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Cross-Linking of EphB6 Resulting in Signal Transduction and Apoptosis in Jurkat Cells

Hongyu Luo,* Xiaochun Wan,* Yulian Wu,*† and Jiangping Wu2*‡§

Eph kinases are the largest family of receptor tyrosine kinases (RTK), and their ligands are cell surface molecules. The known functions of Eph kinases are mainly pattern formation in the CNS. Although several Eph kinases are expressed at high levels in hemopoietic cells and in the thymus, we have no knowledge of the functions of any Eph kinase in the immune system. In this study, we have demonstrated that an Eph kinase, EphB6, was expressed at high levels in Jurkat leukemic T cells. Co-cross-linking of EphB6 and CD3 led to an altered profile of lymphokine secretion along with proliferation inhibition of Jurkat cells. The cells subsequently underwent Fas-mediated apoptosis. Although EphB6 has no intrinsic kinase activity, its cross-linking triggered general protein tyrosine phosphorylation in Jurkat cells. EphB6 was found to associate with a number of molecules in the signaling pathways, notably Cbl. EphB6 cross-linking resulted in Cbl dephosphorylation and dissociation from Src homology 2 domain-containing tyrosine phosphatase-1 (SHP-1). Our results show that EphB6 has important functions in T cells, and it can transduce signals into the cells via proteins it associates with. The Journal of Immunology, 2001, 167: 1362–1370.

Tyrosine kinases play pivotal roles in signal transduction of lymphocytes. Lymphocyte surface molecules are essential for cell-cell and cell-environment communications. Thus, receptor tyrosine kinases (RTKs)3 bear dual importance in lymphocyte function. Eph RTKs are the largest family of RTKs, comprising ~25% of the known RTKs; they are now named EphAs (EphA1 to EphA8) and EphBs (EphB1 to EphB6) according to their sequence homology (1). At the protein level, EphB shares ~47–60% homology with other members of EphB subgroups (2, 3), whereas homology between human and mouse EphB6 is as high as 91.1% (2, 3). Such a high degree of homology suggests important conserved functions of this molecule.

Eph ligands are mainly expressed on cell surfaces and are now called ephrins. EphrinAs (ephrinA1 to ephrinA5) are ligands of EphAs and are GPI-anchored proteins. EphrinBs (ephrinB1 to ephrinB3) are ligands of EphBs and are transmembrane proteins. EphAs bind ephrinAs and EphBs bind ephrinBs with loose specificity. Generally, they do not bind ligands of the other group. The transmembrane ephrinBs can also function as reciprocal receptors for EphB molecules and transduce signals into cells (4).

Because both Eph receptor kinases and their ligands are all cell surface molecules, they can only interact with each other if expressed on adjacent cells. Not surprisingly, the clearly demonstrated function of these receptors and ligands is to control accurate spatial pattern and cell positioning. Most of these findings are derived from studies on the CNS where most Eph kinases have high expression levels (5, 6). Recently, it has been found that ephrinB2 and its ligand EphB4 are involved in angiogenesis, and such a function is consistent with the known roles of Eph kinases in controlling spatial structure formation (7).

A few Eph RTK members, such as EphB6 and EphB4, have high levels of expression in hemopoietic cells and in the thymus (2, 3). However, despite their expression in these tissues and their presumed importance as both cell surface receptors and tyrosine kinases, we have no knowledge to the function of any member of this important RTK family in the immune system. In this paper, we report a novel finding on functional changes of Jurkat T cells after EphB6 and CD3 co-cross-linking. Signal transduction after EphB6 cross-linking was also examined. This work represents the first endeavor in exploring the roles of Eph kinases in the immune system.

Materials and Methods

Cell culture and thymidine uptake assays

Cells were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics (8). Tonsillar T cells, B cells, and PBMC were prepared as described before (8, 9). Thymidine uptake was measured for cell proliferation as described before (8). In some experiments, the culture wells were coated with mAbs. For coating, mAbs of indicated amount in PBS were added to wells (50 µl/well), and the plates were incubated overnight at 4°C. The wells were washed three times with PBS before use.

Northern blot analysis

Total cellular RNA, extracted from different cell lines, tissues, PBMC, or tonsillar B lymphocytes, was analyzed by Northern blotting as described in a previous publication (9). In some experiments, mRNA isolated with Fast-Track 2.0 mRNA isolation kits (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions was used for Northern blotting. A 1.25-kb Smal/EcoRI fragment of human EphB6 cDNA (from positions 2741–3989) was used as a probe.
Preparation of recombinant proteins corresponding to the extracellular domain of human EphB6

A 0.5-kb PCR fragment coding for part of the extracellular domain of human EphB6 (from amino acid positions 31 to 202) was cloned into a plasmid vector, pGEX-2TK. The resulting construct, pGST-2TK-human protein tyrosine kinase (HPTK), encoded a recombinant fusion protein between GST and the Eph extracellular domain. The recombinant protein, designated as GST-HPTK, was expressed in Escherichia coli BL21 and was purified with glutathione-Sepharose beads.

Preparation of mAb against the extracellular domain of EphB6

Basic procedures for mAb preparation have been described by Coligan et al. (10). Briefly, a BALB/c mouse was immunized with the recombinant protein GST-HPTK. Spleen cells of the mouse were fused to SP2/0-Ag14 myeloma cells. Culture supernatants of hybridomas were screened by flow cytometry according to their binding to Jurkat cells. Limiting dilutions were performed to obtain hybridoma clones.

Flow cytometry

To detect EphB6 expression, Jurkat cells were stained with mAb 4F12 followed by PE-conjugated anti-mouse IgG F(ab')2 (Jackson Laboratories, West Grove, PA). Detailed procedures of staining were described in a previous publication (11). To detect apoptotic cells, Jurkat cells were double-stained with annexin V-FITC (BD PharMingen, San Diego, CA) and propidium iodide (PI). The PI-negative population was gated to measure their expression of annexin V. TUNEL was used to monitor Jurkat cell apoptosis with kits from Roche Diagnostics (Laval, Quebec, Canada) according to the manufacturer’s instructions.

Cross-linking of EphB6 and CD3

Jurkat cells were incubated in serum-free RPMI 1640 medium for 45 min on ice in the presence of mAb 4F12 (anti-EphB6), mAb OKT3 (anti-CD3), or both (all at 2 μg per 10^6 cells). The cells were washed and resuspended in 37°C serum-free medium in the presence of rabbit anti-mouse IgG (10 μg/ml final concentration). Cross-linking was terminated by adding 10 ml cold PBS to the cells, which were spun down and lysed in TNE buffer, as detailed in a previous publication (12).

In vitro lymphokine production

To measure lymphokine production, supernatants from Jurkat cell culture were collected 72 h after anti-CD3 and anti-EphB6 cross-linking. IL-4, IFN-γ, and GM-CSF in the supernatants were quantitated by ELISA (Quantikine; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Dot blotting, immunoblotting, and immunoprecipitation (IP)

For dot blotting, different undenatured recombinant GST fusion proteins were spotted on nitrocellulose membranes. The membranes were then hybridized with mAb 4F12 in the absence or presence of competitor recombinant protein GST-HPTK. Signals were revealed by ECL. For immunoblotting, Jurkat cell lysates or immune complexes precipitated by various Abs were resolved in SDS-PAGE and blotted onto polyvinylidene difluoride membranes. The membranes were hybridized first with anti-EphB6 mAb 4F12, anti-phosphotyrosine mAb RC20 (Transduction Laboratories, Lexington, KY), polyclonal goat Ab against EphB6 (Santa Cruz Biotechnology, Santa Cruz, CA), mAb against protein-tyrosine phosphatase 1C/Src homology 2 domain-containing tyrosine phosphatase-1 (SHP-1) (Transduction Laboratories), or polyclonal rabbit Ab against Cbl (Santa Cruz Biotechnology). Signals were detected by ECL. For IP, Jurkat cell lysates were reacted with rabbit Abs against CrkII, CrkL, GRAB2, p85α phosphatidylinositol 3-kinase (PI-3K), Vav, or Cbl (Santa Cruz Biotechnology). Immune complexes were brought down by protein A+G-Sepharose beads. Detailed methods of immunoblotting and IP have been described in a previous publication (12).

Detection of soluble Fas ligand (FasL)

Soluble FasL in culture supernatants was detected using a commercial ELISA from Oncogen Research Products (Boston, MA).

Results

EphB6 expression in human tissues and cell lines according to Northern blot analysis

EphB6 messages are reported to have high expression levels in the adult human brain and pancreas (3) and in the adult mouse brain and thymus (2). Here, its expression was investigated in selected human tissues, tumors, and immune cells. According to total RNA blotting (Fig. 1A), EphB6 had no detectable expression in the heart and muscle, and low expression in the colon, colon tumor, lung, and lung tumor. Its expression in the spleen and B cells was also quite low, whereas Jurkat cells had high expression. Among the cell lines tested (Fig. 1B), Jurkat cells had the highest expression, followed by a chronic myelogenous leukemia line, K562, and Burkitt B cell lymphoma, Namalwa cells. EphB6 expression was at a low level in histiocytic lymphoma U937 cells and promyelocytic leukemia HL-60 cells, but was not detectable in cervical cancer HeLa cells and Burkitt lymphomas Jijoye and Daudi cells.

We next examined EphB6 expression in resting and activated T and B cells (Fig. 1C). According to Northern blotting using total RNA of T cells (Fig. 1C, left), EphB6 expression in T cells was reasonably high. After their activation by PHA, the expression was decreased, and treating the T cells with an immunosuppressant rapamycin during the PHA activation prevented to a certain degree such decrease. The biological significance of such modulation is yet to be understood. Poly-A-enriched RNA from rosette-enriched tonsillar T cells (90% CD3 positive), and resting or activated tonsillar B cells (85% CD19 positive) was also tested in Northern blotting (Fig. 1C, right). In this experiment, EphB6 mRNA could be easily detected, and the T cells had higher expression than B cells. However, EphB6 expression had no apparent modulation in B cells activated by Staphylococcus aureus Cowan I bacteria plus IL-2 for 16 or 40 h. Thus, EphB6 mRNA was expressed in normal T and B cells and was down-regulated after activation in T but not B cells.

Preparation of recombinant EphB6 and mAb against EphB6

To verify EphB6 expression at the protein level as well as for further experimentation on EphB6 function, we produced a mAb against the extracellular domain of EphB6. A GST-HPTK fusion protein (GST fused with a part of the human EphB6 extracellular domain) was expressed in E. coli and purified with glutathione-Sepharose beads (Fig. 2A). The highly purified fusion protein was used to immunize a BALB/c mouse, and hybridomas were produced. The hybridomas were screened with flow cytometry for mAbs that bound on surfaces of Jurkat cells. A positive clone 4F12 (IgG1) was identified, and almost all Jurkat cells, which had high EphB6 mRNA expression, were highly positive for 4F12 staining (Fig. 2B). K562 cells, which had modest EphB6 mRNA expression, were modestly 4F12 positive, whereas Daudi cells, which had no detectable EphB6 mRNA, were 4F12 negative. Thus, the 4F12 staining correlated to the EphB6 mRNA expression. The expression pattern of EphB6 in these malignant cells according to 4F12 staining is consistent with that reported by Shimoyama et al. (13) using their anti-human EphB6 mAb T49-25. Moreover, EphB6 could be detected on 3–5% peripheral T cells according to 4F12 staining (data not shown), and this percentage is also in agreement with that in the publication of Shimoyama using mAb T49-25 (13).

According to Western blotting, 4F12 recognized a major band at 110 kDa, which is the expected size of EphB6 (Fig. 2C). 4F12 bound strongly to undenatured immunogen GST-HPTK, but not to GST, GST fused with a different region of the human EphB6 extracellular domain (GST-control-1), or GST fused with the mouse
EphB6 intracellular domain (GST-control-2) (Fig. 2D). 4F12 binding to solid-phase GST-HPTK could be blocked by soluble GST-HPTK (Fig. 2E). These results illustrate the specificity of mAb 4F12.

The specificity of mAb 4F12 was further confirmed by cell adhesion assay. Jurkat cells grow normally in suspension and form clumps, as shown in the left panel of Fig. 3A. When the cells were cultured in wells coated with 4F12, the mAb anchored the cells to the bottom of the wells and they could no longer form clumps (Fig. 3A, second column). This effect was dose dependent in that when mAb concentrations used for coating were reduced from 1 to 0.2 μg/well/50 μl, small clumps started to appear. Isotypic control mAb (Fig. 3A, third column) or 4F12 in solution (Fig. 3A, fourth column) had no effect on clump formation. When recombinant EphB6 extracellular domain GST-HPTK was added to the coated wells, it reversed the anchoring effect of 4F12, as shown in Fig. 3B. This reversing effect appeared in wells coated with 0.5 μg/50 μl/well 4F12, and was more obvious in wells coated with less 4F12 (0.2 μg/50 μl/well) (Fig. 3B, third column). For all these cultures, Jurkat cell proliferation was not affected (data not shown), indicating that 4F12 or recombinant proteins had not toxic effects on the cells.

Taken together, the results of this section clearly demonstrate that EphB6 protein is expressed on Jurkat cell surfaces and that mAb 4F12 is specific for the extracellular domain of human EphB6.

Co-cross-linking of EphB6 and CD3 results in activation-induced death of Jurkat cells

So far, there are no reports pertaining to the function of any Eph receptor kinase in immune cells, nor there are known functions of EphB6 in any type of tissues or cells. With mAb available against the Ephb6 extracellular domain, we searched for EphB6 function in the immune system, using Jurkat cells as a model.

First, we examined lymphokine production by Jurkat cells after cross-linking their surface CD3, EphB6, or both. Jurkat cells were cultured in wells coated with a suboptimal concentration of OKT3 (anti-CD3, 0.1 μg/50 μl/well), an adequate concentration of 4F12 (anti-EphB6, 0.25 μg/50 μl/well), or both for 3 days. IL-4, IFN-γ, and GM-CSF in the supernatants were assayed with ELISA. As illustrated in Fig. 4A, IL-4 produced by Jurkat cells cross-linked with anti-CD3, anti-EphB6, or both showed no changes. The anti-CD3 alone moderately enhanced IFN-γ production, but anti-EphB6 alone had no effect. However, co-cross-linking of these two molecules led to reduced IFN-γ production. In contrast, such co-cross-linking stimulated GM-CSF production, whereas cross-linking with anti-CD3 or anti-EphB6 alone exerted no effect. The increase of GM-CSF indicates that co-cross-linking was not a null or nonspecific toxic event, but activated a certain cellular program in Jurkat cells.

To further research the functional consequences of EphB6 cross-linking, we assessed the proliferation of Jurkat cells cultured...
in wells coated with the suboptimal concentration of anti-CD3, adequate concentration of anti-EphB6, or both. The cells were pulsed with [3H]thymidine for the last 5 h of their culture. As shown in Fig. 4B, Jurkat cells cultured in wells coated with the anti-CD3 or the anti-EphB6 had proliferation rates comparable to those cultured in uncoated wells. However, cells cultured in wells coated with both the anti-CD3 and anti-EphB6 had significantly reduced proliferation. It is to be emphasized that the anti-CD3 alone had no effect because it was used at a suboptimal concentration. When it was used at a high concentration, it alone caused apoptosis and consequently reduced thymidine uptake of the Jurkat cells (data not shown).

We wondered whether inhibition of proliferation reflected activation-induced cell death. To evaluate this possibility, Jurkat cells were cultured in wells coated with a suboptimal concentration of anti-CD3, an adequate concentration of anti-EphB6, or both for 24 h and stained with anti-annexin V and PI. PI-negative cells were gated for their annexin V expression. As shown in Fig. 4C, cross-linking of Jurkat cells with the anti-CD3 or the anti-EphB6 alone did not cause apoptosis, but co-cross-linking of both molecules did in that annexin V-positive cells increased from <2.4 to 31.4%.

In lymphocytes, apoptosis is often a Fas-mediated process. We thus assessed soluble FasL produced by these Jurkat cells. Supernatants from uncoated wells, wells coated with a suboptimal concentration of anti-CD3 (0.1 μg/50 μl/well for coating) alone, or an adequate concentration of anti-EphB6 (0.25 μg/50 μl/well for coating) alone had very low levels of soluble FasL (<25 ng/ml) (Fig. 4D). When Jurkat cells were cultured in wells coated with the anti-CD3 and anti-EphB6 for 16 h, soluble FasL in the culture supernatant rose to >200 pg/ml, which was at a comparable level by treating Jurkat cells with optimal solid-phase anti-CD3 (1 μg/50 μl/well for coating). To verify that the apoptosis induced by CD3 and EphB6 co-cross-linking was indeed mediated by Fas and FasL interaction, we added soluble recombinant Fas-Fc protein to the above described culture system. As shown in Fig. 4E, anti-CD3 and EphB6 co-cross-linking resulted in apoptosis of 34.7% Jurkat cells after 24 h according to the TUNEL assay. The presence of Fas-Fc (50 μg/ml) significantly suppressed apoptosis with only 17% TUNEL-positive cells. A control recombinant protein with an identical Fc tail had no effect on the apoptosis (Fig. 4E, bottom panel, 33.6%).
The results of this section showed that after co-cross-linking of CD3 and EphB6 on Jurkat cells, a certain cellular program was activated, which leads to altered cytokine production and Fas-mediated apoptosis.

**EphB6 could transduce signals into Jurkat cells**

It has been reported that both human and mouse EphB6 have mutations in the conserved kinase domain, and as a consequence they lack intrinsic tyrosine kinase activity according to tests done using transfected recombinant EphB6 (2, 3). Thus, it has been speculated that EphB6 is a silent receptor with no signaling capability. This speculation was obviously in conflict with our observation that co-cross-linking of CD3 and EphB6 led to functional changes of Jurkat cells. Certain signals must have been transmitted from natural surface EphB6 into the cells. To examine this possibility, tyrosine phosphorylation of total cellular proteins was assayed in Jurkat lysates by immunoblotting using anti-phosphotyrosine mAb RC20. After 4F12 cross-linking, there was an increase in tyrosine phosphorylation of several proteins of ~50, 90, and 200 kDa in size. The phosphorylation declined after 5 min, and at 30 min the intensity of all the phosphoproteins was below that at time 0. CD3 cross-linking induced stronger protein tyrosine phosphorylation in many bands at 1 and 5 min. The overall pattern was similar to that resulted from EphB6 cross-linking, but several bands of ~35, 50, and 90 kDa in size were more prominently phosphorylated between 1 and 5 min. Co-cross-linking of EphB6 with CD3 resulted in pattern similar to that of anti-CD3 alone, with the exception that the 90-kDa band was not strongly phosphorylated and the 200-kDa band had a faster phosphorylation and dephosphorylation kinetics (Fig. 5).

The results of this section indicates that EphB6 can indeed transduce signals into Jurkat cells, that the signaling of EphB6 overlaps with but is not identical with that of CD3, and that costimulation of EphB6 and CD3 results in a tyrosine phosphorylation pattern with certain unique features.

**Interaction between EphB6 and Cbl**

EphB6 had no intrinsic kinase activity, but it could transduce signals and lead to functional changes in Jurkat cells. A logical explanation for this is that EphB6 might associate with molecules involved in signal transduction in T cells. We examined a list of signaling molecules that might form complexes with EphB6. Included in the list are adaptor proteins GRB2 and p85α of PI-3K that are known to associate with other Eph kinases (14, 15). Several other adaptor proteins, such as CrkL, CrkII, and Vav, were also included. Also included was Cbl, which has adaptor function but also has ubiquitin ligase activity (16). Jurkat lysates were immunoprecipitated with rabbit Abs against the above-mentioned molecules, and immune complexes were analyzed by immunoblotting using a rabbit anti-EphB6 Ab. As shown in Fig. 6A, EphB6 was prominently present in Cbl precipitates. EphB6 was also coprecipitated with GRB2, CrkL, and CrkII, but to lesser degrees. No detectable EphB6 was found in immunoprecipitates of p85α PI-3K or Vav. It is to be noted that p85α PI-3K and Vav could be detected by immunoblotting in the p85α PI-3K and Vav immunoprecipitates (data not shown), indicating that the anti-p85α PI-3K and anti-Vav Abs had adequate affinity for IP.

The obvious association between EphB6 and Cbl led us to ask whether it had any relevance to EphB6 signaling. We examined tyrosine phosphorylation of Cbl after cross-linking of EphB6, because such phosphorylation affects Cbl’s association with other downstream signaling molecules (17). As shown in Fig. 6B, Cbl had constitutive phosphorylation, and 3 min after EphB6 cross-linking there was a decline in Cbl tyrosine phosphorylation. By 10 min, most of Cbl was dephosphorylated. In contrast, the level of Cbl protein remained the same. This result indicates that Cbl is actively involved in EphB6 signaling.

Cbl is a substrate of SHP-1 (18), and SHP-1 activity is regulated by allosteric mechanisms involving interaction of its Src homology 2 domain with other proteins (19, 20). We wondered whether dephosphorylation of Cbl after EphB6 cross-linking was related to changes in the interaction between Cbl and SHP-1. Jurkat lysates were immunoprecipitated with anti-Cbl, and SHP-1 in immune complexes was revealed by immunoblotting. As seen in Fig. 6C, SHP-1 could be detected directly in the Jurkat lysate and anti-Cbl IP (first and second lanes, respectively). The SHP-1 level started to diminish 5 min after EphB6 cross-linking, and continued to do so at 15 min after EphB6 cross-linking. This decline was correlated with reduced tyrosine phosphorylation of Cbl as shown in Fig. 6B. Cross-linking of CD3 resulted in a faster disappearance of SHP-1 in the Cbl IP, and co-cross-linking of EphB6 with anti-CD3 had a similar effect as anti-CD3 cross-linking alone. Cbl protein levels showed no apparent changes after different ways of cross-linking as expected (lower panel of Fig. 6C). If SHP-1 is the enzyme responsible for Cbl dephosphorylation in our model as reported in other studies (18), it dissociates from its substrates once its job is done.

We were not able to detect bands corresponding to phospho-SHP-1 in Cbl precipitates (Fig. 6B), whereas SHP-1 protein could
FIGURE 4. Effects of co-cross-linking CD3 and EphB6 on Jurkat cell function. Wells of 96-well plates were coated with mAb OKT3 (anti-CD3, 0.1 µg/50 µl/well), mAb 4F12 (anti-EphB6, 0.25 µg/50 µl/well), or both and then washed. Jurkat cells were cultured in these wells.

A. Effect of co-cross-linking of CD3 and EphB6 on lymphokine production by Jurkat cells. Cell culture supernatants were harvested after 72 h, and duplicate samples were assayed for IL-4, IFN-γ, and GM-CSF by ELISA. Each experiment was performed three times, and means ± SD from a representative experiment are shown.

B. Co-cross-linking of CD3 and EphB6 inhibits Jurkat cell proliferation. Cell proliferation was measured from day 1 to day 4 according to [3 H]thymidine uptake during the last 5 h of culture. The starting cell number was 10⁴ cells/200 µl/well on day 0. Means ± SD of samples in triplicate are shown. The result is representative of five experiments.

C. Co-cross-linking of CD3 and EphB6 induces apoptosis of Jurkat cells. Jurkat cells were cultured in precoated wells as indicated for 24 h and stained with annexin V-FITC and PI. The samples were analyzed with flow cytometry, and histograms of annexin V expression on PI-negative cells are shown. Data are representative of three independent experiments.

D. Co-cross-linking of CD3 and EphB6 induces soluble FasL production by Jurkat cells. Jurkat cells were cultured in precoated wells as indicated for 16 h. The supernatants were harvested and assayed for soluble FasL by ELISA. Wells coated with an optimal concentration of OKT3 (anti-CD3) at 1 µg/50 µl/well were included as a control. The result is representative of three experiments.

E. EphB6/CD3-induced apoptosis in Jurkat cells is Fas mediated. Jurkat cells were incubated in anti-CD3- and anti-EphB6-coated wells in the presence of Fas-Fc (50 µg/ml) or control protein-Fc (50 µg/ml), and apoptosis of the cells was measured with TUNEL 24 h later.
be detected in the anti-Cbl precipitate (Fig. 6C). Because the anti-phospho-protein mAb and anti-SHP-1 mAb are not necessarily comparable in their sensitivity, these results are not self-contradictory. The Cbl-associated SHP-1 proteins could be phosphorylated but were below the detection level by the anti-phospho-Ab RC-20. With that said, we cannot rule out the possibility that most SHP-1 proteins associated with Cbl were not phosphorylated, because SHP-1 is mainly regulated by changing of its tertiary structure depending its association with other proteins.

The results of this section suggest that signal transduction from EphB6 is through proteins it associates with, Cbl being one of them.

**Discussion**

In this report, we have demonstrated for the first time that an Eph kinase has a functional role in immune cells: co-cross-linking of EphB6 and CD3 alters profiles of lymphokine production, suppresses proliferation, and induces Fas-mediated apoptosis of Jurkat cells. Moreover, we reported novel findings that EphB6 transduces signals into Jurkat cells even though it lacks intrinsic kinase activity. This function is achieved through proteins that EphB6 associates with, Cbl being one of them.

Eph family kinases are in the center of attention in neurobiology due to their newly found functions in the guidance of neuron growth and spatial pattern formation, although the function of EphB6 is still unknown. A few of the Eph kinases, including EphB6, are expressed in lymphoid tissues. For example, EphA3 is expressed in pre-B cell lines (21); EphA2 (22), EphB4 (23), and EphB6 (2, 3) are expressed in thymus. The inherent importance of these RTKs, due to their being receptors as well as tyrosine kinases, and due to their expression in lymphoid tissues, is self-evident.

The observed biological function of EphB6 in Jurkat T cells is very interesting. This is the first report that an Eph receptor is involved in inducing apoptosis. We suspected that the apoptosis involved the Fasl/Fas pathway. However, the surface Fasl expression on Jurkat cells was not detectable after stimulation by anti-EphB6 alone, anti-EphB6 in combination with anti-CD3, or even with an optimal concentration of anti-CD3 (data not shown).

It has been reported that Fasl can be rapidly shed from the cells surface, and soluble Fasl can also induce apoptosis (24). Indeed, we could detect a rapid and significant increase of soluble Fasl at 16 h in the supernatant of Jurkat cells stimulated by anti-EphB6 and anti-CD3, and this is correlated to the occurrence of apoptosis of the Jurkat cells as detected by annexin V expression at 24 h. The increase of the soluble Fasl in the supernatant was not due to release of cellular Fasl from dead cells, because at 16 h, few cells were GI positive (data not shown). The apoptosis could be blocked partially by soluble Fas-Fc, and this serves as an additional evidence that the apoptosis is Fas mediated. The partial block is probably due to the fact that the soluble FasL, shed from the Jurkat cells functions as an autocrine or paracrine with high local concentrations, and that the Fas-Fc used was not sufficient to completely neutralize the soluble Fasl. With that said, we cannot exclude the possibility that pathways other than the Fas-mediated one are so involved.

It is noteworthy that with strong cross-linking of TCR, or with strong activation by PMA plus ionomycin, Jurkat cells undergo apoptosis without the help of EphB6. In our model, anti-CD3 was used at a suboptimal concentration that was not sufficient to induce apoptosis alone without the anti-EphB6. Thus, we speculate that one biological function of EphB6 might be to enhance the signal strength of TCR cross-linking. If this is so, then an outcome of EphB6 ligation in normal T cells might be activation or apoptosis, depending on the maturation of T cells and the nature of TCR cross-linking. Indeed, we have found that costimulation of normal T cells with anti-CD3 and anti-EphB6 led to drastic changes in their proliferation and cytokine production (data not shown).

Munthe et al. (25) recently reported that ephrinB2 is a candidate ligand for EphB6 according to cell surface binding assays. In a separate study, we performed in situ hybridization on the expression patterns of mouse ephrinB2 and EphB6 and found that their expression was colocalized in white pulp of the spleen and cortex of the thymus (data not shown), unlike reciprocal expression patterns between other Eph kinases and their ligands in the CNS or vascular system. The colocalization suggests two things. First, EphB6 has relevant functions in lymphocytes, because its putative ligand has chances of interacting with it. Second, a third party might be required to trigger a biological function in immune cells, or else EphB6 will be perennially activated. Consistent with this prediction, we found that cross-linking of CD3 in addition to EphB6 in Jurkat cells was required to trigger GM-CSF production, inhibit proliferation, and induce apoptosis.

Initial reports on sequences and kinase activities of mouse and human EphB6 were perplexing. The two share a very high degree of homology (91% at the peptide level), suggesting that EphB6 is a well-conserved gene across species and must have important functions. Yet several mutations are found in the kinase domain of both human and mouse EphB6 and, consequently, they have no detectable kinase activities according to assays using recombinant proteins (2, 3). We have confirmed that a recombinant human intracellular domain of EphB6 had no kinase activity (data not shown). One might speculate that this RTK is a “dumb” receptor and serves to “damp” other Eph family members that share the same ligands. However, our study demonstrates that EphB6 is a functional receptor and is fully capable of inducing functions and transducing signals in Jurkat cells. Cross-linking of EphB6 results in transient tyrosine phosphorylation of several cellular proteins. Because EphB6 has no intrinsic kinase activity, the kinases responsible for signaling must be the one(s) associated directly or indirectly with EphB6. We found a trace amount of EphB6 in Fyn precipitates (data not shown), and conceivably some tyrosine kinase activities after EphB6 cross-linking might be derived from the
associated Fyn. However, this does not exclude the possibilities that additional signaling molecules are also involved.

Indeed, we have demonstrated that EphB6 could be detected in Cbl precipitates. Conversely, Cbl was found in EphB6 precipitates (data not shown). Therefore, we have convincingly established that Cbl is associated with EphB6. Cbl proto-oncoproteins are primarily expressed in hemopoietic cells (26, 27) and is involved in the signaling of receptor protein tyrosine kinases as well as in the signaling of cell surface receptors that are associated with cytoplasmic protein tyrosine kinases (17). Therefore, the association of Cbl with EphB6 is in keeping with the general property of Cbl and is a novel signaling mechanism for Eph family kinases.

Cbl contains several protein-protein interaction domains such as a Src homology 2 domain, a RING finger domain, a large proline-rich Src homology 3 binding domain, and a leucine zipper. These are the structural basis for its interaction with various signaling proteins, including GRB2, the Crk adaptor family, Vav, p85α of PI-3K (17), and EphB6, which also has a leucine zipper in its carboxy-terminal. In our study, EphB6 could be detected in GRB2, CrkL, and CrkII precipitates, and abundant Cbl was coprecipitated with EphB6. These findings raise a possibility that EphB6 forms multunit complexes with GRB2, CrkI, and/or CrkII using Cbl as an intermediate. This possibility is under further investigation.

Although Cbl has been reported to associate with many other cell surface receptors, it seems that most phosphorylated Cbl molecules in Jurkat cells are involved in the EphB6 signaling pathway, because cross-linking of EphB6 leads to rapid tyrosine dephosphorylation of most Cbl molecules. If the phosphorylated Cbl are at the same time associated with other receptors, then we will arrive at a logical speculation that EphB6 can influence the signaling and functioning of those receptors.

Cross-linking of EphB6 also led to dissociation of SHP-1 from Cbl, which is a known substrate of SHP-1 (18). This finding suggests that EphB6, Cbl, and SHP-1 might form a tricomplex constitutively, and SHP-1 might dephosphorylate Cbl during the course of EphB6 activation and then dissociate itself from Cbl once the dephosphorylation is completed. Such a possibility is supported by our finding that all the three molecules could be detected in the membrane fraction of Jurkat cells (data not shown). We are currently investigating whether the EphB6, Cbl, and SHP-1 tricomplex does exist in Jurkat cells.

Cbl is also known as ubiquitin ligase (16), and its role as a negative regulator in many signaling pathways (28) might related to its function in channeling the kinase receptors it associates with to the proteasome degradation machinery. The association between EphB6 with Cbl certainly raises an interesting question whether EphB6 degradation is via the ubiquitin-proteasome pathway.
When K562 cells were cultured in the presence of a proteasome inhibitor, dipeptide boronic acid (29), their surface expression of EphB6 as measured by 4F12 binding was significantly increased (data not shown), suggesting that EphB6 is degraded via the proteasome.

At present, it is not yet understood how the observed signaling events are related to the functional changes of Jurkat cells after EphB6 cross-linking. Because no prior knowledge is available, our study marks the beginning rather than the end of an exploration into the physiological roles and signaling of Eph kinases in the immune system.

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References