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L-Selectin Tyrosine Phosphorylates Cbl and Induces Association of Tyrosine-Phosphorylated Cbl with CrkL and Grb2

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L-Selectin-mediated rolling of leukocytes on endothelial cells is an important step for lymphocyte homing and an early event in the immune response to pathogens or inflammatory stimuli. We have previously elucidated intracellular signaling cascades upon L-selectin engagement resulting in activation of Ras, Rac and JNK as well as cytoskeletal changes, oxygen release, ceramide synthesis and receptor capping. Activation of the src-tyrosine kinase p56lck is followed by phosphorylation of the L-selectin molecule and MAP-K. Here we show a tyrosine kinase dependent phosphorylation of the Cbl adapter protein after L-selectin engagement in lymphocytes. Phosphorylation of Cbl was absent in Jurkat cells that are pharmacologically treated with tyrosine kinase inhibitors and in lck-deficient JCaM cells. There is an activation induced association of tyrosine phosphorylated Cbl with Grb2 and CrkL, respectively, but not CrkII. Therefore, the adapter protein Cbl plays a role in L-selectin signaling and might modulate immune function by the specific recruitment of signaling molecules to multiprotein complexes. © 2001 Academic Press

Key Words: **adhesion molecule; L-selectin; intracellular signal transduction; Cbl; tyrosine phosphorylation; protein association.**

Leukocyte adhesion to endothelial cells is a crucial step in the immune response to pathogens and in lymphocyte homing (1, 2). The recruitment of leukocytes to sites of inflammation is a multistep process involving different classes of adhesion molecules. Selectins on leukocytes and endothelial cells mediate the initial, transient interaction, known from microscopically studies as rolling. Rolling of leukocytes on endothelial cells is followed by firm adhesion and extravasation which is mediated by integrins and their counter-receptors.

L-Selectin is constitutively expressed on almost all leukocytes (3) and consists in its extracellular part of a calcium dependent lectin binding region important for ligand recognition, followed by an epidermal growth factor like domain and several short consensus repeats. The intracytoplasmic domain of L-Selectin lacks any enzymatic activity (4).

There is increasing evidence that adhesion molecules also function as signaling receptors thereby regulating adhesion related processes.

For the L-selectin molecule, we and others have recently demonstrated a role as signal transducing receptor in neutrophils and lymphocytes (5–7). Briefly, L-selectin stimulation activates the tyrosine kinase p56lck, the small G-proteins Ras and Rac1/2 and results in the release of oxygen radicals, cytoskeletal changes and activation of stress activated protein kinases (7–9). In neutrophils there is a stimulation dependent increase in synthesis of mRNA from TNFα and IL8 (5).

During the course of our studies aimed at further defining L-selectin-mediated signal transduction we detected tyrosine phosphorylation of a 120-kDa protein in Jurkat lymphocytes and PBL. By immunoprecipitation we could identify the 120-kDa protein as the adapter molecule Cbl.

Adapter proteins have been implicated in signal transduction pathways of many receptor systems. They have no known catalytic function but serve to transiently link tyrosine kinases to effectors or tyrosine phosphorylated proteins to other proteins with enzymatic function (10).

The proto-oncogene product c-Cbl (Casitas B-lineage lymphoma) is an ubiquitously expressed complex adapter protein that associates with numerous signaling molecules in a variety of cell types (11, 12).

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C-Cbl has no known catalytic activity but contains a highly basic amino-terminal region, a stretch of seven histidine residues, a potential nuclear localisation signal, a zinc ring finger and a leucin zipper motif, a motif known to promote homo- and hetero-dimerization of other proteins (13). Furthermore, Cbl possess multiple proline-rich sequences and several tyrosine phosphorylation sites, which associate with SH3 or SH2 domain containing polypeptides (13). Cbl is rapidly tyrosine phosphorylated in response to engagement of receptor protein tyrosine kinases, receptors that activate protein tyrosine kinases, including immunoreceptors and hematopoietic growth factor receptors (11, 12). In signal transduction of the adhesion molecules β1 and β2 integrins Cbl was found to form a complex with the src-kinase and the PI-3-K (14, 15).

This points to a pivotal role of the Cbl protein in early signaling events across the cell membrane into the cytoplasm following receptor ligation.

Cbl has been found to associate constitutively or inductively with adapter proteins like Grb2 and Crk (11–13). There are three different Crk homologues (CrkI, CrkII and CrkL) with different composition of SH2 and SH3 domains (16). CrkL binds to tyrosine phosphorylated Cbl after T cell activation and epidermal growth factor receptor stimulation (17) and associates constitutively with the guanine nucleotide exchange factor C3G (18). Grb2 binds via its SH2 domain to a number of activated, tyrosine phosphorylated growth factor receptors, thereby bringing the guanine nucleotide exchange protein Sos1, bound to the Grb2 SH2 domain, to the plasma membrane, where Sos activates Ras (19).

Recently, we could demonstrate for L-selectin triggering in coimmunoprecipitation experiments a stimulation dependent association of the Grb2-Sos complex with the L-selectin molecule (7). In the present study, we further elucidated early signaling events upon L-selectin triggering by identifying an activation induced tyrosine phosphorylated protein as the Cbl adapter protein. Subsequently, we studied the protein interaction of the Cbl adapter protein with other signaling molecules involved in L-selectin signal transduction.

MATERIALS AND METHODS

Cells and reagents. All reagents were purchased from Sigma, Taufkirchen, Germany, if not otherwise cited. The human leukemic T cell lines Jurkat and p56lck-deficient JCaM1.6 (both from ATCC, Rockville, MD) were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 10 mM Hepes [pH 7.4], 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin (all purchased by GIBCO BRL, Life Technologies, Karlsruhe, Germany) and 50 μM β-mercaptoethanol (complete RPMI-1640). The JCaM1.6 cells were generated from Jurkat cells by chemical mutation and have been previously shown to be p56lck-deficient (20).

Peripheral blood mononuclear cells were prepared by Ficoll–Histopaque. The L-selectin antibody Dreg56 is from BD Pharmingen (Heidelberg, Germany). The Dreg55 and Dreg200 antibodies are kindly provided by Dr. C. Buehrer; C-Cbl, Grb2, CrkL, Sos and CrkII are from Santa Cruz (Heidelberg, Germany). The anti-Phosphotyrosine antibody 4G10 was from UBI, Biozol (Eching, Germany). Lavendustin A (30 nM for 5 min), a tyrosine kinase inhibitor and PP_2 (50 mM for 15 min), a selective inhibitor of the Src-family of protein tyrosine kinases (both from Calbiochem-Novabiochem, Bad Soden, Germany), were used to study the role of tyrosine kinases in phosphorylation of Cbl.

Cell stimulation. For activation, cells (20×10^6/100 μl per sample for immunoprecipitations) were washed twice in sterile Hepes/saline (H/S, 132 mM NaCl, 20 mM Hepes, 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, 0.8 mM $MgSO_4$) and stimulated at 37°C with NaN_3-free, low endotoxin, monoclonal mouse anti-human L-selectin antibody Dreg56, Dreg55 and Dreg200 (2 μg/ml).

Immunoprecipitation and immunoblotting. Cell stimulation was terminated by lysis in 25 mM Hepes [pH 7.4], 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM NaCl, 10 mM each NaF, Na_3VO_4 and sodium pyrophosphate and 20 μg/ml of aprotinin/leupeptin (RIPA-buffer) for immunoprecipitation of Cbl and blotting with 4G10. For co-immunoprecipitation of Sos, Grb2, Crk and Cbl, cells were lysed in the same buffer as above with 3% NP-40 as detergent (TN3-buffer). After lysis and centrifugation (20.000*g*, 15 min) proteins were immunoprecipitated (3 μg antibody). Immunoprecipitates (IPs) were immobilized by Protein A/G Plus Agarose (Santa Cruz, Heidelberg, Germany), washed six times in lysis buffer and resuspended in SDS-sample buffer. Proteins were separated by SDS/PAGE, transferred to PVDF membranes (Bio-Rad, Munich, Germany). Membranes were blocked by incubation with 4% BSA in TBS for 1 h at room temperature and were subsequently blotted with the appropriate primary antibody (1 μg/ml) in TBS. After washing the immunoblots 6 times in TBS-Tween the blots were developed by horseradish peroxidase-conjugated protein G (Dako, Hamburg, Germany) with a chemoluminescence kit (Amersham Life Science, Braunschweig, Germany), followed by autoluminography by exposure to Hyperfilm ECL (Amersham Life Science, Braunschweig, Germany). To test for equal amounts of immunoprecipitated protein blots were stripped (45 min in 20 mM Tris [pH 6.8], 2% SDS, 70 mM β-mercaptoethanol at 70°C) and reprobed, or an aliquot of the IPs was analyzed by Western blotting. Association of CrkL, Grb2 with Cbl was tested by co-immunoprecipitation and blotting the IPs with the corresponding antibody.

RESULTS

L-Selectin Triggering Results in Tyrosine Phosphorylation of the Adapter Protein Cbl in Jurkat Cells

Jurkat cells were incubated for different time periods with the monoclonal L-selectin antibody Dreg56, followed by immunoprecipitation of the Cbl protein and incubating the Western blots with an anti-phosphotyrosine antibody 4G10. There was a time dependent increase in tyrosine phosphorylation of a protein with a molecular weight of 120 kDa with a maximum at 5–10 min (Fig. 1). In order to test for equal amounts of protein in each lane and to confirm that the band at 120 kDa is indeed the Cbl protein, membranes were stripped and reprobed with an anti-Cbl antibody. In addition we also detected an increase of tyrosine phosphorylated Cbl protein after

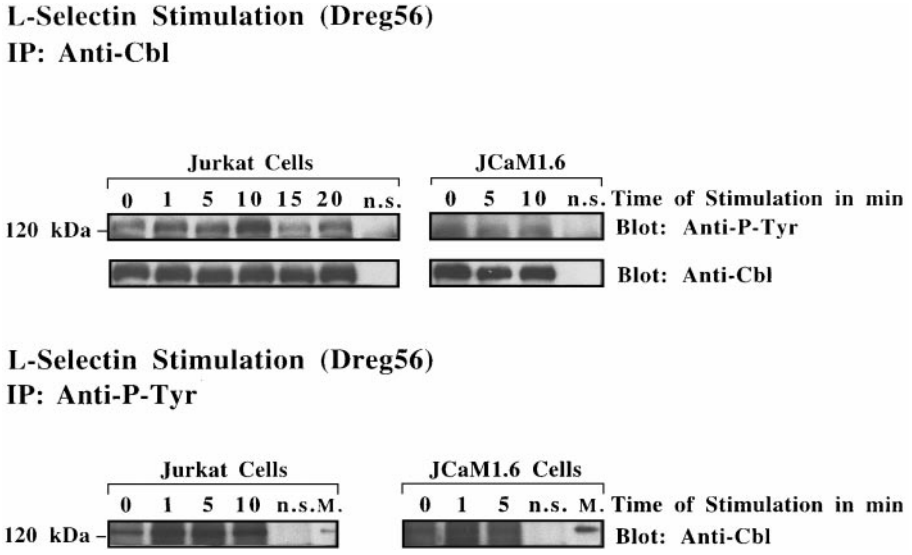

FIG. 1. L-selectin stimulation using the Dreg56 L-selectin antibody results in tyrosine phosphorylation of the adapter molecule Cbl in Jurkat cells, but not in p56lck-deficient JCaM1.6 cells, with a maximum at 10 min. (A) Jurkat and JCaM1.6 cells were incubated with Dreg56 for different time intervals, Cbl was immunoprecipitated, and immunoprecipitates were separated by SDS/PAGE and blotted with an anti-Phosphotyrosine antibody (4G10). In the small blots, filters were stripped and reprobed with an anti-Cbl antibody to check for an equal amount of protein in each lane. To prove the specificity of the IPs, a nonspecific (n.s.) probe was included. (B) Jurkat and JCaM1.6 cells were incubated with Dreg56 as described above. Immunoprecipitation was done with an anti-phosphotyrosine antibody. Membranes were blotted with an anti-Cbl antibody. The increase in tyrosine phosphorylation of a 120 kDa protein after L-selectin triggering was identified as the Cbl protein.

L-selectin stimulation when immunoprecipitating tyrosine phosphorylated proteins and incubating the membranes with an anti-Cbl antibody in Jurkat cells (Fig. 1).

To further investigate L-selectin induced phosphorylation of the Cbl protein we used two other L-selectin antibodies of the Dreg series. Dreg55 and Dreg200 also induced tyrosine phosphorylation of the Cbl protein in Jurkat but not in JCaM1.6 cells. Again, we stripped and reprobed the membranes with an anti-Cbl antibody to test for equal amounts of protein in each lane (Fig. 2).

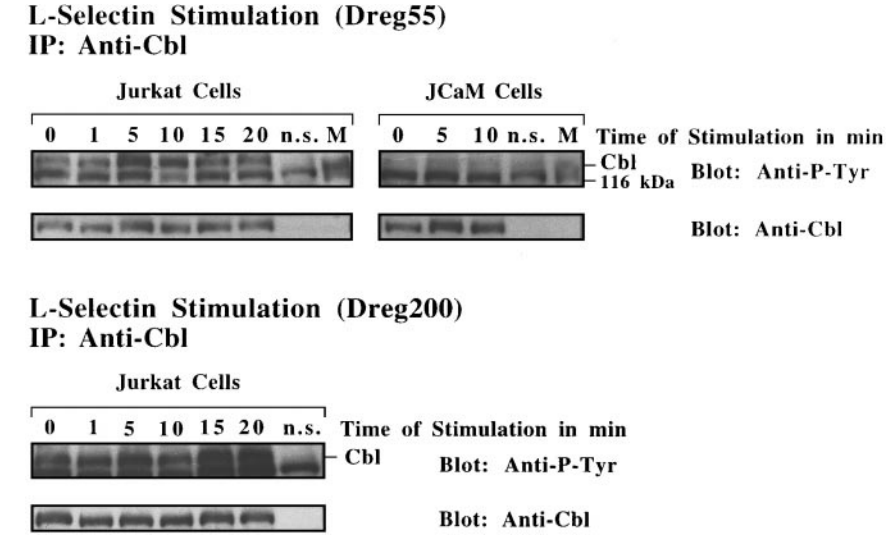

FIG. 2. L-selectin stimulation of Jurkat and JCaM1.6 cells using two different L-selectin antibodies from the Dreg series (Dreg55 and Dreg200) reveal a stimulation-dependent increase in tyrosine phosphorylation of the Cbl protein in Jurkat but not in JCaM1.6 cells. In the blots below membranes were stripped from the primary antibody and reprobed with an anti-Cbl antibody to test for an equal amount of protein in each lane.

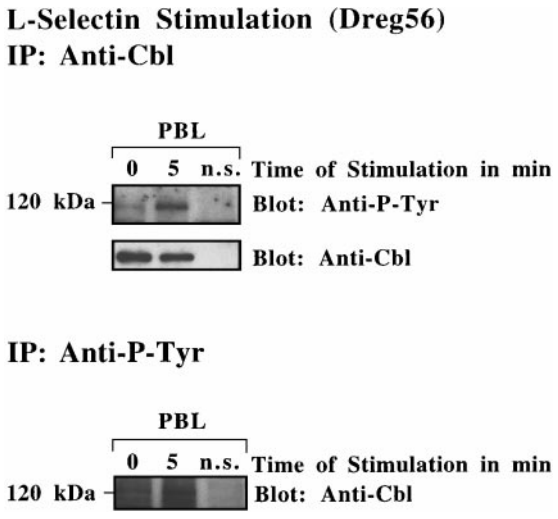

FIG. 3. L-Selectin stimulation of peripheral blood lymphocytes leads to a similar increase in tyrosine phosphorylation of the Cbl protein compared to Jurkat cells. PBL were stimulated by Dreg56, Cbl, or tyrosine-phosphorylated proteins were immunoprecipitated and blotted with anti-P-Tyr or Cbl, respectively. By stripping off the primary antibody and reprobing the membrane with an anti-Cbl antibody an equal amount of protein in each lane was confirmed in Cbl-IPs.

L-Selectin-Induced Phosphorylation of Cbl Is Also Detectable in Peripheral Blood Lymphocytes (PBL)

The physiological significance of L-selectin-mediated tyrosine phosphorylation is underscored by the finding that PBL responded to L-selectin triggering with an increase in phosphorylation of the Cbl protein (Fig. 3). Immunoprecipitating tyrosine phosphorylated proteins and incubating the membranes with an anti-Cbl antibody also exhibited an increase of the tyrosine phosphorylated protein and incubating the membranes with an anti-Cbl antibody also exhibited an increase of the tyrosine phosphorylated Cbl with a maximum at 5 min (Fig. 3).

Tyrosine Phosphorylation of Cbl Depends on the Functional Expression of the Tyrosine Kinase p56lck

To further investigate the molecular mechanism how Cbl gets tyrosine phosphorylated, we compared tyrosine phosphorylation of Cbl in Cbl immunoprecipitates from Jurkat and JCaM1.6 cells. No tyrosine phosphorylation of Cbl upon L-selectin triggering was detected in JCaM1.6 cells (Fig. 1). However, Cbl is expressed in a similar amount compared to Jurkat cells (Fig. 1, small panel). In addition, preincubation of Jurkat cells with PP_2, a selective src-tyrosine kinase inhibitor (Fig. 4) or Lavendustin A, a tyrosine kinase inhibitor (data not shown), also prevented L-selectin-induced tyrosine phosphorylation of Cbl.

Constitutive Association of the Adapter Molecule Grb2 with Cbl and an L-Selectin Induced Increase in Tyrosine Phosphorylated Cbl Binding to Grb2

We have previously shown that Grb2 and Sos associate stimulation dependent with the intracytoplasmic domain of L-selectin (7). Therefore the adapter protein Grb2 was immunoprecipitated after different stimulation periods with Dreg56. Immunoblotting was performed with an anti-Cbl antibody. Grb2 and Cbl constitutively associated and showed no increase in association over time (Fig. 5). However, by immunoprecipitating Grb2 and testing these immunoprecipitates for tyrosine phosphorylation we identified a stimulation-dependent increase in the amount of tyrosine phosphorylated Cbl associating with Grb2 (Fig. 5).

Immunoprecipitating Cbl and blotting the membrane with anti-Sos or vice versa, revealed no association of these two proteins in unstimulated or stimulated Jurkat cells (data not shown).

CrkL but Not CrkII and Tyr-P-Cbl Exhibit a Stimulation-Dependent Increase in Association

Since the Crk family are known associating proteins of Cbl we investigated the potential role of the adapter molecules CrkL and CrkII in L-selectin signaling. We tested if CrkII or CrkL, respectively, associate with Cbl in a stimulation-dependent manner by immunoprecipitating CrkII or CrkL and incubating the membrane with an anti-Cbl or anti-P-Tyr antibody. In CrkL immunoprecipitates association of Tyr-P-Cbl increased in a stimulation-dependent manner (Fig. 6). No activation-induced association of CrkII and Cbl could be detected (not shown).

DISCUSSION

Crosslinking of the L-selectin receptor by natural ligands or monoclonal antibodies initiates several in-

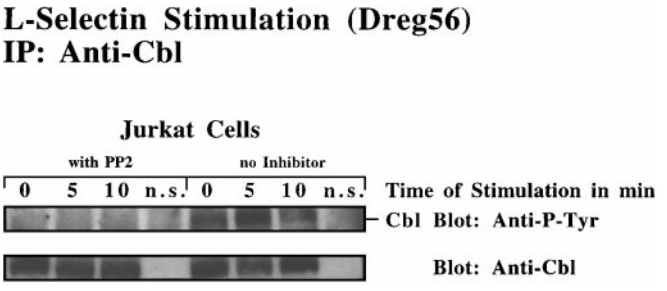

FIG. 4. Tyrosine phosphorylation of Cbl upon L-selectin triggering is pharmacologically inhibited by the src-tyrosine kinase inhibitor PP_2. Jurkat cells were preincubated with 50 μM PP_2 for 15 min at 37°C before stimulation by Dreg56. PP_2 prevented L-selectin-induced tyrosine phosphorylation of the Cbl protein compared to no treatment. Basal expression of Cbl was not influenced by PP_2 incubation as seen in the blot below.

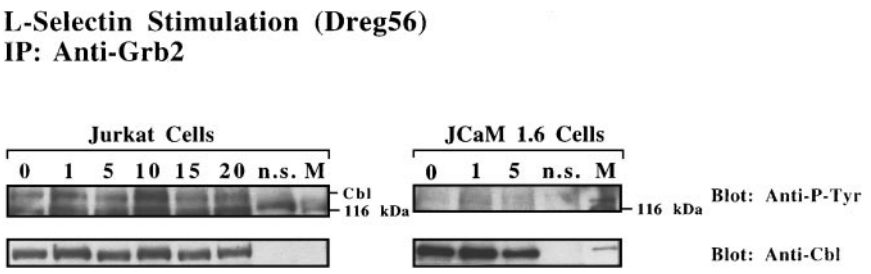

FIG. 5. The adapter molecules Cbl and Grb2 associate constitutively. L-Selectin stimulation leads to an increase of tyrosine phosphorylated Cbl binding to Grb2 in Jurkat but not in JCaM1.6 cells. Jurkat and JCaM1.6 cells were stimulated with Dreg56, Grb2 was immunoprecipitated, and blots were incubated with an anti-Cbl and an anti-P-Tyr antibody. Blotting with an anti-Cbl antibody demonstrates the constitutive association of Grb2 with Cbl. Incubating the membrane with an anti-P-Tyr antibody reveals that in spite of the constitutive association of these two adapter molecules. L-selectin stimulation leads to an increase of tyrosine phosphorylated Cbl interacting with Grb2 in Jurkat cells. In JCaM1.6 cells Cbl also associates with Grb2 constitutively. However, no tyrosine-phosphorylated Cbl could be detected associating with Grb2 after L-selectin stimulation.

tracellular signaling cascades. We reported recently that the engagement of L-selectin on Jurkat T lymphocytes and on PBL induced a p56lck kinase dependent tyrosine phosphorylation of the L-selectin molecule and MAP-kinase. L-selectin triggering resulted in activation of the small G-proteins Ras and Rac (7). In dependency on the activation of the small G-proteins we identified an L-selectin induced release of oxygen radicals (7), activation of stress-activated protein kinases (9) and reorganization of the cytoskeleton (8).

In contrast to the receptor tyrosine kinases, whose autophosphorylation creates multiple distinct phosphopeptide motifs as docking sites for SH2 domain containing signaling molecules, L-selectin lacks any known intrinsic enzymatic activity. To further elucidate signal transduction of L-selectin it is crucial to identify how signaling molecules with docking functions are recruited.

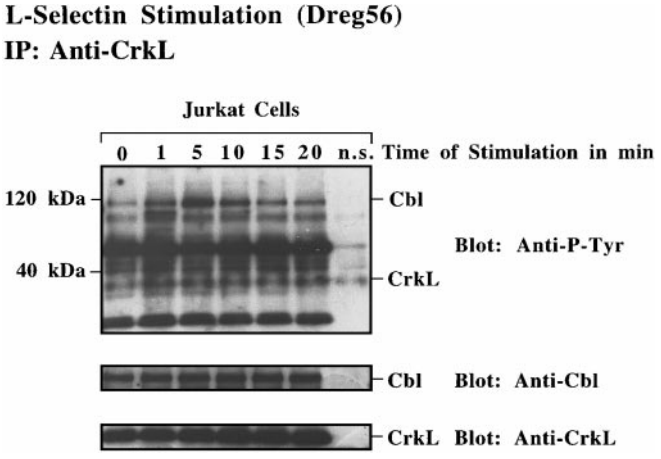

FIG. 6. There is a stimulation-induced increase in binding of tyrosine-phosphorylated Cbl to CrkL. Jurkat cells are stimulated with Dreg56, CrkL immunoprecipitates were separated on SDS/PAGE and blotted with an anti-P-Tyr antibody. L-Selectin stimulation induces an increase in tyrosine phosphorylated Cbl coprecipitating in CrkL IPs. In the two blots below membranes were reprobed with anti-Cbl or anti-CrkL, respectively, to test for equal amount of protein in each lane.

In this study we therefore defined early signal transduction events in L-selectin signaling in lymphocytes.

First, we identified a 120-kDa protein that gets tyrosine phosphorylated upon L-selectin triggering as the Cbl adapter molecule. We showed that the Cbl protein gets tyrosine phosphorylated upon L-selectin triggering using three L-selectin antibodies recognizing different epitopes of L-selectin as characterised by binding studies (21). Tyrosine phosphorylation of Cbl after L-selectin stimulation was dependent on the functional expression of the src-tyrosine kinase p56lck, since no tyrosine phosphorylation of Cbl was detected in p56lck-deficient JCaM1.6 cells. These findings are further encouraged by the pharmacological inhibition of tyrosine phosphorylation of Cbl by PP_2 and Lavendustin A, inhibitors of tyrosine kinases. To confirm the biological significance of the L-selectin-induced tyrosine phosphorylation of Cbl, PBL were isolated from healthy volunteers, stimulated with L-selectin antibodies and Cbl IPs were tested for tyrosine phosphorylation. L-selectin induced tyrosine phosphorylation of Cbl to a comparable extent as in Jurkat cells encouraging the general significance of the observed signaling events.

Second, we showed an activation induced increase in binding of tyrosine phosphorylated Cbl with Grb2. The Cbl-Grb2 association, which is mediated by the SH3 domain of Grb2 and the proline-rich region of Cbl was constitutively. This association appears to be of functional relevance because about 30–50% of the total Cbl protein interacts with Grb2 in T cells (22). In general, the Grb2 adapter molecule plays an important role in coupling cell-surface receptors to the Ras signaling pathway through its binding to the Ras guanine nucleotide exchange factor Sos (23). For L-selectin signal transduction we recently demonstrated in coimmunoprecipitation experiments an L-selectin-induced association of the L-selectin receptor with the Sos-Grb2

complex (7). However, Sos could not be detected in Cbl IPs and no Cbl was coprecipitated with Sos IPs, suggesting that Cbl-Grb2 and Sos-Grb2 are forming two different complexes. This is in accordance with findings after T cell receptor cross-linking where Cbl could be coprecipitated with Grb2 but not with Sos (24).

Third, we could demonstrate after L-selectin stimulation in CrkL, but not in CrkII IPs, an increase of tyrosine phosphorylated Cbl. Studies on T cell activation also demonstrated that tyrosine phosphorylated Cbl rapidly complexes with the Crk adapter protein via its SH2 domain (17, 18). Furthermore, in CrkL IPs several phosphorylated proteins with apparent masses of 30, 70, 90, and 170 kDa were coprecipitated in addition to Cbl which have to be identified. This suggests that CrkL might serve additional adapter functions in L-selectin signal transduction. However, we were unable to detect CrkII or CrkL, respectively, in Cbl IPs (not shown). As recently reported by other groups this might be due to the stochiometry of the complexes (25).

The activation dependent association between Cbl and CrkL in L-selectin signal transduction may bring several functional signaling molecules together to form a potential supramolecular complex. The role of constitutive binding of p120Cbl to Grb2 and CrkL is speculative. Cbl binds to the SH3 domain of Grb2 coming in close contact to membrane proteins that bind Grb2 via the SH2 domain. A possible function of this event could be to deliver p120Cbl to active tyrosine kinases resulting in its phosphorylation. Tyrosine phosphorylated Cbl could then bind to CrkL. The interaction of tyrosine phosphorylated Cbl with CrkL might link these adapter molecules to the activation of Ras and related proteins. CrkL constitutively forms a complex with C3G, a guanine nucleotide exchange factor for the Ras related protein Rap1 (26), which is thought to negatively regulate Ras signaling (27). Thus CrkL and Grb2 seem to couple to distinct guanine nucleotide exchange factors that regulate Rap1 and Ras, respectively. The association of Cbl with these two adapter proteins might be involved in the balance between these two pathways.

The biological function of Cbl is not well defined yet. However, a recent study elucidating the potential role of Cbl in TCR-initiated signaling demonstrated that Cbl is required for a Ras-dependent signaling pathway that leads to NFAT activation, a transcriptional element that plays a key role in the induction of the IL-2 gene (28). Furthermore, the Cbl induced activation of NFAT seems to be dependent on Ras activation. From experiments with Cbl mutant mice it is thought that Cbl might play a role as a negative regulator in tyrosine kinase mediated signaling pathways (29–31). An additional role of Cbl is suggested in transducing stress-activated signals by activating the MAP-K JNK after hepatocyte growth factor triggering (32) and by inhibition of the JAK-STAT pathway in EGF receptor mediated signaling (33).

Furthermore, there is recent evidence, that Cbl down-regulates growth factor receptors by helping to ubiquitinate them, thereby marking them to destruction or internalization (34).

Since the expression of Cbl is downregulated during cellular differentiation, but not in response to mitogenic stimuli, Cbl may have different roles in regulating growth versus differentiation events. Therefore, Cbl seems to display a versatile regulatory activity dependent on the specific cellular context and the proteins that associate with Cbl.

In summary, the present study further elucidates L-selectin induced signal transduction in lymphocytic cell lines and peripheral blood lymphocytes. Stimulation of these cells using different L-selectin antibodies revealed tyrosine phosphorylation of Cbl and an increase in binding of tyrosine phosphorylated Cbl to Grb2 and CrkL. Therefore, the adapter molecule c-Cbl seems to play a significant role in early signaling events in L-selectin triggering recruiting other signaling molecules to multiprotein complexes. The multiprotein complex of CrkL and Grb2 with Cbl may serve as molecular linkers that couple L-selectin generated signals to signaling pathways of small G-proteins.

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