Clnk, a Novel SLP-76-related Adaptor Molecule Expressed in Cytokine-stimulated Hemopoietic Cells

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Summary

We have identified a novel Src homology 2 domain–containing leukocyte protein of 76 kD (SLP-76)–related molecule which we have termed Clnk (for cytokine-dependent hemopoietic cell linker). Unlike its relatives SLP-76 and B cell linker protein (Blnk), Clnk is not expressed uniformly within a given hemopoietic cell lineage. Even though it can be detected in several cell types, including T cells, natural killer cells, and mast cells, its expression seems to be strictly dependent on sustained exposure to cytokines such as interleukin (IL)-2 and IL-3. Strong support for the notion that Clnk is involved in immunoreceptor signaling was provided by the observation that it inducibly associated with at least one tyrosine-phosphorylated polypeptide (p92) in response to immunoreceptor stimulation. Moreover, transient expression of Clnk caused an increase in immunoreceptor-mediated signaling events in a T cell line. Taken together, these results show that Clnk is a novel member of the SLP-76 family selectively expressed in cytokine-stimulated hemopoietic cells. Furthermore, they suggest that Clnk may be involved in a cross-talk mechanism between cytokine receptor and immunoreceptor signaling.

Key words: adaptor • SLP-76 • Blnk • signaling • lymphocytes

The Src homology 2 (SH2) domain–containing leukocyte protein of 76 kD (SLP-76) family of adaptors includes two known members named SLP-76 and B linker protein (Blnk)/SLP-65/B cell adaptor containing the SH2 domain (BASH) (1–4). Whereas SLP-76 is expressed in T cells, NK cells, mast cells, and platelets (5), Blnk/SLP-65/BASH is solely found in B cells (1). These two molecules share a common structure, including from the NH₂ to the COOH terminus: (a) a basic region; (b) an acidic region containing several sites of tyrosine phosphorylation and proline-rich motifs able to associate with SH2 and SH3 domain–bearing molecules, respectively; (c) an SH2 domain; and (d) a short COOH-terminal sequence of unknown function.

Several lines of evidence demonstrate that SLP-76 plays an essential role in T cells, platelets, and mast cells. Most importantly, SLP-76-deficient mice were found to exhibit a profound block in T cell maturation at a very early (double-negative) stage, presumably due to a defect in pre-TCR signaling (6, 7). Functional defects have also been noted in platelets and mast cells, but not in NK cells, derived from these animals (6, 9). Additionally, characterization of an SLP-76-deficient variant of the T cell line Jurkat strongly suggested that SLP-76 is necessary for TCR-induced tyrosine phosphorylation of phospholipase C (PLC)-γ1, mitogen-activated protein (MAP) kinase activation, and IL-2 production in mature T cells (10). In a similar way, it is likely that Blnk plays a central role in B cell development and activation. Although Blnk-deficient mice have not yet been described, overexpression of Blnk in B cell lines was reported to cause an increase in B cell receptor (BCR)-induced tyrosine phosphorylation of PLC-γ1 and PLC-γ2, intracellular calcium flux, and nuclear factor of activated T cells (NFAT) activation (1). Conversely, a Blnk-deficient B cell line exhibited dramatically reduced BCR-induced tyrosine phosphorylation of PLC-γ2, intracellular calcium flux, and activation of c-Jun NH₂-terminal kinase (JNK) (11).

Even though the exact mechanisms of action of SLP-76 and Blnk remain to be elucidated, it is noteworthy that SLP-76 can physically associate with several other proteins, such as Vav, Gads, Nck, and Fyb/SLP-76–associated phosphoprotein of 130 kD (SLAP-130) (12–19). Furthermore, Blnk can bind to Vav, PLC-γ2, and Nck (1). Hence, it is probable that these adaptors function by or-
chestrating interactions between molecules playing a critical role in immunoreceptor signaling.

In this paper, we report the cloning and characterization of a novel molecule, which appears to represent a third member of the SLP-76 family. Contrary to SLP-76 and Blnk, this polypeptide is selectively expressed in cytokine-stimulated hematopoietic cells. Based on this observation, it was termed Clnk (for cytokine-dependent hematopoietic cell linker).

Materials and Methods

dDNA Cloning. A partial dDNA cDNA was initially cloned from a mouse primitive hematopoietic cell (EML-16) cDNA library, during a yeast two-hybrid system screen using the cytoplasmic domain of platelet endothelial cell adhesion molecule (PECAM)-1 as a bait (our unpublished results). Several full-length cDNAs were subsequently isolated from a cDNA library made from day 16 fetal mouse thymus (provided by Dr. L. Matis, Alexion Pharmaceuticals, New Haven, CT), taking the partial cDNA as a probe. The 5′ end of dnk was also verified by 5′ rapid amplification of cDNA ends (RACE; data not shown). Both strands of a representative full-length cDNA clone were sequenced. The dnk cDNA sequence data are available from EMBL/GenBank/DDBJ under accession no. AF187819.

Cells and Tissues. The various hematopoietic cell lines used herein were described elsewhere (20–22). Splenic T cells were isolated from 6–8-wk-old C57BL/6 mice using T cell columns (Cytovax Biotechnologies Inc.). More than 90% of cells obtained were CD3+ (data not shown). Splenic T cells were stimulated for 48 h with anti-CD3 mAb 145-2C11 (1 μg/ml,井曼 Pharmaceuticals, New Haven, CT), taking the partial cDNA as a probe. The 5′ end of dnk was also verified by 5′ rapid amplification of cDNA ends (RACE; data not shown). Both strands of a representative full-length cDNA clone were sequenced. The dnk cDNA sequence data are available from EMBL/GenBank/DDBJ under accession no. AF187819.

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Ribonuclease Protection Assays. Ribonuclease protection assays were performed as described elsewhere (20, 25), using a radiolabeled antisense riboprobe corresponding to nucleotides 965–1231 of mouse dnk (sequence data available from EMBL/GenBank/DDBJ under accession no. AF187819). The integrity of the various RNAs used in these assays was confirmed by electrophoresis of samples in agarose-formaldehyde gels, and subsequent staining of the gel with ethidium bromide (data not shown).

Antibodies. Polyclonal antibodies against Clnk were produced by immunizing rabbits with a trpE fusion protein encompassing amino acids 199–301 of Clnk. These antibodies did not cross-react with SLP-76 (data not shown). Affinity purification was achieved by passing the crude serum over a column containing the immunogen immobilized on Affigel (Bio-Rad). Anti-phosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology. Rabbit antibodies directed against Vav have been reported elsewhere (26).

Cell Stimulation. The IL-2–dependent mouse T cell line 5.32.10 (22) was activated via the TCR by stimulation for 3 min at 37°C in the presence of anti-CD3 mAb 145-2C11 and rabbit anti-

hamster (RAH) IgG. The IL-3-dependent mouse myeloid cell line B6SuA3 (21) was activated via FcγRI by incubation for 3 min at 37°C with mouse IgG2a followed by F(ab′)2 fragments of sheep anti-mouse (SAM) IgG. After stimulation, cells were lysed in TNE buffer (1× TNE: 50 mM Tris pH 8.0, 1% NP-40, 2 mM EDTA), supplemented with protease and phosphatase inhibitors as detailed elsewhere (27).

Immunoprecipitations and Immunoblots. For immunoprecipitation, postnuclear lysates were incubated with the indicated antibodies for 2 h. Immune complexes were then recovered by the addition of formalin-fixed Staphylococcus aureus (Pansorbin; Calbiochem-Novabiochem). After several washes, proteins were eluted in sample buffer and resolved by SDS-PAGE. For analysis of Clnk expression, cells were lysed directly in boiling SDS-containing sample buffer, and lysates corresponding to equivalent cell numbers were resolved by gel electrophoresis. Immunoblots were done according to a previously described protocol (28), using either 32P-labeled goat anti–mouse IgG (ICN Biomedicals) or protein A–horseradish peroxidase and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Translations. For transfections, the mouse dnk and slp-76 cDNAs were individually cloned in the mammalian expression vector pX M 139, which contains the adenovirus major late promoter and the SV40 origin of replication. Cos-1 cells were transfected by the DEAE-dextran method with either pX M 139 alone or pX M 139-dnk (4 μg), as outlined previously (29). Jurkat TAg cells were transfected by electroporation with pX M 139 alone, pX M 139-dnk, or pX M 139-slp-76, in the presence of either pNFAT–luciferase, pAP-1–luciferase, or pIL2 promoter–luciferase, according to a protocol detailed elsewhere (30). After 40 h, 106 viable cells were stimulated for 7 h with anti-CD3 mAb OKT3 (10 μg/ml) alone, OKT3 plus PM A (50 ng/ml), or PM A plus ionomycin (0.75 μg/ml). Cells were then lysed and assayed for luciferase activity using the luciferase reporter assay system (Promega) and a luminometer (EG&G Berthold). Results are presented as percentage of luciferase activity induced by PM A plus ionomycin.

Results

Identification of a dDNA Encoding a Novel SH2 Domain–containing Molecule Related to SLP-76. During an attempt to identify new ligands for the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) of PECAM-1 (30, 31) using the yeast two-hybrid system, we cloned a novel mouse cDNA (see Materials and Methods). Preliminary sequence analysis indicated that this partial cDNA encoded a new molecule in mammalian cells (our unpublished results), its characterization was pursued as it constituted a novel molecule in mammalian cells (our unpublished results). During an attempt to identify new ligands for the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) of PECAM-1 (30, 31) using the yeast two-hybrid system, we cloned a novel mouse cDNA (see Materials and Methods). Preliminary sequence analysis indicated that this partial cDNA encoded a new molecule in mammalian cells (our unpublished results), its characterization was pursued as it constituted a novel molecule in mammalian cells (our unpublished results).
/www.ncbi.nlm.nih.gov/BLAST/) searches showed that the SH2 domain of Clnk is most closely related to those of SLP-76 and Blnk (40–53% identity, 62–69% similarity; references 1–4) (Fig. 1 B). This degree of homology is typical for proteins belonging to the same family. While the overall structures of Clnk, SLP-76, and Blnk are also similar, it should be pointed out that the actual sequences outside the SH2 region of Clnk are quite distinct from those of the two other molecules. Despite this difference, it remains likely that Clnk represents a novel member of the SLP-76 family. Further support for this notion will require comparison of the exon–intron structures of the three slp-76–related genes.

clnk Is Selectively Expressed in Cytokine-stimulated Hemopoietic Cells. The expression pattern of Clnk was analyzed by ribonuclease protection assay, as detailed in Materials and Methods (Fig. 2). Analysis of various mouse tissues (Fig. 2 A) indicated that clnk RNA was low or undetectable in most tissues, including bone marrow (lane 3), lymph node (lane 4), spleen (lane 8), and thymus (lane 10). Slightly greater quantities were present in kidney (lane 11). Evaluation of a panel of mouse hemopoietic cell lines (Fig. 2 B) revealed that most T cell (lanes 2–5), B cell (lanes 6–11), and macrophage (lanes 12 and 13) cell lines did not contain clnk transcripts. However, a subset expressed easily appreciable amounts of clnk RNA. These included the IL-2–dependent T cell line HT-2 (lane 5), the IL-3–dependent myeloid cell lines B6SutA (lane 15) and FDC-P1 (lane 16), and the primitive leukemia cell line L1210 (lane 17). clnk was also expressed in the IL-2–dependent T cell lines CTLL-2 (Fig. 2 C, lane 6) and 5.32.10 (lane 7), and in the IL-3–dependent myeloid cell line 32D (lane 11). Strikingly, most of the cell lines expressing clnk were dependent on cytokines for sustained growth. On this basis, our novel cDNA was named clnk, for cytokine-dependent hemopoietic cell linker.

The distribution of clnk was also characterized in normal hemopoietic cells. Considering our results with the factor-dependent cell lines, it was plausible that cytokine stimulation was required to induce accumulation of clnk in these cell types. To test this possibility, T cells were purified from mouse spleen, and the impact of IL-2 stimulation on clnk expression was evaluated. For these experiments, splenic T cells were pretreated with anti-CD3 antibodies in order to allow expression of functional IL-2 receptors. As expected, resting splenic T cells (Fig. 3 A, lane 1) expressed no appreciable amounts of clnk RNA. However, a substantial increase was observed in IL-2–stimulated T cells (Fig. 3 A, lane 2), indicating that clnk expression was induced by cytokine stimulation.

Figure 1. Predicted sequence and structure of Clnk. (A) The predicted amino acid sequence of Clnk is shown. Amino acid numbering is indicated on the right. The location of the SH2 domain is highlighted by a thick continuous line. A schematic representation of the predicted structure of Clnk is depicted at the bottom. (B) Comparison of the SH2 domains of mouse Clnk, mouse SLP-76, and mouse Blnk. Identical amino acids are shown as dots.

Figure 2. Expression of clnk RNA in various mouse tissues and cell lines. The presence of clnk transcripts in a variety of mouse tissues and cell lines was ascertained by ribonuclease protection assay. In these assays, the undigested riboprobe was ~345 nucleotides in length, whereas the fragment protected by cellular clnk transcripts was ~266 nucleotides long. End-labeled MspI-digested fragments of pBR 322 were used as markers. The positions of size markers, the undigested riboprobe, and the protected fragment of the riboprobe are indicated. Exposures (A) 3 d; (B) 4 d; (C) 4 d.

(lane 6), the mastocytoma cell line P815 (lane 14), the IL-3–dependent myeloid cell lines B6SuA1 (lane 15) and FDC-P1 (lane 16), and the primitive leukemia cell line L1210 (lane 17). clnk was also expressed in the IL-2–dependent T cell lines CTLL-2 (Fig. 2 C, lane 6) and 5.32.10 (lane 7), and in the IL-3–dependent myeloid cell line 32D (lane 11). Strikingly, most of the cell lines expressing clnk were dependent on cytokines for sustained growth. On this basis, our novel cDNA was named clnk, for cytokine-dependent hemopoietic cell linker.

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To help resolve this issue, the impact of IL-2 on a small increase in the levels of Clnk protein. The nature of the additional immunoreactive product consistent with Clnk. A similar polypeptide was observed in Cos-1 cells transfected with a dnk cDNA (lane 7), but not in control Cos-1 cells (lane 6). The presence of the Clnk protein was also examined in normal mouse hemopoietic cells (Fig. 4 B). This assay demonstrated that IL-3–propagated BM M Cs (lane 2), IL-2–stimulated T cells (lane 3), and IL-2–activated NK cells (lane 4) contained easily appreciable amounts of the 54-kD Clnk protein. The nature of the additional immunoreactive products of ~50 and 44 kD in dnk-expressing hemopoietic cells (lanes 2–4) remains to be determined.

To obtain evidence for the participation of Clnk in immunoreceptor signaling, the impact of immunoreceptor

Figure 3. Accumulation of dnk RNA in normal mouse hemopoietic cells. The expression of dnk in normal mouse hemopoietic cells was measured by ribonuclease protection assay. Whereas the undigested riboprobe contained ~345 nucleotides, the fragment protected by cellular dnk transcripts was expected to be ~266 nucleotides in length. End-labeled MspI fragments of pBR322 were used as size markers. The migrations of size markers, the undigested riboprobe, and the protected fragment of the riboprobe are highlighted. (A) Induction of dnk expression by IL-2 in mouse splenic T cells. For activation with anti-CD3 antibodies (lane 2), cells were stimulated with plate-bound anti-CD3 mAb 145-2C11 for 48 h. For stimulation with IL-2 (lane 3), cells were reactivated with anti-CD3 antibodies as above, harvested, and replated for 5 d in growth medium supplemented with high concentrations of recombinant IL-2 (~50 U/ml). Exposure: 3 d. (B) Time course of IL-2 stimulation. Cells were activated with anti-CD3 mAbs as detailed in A.

Clnk, a Novel SLP-76–related Adaptor

AAfter washing, they were replated for the indicated periods of time in medium containing or not recombinant IL-2. Exposure: 31 h. (C) Effects of IL-2 on dnk expression in HT-2 T cells. Cells were harvested, washed, and replated in growth medium lacking IL-2 for 12 h. IL-2 was then added for the indicated periods of time, before final harvesting. Exposure: 6 h. (D) Expression of dnk in mouse BM M Cs and NK cells. Cells were generated as detailed in Materials and Methods. Exposure: 16 h.
stimulation on its state of tyrosine phosphorylation was examined (Fig. 5). The IL-2–dependent mouse T cell line 5.32.10 was activated through the TCR by a combination of anti-CD3 mAb 145-2C11 and RAH IgG, and the changes in Clnk tyrosine phosphorylation were monitored by immunoblotting of anti-Clnk immunoprecipitates with antiphosphotyrosine mAb 4G10 (Fig. 5 A). We found that TCR triggering (lane 2) induced an increase in tyrosine phosphorylation of a 54-kD protein (p54) consistent with Clnk. Interestingly, it also provoked the appearance of an ∼92-kD phosphotyrosine-containing molecule (p92) in Clnk immunoprecipitates. Neither p54 nor p92 was present in immunoprecipitates generated with normal rabbit serum (NRS, lanes 3 and 4). The regulation of Clnk was also studied in the IL-3–dependent cell line B6SutA1, which can be activated via its high-affinity receptor for IgG (FcγRI) by incubation with mouse IgG 2a followed by F(ab′)2 fragments of SAM IgG (Fig. 5 B). As seen in 5.32.10 T cells, Clnk became associated with a tyrosine-phosphorylated p92 in response to activation of B6SutA1 cells (top panel, lane 2). However, it is noteworthy that, in contrast to 5.32.10, the Clnk protein found in B6SutA1 was constitutively tyrosine phosphorylated (lane 1). While the basis for this difference is not known, it may reflect cell type–specific variations in Clnk regulation.

Given the ability of SLP-76 and Blnk to associate with p95vav (1, 17, 18), we wanted to determine whether the Clnk–associated p92 represented Vav. For this purpose, parallel immunoprecipitates from the experiment depicted in Fig. 5 B were probed by immunoblotting with anti-Vav antibodies (Fig. 5 B, bottom panel). Even though Vav could easily be seen in total cell lysates (lanes 5 and 6), we were unable to detect any amount of Vav in Clnk immunoprecipitates (lanes 1 and 2). Thus, it seemed probable that the 92-kD tyrosine-phosphorylated protein interacting with Clnk in response to immunoreceptor stimulation was distinct from Vav.

Clnk Enhances Antigen Receptor–induced Activation of NFAT, AP-1, and IL-2 Promoter in T Cells. Taking into consideration its relatedness to SLP-76 and Blnk, we wished to assess whether Clnk was able to impact on the outcome of immunoreceptor signaling. For this purpose, the effect of Clnk expression on antigen receptor–mediated activation of NFAT, AP-1, and IL-2 promoter was evaluated (Fig. 6). Jurkat T cells were transiently transfected by electroporation with a construct encoding either Clnk or SLP-76, in the presence of NFAT–luciferase, AP-1–luciferase, or IL-2 promoter–luciferase reporter plasmids. 40 h after transfection, cells were stimulated with anti-CD3 mAb OKT3 in the absence or presence of the phorbol ester PMA. After cell lysis, changes in luciferase activity were determined using a luminometer. All results were normalized according to the luciferase activity induced by the combination of nonprecipitates. N either p54 nor p92 was present in immunoprecipitates generated with normal rabbit serum (NRS, lanes 3 and 4). The regulation of Clnk was also studied in the IL-3–dependent cell line B6SutA1, which can be activated via its high-affinity receptor for IgG (FcγRI) by incubation with mouse IgG 2a followed by F(ab′)2 fragments of SAM IgG (Fig. 5 B). As seen in 5.32.10 T cells, Clnk became associated with a tyrosine-phosphorylated p92 in response to activation of B6SutA1 cells (top panel, lane 2). However, it is noteworthy that, in contrast to 5.32.10, the Clnk protein found in B6SutA1 was constitutively tyrosine phosphorylated (lane 1). While the basis for this difference is not known, it may reflect cell type–specific variations in Clnk regulation.

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Methods. Luciferase activity is expressed as percentage of the activity induced by PMA plus ionomycin (which represents 100%).

Discussion

In this manuscript, we report the identification of a novel SLP-76–related molecule which we have termed Clnk. At this time, the most unique feature of Clnk is its expression pattern. While SLP-76 is found in most, and perhaps all, T cells, NK cells, mast cells, and myeloid cells (5), and Blnk is seemingly contained in all B cells (1, 2, 4), the Clnk protein is absent from most hemopoietic cells. However, significant quantities were uncovered in IL-2–stimulated splenic T cells, IL-2–activated NK cells, and IL-3–propagated BMMCs. In a similar way, expression of Clnk was documented in a variety of IL-2–dependent and IL-3–dependent cell lines. By contrast, it was not found in a large number of cytokine-independent cell lines, with the exception of P815 and L1210.

Interestingly, P815 is a mastocytoma cell line carrying an activated mutant of the Kit receptor protein tyrosine kinase (34). Because this mutation mimics the effects of constitutive stimulation by the Kit ligand, this finding added further credence to the notion that Clnk expression is induced after sustained exposure to cytokines. Furthermore, it raises the possibility that growth factors other than IL-2 and IL-3 may have the capacity to induce Clnk expression. Future studies should be aimed at testing this possibility.

The involvement of Clnk in immunoreceptor signaling was first implied by the observation that it became acutely associated with a tyrosine-phosphorylated molecule (p92) in response to stimulation of either TCR or FcyRI. Whereas the identity of p92 remains to be determined, it is likely that this molecule is an effector or a regulator of Clnk. Possibly, p92 allows Clnk to become functionally active in immunoreceptor-stimulated cells. More definitive evidence for the participation of Clnk in immunoreceptor-mediated signal transduction was lent by the finding that Clnk, like SLP-76, was capable of augmenting antigen receptor–induced activation of NFAT, AP-1, and IL-2 promoter in transiently transfected T cells. At first glance, this result may suggest that Clnk and SLP-76 actually have redundant functions in hemopoietic cells. The presence of Clnk could explain the lack of functional abnormalities noted in IL-2–activated NK cells from SLP-76–deficient mice (35). Nonetheless, it should be pointed out that Clnk and SLP-76 are likely to have specialized roles. Clnk lacks the two DYESP motifs present in the NH2-terminal portion of SLP-76, which mediate binding to the exchange factor Vav and the adaptor molecule Gads (12, 17–19, 33, 36). Accordingly, we have been unable to show binding of Clnk to either Vav or Gads (this report; our unpublished results). Even though these interactions are not necessary for SLP-76–mediated activation of NFAT (33, 36), they appear to be required for proper reorganization of the actin cytoskeleton during T cell activation (12). Likewise, the binding motif for the Gads adaptor molecule in SLP-76 (residues 224–244 [15]) is not strictly conserved in Clnk, raising the possibility that Clnk does not associate with Gads. Instead, Clnk possesses other sites of tyrosine phosphorylation and proline-rich motifs, as well as an SH2 domain, which presumably allow associations with an alternative set of partners. One of these molecules may be p92, which was tyrosine phosphorylated and became associated with Clnk in response to immunoreceptor stimulation. Clearly, a better understanding of the role of Clnk will come with the identification of these partners.

In summary, we have identified a novel SLP-76–related adaptor molecule named Clnk. While Clnk is absent in most hemopoietic cells, it is abundantly expressed in a variety of hemopoietic cell types after sustained exposure to cytokines. Taking into consideration our finding that Clnk was able to regulate immunoreceptor signaling, these results suggest that Clnk may provide a mechanism that modulates immunoreceptor signaling in response to cytokine receptor stimulation.
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