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Transcriptional Regulation of Src Homology 2 Domain-Containing Leukocyte Phosphoprotein of 76 kDa: Dissection of Key Promoter Elements

Xiao-Ping Zhong,*† Jonathan S. Maltzman,*‡ Ehmonie A. Hainey,*§ and Gary A. Koretzky‡§

SLP-76 (Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa) is an adaptor molecule expressed in all hemopoietic cell lineages except mature B cells and is known to play critical roles in the function of T cells, mast cells, and platelets and in vascular differentiation. Although great progress has been achieved in our understanding of SLP-76 function, little is known about the mechanisms regulating its expression. In this study we report the initial characterization of essential elements that control SLP-76 transcription. We identify several DNase I-hypersensitive sites in the SLP-76 locus, with a prominent site located in its promoter region. This site exists in T cells and monocyctic cells, but not in B cells or fibroblasts. Using transient transfection assays, we identify a 507-bp fragment containing the 5′-untranslated region of the first exon and the immediate upstream sequence that confers transcriptional activation in T cells and monocyctic cells, but not in B cells. Analysis of the 5′ ends of SLP-76 transcripts reveals differential regulation of SLP-76 transcription initiation between T cells and monocyctic cells. Mutational and gel-shift analyses further indicate a critical role within this region for a binding site for Ets family transcription factors. The present study provides the first data to address the mechanisms controlling SLP-76 transcription by providing evidence for several key cis-regulatory elements in the promoter region. The Journal of Immunology, 2003, 171: 6621–6629.

Adaptor molecules play important roles in cellular activation by integrating components of signaling complexes. Recently, a number of hemopoietic lineage-specific adaptor molecules have been identified that are involved in signal transduction by numerous cell surface receptors, including those binding Ag, cytokines, growth factors, and apoptosis-inducing molecules (1–3). One such adaptor, Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76), is expressed in T cells, NK cells, monocytes, macrophages, granulocytes, mast cells, platelets, and megakaryocytes (4, 5). However, normal mature B cells do not express SLP-76. Biochemical and genetic evidence has clearly established important roles for SLP-76 in signal transduction by several cell surface receptors in the hemopoietic system. Mice deficient in SLP-76 demonstrate a complete blockade of T cell development by several cell surface receptors in the hemopoietic system. Mice deficient in SLP-76 demonstrate a complete blockade of T cell development at the CD4+CD8+ double-negative (DN) stage (5, 6), documenting a pivotal role for SLP-76 in pre-TCR signaling. In SLP-76-deficient mice reconstituted with SLP-76 mutants, mature T cells develop, but are defective in TCR-induced activation (7, 8). In these T cells as well as in SLP-76-deficient Jurkat T cells, TCR-induced activation of phospholipase Cγ1 (PLCγ1), NFAT, and extracellular signal-regulated kinase is impaired, indicating that SLP-76 is also critical for TCR signaling in mature T cells (7–9).

SLP-76 is critical for signal transduction in other cell lineages. For example, SLP-76-deficient mice manifest hemorrhage despite normal megakaryocyte and platelet development. Collagen-induced platelet aggregation, granule release, and tyrosine phosphorylation of PLCγ2 are markedly impaired (6, 10, 11). More recent studies of SLP-76-deficient mice indicate that SLP-76 is also involved in the segregation of blood and lymphatic vessels (12). Bone marrow–derived mast cells from SLP-76-deficient mice manifest reduced tyrosine phosphorylation of PLCγ and calcium mobilization and fail to release β-hexosaminidase and to secrete IL-6 after FcεRI cross-linking (13, 14). Consistent with the biochemical deficiency, SLP-76-deficient mice are resistant to IgE-mediated passive anaphylaxis, although they develop normal numbers of mast cells.

Although the functional importance of SLP-76 in diverse signaling pathways in different cells of hemopoietic origin has been well established, mechanisms that determine its lineage-specific expression are unknown. Furthermore, SLP-76 is regulated differentially during T cell development and differentiation. SLP-76 protein is first expressed at low levels in the CD25+CD44+ subset of DN thymocytes, is up-regulated during maturation of the DN thymocytes, and is decreased again at the CD4+CD8+ double-positive stage. SLP-76 is then up-regulated again after selection and commitment to mature single-positive T cells (15). In peripheral T cells, TCR stimulation increases SLP-76 protein expression (15, 16). More recent evidence demonstrates that SLP-76 expression is also differentially regulated during immune responses. Compared with naive T cells, SLP-76 protein is up-regulated in effector T cells, but is then dramatically down-regulated in memory T cells (16). The mechanisms determining this regulation are not yet clear. Overexpression studies have indicated that the levels of SLP-76 expression as well as those of many other signaling molecules are controlled by multiple mechanisms at different developmental stages. In this report, we describe the initial characterization of cis-regulatory elements that control SLP-76 expression in T cells and provide evidence that the transcription of this gene is under the control of Ets family members.

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2 Address correspondence and reprint requests to Dr. Gary Koretzky, 415 BRRBU/III, The Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, 421 Curie Boulevard, Philadelphia, PA 19104. E-mail address: koretzky@mail.med.upenn.edu

3 Abbreviations used in this paper: SLP-76, Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa; DN, double negative; HS, hypersensitive site; Lac, luciferase; PLC, phospholipase C; RLM-RACE, RNA ligase-mediated RACE.
influence the efficiency of TCR signaling and T cell function (17–19). Thus, an understanding of the ways in which SLP-76 expression is controlled should provide new insight into the complex biology of immune cell development and function. In this study we report the initial identification and characterization of cis-regulatory elements that are important for SLP-76 transcription.

**Materials and Methods**

**Cell lines and primary lymphocytes**

The Jurkat human leukemia T cell line (provided by Dr. A. Weiss, University of California, San Francisco, San Francisco, CA) was grown in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin G, 100 U/ml streptomycin, and 292 μg/ml L-glutamine. Primary T lymphocytes were isolated by negative selection from RBC-depleted lymph node cells and splenocytes using Biomag sheep anti-B220, anti-Mac1, and anti-DX5 (BD PharMingen, San Diego, CA). B lymphocytes were purified in a similar manner, except that anti-CD3 was substituted for anti-B220. After purification, cells were >90% pure as measured by flow cytometry.

**Plasmids**

The 3.44-kb BamHI-SacI genomic DNA fragment spanning −3360 to +81 relative to the first nucleotide of the mouse CDNA (position 1) was released from pZeroB1(5′), which contains the 13-kb BamHI fragment of the 5′-portion of the SLP-76 gene (5), with SacI, blunted with T4 DNA polymerase and then digested by XhoI. The released fragment was ligated into the pGL3-Basic vector (GL3B; Promega, Madison, WI) that was already sequentially digested with HindIII, BluntII, and Klenow, and ligated with XhoI in the sense orientation to create pSLP-3360Luciferase (pSLP-3360Luc). The same 3.44-kb fragment was released from pZeroB1(5′) with XhoI and SacI and cloned into the corresponding sites of the pGL3-Basic in the antisense orientation to create pSLP-3360RLuc. Regions within the 3.44-kb fragment in pSLP-3360Luc were deleted after digesting with different restriction enzymes, blunted with Klenow if necessary, and religated to generate pSLP-2082Luc (−2082 to +81, with SacI, blunted with T4 DNA polymerase and then digested by XhoI). The released fragment was ligated into the pGL3-Basic vector (GL3B; Promega, Madison, WI) that was already sequentially digested with HindIII, BluntII, and Klenow, and ligated with XhoI in the sense orientation to create pSLP-3360Luciferase (pSLP-3360Luc). The same 3.44-kb fragment was released from pZeroB1(5′) with XhoI and SacI and cloned into the corresponding sites of the pGL3-Basic in the antisense orientation to create pSLP-3360Luc. Regions within the 3.44-kb fragment in pSLP-3360Luc were deleted after digesting with different restriction enzymes, blunted with Klenow if necessary, and religated to generate pSLP-2082Luc (−2082 to +81, with SacI, blunted with T4 DNA polymerase and then digested by XhoI).

**Construction of reporter plasmids**

The PCR product from the 3.44-kb fragment in pSLP-3360Luc was deleted after digesting with XhoI and HindIII, blunted with Klenow, and digested with PvuII and PstI to generate pSLP-169Luc. This fragment was sequenced, and the potential Ets site mutated from to GAGGAA to GAGAGA, and cloned into the corresponding site of the pGL3-Basic vector (GL3B; Promega, Madison, WI) that was already sequentially digested with HindIII, BluntII, and Klenow, and ligated with PvuII and PstI. The released fragment was ligated into the pGL3-Basic vector (GL3B; Promega, Madison, WI) that was already sequentially digested with HindIII, BluntII, and Klenow, and ligated with PvuII and PstI. The released fragment was ligated into the pGL3-Basic vector (GL3B; Promega, Madison, WI) that was already sequentially digested with HindIII, BluntII, and Klenow, and ligated with PvuII and PstI.

**Oligonucleotides used in this study**

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<th>Designation</th>
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<td>5′–CTC TCT CCC CCT TCT TCT CT</td>
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* These primers were used for circular PCR to clone the Ets-1 and YY1 mutant constructs.

* The underlined sequences are the WT or mutant binding sites for Ets transcription factors and for YY1.

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**Transcriptional regulation of SLP-76**

**DNAse I-hypersensitive site (HS) assay**

For each transfection, 20 μg of test plasmid and 5 μg of pCMV-β-gal were added to 0.5 ml of cells at 5 × 10^6 cells/ml in RPMI 1640 medium without serum in a 4-mm gap cuvette. Cells were electroporated (300 V/950 μF for Jurkat and 2B4 cells, 400 V/825 μF for Raw cells, and 300 V/700 μF for WEHI-231 cells). Transfected cells were harvested at 10 ml of RPMI 10 and incubated at 37°C for 24 h before harvesting for Luc activity. Luc and β-galactosidase activities were determined as previously described (20). In TCR stimulation experiments, Jurkat cells were harvested 16 h after transfection and seeded in 96-well plates in triplicate at 2 × 10^5 cells/well in 200 μl of RPMI 10 with or without 1/20,000 diluted C305 antigens (an IgM mAb specific for TCR on Jurkat cells) (21). Cells were harvested 8 h later for Luc activity.

**Northern blot analysis**

Purified mouse primary T cells were left unstimulated or were stimulated with a plate-bound anti-CD3 Ab (clone 500A2; BD PharMingen) for 16, 24, and 48 h. Total RNA from these cells was isolated using TRizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The level of SLP-76 transcripts was determined by Northern blot analysis using a mouse SLP-76 cDNA probe and was quantified by a phosphorimager.
Primer extension

Primer extension to analyze the 5' end of the SLP-76 transcript was performed as previously described (22). Briefly, an SLP-76-specific oligonucleotide, mSLP-PE1, was labeled with [32P]phosphate at the 5' end, annealed to total RNA templates isolated from thymus and Raw267.4, and then extended by RT using Superscript II polymerase (Invitrogen). The products were denatured and resolved by electrophoresis in a 10% TBE (89 mM Tris, pH 8.3, 89 mM boric acid, 2 mM EDTA) urea polyacrylamide gel and revealed by autoradiography.

**RNA ligase-mediated RACE (RLM-RACE)**

RLM-RACE for SLP-76 was performed using the GeneRacer kit (Invitrogen) according to the manufacturer's protocol. Briefly, total thymus RNA was treated with calf intestinal phosphatase to remove the 5' phosphate of truncated mRNA and other RNA species. After dephosphorylation, the 5' cap structure of intact, full-length mRNA was removed by treatment with tobacco acid pyrophosphatase. This treatment exposed the 5' cap structure of intact, full-length mRNA for ligation to the GeneRacer RNA oligo. The 5' portion of SLP-76 cDNA was then generated by RT-PCR with GeneRacer primers and SLP-76-specific primers. The amplified products were cloned into pBluescript II KS' (Stratagene, La Jolla, CA) and subsequently sequenced.

**EMSA**

Preparation of nuclear extracts and EMSAs were performed essentially as previously described (23). Briefly, cells (primary B or T lymphocytes) were washed twice in PBS at 4°C, then nuclear extracts were prepared by hypotonic lysis and high salt extraction. Double-stranded oligonucleotide probes (see Table I) were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) according to the manufacturer's instructions. Probes were purified using MicroSpin G-25 columns according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ). Binding reactions were conducted for 30 min at room temperature in a 20-μl volume containing 50,000 dpm of labeled probe, nuclear extract containing 2.5–5 μg of protein, 1 μg of poly(dI-dC) (Roche), 10 mM Tris (pH 7.5), 40 mM NaCl, 1 mM EDTA, 1 mM 2-ME, and 2% glycerol. Competing oligonucleotides, anti-Ets1 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-PU.1 Abs (a gift from C. Simon, University of Pennsylvania) were preincubated at room temperature 15 min before addition of radiolabeled probe. Binding reactions were size-fractionated using a 6% polyacrylamide gel in 0.5× TBE and electrophoresed at 150 V for 2 h at room temperature.

**Results**

**Identification of DNase I HSs in the SLP-76 locus**

DNase I HSs often correlate with important transcriptional regulatory regions within genes (24). Therefore, to facilitate the identification of cis-regulatory elements controlling SLP-76 transcription, we first determined the DNase I HSs in the 5' region of the locus by comparing such sites in SLP-76-expressing and -nonexpressing cells. The mouse B4.11 T cell hybridoma (B4) and Raw 264.7 macrophage cell line (Raw) express SLP-76 and were used to represent T and monocyctic cell lineages. Bal-17, a mouse B cell line, and NIH-3T3 mouse fibroblast cells do not express SLP-76 (data not shown). Nuclei from each cell line were isolated and digested with limited dilutions of DNase I. DNA was then purified and subjected to BamHI digestion. DNase I HSs were identified by Southern blot analysis with a 5' genomic probe. As shown in Fig. 1, several DNase I HSs were identified in the 5' region of the mouse SLP-76 locus. Among these, HS2 is most prominent and is localized ~3.3 kb downstream of the 5' BamHI site, where the SLP-76 promoter is probably located. Importantly, HS2 exists only in B4 and Raw cells, not in Bal-17 or NIH-3T3 cells, correlating with SLP-76 expression. The other HSs are weaker compared with HS2. HS1 exists only in B4 cells. HS3, HS4, and HS5 exist in both B4 and Raw cells. HS5 appears stronger in Raw cells than in B4 cells. In contrast, HS4 is much weaker in Raw cells than in B4 cells and can be seen only in longer exposures (data not shown), suggesting that these HSs may be differentially important in different lineages that express SLP-76. No HSs were detected in either Bal-17 or fibroblast cells. Thus, in the cell lines we tested, the DNase I HSs, particularly HS2, correlate well with the pattern of SLP-76 expression.

**A 507-bp core promoter of SLP-76 confers lineage-specific expression of a reporter in vitro**

The prominence and location of HS2 suggest a potential role of this region in the regulation of SLP-76 transcription. We first tested whether a 3.44-kb BamHI-SacI DNA fragment, which includes the 5'-untranslated region of the first exon as well as the 5' upstream sequence of the mouse SLP-76 gene (and contains both HS1 and HS2), possesses promoter activity. This fragment was linked to the promoterless Luc reporter, pGL3B, in the sense orientation to create pSLP-3360 Luc. This construct was transiently cotransfected with pCMV-β-galactosidase into Jurkat, 2B4, and Raw cells. Twenty-four hours later, cells were harvested and evaluated for Luc activity. As shown in Fig. 2A, insertion of this fragment upstream of the Luc gene in the sense orientation (~3360) leads to significant increases in Luc reporter expression in each of these three cell lines. An average of a 35.1-, 11.8-, and 8.1-fold increase in Luc activity from multiple experiments was observed compared with the promoterless Luc reporter in Jurkat, 2B4, and Raw cells. Twenty-four hours later, cells were harvested and evaluated for Luc activity. As shown in Fig. 2A, insertion of this fragment upstream of the Luc gene in the sense orientation (~3360) leads to significant increases in Luc reporter expression in each of these three cell lines. An average of a 35.1-, 11.8-, and 8.1-fold increase in Luc activity from multiple experiments was observed compared with the promoterless Luc reporter in Jurkat, 2B4, and Raw cells.}

![FIGURE 1](http://www.jimmunol.org/.../figure1.png)

Identification of DNase I HSs in the SLP-76 locus. A, Schematic genomic organization of the 5' portion of the mouse SLP-76 locus. Arrows represent DNase I HSs. B and S represent restriction sites for BamHI and SacI, respectively. Rectangles represent exons. B, DNase I HSs in the SLP-76 locus in cells of different lineages. Nuclei were isolated and digested with serial dilutions of DNase I. DNA from these nuclei was then purified and digested with BamHI. Digested DNA was separated on an agarose gel and transferred to a nylon membrane. The membrane was hybridized with a 32P-labeled probe. B4 is a mouse T cell hybridoma; Bal-17 is a mouse B cell line; Raw is a mouse monocyctic cell line; NIH-3T3 is a mouse fibroblastic cell line.
FIGURE 2. Activation of transcription in T and monocytic cells by the putative SLP-76 promoter. A, Jurkat, 2B4 and Raw cells were cotransfected in triplicate with different SLP-76 promoter test constructs and a β-galactosidase construct (pCMV-β-gal) for control of transfection efficiency. Luc and β-galactosidase activities in cell lysates were determined 24 h after transfection. Values shown are the fold increase in Luc activity in cells transfected with test constructs relative to those transfected with the promoterless pGL3B plasmid after normalization with pGL3-Control (pGL3-Control; Promega), and the relative Luc activity in cell lysates was determined as described in A. Data shown are representative of three experiments.

or were stimulated with an anti-CD3 Ab for various times. The level of SLP-76 transcript in RNA isolated from these cells was determined by Northern blot. As shown in Fig. 3A, SLP-76 mRNA levels in T cells stimulated for 16 and 24 h was ~3-fold higher than that in unstimulated T cells. The elevated levels of the SLP-76 transcript began to decline after 48 h of stimulation (top panel). These data indicate that the increase in SLP-76 mRNA may at least partially account for the increase in SLP-76 protein during T cell activation.

We next asked whether the SLP-76 core promoter is regulated by signals mediated via the TCR. Jurkat cells were transfected with GL3B and pSLP-426Luc. Sixteen hours later, transfected cells were stimulated with an anti-TCR Ab for 8 h. As shown in Fig. 3B, an ~2-fold increase in Luc activity was observed in cells transfected with the reporter construct controlled by the SLP-76 core promoter. In contrast, TCR stimulation did not induce reporter expression in GL3B-transfected cells. Thus, it appears that the 507-bp core promoter contains elements that confer inducible transcription of SLP-76 during T cell activation.

**Determination of SLP-76 transcription initiation site**

Sequence alignment of the 14-kb BamHI fragment of mouse SLP-76 genomic DNA (GenBank accession no. NT 039515.1: 31404730-31418634) with the human SLP-76 genomic sequences (GenBank accession no. NT 023133.11:14532000-14485381) from public databases reveals high conservation within the 507-bp

**TCR induced up-regulation of SLP-76 core promoter activity**

Previous studies have shown that SLP-76 protein is up-regulated during T cell activation (15, 16). To determine whether changes in steady state levels of SLP-76 mRNA are involved in such up-regulation, purified mouse primary T cells were left unstimulated or were stimulated with an anti-CD3 Ab for various times. The level of SLP-76 transcript in RNA isolated from these cells was determined by Northern blot. As shown in Fig. 3A, SLP-76 mRNA levels in T cells stimulated for 16 and 24 h was ~3-fold higher than that in unstimulated T cells. The elevated levels of the SLP-76 transcript began to decline after 48 h of stimulation (top panel). These data indicate that the increase in SLP-76 mRNA may at least partially account for the increase in SLP-76 protein during T cell activation.

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the 5' and 3' ends. As shown in Fig. 2B, truncation of the 1619-bp region from −1538 to +81 of the 3.44-kb promoter fragment (construct −3360 to −1539) resulted in a complete loss of promoter activity, indicating that this region is probably essential for transcriptional activation. In contrast, truncating a 1278-bp region from the 5' portion of the 3.44-kb fragment (−2082 to +81) did not reduce promoter activity in either Jurkat or Raw cells, indicating that this region is not important for SLP-76 expression in this experimental setting. Further deletion of 511 bp toward the 3' direction (−1571 to +81) resulted in an ~2-fold increase in promoter activity in both Jurkat or Raw cells, suggesting the existence of a potential negative regulatory element in this region. Additional deletion mapping of the SLP-76 promoter revealed that the 507-bp SpeI-Sacl (−426 to +81) fragment contains maximal transcriptional activating capability in both Jurkat and Raw cells. Importantly, the same 507-bp region as well as the 3.44-kb SLP-76 promoter is not active in B cells. WEHI-231 B cells were transfected with the Luc reporter driven by the 3.44-kb and the 507-bp SLP-76 promoter as well as a positive control vector pGL3C (pGL3-Control; Promega), and the relative Luc activity in cell lysates was determined as described in A. Data shown are representative of three experiments.

**FIGURE 2.** Activation of transcription in T and monocytic cells by the putative SLP-76 promoter. A, Jurkat, 2B4 and Raw cells were cotransfected in triplicate with different SLP-76 promoter test constructs and a β-galactosidase construct (pCMV-β-gal) for control of transfection efficiency. Luc and β-galactosidase activities in cell lysates were determined 24 h after transfection. Values shown are the fold increase in Luc activity in cells transfected with test constructs relative to those transfected with the promoterless pGL3B plasmid after normalization against β-galactosidase activity. A representative of three experiments is shown. B, Characterization of the SLP-76 core promoter. A series of Luc reporter constructs with the 3.44-kb SLP-76 promoter truncated at the 5' and 3' ends was tested as described in A. Data shown are representative of four experiments. C, The SLP-76 promoter is not active in B cells. WEHI-231 B cells were transfected with the Luc reporter driven by the 3.44-kb and the 507-bp SLP-76 promoter as well as a positive control vector pGL3C (pGL3-Control; Promega), and the relative Luc activity in cell lysates was determined as described in A. Data shown are representative of three experiments.
region described above (Fig. 4A), arguing further the likely importance of this sequence as a bona fide SLP-76 promoter. In contrast, with the exception of the three exons, no significant homology is observed in other regions. Within the 507-bp region, there is a 54-bp TC repeat that separates the core promoter into a 5’ 208-bp region (~426 to ~217, designated region A) and a 3’ 250-bp region (~169 to ~81, designated region B). The TC repeats do not exist in the human SLP-76 promoter. Deletion of the TC repeats from the 507-bp mouse SLP-76 promoter did not affect its activity in either Jurkat or Raw cells (data not shown), indicating that the TC repeats are dispensable for promoter activity.

Searching the Transfac database using the MatInspector V2.2 program (http://transfac.gbf.de/TRANSFAC/) (25) revealed several potential binding sites for transcription factors such as GATA, MZF1, Ets, NFAT, YY1, and c-Rel (Fig. 4A). However, no canonical TATA box, CCAAT box, or Sp-1 binding site was identified.

Before studying the putative SLP-76 promoter elements further, two approaches were used to define the transcription initiation site of the SLP-76 gene. We first performed RNA primer extension using an SLP-76-specific oligonucleotide (mSLP-PE1; Table I) to examine RNA isolated from mouse thymus or Raw cells. As shown in Fig. 4B, a band of ~130 bases was revealed when using RNA from both sources as a template. Interestingly, an additional band of ~180 bases was revealed in thymus RNA, but not in RNA from Raw cells, indicating the existence of an additional transcription initiation site that appears active in T cells, but not in the monocytic cell line.

The presence of two primer extension products in T cells could be due to two initiation sites in the same promoter or to the presence of two promoters that control SLP-76 transcription. To differentiate between these possibilities and to further determine the exact 5’ end of the SLP-76 transcript, we employed RLM-RACE of SLP-76 mRNA that requires full-length, capped mRNA template (26, 27). SLP-76 cDNA fragments amplified by this method were cloned. We sequenced 15 clones and found that five clones start at the same nucleotide, which we define as +1 as the major transcription initiation site (Fig. 4A). This initiation site is 134 bases away from the 5’ end of the SLP-PE1 oligonucleotide used for the primer extension assay and is apparently used to generate the smaller band in that assay. Additional clones all start within the 507-bp region (Fig. 4A, each star above the nucleotide represents the 5’ end of one clone). Two of these clones start close to the YY1 site, 181 and 185 bases away from the 5’ end of the mSLP-PE1 oligonucleotide. Thus, it appears that the larger (180-base) band in the primer extension experiment may be generated using the potential YY1 site, because YY1 has been reported to function as an initiator element-binding protein to promote transcription initiation in certain promoters (28). The presence of multiple transcription initiation sites in the SLP-76 core promoter probably reflects the nature of TATA-less promoters being less precise in transcription initiation than TATA promoters (29). Taken together, these results and results from the transfection experiments demonstrated that the SLP-76 promoter lie within the 507-bp region we have defined.

### Requirement of multiple cis-regulatory elements for SLP-76 core promoter activity

Within the human and mouse SLP-76 core promoter, the homology in region B is higher than that in region A, with 85 vs 67% identity, respectively. We further assessed the importance of these regions for SLP-76 core promoter activity. As shown in Fig. 5, deletion of region A and the TC repeats from the core promoter with region B intact (~169 to +81) results in an ~50% decrease in the ability of SLP-76 core promoter to activate Luc reporter expression in both Jurkat and Raw cells, indicating that both regions A and B contribute to the core promoter activity in these cell types. A potential binding site for MZF1, a zinc finger transcription factor preferentially expressed in the hematopoietic system (especially in myeloid lineages (30, 31)), is located at the 5’ end of region B. Further deletion of this potential MZF1 site from region B (~155 to +81) results in a more dramatic decrease in promoter activity, suggesting an important role of this site in the activation of SLP-76 transcription.

Members of the GATA transcription factor family regulate the expression of many genes and play important and differential roles in the development of various lineages of the hematopoietic system (32). For example, GATA-3 is essential for the development of T cells and regulates the expression of many genes important for T cell function (33) as well as Th1/Th2 differentiation (34–36). Two potential GATA sites are located in region A of the SLP-76 core promoter. As shown above, region A contributes significantly to the full activity of the SLP-76 core promoter. However, mutation of both potential GATA sites in pSLP-426Luc does not impair the ability of the SLP-76 core promoter to activate the Luc reporter (data not shown), suggesting that in transient transfection assays these GATA sites are dispensable.

### Essential role of an Ets transcription factor-binding site for SLP-76 promoter activity

Potential binding sites for the Ets and YY1 transcription factors are also identified in region B of the SLP-76 core promoter (Fig. 4A). Similar to GATA transcription factors, members of the Ets family are differentially expressed among lineages within the hematopoietic
system and may play distinct roles in these cells. For example, Ets-1 is required for NK cell development (37) and regulates T cell survival and activation (38, 39), whereas PU.1 is critical for myeloid and B cell development as well as B cell activation (40–42).

YY1 is a multifunctional factor that is ubiquitously expressed and can both positively and negatively regulate gene transcription (43). The importance of YY1 in the immune system is much less clear compared with that of the GATA and Ets transcription factors.

FIGURE 5. Multiple cis-regulatory elements in the SLP-76 promoter. G (circle), M (hexagon), CT (octagon), E (oval), N (diamond), Y (square), and rectangle represent GATA, MZF1, CT repeats, Ets, NFAT, YY1, and the 5′-untranslated region, respectively. The 507-bp SLP-76 core promoter in pSLP-426Luc was further deleted from the 5′ end, and these deletion constructs were tested as described in Fig. 2. Data shown are representative of three experiments.
To investigate whether these potential Ets and YY1 binding sites are important for SLP-76 promoter activity, site-directed mutagenesis analysis was conducted on the 507-bp core promoter. Mutation of the potential Ets binding site from GAGGAA to GAGAGA results in an ~80–90% decrease in promoter activity in the Luc reporter assay in both Jurkat and Raw cells, indicating that this Ets binding site is critical for maximal SLP-76 transcriptional activation (Fig. 6A). In contrast, mutation of the YY1 binding site from CCATATT to CGCTAGC has no significant effect on SLP-76 promoter activity in Raw cells and inhibits the promoter activity by 30% in Jurkat cells. These results are consistent with the data from primer extension experiments suggesting a minimal role of this potential YY1 binding site for SLP-76 transcription in Raw cells, but that it may serve as an alternative transcription initiation site in T cells.

Given the importance of the potential Ets binding site for SLP-76 promoter activity, we sought to determine the identities of transcription factors binding to this site by EMSAs. Nuclear extracts were made from purified primary mouse T cells and B cells. \(^{32}\)P-labeled double-stranded oligonucleotide containing the Ets site from the SLP-76 promoter (mSLPEtsa) were incubated with nuclear extracts in the presence or the absence of excess unlabeled competitors. Protein-DNA complexes were separated by PAGE and detected by autoradiography. In supershift (ss) experiments, nuclear extracts were preincubated with specific Abs at room temperature before incubation with the oligonucleotide.

**FIGURE 6.** Regulation of SLP-76 transcription by Ets family transcription factors. A, A critical role of a potential Ets transcription factor binding site for SLP-76 promoter activity. The potential Ets and YY1 binding sites in pSLP-426Luc were mutated from GAGGAA to GAGAGA and from CCATATT to CGCTAGC to generate pSLP-426EtsM and pSLP-426YY1M, respectively. These constructs were tested as described in Fig. 2. Data shown are representative of four experiments. B, Association of Ets-1 and PU.1 to the potential Ets binding site of the SLP-76 core promoter. \(^{32}\)P-labeled, double-stranded oligonucleotides containing the Ets site from the SLP-76 promoter (mSLPEtsa) were incubated with nuclear extracts made from purified primary mouse T cells and B cells in the presence or the absence of excess unlabeled competitors. Protein-DNA complexes were separated by PAGE and detected by autoradiography. In supershift (ss) experiments, nuclear extracts were preincubated with specific Abs at room temperature before incubation with the oligonucleotide. C, Dose responses of SLP-76 core promoter activity to Ets-1 overexpression. pSLP-426Luc was cotransfected with different amounts of pEF-IREs-NGFR-hEts1 expression vector or the control vector pEF-IREs-NGFR into Jurkat and Raw cells. Twenty-four hours later, Luc activity in cell lysates was determined as described in Fig. 2. A representative of three experiments is shown. D, Requirement for the Ets binding site in SLP-76 core promoter for Ets-1 action. pSLP-426Luc and pSLP-426EtsmLuc were cotransfected with pEF-IREs-NGFR-hEts1 into Jurkat and Raw cells. Twenty-four hours later, Luc activity in cell lysates was determined as described in Fig. 2. A representative of three experiments is shown.
competitors. DNA-protein complexes were separated by PAGE and detected by autoradiography. As shown in Fig. 6B, multiple DNA-protein complexes were revealed (lanes 2 and 7). Among them, complexes I, II, III, and IV were specific, as these were greatly inhibited by excess unlabeled wild-type competitor (mSLP-PEIsa; lanes 3 and 8) or competitor containing a consensus Ets binding site (Ets-C; lanes 5 and 10) from the human c-erbB2/neu promoter (44, 45), but not by an oligonucleotide with the Ets sites mutated (mSLP-PEIsaM; lanes 4 and 9). Complex I is abundant in extracts from both T and B cells, whereas complex IV is abundant in B cells, but is undetectable in T cells. Complexes II and III are less abundant and exist in both lineages. A pattern similar to that in B cells was revealed using nuclear extract from Raw cells (data not shown). Supershift experiments with Abs revealed that complexes III and IV contain Ets-1 and PU.1, respectively (lanes 6, 11, 14, and 15), suggesting a role of these transcription factors in SLP-76 expression. In support of this idea, we found that overexpression of Ets-1 significantly enhanced SLP-76 core promoter activity in both Jurkat and Raw cells in a dosage-dependent manner (Fig. 6C). The effect of Ets-1 overexpression on enhancement of SLP-76 core promoter activity appeared direct, as it failed to induce the promoter activity when this site was mutated (Fig. 6D). Similarly, overexpression of PU.1 in Jurkat cells enhanced the activity of the SLP-76 reporter construct in this in vitro assay system (data not shown).

Discussion

SLP-76 plays critical roles in the development and function of multiple hematopoietic lineages as well as in vascular specification. In this study we have identified several DNases I HSs in the SLP-76 locus. The pattern of these HSs correlates well with SLP-76 expression in cells representing different lineages of the hematopoietic system. Using transient transfection assays and analysis of transcription initiation, we further identified the SLP-76 core promoter activity as a 507-bp region that is colocalized with HS2, the most prominent HS in the S5' portion of the SLP-76 locus. In the assay systems we employed in this study, the SLP-76 core promoter is able to activate high levels of reporter expression in T and monocytic cells, but not in B cells. In addition, the core promoter activity can be further induced after TCR stimulation. Thus, it appears that the core promoter contains elements that are sufficient to confer lineage-specific and inducible transcription of SLP-76 in these cell types. Further studies will determine whether the SLP-76 core promoter is also active in other cell types known to express SLP-76. As there are major limitations of transient transfection reporter assays, additional efforts will determine whether the core promoter is sufficient to activate SLP-76 transcription and to confer lineage specificity in vivo or if as yet unidentified additional elements also play important roles in SLP-76 transcriptional regulation.

Multiple potential transcription factor binding sites exist within the 507-bp region. Mutational analysis has suggested that the two potential GATA binding sites are not essential for SLP-76 promoter activity. However, because region A, where these two GATA sites are located, does contribute significantly to the SLP-76 core promoter activity, additional elements in this region must be important for SLP-76 promoter activity. Studies are underway to identify the appropriate sites and binding factors.

Several pieces of evidence suggest a critical role for Ets family transcription factors in activation of the SLP-76 promoter. These include a dramatic reduction of SLP-76 promoter activity when the Ets binding site is mutated in the core promoter, the association of Ets-1 and PU.1 to the potential Ets binding site, and the enhancement of SLP-76 promoter activity when Ets-1 and PU.1 are over-expressed. Although the results suggest that both Ets-1 and PU.1 can impact SLP-76 promoter activity, at present we do not know the exact role of either factor in vivo for SLP-76 transcriptional regulation, as additional protein-DNA complexes have also been shown to associate with the Ets binding site. The identities of the transcription factors in these complexes remain to be determined.

Among the four specific protein-DNA complexes found at the Ets-binding site in the SLP-76 core promoter, three complexes (I, II, and III) are similar among T, B, and myeloid cells. The other complex (complex IV, PU.1) is present only in B and myeloid cells, not in T cells. Thus, although the Ets binding site is critical for SLP-76 promoter activity, Ets transcription factors do not appear to be the determinants of lineage-specific expression of SLP-76. It has been documented that T cells express Ets-1, but not PU.1 (46). Thus, PU.1 is not absolutely required for SLP-76 expression. It has also been reported that forced expression of PU.1 in developing T cells interferes with the differentiation of precursors to T cell lineages (47). However, we do not believe this effect to be due to suppression of SLP-76 expression, as PU.1 overexpression can enhance SLP-76 promoter activity in Jurkat cells. In addition, Raw cells expressing a high level of PU.1 still express SLP-76. As SLP-76 is essential for T cell development (5, 6), it seems likely that Ets-1 is not absolutely required for SLP-76 expression, as T cells develop in Ets-1-deficient mice (38, 39). As additional members of the Ets transcriptional factors, such as Elf and Elk, are also expressed in T cells, these factors may function redundantly with Ets-1 and may compensate for its deficiency in activating the SLP-76 promoter (46). This is consistent with our EMSA data, which showed that additional transcription factors may associate with the Ets binding site and may regulate SLP-76 transcription. It was noted, however, that Ets-1-deficient T cells do manifest impairment of T cell activation (38, 39). It would be interesting to learn whether deficiency of Ets-1 and/or other Ets family members results in defective or dysregulated SLP-76 expression, and if so, whether abnormal expression of SLP-76 is causal (at least in part) for the observed impairment in T cell activation.

SLP-76 expression is tightly regulated during T cell development and at different stages of immune responses (15, 16). The importance of such modulation of SLP-76 expression is not clear, but probably impacts the efficiency of TCR signaling. In addition to the Ets binding sites, potential sites for other transcription factors, such as NFAT and c-Rel, appear in the SLP-76 core promoter. As the activities of Ets-1, NFAT, and NF-xB are modulated by signaling from cell surface receptors, including the TCR (48–50), this may provide a possible mechanism for regulation of SLP-76 expression during immune responses. Additionally, it has been reported that these factors can directly associate to form a multimolecular enhancerosome in several inducible promoters (51). It is of particular interest that the Ets binding site and the potential NFAT binding site are adjacent to each other. Studies are underway to determine the importance of the NFAT and c-Rel binding sites as well as other transcription factor binding sites for SLP-76 promoter activity, and whether these factors cooperate with each other for efficient activation of SLP-76 transcription. Multiple cis-regulatory elements and transcription factors that are involved in transcription initiation have been documented. The SLP-76 core promoter lacks a TATA box, a CCAAT box, and SP1 sites. Thus, the major transcription initiation site for this gene does not contain obvious known motifs involved in transcriptional regulation, suggesting that less conserved mechanisms must be involved in SLP-76 transcription initiation. The SLP-76 promoter does contain a potential YY1 site, which has been reported to be involved in transcription initiation in some TATA-less promoters. Interestingly, the YY1 site appears to serve as an alternative transcription initiation site for SLP-76 in T cells, but not in Raw cells (Fig. 4A).
In summary, the present study provides the first characterization of cis-regulatory elements in the SLP-76 promoter. It appears that the 507-bp SLP-76 core promoter contains elements that are able to confer both lineage-specific and inducible expression of SLP-76. We also found that transcription initiation within this promoter is differentially regulated between T cells and Raw cells. Further studies are needed to evaluate additional cis- and trans-acting elements involved at the core promoter as well as the other HSs we have localized at the SLP-76 locus. These studies will improve our understanding regarding how lineage-specific and developmentally regulated expression of SLP-76 is achieved. Understanding such mechanisms may provide tools to manipulate SLP-76 expression through genetic modification at its locus and to further evaluate the physiological role of variable levels of SLP-76 expression during T cell development, activation, and immune responses.

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References


