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Molecular Cloning of the Mouse APS as a Member of the Lnk Family Adaptor Proteins

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Engagement of cell-surface receptors leads to activation of protein tyrosine kinases, which in turn phosphorylate various downstream enzymes and adaptor proteins. Lnk is an adaptor protein that appears to be involved in signal transduction in lymphocytes, and forms an adaptor protein family with SH2-B. We tried to identify another member of the adaptor protein family and isolated the mouse APS (adaptor molecule containing PH and SH2 domains). APS contains a proline-rich region, PH and SH2 domains, and a putative tyrosine phosphorylation site at the C-terminal, and the overall structure resembles those of Lnk and SH2-B. APS is expressed in brain, kidney, muscle, and mature B cells in spleen. Mouse *APS* gene consists of 8 coding exons and is deduced to map to chromosome 5. APS is tyrosine phosphorylated at the C-terminal phosphorylation site conserved among the Lnk family adaptor proteins by stimulation of IL-5 or IL-3 as well as by crosslinking of B cell receptor complex. These results suggest that APS is a member of the Lnk family adaptor protein and likely plays a role in signaling in B cells.

The binding of extracellular ligands to their respective polypeptide receptors initiates a cascade of events that are critical for mitosis, differentiation and death, as well as other cellular responses. These signal transduction cascades involve activation of numerous intracellular molecules including protein kinases, lipid kinases, GTP-binding proteins, and other various effector enzymes. The protein-protein interactions are critical not only for the activation of effector enzymes themselves but also for their recruitment to activated receptor complexes. While the details of how signaling molecules are recruited to activated receptors remains an enigma, it is becoming increasingly appreciated that a group of cellular proteins called adaptor proteins regulate the interaction of effector enzymes with surface receptors.

Adaptor proteins lack catalytic function (insofar as is known) but still possess interaction domains; *e.g.*, SH2 and PTB domains that bind tightly to phosphotyrosine residues, SH3 domain that binds proline-rich residues or PH domain that has been implicated in interaction of proteins with lipid membranes (1–3). An appreciation for the importance of adaptor proteins in signal transduction has been derived largely from studies of growth factor receptors, where initial binding and phosphorylation of the adaptor protein Shc creates a binding site for a second adaptor, Grb2, that then directs Sos, a guanine nucleotide exchange factor, to the membrane where it can act upon Ras (4). Since then, growing evidence supporting the importance of adaptor proteins has been presented in various signal transduction cascades. A thorough enumeration of the normal complement of adaptor proteins, and an analysis of how these proteins behave should therefore help to illuminate mechanisms of signal transduction in cell interior.

Lnk has been reported as a 38-kDa adaptor protein expressed in lymphocytes (5, 6). Lnk consists of a single SH2 domain and several candidate tyrosine phosphorylation sites for possible interaction with Grb2, phospholipase C-γ1 and phosphatidylinositol 3-kinase (5). The observation that Lnk becomes phosphorylated on tyrosine residue after crosslinking of T cell receptor (TCR) complex suggested that Lnk might play an important role in TCR-mediated signaling. However, the transgenic mice over-expressing Lnk at high levels in thymocytes demonstrated that the availability of Lnk does not restrict normal T cell development or respon-

Nucleotide and translated amino acid sequences have been submitted to GenBank under Accession No. AF234838.

Abbreviations used: APS, adaptor molecule containing PH and SH2 domains; BCR, B cell receptor; TCR, T cell receptor; PH, pleckstrin homology; SH2, Src homology 2; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; GST, glutathione *S*-transferase; ITAM, immunoreceptor tyrosine-based activation motif.

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siveness at all (6). Thymocytes from transgenic mice proliferated normally and showed an equivalent pattern of tyrosine phosphorylated proteins when compared with normal thymocytes stimulated via TCR crosslinking. Thus Lnk almost certainly interacts with phosphoproteins other than those that comprise the TCR.

We noticed that Lnk has a significant homology with SH2-B, an adaptor protein which interacts with the immunoreceptor tyrosine-based activation motif (ITAM) of FcεRIγ chain in a phosphorylation dependent manner (7). SH2 domains of Lnk and SH2-B share highly conserved amino acid sequence among other various SH2 domains. The N-terminal region of the reported Lnk has a significant homology with a part of SH2-B (6). These strongly indicate that Lnk and SH2-B are likely to be members of a new adaptor protein family.

In this report, we tried to identify another member of the new adaptor protein family represented by Lnk, and isolated the mouse APS (mAPS), adaptor molecule containing PH and SH2 domains that was recently identified as a substrate for c-kit, Trk, insulin receptor or insulin-like growth factor receptor (8–12). APS is expressed mainly in B cells in lymphoid organs, as well as in brain, kidney and muscle. APS is tyrosine phosphorylated by various protein tyrosine kinases (PTKs), and the C-terminal tyrosine, which is conserved among the Lnk family adaptor proteins, is a main target of various PTKs. APS becomes phosphorylated after crosslinking of B cell receptor (BCR) and by the stimulation of various cytokines, indicating APS may play a role in BCR or cytokine signaling pathways.

MATERIALS AND METHODS

Isolation of cDNA and genomic DNA clones. The amino acid sequences of mouse Lnk (aa 239-288: GenBank Accession Number [Acc. No.] U89992) and rat SH2-B (aa 337-408: Acc. No. U57391) was used to search the GenBank database with BLAST algorithm, and an EST clone from human germinal center B cell (Acc. No. AA292710) was found to encode an amino acid sequence homologous to Lnk and SH2-B. A cDNA fragments was amplified from mouse spleen by nested PCR using following primers that were synthesized based on the human cDNA sequence from EST database; sense primers: 5′-CTCCAGCCCAAGGTCAGCATCC-3′, 5′-AGCCCAAGGTCAGCATCCCACT-3′, antisense primers: 5′-TGTCTTCCTCACTGTCACCGGG-3′, 5′-AGCCCTGGATGTCAGCTACCCA-3′. The cDNA fragment was labeled using the random-priming method with [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Uppsala, Sweden), and used as a probe to screen a library previously constructed with mouse spleen cDNA in the λZAPII vector (Stratagene, La Jolla, CA) by plaque hybridization. Five positive plaques were isolated from 1×10^6 plaques screened, and those phage DNAs were excised *in vivo* to rescue phagemids containing the cDNA fragments. The DNA sequences were determined using the dideoxy chain termination method employing AutoCycle Sequencing Kit (Amersham Pharmacia Biotech). Genomic clones were isolated from a λFIXII library of 129SVJ liver DNA partially digested with *Mbo*I (Stratagene) using [α-^{32}P]dCTP-labeled mAPS cDNA fragments as probes. By screening 1×10^6 plaques, 3 positive clones were isolated. The inserts of those independent clones were subcloned into pBluescript SK(−) (Stratagene). Physical maps of subcloned plasmids were determined by restriction enzyme digestion, Southern blotting, PCR and DNA sequencing.

Cells. COS7 cells were maintained in RPMI 1640 supplemented with 8% fetal calf serum (FCS), penicillin (100 IU/ml) and streptomycin (50 μg/ml). Y16 cells were maintained in RPMI 1640 supplemented with 4% FCS, penicillin, streptomycin and 50 μM 2-mercaptoethanol (2-ME), containing 5 U/ml mIL-5. MC9 cells were maintained in RPMI 1640 supplemented with 8% FCS, penicillin, streptomycin and 50 μM 2-ME, containing 20 U/ml mIL-3. BAL17 cells were maintained in RPMI 1640 supplemented with 8% FCS, penicillin, streptomycin and 50 μM 2-ME.

Northern blotting and reverse transcriptase-PCR. Total RNA was isolated from cell lines by the acid guanidine isothiocyanate-phenol-chloroform method (13) and poly(A)$^+$ RNA was isolated from mouse tissues using the Micro-FastTrack kit (Invitrogen, Carlsbad, CA). RNAs were separated on a 1% agarose/formaldehyde gel and transferred to nylon membranes (NEN, Boston, MA). The Multiple Choice Northern Blots were purchased from OriGene (Rockville, MD). The membranes were hybridized with ^{32}P-labeled cDNA fragments encoding mAPS (amino acids 240-306) or mouse β-actin (amino acids 160-375). For reverse transcriptase-PCR analysis, splenocytes were separated by FACStar (Becton-Dickinson, Mountain View, CA) on basis of the expression of CD3 or B220. The first-strand cDNA was synthesized using SuperScript Reverse transcriptase (GIBCO BRL, Rockville, MD) and random hexamers (TAKARA, Shiga, Japan) from poly(A)$^+$ RNA isolated from separated cells by the Micro-FastTrack kit. The cDNA templates were subjected to PCR amplification using sets of primers for APS (forward 5′-TTTCTCTGAACGGTCACGGC-3′; reverse 5′-AGGCTGGAGAAGTAGTGCTG-3′) or for β-actin (forward 5′-ACACTGTGCCCATCTACGAG-3′; reverse 5′-CTAGAAGCACTTGCGGTGCA-3′). Cycling parameters were 1 min at 94°C, 2 min at 56°C, 2 min at 72°C for 34 cycles to detect mAPS mRNA, and 27 cycles for β-actin. PCR products were separated by electrophoresis on a 1.0% agarose gel and visualized by staining with ethidium bromide.

GST fusion proteins. The cDNA fragments encoding the N-terminal domain (mAPS-N; amino acids 1-102), and the C-terminal domain (mAPS-C: amino acids 534-621) were amplified by PCR using the following primers: (mAPS-N: forward 5′-AAGGATCCATGAATGGTGCCACCCCCA-3′, reverse 5′-GTCGTCTGCCACCATGAAGC-3′; mAPS-C: forward 5′-CTGATATCACCCTAAGAAGC-3′, reverse 5′-TAGCGGCCGCTTAGGTGTTGCTGACCATCG-3′). PCR fragments were digested by restriction enzymes and subcloned into the pGEX-4T-1 (Amersham Pharmacia Biotech), and the plasmids were used to transform *E. coli* BL21. Induction of the recombinant proteins was performed by incubating host *E. coli* with 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested and lysed, and the fusion proteins were purified by absorption to glutathione-coupled Sepharose-4B (Amersham Pharmacia Biotech).

Generation of anti-mAPS antibodies. Rabbit polyclonal antisera were raised against the GST-mAPS-N and -C fusion proteins. The antibodies were purified by chromatography on CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech), conjugated with either GST-mAPS-N or GST-mAPS-C fusion protein, followed by elution using 0.1 M glycine, pH 2.5. Antibodies against GST were absorbed from the affinity purified antibodies using GST-coupled CNBr-activated Sepharose 4B.

Construction of mAPS expression vectors. To create Myc-tagged mAPS (Myc-mAPS), the *Eag* I fragment of mAPS (codon 9-621) was subcloned into pMEMyc2 (gift from Dr. T. Yamamoto) in frame. Resulting fusion protein contained Myc-tag (SMEQKLISEEDLN) at the N-terminus. Full-length mAPS expression vector (pcDNA3APS) was constructed by subcloning the cDNA fragment encoding full

length mAPS into *Eco*RI/*Not*I site of pcDNA3 (Invitrogen). Deletion mutant lacking the C-terminal region was constructed by eliminating the *Apa*I fragment (0.5 kb) and re-ligation of pcDNA3APS. Resulting mutant protein (ΔC) consists of amino acids 1M-512P of mAPS and 2 extra residues (I and L) added by the deletion.

Transient and stable transfections. COS7 cells were transiently transfected by using SuperFect (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. For stable transfection of Y16 or MC9 cells, 10^7 cells in 0.4 ml cytomix (14) (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM HEPES, 2 mM EGTA and 5 mM $MgCl_2$, pH 7.6) were electroporated with 10 μg of linearized pcDNA3APS or control pcDNA3 vector. Stable transfectants were selected in the presence of 0.5 mg/ml G418, and screened for the expression of the recombinant APS by immunoblotting.

Immunoprecipitation and Western blotting. COS7 cells were harvested at 48 h after transfection and lysed with 0.5 ml lysis buffer (1.0% Triton X-100 in Tris-buffered saline containing 10% glycerol, 5 mM EDTA, 100 U/ml aprotinin, 1 mM sodium vanadate, 10 mM sodium fluoride, 25 μg/ml *p*-nitrophenyl-*p'*-guanidinobenzoate, 10 mM iodoacetoamide, 10 μg/ml leupeptin), and the lysates were clarified by centrifugation. For SCF stimulation, COS7 cells were starved for 16 h (from 48 h to 64 h after transfection) in medium containing 0.1% FCS, then stimulated with 100 ng/ml stem cell factor (SCF, Pepro Tech EC, London, UK) in the presence of 0.1 mM sodium vanadate for 10 min at 37°C. The lysates were incubated with anti-Myc monoclonal antibodies (9E10) or anti-mAPS antibodies, and protein G Sepharose 4B (Amersham Pharmacia Biotech) at 4°C for 1 h. Then, the Sepharose beads were washed five times with lysis buffer. The precipitated proteins were resolved by SDS-10% PAGE under reducing conditions, and transferred to a PVDF membrane (Millipore, Bedford, MA). After blocking with 5% BSA/Tris-buffered saline pH 7.6, blots were probed with anti-phosphotyrosine mAb (4G10, Upstate Biotechnology, Lake Placid, NY), or anti-mAPS antibodies, and incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, Santa Cruz, CA). Filters were washed in 0.05% Tween 20/Tris-buffered saline and visualized by enhanced chemiluminescence (NEN).

For Y16, MC9 transfectants or BAL17, cells were washed three times with Hanks' balanced salt solution and cultured in RPMI 1640 containing 4% FCS for 8 to 12 h. The cells were then collected and stimulated with 10 nM mIL-5 (for Y16), 30000 U/ml mIL-3 (for Y16 and MC9), 1 μg/ml SCF (for MC9) or 100 μg/ml anti-IgM F(ab′)2 fragment (ICN Pharmaceuticals, Aurora, OH). Then, cells were collected and lysed with lysis buffer, and the lysates were subjected to immunoprecipitation and Western blot analysis as described above.

RESULTS

Identification of a protein homologous to Lnk and SH2-B. We reported that the N-terminal region of the mouse Lnk was homologous to a part of SH2-B (6). The region turned out to be a part of the pleckstrin homology (PH) domain as the full-length Lnk was identified (S.T. will describe elsewhere. The updated mouse Lnk cDNA sequence is available through GenBank, Acc. No. U89992). We searched GenBank database for proteins homologous to Lnk and SH2-B by BLAST algorithm using the amino acid sequences corresponding to a part of PH domains of Lnk and SH2-B as queries (see Materials and Methods). An EST clone (Acc. No. AA292710) generated from human germinal center B cells was found, and the corresponding mouse cDNA fragment was successfully amplified by PCR from spleen. To obtain the full-length cDNA, we screened a mouse spleen cDNA library using the cDNA fragment as a probe, and isolated three independent clones. The sequences of those clones were analyzed and a fused cDNA sequence was composed from the three overlapping cDNA sequences. The composed cDNA sequence contained an open reading frame (ORF) encoding a protein that consists of 621 amino acids with the predicted molecular weight of 67 kDa, carrying a PH domain, a Src homology 2 (SH2) domain, and a possible tyrosine phosphorylation site at the C-terminal end (Fig. 1). The overall structure of the protein highly resembles to those of Lnk and SH2-B. During our analysis of the cDNA, a human adaptor protein, APS (hAPS) which shares similar structure with SH2-B was reported (8). A homology search revealed that our cDNA encodes the mouse APS. The amino acid sequence is 82% identical to hAPS, and particularly its PH and SH2 domains share 96% identity with hAPS (data not shown). The PH domain located between amino acid 197-299 is 40% and 61% identical to those of mLnk and mSH2-B, respectively. The SH2 domain located between amino acid 408–507 is 65% and 79% identical to those of mLnk and mSH2-B, respectively (Fig. 1B). The N-terminal region of mAPS is also significantly homologous to those of mLnk and mSH2-B (28% and 38% identity, respectively). Two possible tyrosine phosphorylation sites matching to [R/K]XX(X)[D/E]XX(X)Y motif (PROSITE database, Medical Biochemistry Department, University of Geneva) are located at amino acids 47 (RFLRDNPTY) and 618 (RAVENQY). The C-terminal phosphorylation site is conserved among all three related proteins, whereas the N-terminal one is conserved only between APS and SH2-B. These observation confirmed that those three proteins form an adaptor protein family and that we identified a mouse counterpart of the family member, mAPS.

Expression of mAPS mRNA. The distribution of APS mRNA in various mouse tissues was analyzed by Northern blotting. A single transcript of 2.9 kb was strongly expressed in brain, and found at detectable levels in spleen, kidney and skeletal muscle (Fig. 2A). Low levels of mAPS expression were also detected in small intestine and bone marrow. Among various hematopoietic cell lines, mAPS was strongly expressed in B cell lines (WEHI231, BAL17 and BCL1), and at marginal levels in B-precursor cell lines (LyD9, Y16, and 40E1) except one pre-B cell line (18-81) (Fig. 2B). T cell lines (MTH and CTLL-2) did not express mAPS, although low level expression was detected in a thymoma cell line (LSTRA). APS expression was also detected in myeloid and fibroblast cell lines (FDC-P1 and NIH3T3). Since a fair amount of mRNA was expressed in spleen and the hAPS EST clone we found was derived from germinal center B cells as described above, we assessed mAPS mRNA expression in lymphocyte subpopulations. Splenic B cells ($B220^+$) and T cells ($CD3^+$) were purified by cell sorting, and expression of

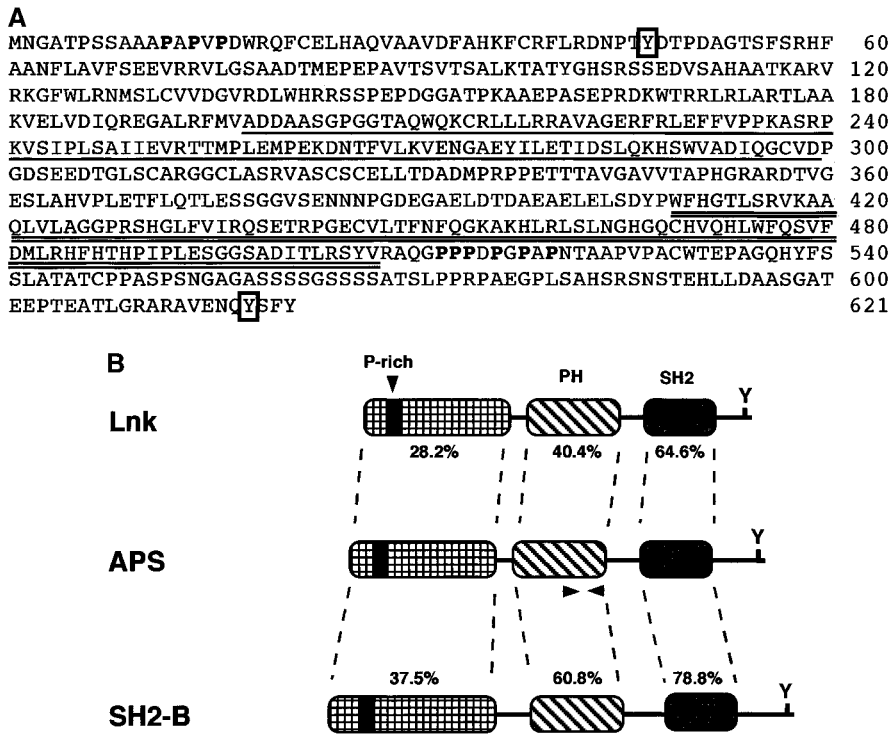

FIG. 1. Structure of mouse APS. (A) Amino acid sequence of mAPS. Proline residues in the two proline rich regions indicated by bold letters. PH domain and SH2 domain are indicated by underline and double underline, respectively. Two putative tyrosine phosphorylation sites are indicated by box. (B) Schematic representation of mAPS, mLnk and mSH2-B showing location of N-terminal conserved domain, proline rich region, PH domain and SH2 domain. Percentages indicate amino acid identity between respective mouse proteins. Arrowheads indicate PCR primers synthesized based on sequence of the EST clone (Acc. No. AA292710).

mAPS in each fraction was analyzed by a semi-quantitative reverse transcriptase-PCR (RT-PCR) analysis. APS mRNA was detected in B cells but not in T cells (Fig. 2C, right panels). In consistent with the result of our Northern blotting, APS mRNA was not amplified from thymus (Fig. 2C, left panels). These observations suggest that mAPS is expressed mainly in mature B cells in lymphoid tissues, although its expression is not limited in lymphoid organs.

Genomic structure and chromosomal localization of mouse APS gene. We also obtained clones of the mouse *APS* gene to gain a better insight into the function and structure of APS. We screened a 129SVJ genomic library using the mAPS cDNA fragment as probes, and isolated three overlapping genomic DNA clones. Physical map of a 20 kb region of the mouse *APS* gene was constructed (Fig. 3). Restriction map was determined and exon-intron boundaries were analyzed. The mouse *APS* gene consists of 8 coding exons that extended over 13 kb (Fig. 3). Comparison of genomic and cDNA sequences indicated there exists extra exon(s) encoding 5′ untranslated region of m*APS* (data not shown).

A database search using APS cDNAs revealed that a 125-kb high throughput genomic sequence (GenBank Acc. No. AF030453) contains DNA sequences for exons of h*APS* gene. The sequence was obtained from a human chromosome 7q22 PAC clone, indicating the h*APS* gene localizes at chromosome 7q22. We found the same PAC clone contains *CUTL1* (CUT-LIKE 1) gene about 50 kb apart from human *APS* gene. Mouse *Cutl1* has been mapped to distal region of the mouse chromosome 5 (15). Genes surrounding *Cutl1* gene (from *Epo* to *Cyp3a11*) perfectly co-localize and align in human as well as in mouse, according to Human/Mouse Homology Region Map (National Center for Biotechnology Information: NCBI). Therefore, it is deduced that mouse *APS* gene also co-localized to the chromosome 5 with *Cutl1* and other surrounding genes (Fig. 4).

Tyrosine phosphorylation of mAPS. APS contains tyrosine kinase phosphorylation sites and is expressed in B cells. We investigated whether mAPS function as a substrate of tyrosine kinases expressed in B cells by overexpressing mAPS with various tyrosine kinases in COS7 cells. After immunoprecipitation, tyrosine phosphorylation of mAPS was examined by immunoblotting. APS became strongly phosphorylated on tyrosine residues when co-expressed with JAK2, JAK3, Lyn, Fyn, and Syk (Fig. 5A). Weak but significant levels of phosphorylation by Btk and JAK1 were detected. APS migrates as a doublet band both in phosphorylated and non-phosphorylated forms (see Figs. 5, 6 and 7), al-

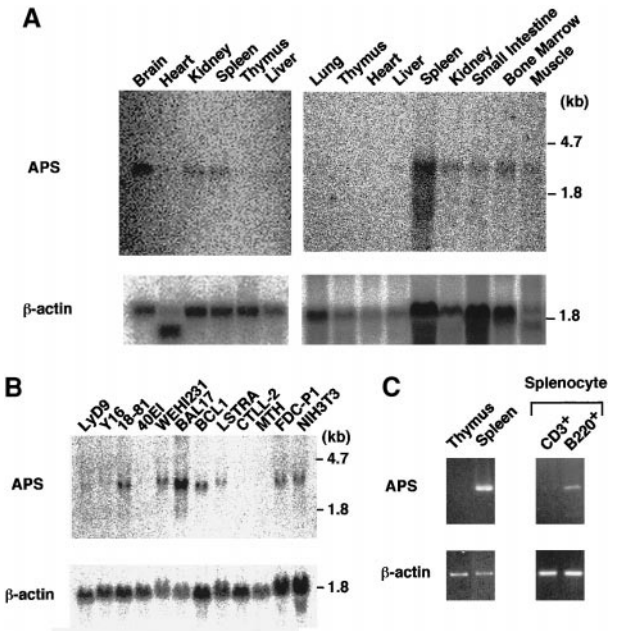

FIG. 2. Expression of mAPS mRNA. Membranes carrying poly(A)$^+$ RNA from indicated mouse tissues (A) or total RNA from various mouse cell lines (B) were hybridized with a ^{32}P-labeled cDNA fragment of mAPS (upper panel). The blot was stripped and reprobed with β-actin to estimate amounts of loaded RNA (lower panel). LyD9, Y16 and 40EI; pro-B cell line, 18-81; pre-B cell line, WEH1I231, BAL17 and BCL1; B cell line, LSTRA; immature T cell line, CTLL-2 and MTH; mature T cell line, FDC-P1; myeloid cell line, NIH3T3; fibroblast cell line. (C) Semiquantitative RT-PCR analysis. cDNAs were prepared from thymocytes, total splenocytes, CD3$^+$ or B220$^+$ splenocytes and APS expression was analyzed by RT-PCR.

though the molecular basis explaining the difference between two bands is currently unknown. Of note, JAK2, JAK3, Syk, and Fyn were co-immunoprecipitated with mAPS (Fig. 5A, upper panel). When mAPS was co-expressed with c-kit in COS7 cells, mAPS was weakly phosphorylated and the level of tyrosine phosphorylation was significantly enhanced by the stimulation of SCF, the ligand for c-kit (Fig. 5B). C-kit was also co-immunoprecipitated with mAPS (Fig. 5B). Thus, at least in overexpressed cells, mAPS functions as a substrate for various tyrosine kinases.

It has been reported that SH2-B is a substrate of JAK2 in growth hormone signaling (16). As we also observed phosphorylation of mAPS by JAK2 in overexpressed COS7 cells, we investigated whether mAPS is a substrate of JAK2 by overexpressing mAPS in Y16, IL-5 and IL-3-dependent pro-B cell line. Receptors for IL-5 and IL-3 use the βc subunit as a common component, and activate JAK2 (17, 18). As observed by Northern blotting, Y16 cells expressed very small amount of mAPS and the mAPS protein was undetectable by our immunoblot. Y16 cells were stably transfected with mAPS, and phosphorylation of mAPS by the stimulation of IL-5 or IL-3 was examined. Both IL-5 and IL-3 induced tyrosine phosphorylation of mAPS and the level of phosphorylation peaked in 1–5 min (Fig. 6A, left panels and data not shown). Some other undefined tyrosine-phosphorylated proteins co-precipitated with mAPS. Next, we examined phosphorylation of mAPS by c-kit in an SCF-responsive cell line, MC9. Since MC9 cells also express very low amount of APS, we used stable transfectant overexpressing mAPS. Tyrosine phosphorylation of mAPS reached at a peak by 1 min was observed by the stimulation with SCF (Fig. 6A, right panels). The phosphorylation of p140, probably c-kit, was co-immunoprecipitated with mAPS. MC9 cells respond to IL-3 as well as SCF. IL-3-induced phosphorylation of APS is also observed in MC9 transfectants (Fig. 6A, middle panels).

A mouse B cell line, BAL17 strongly expressed mAPS as demonstrated by Northern blotting. We examined the phosphorylation of endogenous mAPS induced by BCR stimulation using BAL17 cells (Fig. 6B). Crosslinking of BCR by anti-IgM induced phosphorylation of mAPS, which reached a peak at 5 to 15 min. The phosphorylation level of mAPS was decreased over 15 min after stimulation. These observations indicated that mAPS is phosphorylated by JAK2, c-kit and kinases activated by BCR in the stimulation dependent manner.

The tyrosine phosphorylation site of mAPS is at C-terminal. The C-terminal possible phosphorylation site is conserved among all three Lnk family adaptor proteins. To clarify whether the tyrosine residue in C-terminal phosphorylation site play a critical role, we constructed a mutant form (ΔC) of mAPS cDNA lacking the C-terminal tail harboring the conserved tyrosine phosphorylated site, and transfected it into MC9 cells. Wild type mAPS was efficiently tyrosine phosphorylated by the stimulation of both IL-3 and SCF as described above. In contrast, the ΔC form mAPS was not phosphorylated at all by both stimuli (Fig. 7). Association of ΔC with other phosphoproteins including p140 was essentially maintained. From these results, we concluded that a major tyrosine phosphorylation site of mAPS by JAK2 and c-kit is the C-terminal

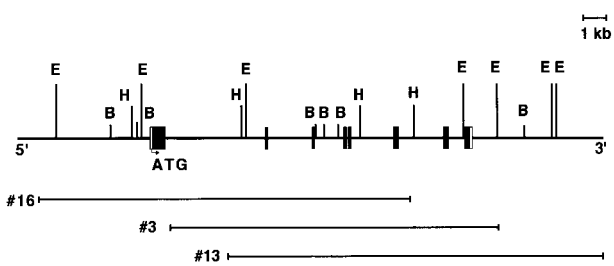

FIG. 3. Genomic structure of the mouse *APS* gene. Exons are indicated by boxes. Filled boxes indicate amino acid coding region. *Eco*RI(E), *Bam*HI(B) and *Hin*dIII(H) restriction sites are shown. Three independent and overlapping genomic DNA clones are indicated below.

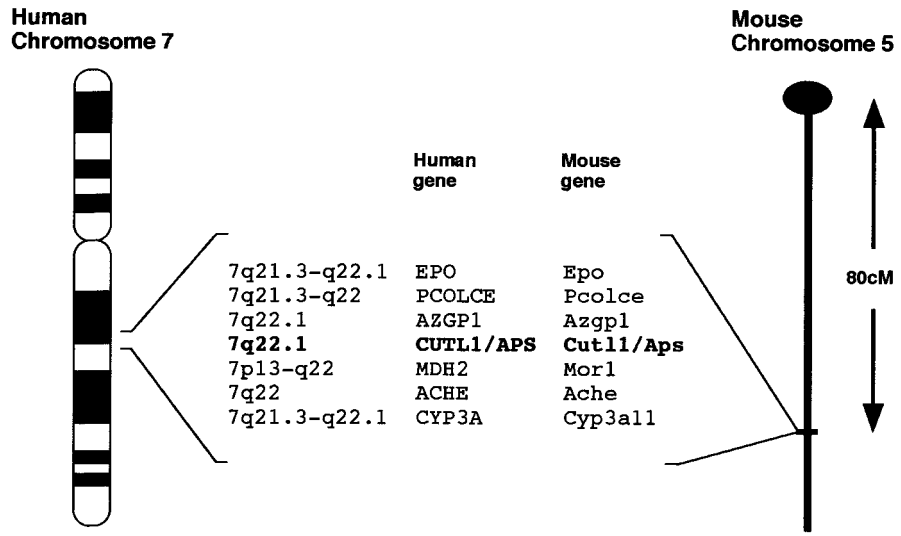

FIG. 4. Deduced chromosomal map position of mouse *APS* gene. From database search, human 7q22 PAC clone containing human *APS* and *CUTL1* was found. *Cutl1*, the mouse counterpart of *CUTL1*, has been already mapped on the mouse chromosome 5. The distance between human *APS* and *CUTL1* loci is about 50 kb, and several genes (*Epo, Pcolce, Azgp1, Mor1, Ache,* and *Cyp3a11*) surrounding the two genes are colocalized in both human and mouse. Therefore, it is deduced that mouse *APS* gene is also mapped on the mouse chromosome 5.

tyrosine, which is conserved among all Lnk family adaptor proteins.

Consequence of overexpression of mAPS and its mutant in various cell lines. We investigated the consequence of overexpression of the wildtype APS or ΔC mutant form of mAPS lacking the C-terminal major phosphorylation site on cell proliferation in Y16 and MC9 cells. Overexpression of APS or APSΔC also did not show any effect on IL-5 or IL-3-induced proliferation of Y16 cells (data not shown). Neither the wildtype APS nor APSΔC had effect on SCF-induced proliferation of MC9 transfectant by their overexpression (data not shown). Although IL-5, IL-3 or SCF induced tyrosine phosphorylation of APS, overexpression of the wildtype APS or APSΔC mutant had no effect on the cell proliferation induced by IL-5, IL-3 or SCF.

DISCUSSION

In this study we tried to identify a new member of the Lnk family adaptor proteins, and cloned a cDNA for the mouse APS (adaptor molecule containing PH and SH2 domains). APS consists of an N-terminal proline-rich region, PH and SH2 domains, and a tyrosine phosphorylation site at the C-terminal end. The overall structure of APS remarkably resembles to that of SH2-B. Lnk was originally reported as a 38-kDa adaptor protein, which may function in signaling through T cell receptor (TCR) complex because of its possible interaction with Grb2, PLCγ and PI3-K (5). However, the full length Lnk is nearly twice the size (68 kDa) of the previously published protein and contains a proline-rich region, PH and SH2 domains, and a tyrosine phosphorylation site at the C-terminal end (S. T. will report elsewhere). The N-terminal proline-rich region of these three adaptor proteins share significant homology with each other. Thus, the structures of Lnk, APS and SH2-B proteins turned out to be highly homologous to each other, and these three proteins forms a unique adaptor family. So far, only those three proteins are identified as the family members in mammals. We tried to amplify genomic DNA fragments using degenerative oligomers corresponding to the PH domain of Lnk. The fragments from *SH2-B* and *lnk* genes were amplified from mouse genomic DNA, however, no other fragment carrying ORF was obtained (M. I. and S. T., unpublished observation).

There are splicing variant forms of SH2-B, α and β in mouse, rat (16, 19) and human (EST clone Acc. No. AW374366). SH2-Bβ cDNA carries a 100 bp insertional sequence that juxtapose the SH2 domain coding region, which resulted in a reading frame shift and a deletion of the C-terminal tyrosine residue conserved among the Lnk family proteins. In our screening procedures to pick the full length mAPS cDNA, we obtained a cDNA clone with a 25 bp deletion at the 3′ end of SH2 coding sequence resulting in a flame shift and replacement of the conserved C-terminal sequence with more than 34 amino acids stretch without tyrosine phosphorylation motif. We evaluated the amounts of the variant form of mAPS mRNA in spleen by RT-PCR, and found that only the cDNA fragment without the deletion was successfully amplified (data not shown). We concluded the variant forms was generated from an abnormal splicing event which targeted the cryptic splicing donor site of mAPS transcript, and

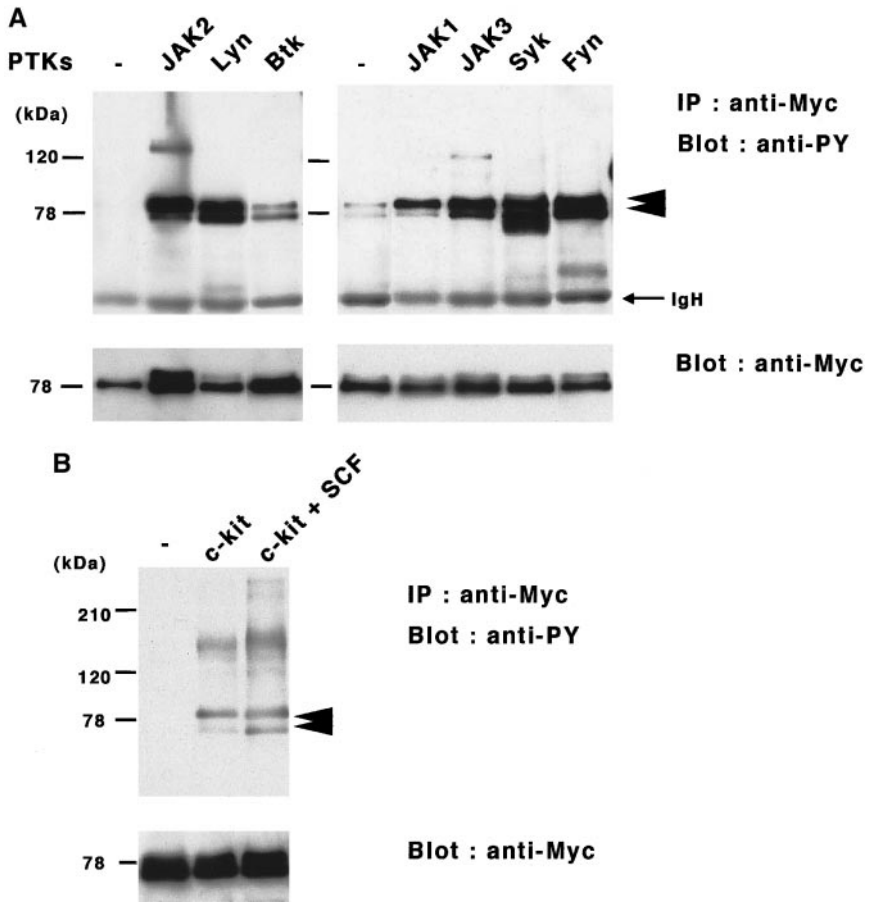

FIG. 5. Tyrosine phosphorylation of mAPS by various protein tyrosine kinases. (A) Myc-tagged mAPS and various protein tyrosine kinases were co-expressed in COS7 cells. APS was immunoprecipitated from cell lysates with anti-Myc antibody and immunoblotted with anti-phosphotyrosine (upper panel) and anti-Myc antibodies (lower panel). Arrowhead indicates phosphorylated Myc-mAPS. Thin arrow indicates the immunoglobulin heavy chain. (B) COS7 cells co-expressing Myc-tagged mAPS and c-kit were stimulated by SCF. APS was immunoprecipitated with anti-Myc antibody proved by anti-phosphotyrosine (upper panel) and anti-Myc antibodies (lower panel). Phosphorylated mAPS was indicated by arrowheads.

that the variant of mAPS protein was made only negligibly *in vivo*. This is also supported by the exon-intron boundaries of APS genes. The genomic sequence of the cryptic splicing site used in the variant cDNA form does not match to the consensus splicing donor sequence (data not shown).

Analysis of APS expression in various cell lines suggested that APS is expressed mainly in B cell lines, especially mature B cell lines. This is consistent with the observation that APS transcripts were accumulated more in spleen than in bone marrow. In contrast, Lnk was expressed in all B-lineage cell lines at various differentiation stages from pro B to mature B cells (S. T. unpublished observations), whereas SH2-B was ubiquitously expressed (data not shown). In addition to the APS expression in B cells, APS was tyrosine-phosphorylated by BCR crosslinking in a mature B cell line, BAL17. A similar result has been observed using Raji, a human B cell line (8). These observations support that APS may play a certain role in BCR signaling. The APS expression is, however, not restricted in B cells. In brain, kidney and other tissues like muscle, APS presumably functions in other signaling pathways. It has been reported that APS become tyrosine phosphorylated by the stimulation of NGF, PDGF, erythropoietin and insulin (9–12, 20).

Although it has been demonstrated that various stimuli induce tyrosine phosphorylation of APS, physiological functions of APS are not well understood. In NGF-Trk signaling pathway, APS works as a substrate of Trk and mediates NGF-induced activation of MAPK and neurite outgrowth (9). On the other hand, APS inhibits PDGF-R-mediated activation of MAPK pathway and mitogenesis, and EPO-R mediated activation of the JAK-STAT pathway (12, 20). APS is tyrosine phosphorylated by stimulation of insulin, but a function in this signaling pathway has not been elucidated (10, 11). Since APS has a structure conserved among Lnk and SH2-B, these three molecules are likely to manifest common features in their functions. SH2-B

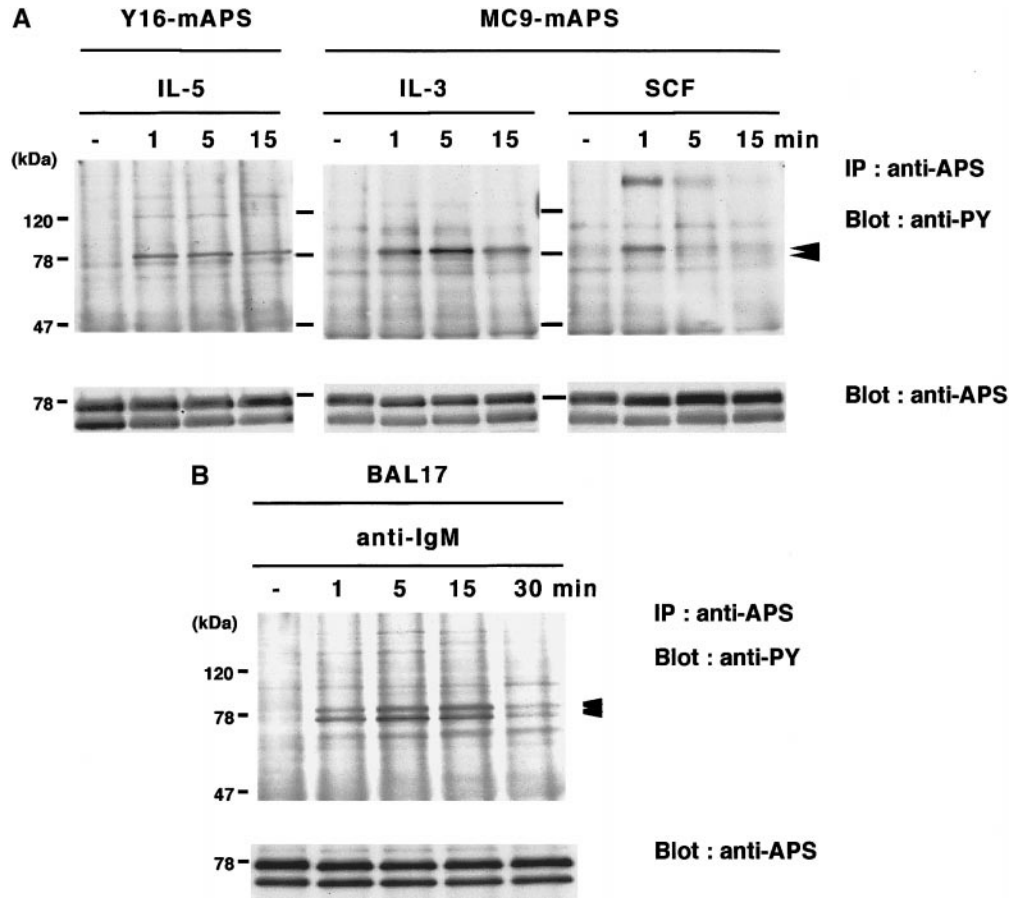

FIG. 6. Induction of mAPS tyrosine phosphorylation by the stimulation of IL-5, IL-3, SCF and BCR crosslinking. (A) Y16 cells overexpressing mAPS were stimulated with IL-5 (left panels), or MC9 transfectants overexpressing mAPS were stimulated with IL-3 or SCF (middle or right panel) for indicated periods. APS was immunoprecipitated and probed with anti-phosphotyrosine (upper panels) or anti-mAPS antibodies (lower panels). Arrowhead indicates phosphorylated mAPS. (B) BAL17 cells were stimulated with anti-IgM antibody for indicated periods. Endogenously expressed mAPS was immunoprecipitated and probed by anti-phosphotyrosine (upper panel) or anti-mAPS antibodies (lower panel). Phosphorylated mAPS was indicated by arrowheads.

was cloned as an association molecule to tyrosine phosphorylated ITAM motif of γ chain of the high-affinity IgE receptor, FcϵRI. In sympathetic neurons, SH2-B is a substrate of Trk and mediates NGF-dependent MAPK activation (9). In PC12 cells, SH2-Bβ mediates NGF-induced neural outgrowth (21). SH2-B is tyrosine phosphorylated by the stimulation of GH, IFNγ (16, 22), IGF-I (23), insulin (24), and PDGF (25), however, its role in those signaling cascades is still obscure. Mice lacking *lnk* gene, showed massive accumulation of B-lineage cells caused by overproduction of B cell precursors, indicating the function of Lnk as a negative regulator of cell proliferation (S. T. in preparation). Generation of *APS*-deficient mice is important to clarify physiological roles of APS *in vivo.*

Deletion of the C-terminal tyrosine phosphorylation site (ΔC mutant) confirmed that the phosphorylation site of APS conserved among the Lnk family adaptor proteins is a one of major targets recognized by various tyrosine kinases. Meanwhile, the association of tyrosine kinases with APS was not abolished by the deletion of the C-terminal phosphorylation site in analogy with the association of SH2-Bβ and various tyrosine kinases (16, 21, 22, 25). It is therefore, likely that the C-terminal phosphorylation site plays an important role in signal transduction, probably serving as a docking site for downstream signaling molecules carrying a SH2 or PTB domains that recognize protein stretch carrying a phosphotyrosine. Yokouchi *et al.* have showed that c-Cbl associates with APS through its C-terminal phosphorylated tyrosine residues, and overexpression of c-Cbl with APS cooperatively suppresses MAPK activation and STAT-mediated transcription (12, 20). Thus the function of the Lnk family adaptor proteins seems to be controlled by their phosphorylation and expression of signaling molecules interacting with the C-terminal tyrosine residues like c-Cbl. However, there may exist a positive regulatory role for the Lnk family adaptor proteins, that is independent of the phosphorylation of the C-terminal phos-

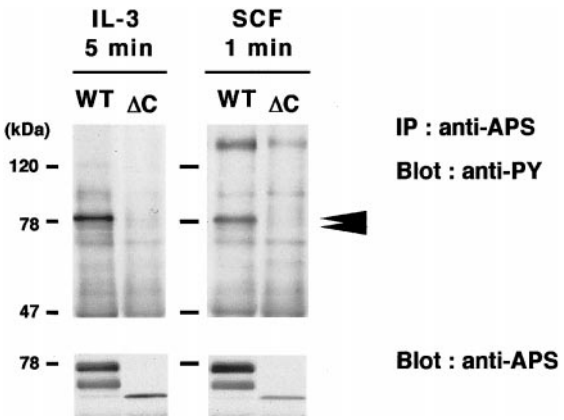

FIG. 7. The C-terminal tyrosine residue is the major phosphorylation site of mAPS. MC9 cells overexpressing the wildtype (WT) or the mutant form (ΔC) lacking the C-terminal portion of mAPS were stimulated with IL-3 for 5 min (left panels) or with SCF for 1 min (right panels). Immunoprecipitated mAPS was blotted with anti-phosphotyrosine (upper panel) or anti-mAPS antibodies (lower panel). Phosphorylated mAPS is indicated by arrowheads.

phorylation motif. SH2-Bβ lacking the conserved tyrosine phosphorylation site is still phosphorylated by GH, IFNγ (16, 22), TrkA (21) and PDGF (25), and is shown to mediate the NGF-dependent neural outgrowth of PC12 cells and JAK2 activation in overexpressed COS cells (21, 22). Our trials to overexpress the wild-type or ΔC forms of APS in IL-5 and IL-3 dependent Y16 cell line or SCF-responsive MC9 cell line were not able to demonstrate measurable changes in cell proliferation in each cell line. Further biochemical experiments including identification of associating proteins with APS will be required to clarify how and where APS functions.

The *APS* gene locates on the chromosome 7, q22 region in human, and presumably on the chromosome 5 in mouse. Structural abnormalities of this region are common in myeloid malignancies, but reported in chronic lymphoid disorders. Deletions or translocations involving 7q22 has been reported in primary and therapy-induced myelodysplastic syndrome, acute myeloid leukemia (26, 27), acute lymphoblastic leukemia (28) and chronic lymphoproliferative disorders (29). In non-lymphoid tissue, deletion of 7q22 was also found in uterine leiomyomas (30). It is an interesting issue to study whether the deletions or translocations involve the *APS* gene or not, since APS is expressed in myeloid cell lines, bone marrow, and muscle. Analysis of *APS* gene in those diseases may also give us an important clue to elucidate the physiological functions of APS.

In conclusion, we have identified mouse APS as a homologous protein with Lnk and SH2-B, and confirmed that these three proteins forms a new adaptor protein family. Although the physiological function of APS remains obscure, our result indicate APS appears to play, at least in part, a role downstream of BCR in B cells.

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