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

By Ming Yu Cao,* Dominique Davidson,* Jie Yu,* Sylvain Latour,* and André Veillette*‡§‖

*From the *McGill Cancer Centre, and the ‡Department of Biochemistry, the §Department of Oncology, and the ‖Department of Medicine, McGill University, Montréal, Québec, Canada H3G 1Y6*

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)[1] 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 NH_2 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 (8, 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 NH_2-terminal kinase (JNK) (11).

Even though the exact mechanisms of action of SLP-76 and Blnk remain to be established, 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-γ, Grb2, and Nck (1). Hence, it is probable that these adaptors function by or-

M.Y. Cao and D. Davidson contributed work of equal importance to this paper and both should be viewed as first author.

[1]*Abbreviations used in this paper:* BCR, B cell antigen receptor; Blnk, B cell linker protein; BMMC, bone marrow–derived mast cell; Clnk, cytokine-dependent hemopoietic cell linker; Gads, Grb2-related adaptor downstream of Shc; NFAT, nuclear factor of activated T cells; PECAM, platelet-endothelial cell adhesion molecule; PLC, phospholipase C; RAH, rabbit anti–hamster; SAM, sheep anti–mouse; SH2, Src homology 2; SLP, SH2 domain–containing leukocyte protein of 76 kD.

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 hemopoietic cells. Based on this observation, it was termed Clnk (for cytokine-dependent hemopoietic cell linker).

Materials and Methods

cDNA Cloning. A partial *clnk* cDNA was initially cloned from a mouse primitive hemopoietic 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 *clnk* 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 *clnk* cDNA sequence data are available from EMBL/GenBank/DDBJ under accession no. AF187819.

Cells and Tissues. The various hemopoietic 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 [23]) immobilized on plastic. They were then harvested, washed extensively, and replated for the indicated periods of time in the absence or presence of recombinant mouse IL-2. NK cells were obtained from splenic tissue of 6–8-wk-old Nude CD-1 mice (Charles River Canada), as described previously (24). Resting spleen cells were expanded for ~8 d in growth medium containing recombinant IL-2. After this period, nearly 100% of cells recovered were $CD16^+$ and $CD3^-$ (data not shown). Bone marrow–derived mast cells (BMMCs) were established from the femurs of 8-wk-old C57BL/6 mice by prolonged culture (>3 wk) of bone marrow–derived cells in IL-3–containing medium. Approximately 100% of cells obtained with this protocol were positive for FcεRI (data not shown).

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 *clnk* (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). Antiphosphotyrosine 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 $B6SutA_1$ (21) was activated via FcγRI by incubation for 3 min at 37°C with mouse IgG_{2a} 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 ^{125}I-labeled goat anti–mouse IgG (ICN Biomedicals) or protein A–horseradish peroxidase and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Transfections. For transfections, the mouse *clnk* and *slp-76* cDNAs were individually cloned in the mammalian expression vector pXM139, 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 pXM139 alone or pXM139-*clnk* (4 μg), as outlined previously (29). Jurkat TAg cells were transfected by electroporation with pXM139 alone, pXM139-*clnk*, or pXM139-*slp-76*, in the presence of either pNFAT-luciferase, pAP-1–luciferase, or pIL2 promoter–luciferase, according to a protocol detailed elsewhere (15). After 40 h, 10^6 viable cells were stimulated for 7 h with anti-CD3 mAb OKT3 (10 μg/ml) alone, OKT3 plus PMA (50 ng/ml), or PMA 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 PMA plus ionomycin.

Results

Identification of a cDNA 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 SH2 domain–containing molecule (data not shown). Full-length cDNA clones were subsequently obtained through screening of a mouse thymus cDNA library and 5′ rapid amplification of cDNA ends (RACE). Although we were unable to show an association between PECAM-1 and this novel molecule in mammalian cells (our unpublished results), its characterization was pursued as it constituted a potentially interesting novel signal transduction molecule. The deduced amino acid sequence of a representative cDNA clone is shown in Fig. 1 A.

This molecule, termed Clnk (see below), is predicted to be a 435 amino acid polypeptide. It contains, from the NH_2 to the COOH terminus: a basic domain, a portion rich in tyrosines and prolines, an SH2 domain, and a COOH-terminal tail (Fig. 1 A). BLAST (available at http:/

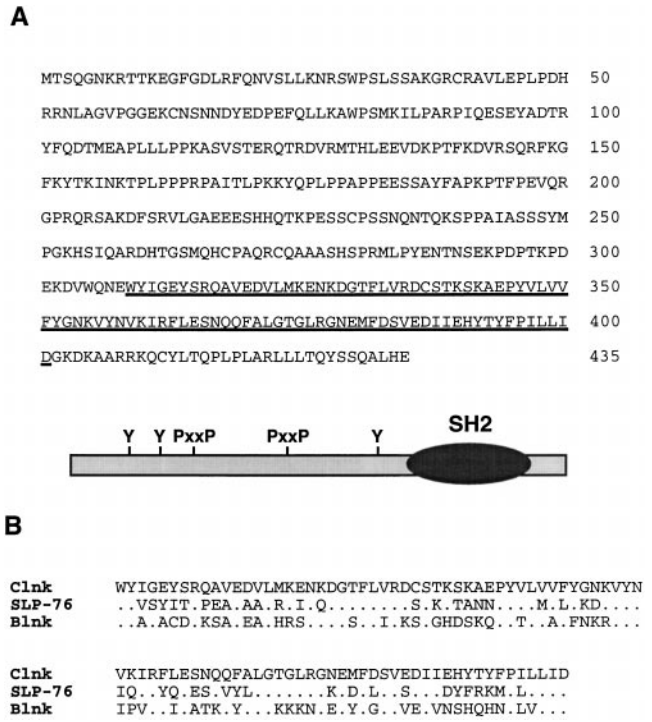

Figure 1. Predicted sequence and structure of Clnk. (A) The predicted amino acid sequence of Clnk is shown. Amino acid numbering is indicated on the right. The location of the SH2 domain is highlighted by a thick continuous line. A schematic representation of the predicted structure of Clnk is depicted 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.

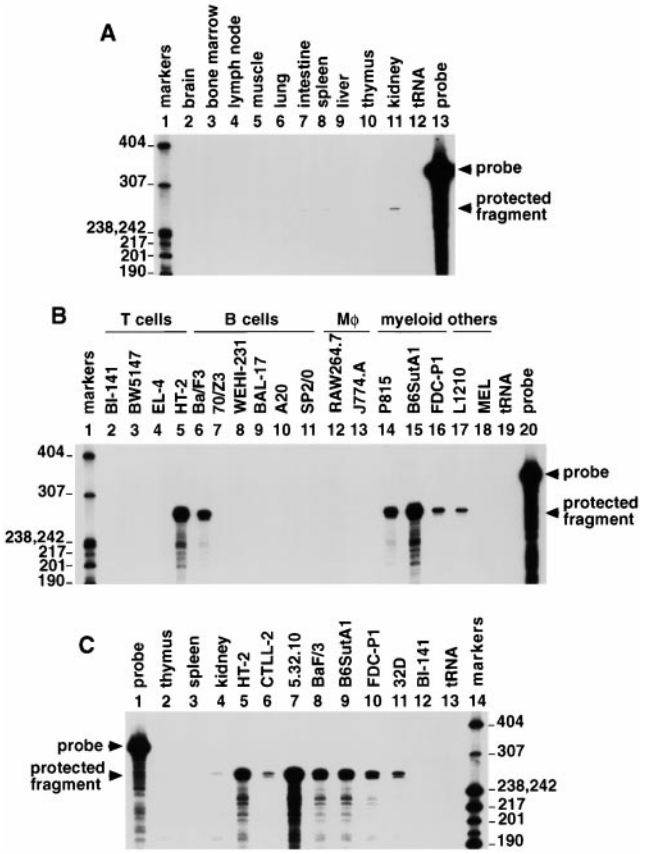

Figure 2. Expression of *clnk* RNA in various mouse tissues and cell lines. The presence of *clnk* transcripts in a variety of mouse tissues and cell lines was ascertained by ribonuclease protection assay. In these assays, the undigested riboprobe was ~345 nucleotides in length, whereas the fragment protected by cellular *clnk* transcripts was ~266 nucleotides long. End-labeled MspI-digested fragments of pBR322 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.

/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 pro-B cell line Ba/F3 (lane 6), the mastocytoma cell line P815 (lane 14), the IL-3–dependent myeloid cell lines $B6SutA_1$ (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 appre-

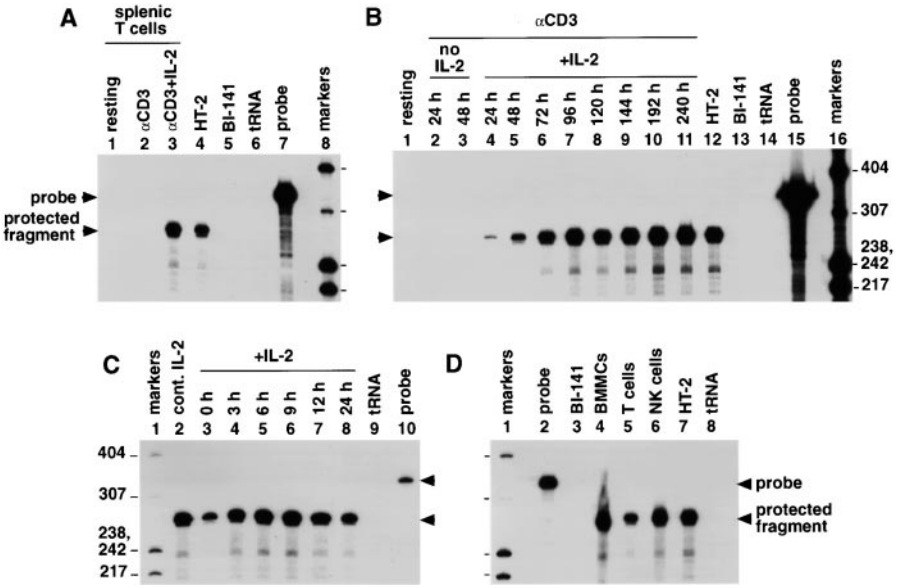

Figure 3. Accumulation of *clnk* RNA in normal mouse hemopoietic cells. The expression of *clnk* in normal mouse hemopoietic cells was measured by ribonuclease protection assay. Whereas the undigested riboprobe contained ~345 nucleotides, the fragment protected by cellular *clnk* transcripts was expected 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 *clnk* 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 preactivated with anti-CD3 antibodies as above, harvested, washed, 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 mAb as detailed in A. After 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 *clnk* 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 *clnk* in mouse BMMCs and NK cells. Cells were generated as detailed in Materials and Methods. Exposure: 16 h.

ciable amount of *clnk* transcripts. In a similar way, cells preactivated with anti-CD3 antibodies for 48 h (lane 2) did not contain *clnk*, even though they exhibited robust thymidine incorporation (data not shown). However, additional stimulation with exogenous IL-2 (lane 3) induced strong expression of *clnk*, in a manner comparable to that seen in IL-2–propagated HT-2 cells (lane 4).

Next, a time course of IL-2 stimulation was performed (Fig. 3 B). After activation with anti-CD3 antibodies, splenic T cells were washed extensively and replated in growth medium with or without IL-2 for various periods of time. In the absence of exogenous IL-2 (lanes 2 and 3), there was a small increase in the levels of *clnk* RNA, which was only visible on longer autoradiographic exposures of this gel (data not shown). In the presence of IL-2 (lanes 4–11), though, there was a progressive induction of *clnk* RNA expression. The enhancement of *clnk* expression could be observed as early as 24 h after addition of IL-2 (lane 4), and was maintained for at least 10 d (lane 11).

Coupled with the findings shown in Fig. 2, these data strongly suggested that sustained IL-2 stimulation was necessary for induction of *clnk* expression in T cells. But it was also possible that a small subset of T cells constitutively expressing *clnk* expanded preferentially in the presence of IL-2. To help resolve this issue, the impact of IL-2 on *clnk* expression was ascertained in an established T cell line (HT-2). After depriving them of IL-2 for 12 h, HT-2 cells were restimulated with IL-2 for various periods of time, and the levels of *clnk* RNA were monitored by ribonuclease protection assay (Fig. 3 C). First, this analysis showed that removal of IL-2 (lane 3) caused an approximately fourfold reduction in the abundance of *clnk* RNA in HT-2 cells, compared with cells grown in the continuous presence of IL-2 (lane 2). Furthermore, it demonstrated that reintroduction of the cytokine (lanes 4–8) provoked a rapid increase (approximately sixfold) in *clnk* expression, which was maximal after 6–9 h of stimulation (lanes 5 and 6). Thus, these observations were consistent with the idea that *clnk* expression in T lymphocytes was a consequence of IL-2 stimulation.

Finally, the expression of *clnk* was measured in other cytokine-induced normal hemopoietic cell types (Fig. 3 D). As was the case for IL-2–stimulated splenic T cells (lane 5), we found that *clnk* RNA accumulated in high amounts in IL-3–propagated BMMCs (lane 4) and IL-2–activated NK cells (lane 6).

Regulation of the Clnk Protein by Immunoreceptor Stimulation. To identify the protein product of *clnk*, a polyclonal rabbit antiserum was generated against a bacterial fusion protein encompassing amino acids 199–301 of Clnk. When lysates from various hemopoietic cell lines were probed by immunoblotting with this antibody (Fig. 4 A), we found that HT-2 (lane 1), CTLL-2 (lane 2), Ba/F3 (lane 3), and B6SutA$_1$ (lane 4), but not BI-141 T cells (lane 5), contained an ~54-kD immunoreactive product consistent with Clnk. A similar polypeptide was observed in Cos-1 cells transfected with a *clnk* cDNA (lane 7), but not in control Cos-1 cells (lane 6). The presence of the Clnk protein was also examined in normal hemopoietic cells (Fig. 4 B). This assay demonstrated that IL-3–propagated BMMCs (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 *clnk*-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

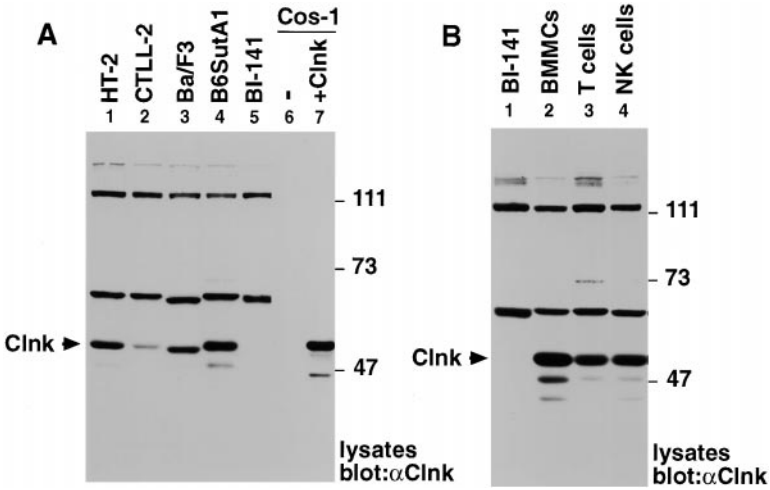

Figure 4. Detection of the 54-kD Clnk protein in mouse hemopoietic cells. Lysates from the indicated cells were probed by immunoblotting with affinity-purified rabbit anti-Clnk antibodies. It is noticeable that the electrophoretic mobility of Clnk in Ba/F3 cells (A, lane 3) was faster than that observed in the other cells. The basis for this difference is not known. The detection of immunoreactive products at ~140, 120, and 68 kD is likely due to nonspecific cross-reactivity of the antiserum, since these bands are also present in cells lacking *clnk* RNA (A, lane 5; B, lane 1). The migrations of prestained molecular mass markers are indicated on the right, whereas that of Clnk is shown on the left. Exposures: (A) lanes 1–5, 1 min; lanes 6 and 7, 5 s; (B) 10 min (enhanced chemiluminescence).

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 $B6SutA_1$, 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 $B6SutA_1$ cells (top panel, lane 2). However, it is noteworthy that, in contrast to 5.32.10, the Clnk protein found in $B6SutA_1$ 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.

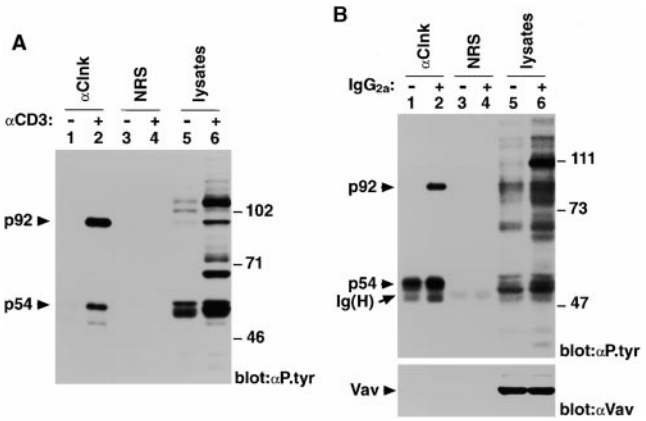

Figure 5. Regulation of Clnk by immunoreceptor stimulation. (A) Effects of TCR stimulation on Clnk tyrosine phosphorylation. 5.32.10 was grown in the presence of IL-2 and activated by anti-CD3 mAb 145-2C11 and RAH IgG. The nature of the 50-kD tyrosine-phosphorylated product seen in anti-Clnk immunoprecipitates (lane 2) is not known. It may represent an alternative form of Clnk protein. The migrations of p54 and p92 are indicated on the left; those of prestained molecular mass markers are shown on the right. Exposure: 16 h. (B) Effects of FcγRI stimulation on Clnk tyrosine phosphorylation. $B6SutA_1$ was propagated in medium containing IL-3 and activated by mouse IgG_{2a} and $F(ab')_2$ fragments of SAM IgG. The identity of the 50-kD tyrosine-phosphorylated product seen in anti-Clnk immunoprecipitates (lanes 1 and 2) is not known. It may represent an alternative form of Clnk. The migrations of p54, p92, and Vav are shown on the left; those of prestained molecular mass markers are indicated on the right. Exposures: top panel, 16 h; bottom panel, 30 s (enhanced chemiluminescence).

Given the ability of SLP-76 and Blnk to associate with $p95^{vav}$ (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

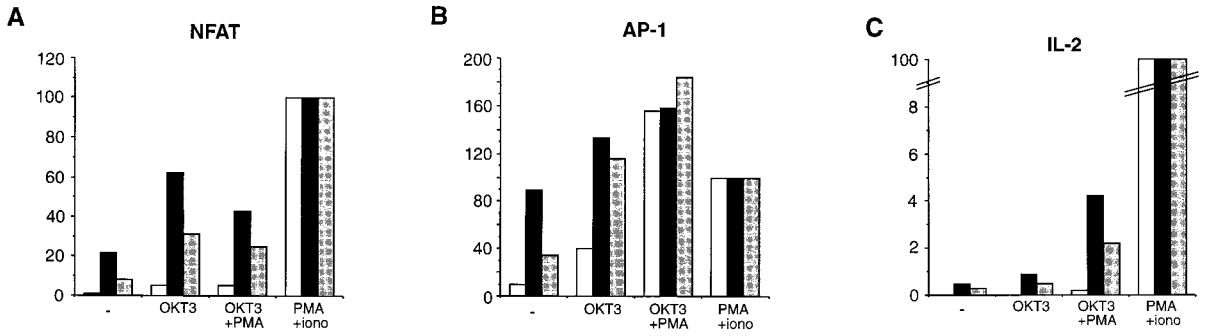

Figure 6. Regulation of immunoreceptor signaling by Clnk. Jurkat TAg cells were transiently transfected with the indicated DNAs (empty vector, white bars; Clnk, black bars; SLP-76, gray bars) in the presence of NFAT- (A), AP-1– (B), or IL-2 promoter (C) luciferase reporter plasmids. 40 h after transfection, cells were activated with the indicated stimuli for 7 h, and the changes in luciferase activity were measured as outlined in Materials and Methods. Luciferase activity is expressed as percentage of the activity induced by PMA plus ionomycin (which represents 100%).

PMA and ionomycin. This experiment showed that, like SLP-76 (18, 32, 33; Fig. 6), the Clnk protein markedly enhanced the activation of NFAT (Fig. 6 A), AP-1 (Fig. 6 B), and IL-2 promoter (Fig. 6 C) in response to stimulation with anti-CD3 antibodies. An analogous effect was seen in cells treated with anti-CD3 plus PMA. It is of note that both Clnk and SLP-76 were able to induce some extent of transcriptional activation of these promoters in the absence of CD3 stimulation. While the exact significance of this observation is unclear, it may reflect the high levels of protein expression typically achieved in these systems.

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 FcγRI. 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 NH_2-terminal portion of SLP-76, which mediate binding to the exchange factor Vav and the adaptor molecule Nck (12, 17–19, 33, 36). Accordingly, we have been unable to show binding of Clnk to either Vav or Nck (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.

We thank Michael Julius for much appreciated discussions, and Steven Lam for technical help. We also acknowledge S. Tsai (University of Washington, Seattle, WA) for the EML-16 cDNA library, L. Matis (Alexion Pharmaceuticals, New Haven, CT) for the mouse thymus cDNA library, M. Julius (University of Toronto, Toronto, Ontario, Canada) and G. Krystal (Terry Fox Laboratory, Vancouver, British Columbia, Canada) for various cell lines, G. Koretzky (University of Iowa, Iowa City, IA) for the mouse *slp-76* cDNA, and G. Crabtree (Stanford University, Stanford, CA) for Jurkat TAg cells and the various luciferase plasmids.

This work was supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada to A. Veillette. M.Y. Cao was supported by a Joseph Kaufmann Memorial Fellowship, and S. Latour was funded by a Fellowship from the Medical Research Council of Canada. A. Veillette is a Senior Scientist of the Medical Research Council of Canada.

Address correspondence to André Veillette, McGill Cancer Centre, Rm. 715, McIntyre Medical Sciences Bldg., McGill University, 3655 Drummond St., Montréal, Québec, Canada H3G 1Y6. Phone: 514-398-8936; Fax: 514-398-4438; E-mail: andrev@med.mcgill.ca

Submitted: 29 July 1999 Revised: 9 September 1999 Accepted: 10 September 1999

References

1. Fu, C., C.W. Turck, T. Kurosaki, and A.C. Chan. 1998. BLNK: a central linker protein in B cell activation. *Immunity*. 9:93–103.
2. Wienands, J., J. Schweikert, B. Wollscheid, H. Jumaa, P.J. Nielsen, and M. Reth. 1998. SLP-65: a new signaling component in B lymphocytes which requires expression of the antigen receptor for phosphorylation. *J. Exp. Med*. 188: 791–795.
3. Jackman, J.K., D.G. Motto, Q. Sun, M. Tanemoto, C.W. Turck, G.A. Peltz, G.A. Koretzky, and P.R. Findell. 1995. Molecular cloning of SLP-76, a 76-kDa tyrosine phosphoprotein associated with Grb2 in T cells. *J. Biol. Chem*. 270: 7029–7032.
4. Goitsuka, R., Y. Fujimura, H. Mamada, A. Umeda, T. Morimura, K. Uetsuka, K. Doi, S. Tsuji, and D. Kitamura. 1998. BASH, a novel signaling molecule preferentially expressed in B cells of the bursa of Fabricius. *J. Immunol*. 161: 5804–5808.
5. Clements, J.L., S.E. Ross-Barta, L.T. Tygrett, T.J. Waldschmidt, and G.A. Koretzky. 1998. SLP-76 expression is restricted to hemopoietic cells of monocyte, granulocyte, and T lymphocyte lineage and is regulated during T cell maturation and activation. *J. Immunol*. 161:3880–3889.
6. Clements, J.L., B. Yang, S.E. Ross-Barta, S.L. Eliason, R.F. Hrstka, R.A. Williamson, and G.A. Koretzky. 1998. Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development. *Science*. 281:416–419.
7. Pivniouk, V., E. Tsitsikov, P. Swinton, G. Rathbun, F.W. Alt, and R.S. Geha. 1998. Impaired viability and profound block in thymocyte development in mice lacking the adaptor protein SLP-76. *Cell*. 94:229–238.
8. Clements, J.L., J.R. Lee, B. Gross, B. Yang, J.D. Olson, A. Sandra, S.P. Watson, S.R. Lentz, and G.A. Koretzky. 1999. Fetal hemorrhage and platelet dysfunction in SLP-76–deficient mice. *J. Clin. Invest*. 103:19–25.
9. Pivniouk, V.I., T.R. Martin, J.M. Lu-Kuo, H.R. Katz, H.C. Oettgen, and R.S. Geha. 1999. SLP-76 deficiency impairs signaling via the high-affinity IgE receptor in mast cells. *J. Clin. Invest*. 103:1737–1743.
10. Yablonski, D., M.R. Kuhne, T. Kadlecek, and A. Weiss. 1998. Uncoupling of nonreceptor tyrosine kinases from PLC-γ1 in an SLP-76-deficient T cell. *Science*. 281:413–416.
11. Ishiai, M., M. Kurosaki, R. Pappu, K. Okawa, I. Ronko, C. Fu, M. Shibata, A. Iwamatsu, A.C. Chan, and T. Kurosaki. 1999. BLNK required for coupling Syk to PLC gamma 2 and Rac1-JNK in B cells. *Immunity*. 10:117–125.
12. Bubeck Wardenburg, J., R. Pappu, J.Y. Bu, B. Mayer, J. Chernoff, D. Straus, and A.C. Chan. 1998. Regulation of PAK activation and the T cell cytoskeleton by the linker protein SLP-76. *Immunity*. 9:607–616.
13. da Silva, A.J., Z. Li, C. de Vera, E. Canto, P. Findell, and C.E. Rudd. 1997. Cloning of a novel T-cell protein FYB that binds FYN and SH2-domain-containing leukocyte protein 76 and modulates interleukin 2 production. *Proc. Natl. Acad. Sci. USA*. 94:7493–7498.
14. Law, C.L., M.K. Ewings, P.M. Chaudhary, S.A. Solow, T.J. Yun, A.J. Marshall, L. Hood, and E.A. Clark. 1999. GrpL, a Grb2-related adaptor protein, interacts with SLP-76 to regulate nuclear factor of activated T cell activation. *J. Exp. Med*. 189:1243–1253.
15. Liu, S.K., N. Fang, G.A. Koretzky, and C.J. McGlade. 1999. The hematopoietic-specific adaptor protein Gads functions in T-cell signaling via interactions with the SLP-76 and LAT adaptors. *Curr. Biol*. 9:67–75.
16. Musci, M.A., L.R. Hendricks-Taylor, D.G. Motto, M. Paskind, J. Kamens, C.W. Turck, and G.A. Koretzky. 1997. Molecular cloning of SLAP-130, an SLP-76-associated substrate of the T cell antigen receptor-stimulated protein tyrosine kinases. *J. Biol. Chem*. 272:11674–11677.
17. Tuosto, L., F. Michel, and O. Acuto. 1996. $p95^{vav}$ associates with tyrosine-phosphorylated SLP-76 in antigen-stimulated T cells. *J. Exp. Med*. 184:1161–1166.
18. Wu, J., D.G. Motto, G.A. Koretzky, and A. Weiss. 1996. Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation. *Immunity*. 4:593–602.
19. Wunderlich, L., A. Farago, J. Downward, and L. Buday. 1999. Association of Nck with tyrosine-phosphorylated SLP-76 in activated T lymphocytes. *Eur. J. Immunol*. 29:1068–1075.
20. Chow, L.M., D. Davidson, M. Fournel, P. Gosselin, S. Lemieux, M.S. Lyu, C.A. Kozak, L.A. Matis, and A. Veillette. 1994. Two distinct protein isoforms are encoded by *ntk*, a csk-related tyrosine protein kinase gene. *Oncogene*. 9:3437–3448.
21. Damen, J.E., L. Liu, P. Rosten, R.K. Humphries, A.B. Jefferson, P.W. Majerus, and G. Krystal. 1996. The 145-kDa

protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase. *Proc. Natl. Acad. Sci. USA*. 93: 1689–1693.

22. Haughn, L., S. Gratton, L. Caron, R.P. Sekaly, A. Veillette, and M. Julius. 1992. Association of tyrosine kinase $p56^{lck}$ with CD4 inhibits the induction of growth through the alpha beta T-cell receptor. *Nature*. 358:328–331.
23. Leo, O., M. Foo, D.H. Sachs, L.E. Samelson, and J.A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA*. 84: 1374–1378.
24. Amigorena, S., C. Bonnerot, W.H. Fridman, and J.-L. Teillaud. 1990. Recombinant interleukin 2-activated natural killer cells regulate IgG_{2a} production. *Eur. J. Immunol*. 20: 1781–1787.
25. Chow, L.M., M.J. Ratcliffe, and A. Veillette. 1992. *tkl* is the avian homolog of the mammalian *lck* tyrosine protein kinase gene. *Mol. Cell. Biol*. 12:1226–1233.
26. Weil, R., and A. Veillette. 1994. Intramolecular and extramolecular mechanisms repress the catalytic function of $p56^{lck}$ in resting T-lymphocytes. *J. Biol. Chem*. 269:22830–22838.
27. Cloutier, J.F., and A. Veillette. 1996. Association of inhibitory tyrosine protein kinase $p50^{csk}$ with protein tyrosine phosphatase PEP in T cells and other hemopoietic cells. *EMBO (Eur. Mol. Biol. Organ.) J*. 15:4909–4918.
28. Veillette, A., M.A. Bookman, E.M. Horak, and J.B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. *Cell*. 55:301–308.
29. Fournel, M., D. Davidson, R. Weil, and A. Veillette. 1996. Association of tyrosine protein kinase Zap-70 with the protooncogene product $p120^{c\text{-}cbl}$ in T lymphocytes. *J. Exp. Med*. 183:301–306.
30. Cao, M.Y., M. Huber, N. Beauchemin, J. Famiglietti, S.M. Albelda, and A. Veillette. 1998. Regulation of mouse PECAM-1 tyrosine phosphorylation by the Src and Csk families of protein-tyrosine kinases. *J. Biol. Chem*. 273:15765–15772.
31. Newman, P.J., M.C. Berndt, J. Gorski, G.C. White II, S. Lyman, C. Paddock, and W.A. Muller. 1990. PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. *Science*. 247:1219–1222.
32. Motto, D.G., S.E. Ross, J. Wu, L.R. Hendricks-Taylor, and G.A. Koretzky. 1996. Implication of the GRB2-associated phosphoprotein SLP-76 in T cell receptor–mediated interleukin 2 production. *J. Exp. Med*. 183:1937–1943.
33. Raab, M., A.J. da Silva, P.R. Findell, and C.E. Rudd. 1997. Regulation of Vav-SLP-76 binding by ZAP-70 and its relevance to TCR zeta/CD3 induction of interleukin-2. *Immunity*. 6:155–164.
34. Piao, X., R. Paulson, P. van der Geer, T. Pawson, and A. Bernstein. 1996. Oncogenic mutation in the Kit receptor tyrosine kinase alters substrate specificity and induces degradation of the protein tyrosine phosphatase SHP-1. *Proc. Natl. Acad. Sci. USA*. 93:14665–14669.
35. Peterson, E.J., J.L. Clements, Z.K. Ballas, and G.A. Koretzky. 1999. NK cytokine secretion and cytotoxicity occur independently of the SLP-76 adaptor protein. *Eur. J. Immunol*. 29:2223–2232.
36. Fang, N., and G.A. Koretzky. 1999. SLP-76 and Vav function in separate, but overlapping pathways to augment interleukin-2 promoter activity. *J. Biol. Chem*. 274:16206–16212.