and furthermore may reveal a basis by which EphA receptors can cross-talk with other T cell-expressed receptors, most importantly the Ag and chemokine receptors. Eph costimulation has recently been shown to modulate T cell responses to activation of both of these receptors (8, 9, 11). As the TCR and chemokine receptors both regulate Cbl phosphorylation and function (35, 37, 44, 45), the ability to modulate Cbl and its associated proteins may permit EphA receptors to influence the activation of these signaling pathways.

While EphA receptor activation was observed to induce rapid tyrosine phosphorylation of Cbl in Jurkat cells, interestingly Cbl phosphorylation was not induced by ephrin-B1. This is despite the presence of multiple EphB receptors in Jurkat cells and suggests that EphBs may not be subject to regulation by Cbl. Previously, we have shown that the EphB6 receptor, but not EphB1, can bind Cbl in transfected 293-T cells (16), but were unable to observe any effect on Cbl phosphorylation. Luo et al. (8) also examined the linkage between EphB6 and Cbl using Jurkat cells, demonstrating that ligation of EphB6 with specific Ab caused a reduction in basal Cbl phosphorylation. We did not observe a decrease in Cbl phosphorylation upon stimulation of Jurkat with ephrin-B1, although this may reflect the different natures of ephrin-B and Ab stimuli, one activating multiple EphB receptors and the other EphB6 alone.

To date, the potential for EphA down-regulation has received little attention, although down-regulation of the ephrin-A ligand upon engagement by EphA receptor has been reported to occur as the result of proteolytic cleavage of the cell surface-expressed ligand (46). We observed down-regulation of EphA receptors from the surface of Jurkat cells following ephrin-A1 stimulation, with kinetics and magnitude of receptor down-regulation similar to those previously reported for other RTKs, such as the EGF receptor (25, 26). The PDGF, EGF, and CSF receptor tyrosine kinases all induce Cbl phosphorylation and undergo down-regulation as the result of Cbl-mediated ubiquitination (27, 41, 42). Although we could not unambiguously detect association between Cbl and the EphA3 receptors in Jurkat cells, a tyrosine-phosphorylated protein of the correct m.w. was observed in ephrin-A pull-downs upon long exposure of anti-phosphotyrosine Western blots (not shown). As with many RTKs, the isolation of the Cbl-EphA receptor complex required overexpression of both proteins, and we were readily able to identify EphA4-Cbl complexes by cotransfection in HEK-293 cells. Most recently, Wang et al. (47) have also demonstrated EphA2-Cbl association by coexpression in these cells. However, it is not clear that all members of the EphA receptor family should bind Cbl and be subject to similar Cbl-mediated regulation. Cbl binding is known to be selective in the ErbB family of RTKs, with subsequent differences in the regulation of activation and expression (27), and we have previously demonstrated that although EphB6 binds Cbl, EphB1 does not (16). As the site of Cbl binding to EphA receptors has not been identified, association with the different subfamily members is not yet predictable.

While Wang et al. (47) observed ligand-inducible EphA2-Cbl association, they did not report ligand-inducible receptor down-regulation or induction of Cbl phosphorylation. However, it is possible that the preactivated status of the receptors due to persistent contact with neighboring cell-expressed ephrins precludes the observation of these events. Alternatively, the different natures of the relatively immobile adherent epithelial cell and the mobile lymphoid cells may entail different modes of EphA receptor regulation in response to engagement by ephrin-A ligand.

The ability to block ephrin-A1-induced Cbl phosphorylation in Jurkat cells by inhibiting Src family kinase activity with PP2 was surprising. This suggests that either the EphA receptor kinase domain is not responsible for Cbl phosphorylation, or alternatively, that ligand-induced, Src family kinase-mediated phosphorylation is required for EphA-Cbl interaction. Although this observation relies upon the use of a synthetic kinase inhibitor, PP2, and the closely related PP1 are considered to be specific inhibitors of the Src family (34, 48–50) and are commonly used to examine the role of Src family kinases. Providing indirect support for a role for Src family kinase(s) in ephrin-A1-induced Cbl phosphorylation, Fyn kinase activity was observed to be increased following ephrin-A1 stimulation (not shown) and a phosphorylated 100-kDa protein consistent with Cbl was also observed to coimmunoprecipitate with Fyn. It has been suggested that the Src family kinases Fyn and Yes may play a major role in Cbl phosphorylation, while the related Lck phosphorylates Cbl inefficiently (33). In agreement, pp100 was not observed in Lck immunoprecipitates from ephrin-A1-stimulated Jurkat cells, and an absence of Lck expression in the J.Cam.1.6 cell line did not appear to hinder ephrin-A1-induced Cbl phosphorylation (not shown). Our observations are clearly akin to the recent findings of Kassenbrock et al. (51), who demonstrated a role for Src family kinases in EGF-induced phosphorylation of Cbl using the PP1 inhibitor. PP1 inhibited Cbl activation, but not phosphorylation of the EGF receptor itself, observations clearly similar to our findings. While Kassenbrock et al. (51) were also able to observe abrogation of EGF receptor-Cbl association upon PP1 treatment, their observations similarly do not yet permit us to determine the precise role of Src family-mediated phosphorylation.

While the role of Cbl in regulating the expression of activated RTKs is beginning to be reasonably well understood, its precise role in signal transduction is unclear, although interaction with a variety of signaling proteins has been observed. Currently, its role is defined as that of a scaffold protein, serving to juxtapose other proteins in a spatially and temporally regulated manner (23, 24, 43). Following Ag receptor stimulation of T cells, Cbl undergoes rapid tyrosine phosphorylation and associates with the Src homology domain-containing adapters Grb2, Crk, and Crk-L (36, 37, 44). In contrast, ephrin-A1 stimulation of Jurkat cells appeared to result in the association of phosphorylated Cbl with Crk proteins, but not Grb2. Cbl has been suggested to sequester Grb2 away from Sos, thus inhibiting Ras activation (52), and EphA activation has indeed been reported to inhibit Ras-mediated MAPK activation in neuronal and endothelial cells (17, 18). We did not, however, observe decreased MAPK activity in Jurkat cells upon ephrin-A1 stimulation (data not shown), perhaps consistent with unchanged Grb2 association.

While Crk-II was recently shown to link EphA3 activation to a cellular retraction and detachment response in adherent 293T cells (53), the potential function of Crk-L in lymphoid cells in response to ephrin-A stimulation is uncertain. Through the ability to modulate Crk interactions, EphA receptors could potentially regulate...
any aspects of T cell function. Cbl is the predominant phospho-
protein associated with the Crk proteins in activated lymphocytes and in response to Ag receptor stimulation, and this complex appears to have multiple functions (36, 54). The formation of a ter-
nary complex of Cbl-Crk-L and c3G, a guanine nucleotide ex-
change factor for the small G protein Rap1, has been reported upon
Ag receptor stimulation of T cells (24, 35, 55). The role of Rap1
activation is unclear and controversial, but it may antagonize Ras
activation. Crk also associates with p130Cas; this complex plays an important role in regulating the attachment and migration of adherent cells (56). Carter et al. (30) have shown recently that EphA receptor stimulation modulates cytoskeletal rearrangement through Cas in NIH-3T3 cells. In T cells the complex of p105Cas-L and Crk plays an important role in β1 integrin signaling, regulating migration on fibronectin (57).

In summary, Cbl would appear to play multiple roles down-
stream of the EphA receptors, functioning to transduce signals from activated receptors as well as regulating EphA receptor expression.

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