ELSEVIER

EXPERIMENTAL HEMATOLOGY

Experimental Hematology 28 (2000) 1250–1259

Adaptor protein SKAP55R is associated with myeloid differentiation and growth arrest

David J. Curtis[a,d], Stephen M. Jane[b], Douglas J. Hilton[a], Loretta Dougherty[a], David M. Bodine[d], and C. Glenn Begley[a,b,c]

[a]*The Walter and Eliza Hall Institute of Medical Research and The Co-operative Research Centre for Cellular Growth Factors,* [b]*Rotary Bone Marrow Research Laboratory, Royal Melbourne Hospital, Victoria, Australia;* [c]*The Western Australian Institute for Medical Research, Perth, Australia;* [d]*Hematopoiesis Section, Genetics & Molecular Biology Branch, NHGRI, National Institutes of Health, Bethesda, Md., USA*

(Received 4 May 2000; revised 24 July 2000; accepted 25 July 2000)

Objective. **Activation of the SRC family of protein tyrosine kinases is an important component of intracellular signaling in hematopoiesis, but their critical substrates are less well understood. In this report, we describe the cloning and functional characterization of murine SKAP55R (mSKAP55R), an SRC family kinase substrate.**

Materials and Methods. **Expression of mSKAP55R was examined by Northern blot. Phosphorylation of mSKAP55R was examined by transient transfection of COS cells. For overexpression studies, mSKAP55R was cloned into a bicistronic murine stem cell virus–based retrovirus. Transduced cells (FDC-P1 cell line and murine bone marrow) were FACS isolated by expression of the selectable marker green fluorescent protein.**

Results. **mSKAP55R showed 90% amino acid identity to the recently published human SKAP55R. mSKAP55R contained a central pleckstrin homology domain, a C-terminal SH3 domain, and a putative SRC kinase consensus substrate DEIY^{260}. mSKAP55R was expressed in all hematopoietic lineages, with relative mRNA levels greatest in cells of the myeloid and erythroid lineages. Induced myeloid differentiation of M1 and HL-60 cell lines was associated with an eight-fold increase in mSKAP55R mRNA. Transient expression of mSKAP55R in COS cells demonstrated that tyrosine 260 was the predominant site of phosphorylation by FYN kinase. Furthermore, this phosphotyrosine was essential for coimmunoprecipitation of FYN with mSKAP55R. Enforced expression of mSKAP55R inhibited in vitro growth of the myeloid FDC-P1 cell line and primary hematopoietic progenitors. In contrast, a tyrosine 260 mutant mSKAP55R had no effect on in vitro growth.**

Conclusion. **These studies implicate mSKAP55R in the processes of myeloid differentiation and growth arrest.** **© 2000 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

Keywords: **SRC kinase substrate—Signaling—Hematopoiesis—Tyrosine phosphorylation**

Introduction

Cytokine binding to cell surface receptors results in activation of either intrinsic tyrosine kinase function or nonreceptor tyrosine kinases such as the Janus and SRC kinase families [1,2]. In addition to kinase activity, the nonreceptor tyrosine kinases contain conserved protein modules, such as SRC homology (SH2 and SH3) and pleckstrin homology (PH) domains, which mediate critical protein-protein and protein-lipid interactions [3,4].

SRC family kinases (SRC PTK) contain a C-terminal kinase domain in addition to SH2 and SH3 domains. The preferential SRC PTK target sequence and SH2 binding site is D/EXXYXXL/I [5,6]. The SH3 domain binds to proline-rich regions in proteins [7,8]. SRC PTKs are constitutively associated with the cytoplasmic domains of receptors such as FcεRI in mast cells and FcγR in monocytes [9–12]. Upon receptor activation, tyrosine phosphorylation of the cytoplasmic domain of receptors by SRC PTK appears to be a critical step in initiation of the signaling pathway [13].

Gene deletion of the SRC PTK suggests both positive and negative roles for this class of molecules in hematopoiesis. In T cells, several SRC PTKs are essential for nor-

Offprint requests to: David J. Curtis, M.D.,Ph.D., Hematopoiesis Section, Genetics and Molecular Biology Branch, NHGRI, NIH, Bethesda, MD 20892; E-mail: dcurtis@nhgri.nih.gov

PII S0301-472X(00)00537-3

mal T-cell development and response to T-cell receptor activation [14–16]. In contrast, gene deletion of LYN suggests a negative regulatory role in B-cell response to immunoglobulin stimulation [17,18]. LYN also may have a negative proliferative role in myeloid cells because $LYN^{-/-}$ mice develop myeloid hyperplasia [19].

Several immediate downstream targets of SRC PTK following activation of the T-cell receptor have been described. Two of these proteins, SKAP55 and SKAP55-related protein (SKAP55R), were identified using a two-hybrid screen with another FYN binding protein FYB (also known as SLAP130) [20]. These proteins also were identified by immunoprecipitation of human T-cell lysates using a recombinant GST-FYN-SH2 domain [21,22]. SKAP55 and SKAP55R are adaptor proteins containing PH and SH3 domains but no intrinsic kinase activity. Both are tyrosine phosphorylated by FYN and are believed to be involved in T-cell receptor signaling. The precise function of these related proteins is unknown. In this article, we describe the cloning and characterization of the murine homologue of SKAP55R (mSKAP55R).

Materials and methods

Cloning of the cDNA clones encoding mSKAP55R protein

The initial partial cDNA clone encoding mSKAP55R was obtained by polymerase chain reaction (PCR) of genomic DNA from cells that were infected with a retrovirus containing an FDC-P1 cDNA library [23]. An oligonucleotide (5′-TCTCTCCTACCCACCAGCCATATC-3′) derived from the partial cDNA sequence of mSKAP55R was synthesized and used as a probe to screen λZAP cDNA libraries derived from murine testis (Stratagene) as previously described [24]. cDNA cloned into pBluescript were excised and sequenced using an ABI automatic sequencer according to the manufacturer's instructions. The nucleotide and predicted amino acid sequence of mSKAP55R cDNA was compared to DNA databases using the BLASTN and TFASTA algorithms [25–27].

Northern blot analysis

Polyadenylated RNA from mouse tissues and cell lines was extracted and analyzed in Northern blots as described previously [28]. Membranes were hybridized with [α-^{32}P]ATP-labeled 1.5-kbp mSKAP55R cDNA or a 1.2-kbp *Pst* I chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragment. For analysis of gene induction in mouse M1 leukemia cells, the cells were stimulated with interleukin- 6 (IL-6) at a concentration of 100 ng/mL for various times at 37°C.

Construction of vectors

Full-length cDNA clones with 5′ *AscI* and 3′ *MluI* sites were generated by PCR. A tyrosine-to-phenylalanine substitution at position 260 (Y260F) was created by PCR site-directed mutagenesis using the oligonucleotide, 5′-TGATGAGATTTTTGAGGAGC-3′ (sense oligonucleotide is shown with the alteration from wild-type sequence underlined). For transient expression in COS cells, PCR-generated cDNA clones were cloned into a modified pEFBOS mammalian expression vector in frame with a FLAG or MYC epitope at the 5′ or 3′ end of an AscI/MluI cloning site. For cellular localization studies, full-length mSKAP55R cDNA was cloned into pEGFP (Clontech). For expression in FDC-P1 and bone marrow cells, mSKAP55R and tyrosine mutant Y260F were cloned into a murine stem cell virus (MSCV)-based retroviral vector that contained the cDNA encoding the green fluorescent protein (MSCV/GFP) [29]. In this vector, transcription of mSKAP55R is linked by a viral internal ribosomal entry site (ir) to the green fluorescent protein (GFP). All constructs were sequenced using an ABI automatic sequencer according to the manufacturer's instructions.

Cells

COS, 293T, and HL-60 cells were grown in RPMI containing 10% newborn calf serum (NCS) at 37°C in a 5% CO_2 humidified atmosphere. The factor-dependent FDC-P1 cell line was maintained in suspension culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% NCS and 10% WEHI-3BD$^-$ conditioned medium. The ecotropic GPE86 producer cell lines (MGirL22Y, mSKAP55RirGFP, and Y260FirGFP) were maintained in DMEM supplemented with 10% NCS. Bone marrow cells from 5-fluorouracil (5-FU)-treated mice were maintained in DMEM supplemented with 15% fetal bovine serum and a cytokine cocktail of recombinant murine IL-3 (10 ng/mL), murine IL-6 (100 ng/mL), and rat stem cell factor (SCF; 100 ng/mL).

Transient cell transfections

For immunoprecipitation studies, an 80% confluent 175-cm^2 tissue culture flask of COS cells was used for each transfection. After harvesting, cells were resuspended in 800-μL mouse tonicity phosphate-buffered saline with 5 μg of vector DNA in a 0.4-cm gap cuvette. The cuvette was put on ice for 10 minutes and then electroporated using 0.3 kV and a capacitance of 500 μF. Cells were layered over 1 mL of NCS, spun at 1,500 rpm, and the pellet resuspended and transferred to a new 175-cm^2 flask. For cellular localization, HeLa and NIH 3T3 cells were transfected with 10 μg DNA using lipofectamine (Clontech), and cells were visualized 48 hours later by fluorescence microscopy.

Immunoprecipitation, Western blot analysis, and in vitro kinase assay

Forty-eight hours after COS cell transfections, cells were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM TRIS-HCl, pH 7.4, 1 mM EDTA, 1 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulphonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL leupeptin) at 4°C for 20 minutes. When necessary, lysates were incubated with anti-FLAG affinity gel (Eastman Kodak, New Haven, CT) or the anti-MYC monoclonal antibody 9E10 covalently coupled to Affi-Gel 10 (Bio-Rad Laboratories, Hercules, CA) at 4°C for 3 hours. The antibody-protein complexes were washed twice with lysis buffer. Complexes were either eluted by incubation for 1 hour at 4°C with 100 μg/mL FLAG peptide or boiled for 5 minutes in reducing sample buffer (30% glycerol, 7% SDS, 0.125 M TRIS-HCl, 5% 2-mercaptoethanol, and 0.01% bromophenol blue) and then resolved on 10% SDS-PAGE (Bio-Rad Laboratories), transferred to Immobilin polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), and immunoblotted with anti-FLAG antibody, anti-MYC antibody, or horseradish peroxidase-conjugated anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY) as previously described [30].

For in vitro kinase assay, immunoprecipitates were washed twice with wash buffer (50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$ and 10 mM HEPES, pH 7.4) and the protein-antibody complex incubated at room temperature for 30 minutes with 20 μL of wash containing 0.5 μCi [γ-^{32}P]ATP per sample. Unbound [γ-^{32}P]ATP was removed, the pellet heated at 95°C for 5 minutes in sample buffer, and resolved on 10% SDS-PAGE. The gel was fixed in 50% methanol, 7% acetic acid for 30 minutes at room temperature, then at 55°C in 1 M KOH for 2 hours, and finally in 50% methanol, 7% acetic acid for another 30 minutes at room temperature. The gel was dried and analyzed using autoradiography.

Expression of mSKAP55R in FDC-P1 cells

Viral supernatants were derived by $CaPO_4$ cotransfection of 293T cells with the MSCV vectors and a helper plasmid containing the required gag, pol, and env retroviral genes driven by a Moloney Leukemia Virus LTR [28]. Media was harvested and frozen at 24 and 48 hours. Transfection efficiency was assessed at 48 hours by fluorescence detection (530 nm) of a single suspension of 293T cells using a FACScan (Becton Dickinson, San Jose, CA. Viral supernatants where transfection efficiency was >50% were used. FDC-P1 and M1 cells (2×10^4/mL) were cultured with fresh viral supernatant and 6 μg/mL polybrene every 12 hours for 3 days. Cells were grown in culture for another 24–48 hours prior to cell sorting.

Expression of mSKAP55R in bone marrow cells

The retroviral producer cell lines mSKAP55RirGFP and Y260irGFP were made by $CaPO_4$ transfection of GPE86 cells with the mSKAP55RirGFP or Y260F vectors, respectively. GFP-positive cells were isolated by FACS after 72 hours. Clones expressing mSKAP55R mRNA were isolated and expanded. Southern blot analysis of DNA from transduced NIH 3T3 cells confirmed an intact retrovirus. Titers of the producer mSKAP55RirGFP and Y260FirGFP as well as the control MGirL22Y producer were estimated by analysis of GFP expression of transduced NIH 3T3 cells. Bone marrow cells were harvested from mice 2–3 days after subcutaneous injection of 5-FU (150 mg/mL). Cells were cultured for 48 hours and then cocultured with the retroviral producers in the presence of polybrene (6 μg/mL) for 48 hours. Nonadherent cells were harvested. Cells were grown in culture for another 24–48 hours prior to cell sorting.

FACS

GFP-positive and GFP-negative cells were collected by sterile sorting performed on a dual laser FACStar^{Plus} (Becton Dickinson). Negative controls were uninfected cells. Nonviable cells were excluded by forward scatter and staining with propidium iodide (PI).

Agar cultures

Agar cultures of FDC-P1 and bone marrow cells were performed as previously described [30]. After 7 days of incubation, colonies were scored at 35× magnification using a dissection microscope. For bone marrow cultures, plates were fixed and stained as previously described [31].

Results

Cloning of the murine homologue of SKAP55R

FDC-P1 is a factor-dependent, nontumorigenic myeloid cell line [32]. FDC-P1 cells were transduced with an FDC-P1–derived retroviral cDNA library in an effort to identify genes that conferred a tumorigenic phenotype [23]. Pools of transduced FDC-P1 cells (10^7 cells) were injected intravenously into adult DBA/2 mice. Four of 16 mice became moribund after 24 days due to transformed FDC-P1 cells. PCR analysis of genomic DNA from the spleen of one moribund mouse recovered a cDNA encoding a previously unreported SH3 domain (Fig. 1). However, overexpression of this partial cDNA in parental FDC-P1 cells did not confer a tumorigenic phenotype (data not shown). This suggested the transforming event in the original transduced-FDC-P1 cells was most likely due to insertional mutagenesis. Given the hematopoietic expression of the cDNA (Fig. 2), we decided to characterize the full-length cDNA.

In light of the high level of expression in the testis (Fig. 2), a mouse testis cDNA library was screened with a 21-bp oligonucleotide deduced from the incomplete cDNA clone. Four positive clones were identified, of which the longest clone was 1,546 bp. This clone contained a putative initiation methionine at nucleotide 96, preceded by in-frame stop codons. This methionine was followed by an open reading frame predicted to encode a 358 amino acid polypeptide. There was no evidence of alternatively spliced products. The deduced amino acid sequence contained a central PH domain (residues 115-214) and a C-terminal SH3 domain

```
                10        20        30        40        50
mouse   MPNPSCTSSPGPLPEEIRNLLADVETFVADTLKGENLSKKAKEKRESLIK
human   :::::S::::Y:::::::::::::::::::I::::::::::::::::::

                60        70        80        90       100
mouse   KIKDVKSVYLQEFQDKGDAEDGDEYDDPFAGPADTISLASERYDKDDDGP
human   :::::::I::::::::::::::E:::::::::P::::::::::::::EA:

               110       120       130       140       150
mouse   SDGNQFPPIAAQDLPFVIKAGYLEKRRKDHSFLGFEWQKRWCALSKTVFY
human   :::A::::::::::::L::::::::::::::::::::::::::::::::

               160       170       180       190       200
mouse   YYGSDKDKQQKGEFAIDGYDVRMNNTLRKDGKKDCCFEICAPDKRIYQFT
human   ::::::::::::::::::S::::::::::::::::::S::::::::::

               210       220       230       240
mouse   AASPKDAEEWVQQLKFILQDLGSDVIPEDDEERGELYDDVDHPAAVSSP-
human   :::::::::::::::::V:::ME::I::::YD::::::::::::LPI:N:L

      250       260       270       280       290
mouse   QRSQPIDDEIYEELPEEEEDTASVKMDEQGKGSRDSVHHTSGDKSTDYAN
human   TS::::::::::::::::::S:P::VE::R:M:Q:::::::::::::::

      300       310       320       330       340
mouse   FYQGLWDCTGALSDELSFKRGDVIYILSKEYNRYGWWVGEMKGAIGLVPK
human   ::::::::::::F::::::::::::::::::::::::::::::::::::

      350
mouse   AYLMEMYDI
human   ::I::::::
```

Figure 1. Comparison of mouse and human amino acid sequence of SKAP55R cDNA. Alignment was performed using the Multiple Sequence Alignment program of DNASTAR. Identical amino acids are indicated by dots. The amino acid sequence contains a pleckstrin homology domain (underlined), an SH3 domain (boxed), and a putative src kinase tyrosine phosphorylation site (bold).

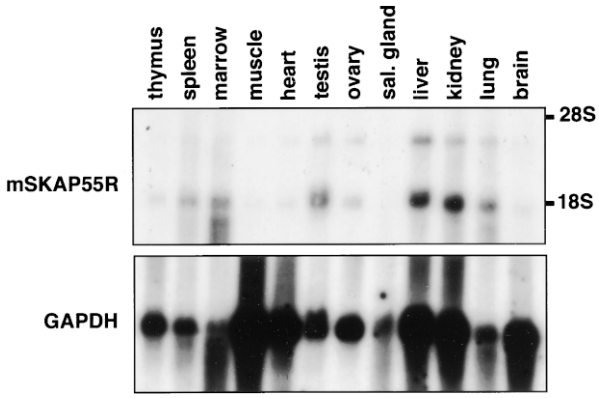

Figure 2. Tissue expression of mSKAP55R. Northern blot of 5 μg poly(A)$^+$ mRNA from murine tissues was probed with radiolabeled full-length mSKAP55R cDNA. Blots were stripped and reprobed with GAPDH. The location of the 28S and 18S ribosomal bands are shown on the right.

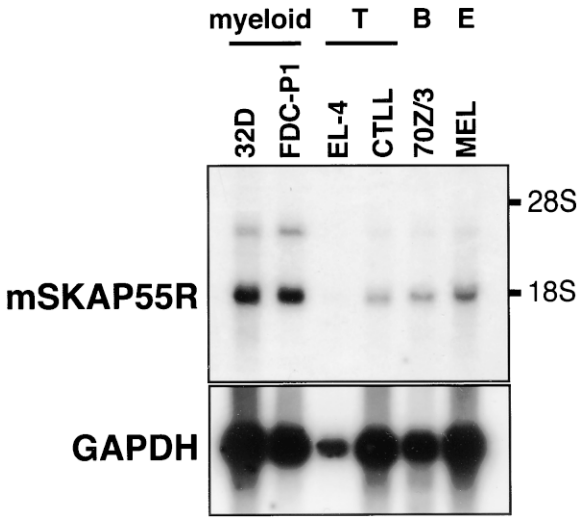

Figure 3. Hematopoietic expression of mSKAP55R. Northern blots of 5 μg poly(A)$^+$ mRNA from murine hematopoietic cell lines. B = B cell; E = erythroid lineage; T = T cell. Blots were probed with radiolabeled full-length mSKAP55R cDNA.

(residues 302-358) identical to the original partial cDNA clone obtained from the FDC-P1 library. In addition, there was a putative src kinase motif DEIY^{260}EEL. The predicted protein sequence was 90% identical to the recently published human SKAP55R (hSKAP55R) sequence, also called SKAP-HOM or RA70 (Fig. 1) [20,21,33]. During preparation of this manuscript, an identical cDNA sequence was deposited in the database: murine RA70 (GenBank Accession AB014485) and SAPS (GenBank Accession AF051324). Based on the amino acid identity, our cDNA is most likely the murine homologue of hSKAP55R. The PH and SH3 domains are highly conserved between species: 97% and 96% respectively. In addition, the tyrosine at position 260 is conserved.

Tissue expression of mSKAP55R

Northern blot analyses were performed with poly(A$^+$) RNA isolated from different murine tissues (Fig. 2). A predominant 1.7-kbp transcript was observed similar in size to the cDNA clones isolated. A less abundant transcript of 3.7 kbp also was evident. Expression was most abundant in bone marrow, testis, liver, kidney, and lung, with lower levels of expression observed in spleen. The mSKAP55R mRNA was barely detectable in other tissues including thymus. In contrast, hSKAP55R is expressed at higher relative levels in muscle, heart, and brain [22,33].

Analysis of mSKAP55R mRNA in hematopoietic cell lines showed most abundant expression in the myeloid cell line 32D and consistent with the initial source of the cDNA clone FDC-P1 (Fig. 3). Expression in the erythroid MEL cell line was two-fold lower than myeloid lines, whereas B- and T-cell lines expressed five- to ten-fold lower levels of mSKAP55R. These results are consistent with the relative levels of mRNA found in bone marrow, spleen, and thymus.

hSKAP55R was independently identified as a retinoic acid (RA)-inducible gene expressed during neuronal differentiation of P19 EC cells [33]. mRNA levels also were shown to increase following RA-induced differentiation of the myeloid cell line U937 [33]. Therefore, we examined mSKAP55R mRNA expression in myeloid cell lines in response to cytokine-induced differentiation. The myeloid M1 leukemic cell line can be induced to differentiate into cells with a macrophage phenotype in response to IL-6 [34,35]. Levels of mSKAP55R mRNA became elevated after only 1-hour exposure of M1 cells to IL-6 and reached a maximum at 4 hours (approximately eight-fold greater than basal levels as estimated by densitometry) (Fig. 4A). This result suggests that mSKAP55R plays an early role in macrophage differentiation well before morphologic changes have occurred. mRNA levels subsequently fell to two-fold above basal levels and remained at this level for up to 4 days following exposure to IL-6 (Fig. 4A).

To further examine SKAP55R during myeloid differentiation, we treated the human myeloid cell line HL-60 with various differentiating agents (Fig. 4B). Northern blots probed for hSKAP55R cDNA showed a single 4-kbp transcript. Similar to the observations in RA-treated U937 cells, HL-60 cells induced to differentiate into granulocytes with RA expressed five-fold higher levels of hSKAP55R than untreated cells. Increased expression of hSKAP55R mRNA also was seen following PMA-induced macrophage differentiation of HL-60 cells. Interestingly, no significant increase of hSKAP55R was seen following exposure to dimethylsulfoxide or interferon-γ.

Cellular localization of mSKAP55R

To examine the cellular localization of mSKAP55R, we utilized transient transfection of HeLa or NIH 3T3 cells with an mSKAP55R/EGFP fusion construct (Fig. 5). Nuclei

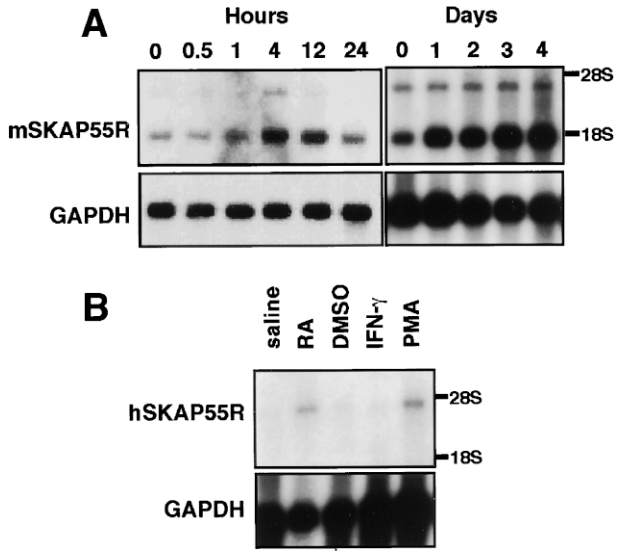

Figure 4. SKAP55R is associated with myeloid differentiation. Northern blots of 5 μg poly(A)$^+$ mRNA from **(A)** murine myeloid leukemia M1 cells grown in the presence of IL-6 for various times. **(B)** Human promyelocytic HL-60 cells grown in the presence of retinoic acid (RA), dimethyl sulfoxide (DMSO), interferon-γ (IFN-γ), or phorbol 12-myristate 13-acetate (PMA). Blots were probed with radiolabeled full-length mSKAP55R cDNA.

were stained with DAPI and cells visualized by fluorescence microscopy. The hSKAP55 protein has been shown to be localized to the cytoplasm [20]. Similarly, in contrast to the control EGFP construct, mSKAP55R was localized to the cytoplasm. Perinuclear deposits occasionally were seen in transfected HeLa but not NIH-3T3 cells. Perinuclear staining also was described in studies of hSKAP55 [20].

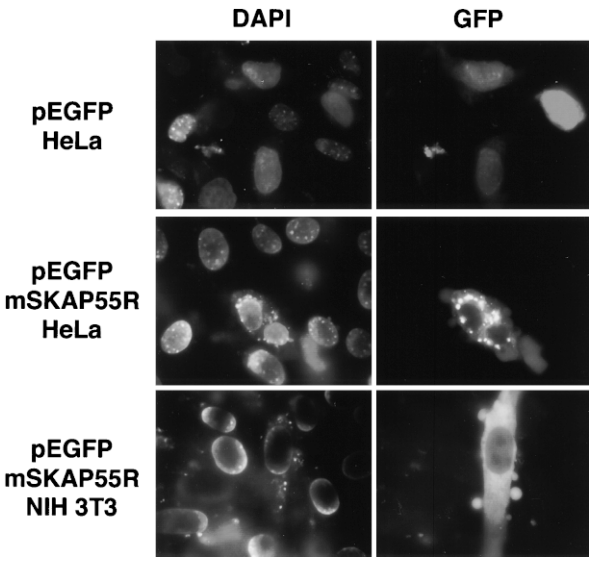

Figure 5. mSKAP55R is a cytoplasmic protein. HeLa or NIH 3T3 cells were transfected transiently with either control plasmid (pEGFP) or mSKAP55R plasmid (pEFGPmSKAP55R). Cells were stained with the nuclear stain DAPI and then visualized with a fluorescence microscope.

However, it is possible that these deposits were an "artifact" of high levels of mSKAP55R protein in HeLa cells rather than a true reflection of perinuclear localization.

Phosphorylation of tyrosine 260 is required for binding to the SRC kinase FYN

hSKAP55R was purified previously from T-cell lysates by binding to recombinant GST-FYN-SH2 fusion protein. Subsequently, hSKAP55R was shown to be phosphorylated by the src kinase FYN but not LCK or ZAP70 [20,22]. The site of phosphorylation was postulated to be the consensus site DEIY^{260}. To examine this possibility, C-terminal FLAG-tagged mSKAP55R or a tyrosine-to-phenylalanine mSKAP55R (Y260F) mutant was expressed in COS cells either alone or with a myc-tagged FYN (Fig. 6). Expression of mSKAP55R and FYN was similar in all transfectants (Figure 6A,B). Mouse immunoglobulin heavy chain (the band of similar molecular size to mSKAP55R seen in COS cells transfected with FYN alone; Fig. 6A, middle lane) was detected because the immunoprecipitaions were performed with covalently coupled antibody. The kinase activity of FYN was confirmed by the presence of phosphoprotein in the anti-myc immunoprecipitates (Fig. 6C). Coimmunoprecipitation of FYN with wild-type mSKAP55R but not Y260F mutant was confirmed by probing anti-FLAG immunoprecipitates with anti-myc antibody (Fig. 6D). Binding of FYN to mSKAP55R is not unique, because cotransfection of mSKAP55R with two other SRC kinases, LYN and HCK, revealed a similar ability of these SRC kinases to bind to mSKAP55R (data not shown). Probing of anti-FLAG immunoprecipitates with anti-phosphotyrosine antibody demonstrated that wild-type mSKAP55R was phosphorylated in the presence of FYN (Fig. 6E, solid arrowhead). Furthermore, a slower migrating phosphoprotein, presumably FYN, also was detected (Fig. 6E, open arrowhead). In contrast, the Y260F mutant mSKAP55R was not phosphorylated by FYN, and the slower migrating band was not seen. Together, these results suggest that Y260 is the major site of mSKAP55R phosphorylation by FYN and this phosphotyrosine is essential for FYN binding to mSKAP55R.

In vitro kinase assays were performed to examine the phosphorylation of mSKAP55R by FYN. Gels were treated for 2 hours in 1M KOH to reduce the detection of phosphorylated serine or threonine residues. In vitro kinase assay of anti-FLAG immunoprecipitates confirmed that wild-type but not Y260F mutant mSKAP55R was phosphorylated by FYN (Fig. 6F). In addition, a lower level of phosphorylation was observed when wild-type mSKAP55R was expressed in COS cells in the absence of exogenous FYN. Because mSKAP55R does not contain a recognizable kinase domain, it seemed likely that the constitutive phosphorylation of tyrosine 260 was due to the presence of an endogenous kinase expressed in serum-stimulated COS cells. In vitro kinase assay of anti-myc immunoprecipitates demonstrated autophosphorylation of FYN (Fig. 6G, upper bands). In addi-

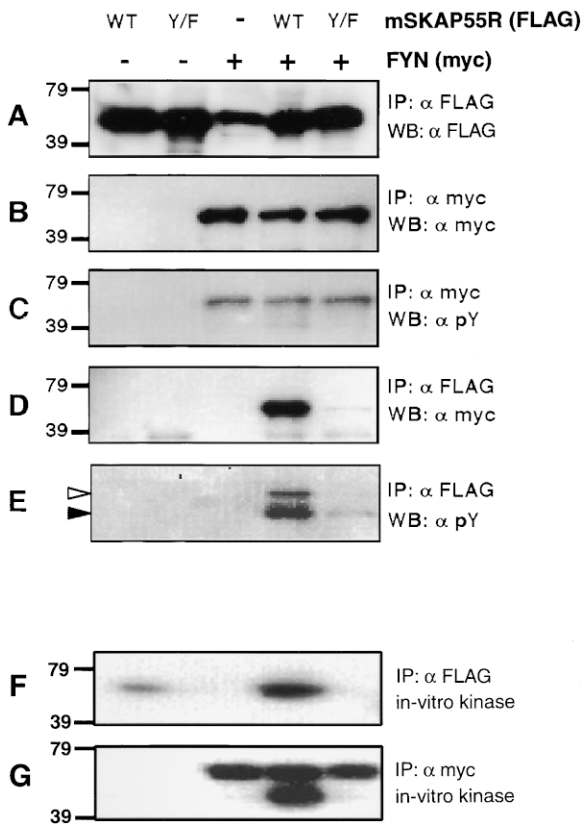

Figure 6. FYN phosphorylates tyrosine 260 of mSKAP55R. COS cells were transfected with FLAG-tagged wild-type (WT) or mutant Y260F (Y/F) mSKAP55R pEF-BOS constructs either alone or cotransfected with a myc-tagged FYN pEF-BOS. Cell lysates were immunoprecipitated with anti-FLAG or anti-myc antibody as indicated. Immunoprecipitates were resolved on 10% SDS-PAGE and Western blots **(A–E)** probed with anti-FLAG, anti-myc, or anti-phosphotyrosine antibody as indicated. The FYN (open arrowhead) and mSKAP55R (solid arrowhead) are indicated in **(E)**. The nonspecific immuoreactive band present in the FYN alone transfection in **(A)** is due to the immunoglobulin heavy chain. In vitro kinase assay **(F,G)** was performed on immunoprecipitates, and radiolabeled proteins were visualized by autoradiography of the dried gel. The position of MW markers is shown on the left.

tion, a lower band was observed in cells transfected with wild-type but not Y260F mutant mSKAP55R (Fig. 6G, lower band). This band presumably represented coimmunoprecipitation of phosphorylated wild-type mSKAP55R. Thus, in vitro kinase assays confirmed the Western blot analyses that binding of mSKAP55R to FYN required phosphorylation of mSKAP55R at tyrosine 260.

mSKAP55R inhibits growth of FDC-P1 cells

The function of SKAP55R is unknown. To examine the function of mSKAP55R in hematopoietic cells, we attempted to establish stable mSKAP55R expressing FDC-P1 cells by coelectroporation of pEFBOS/mSKAP55R/FLAG and pgkPuro. Although multiple puromycin-resistant clones were established, none expressed mSKAP55R as assessed by Western blot. This raised the possibility that enforced expression of mSKAP55R was deleterious to cell proliferation. To examine the effects of mSKAP55R expression on cell growth, FLAG-tagged mSKAP55R was cloned into the MSCV/GFP retrovirus LTR (mSKAP55RirGFP, Fig. 7A) [29]. Viral supernatants from packaging cells transfected with control irGFP or mSKAP55RirGFP vectors were used to transduce FDC-P1 cells. After 3 days, GFP-positive cells were isolated using FACScan^{PLUS} and then grown in liquid culture for 5 days. Western blot analysis of cell lysates confirmed expression of mSKAP55R (Fig. 7B). Cells then were reanalyzed for GFP expression. Consistent with the hypothesis that mSKAP55R was detrimental to cell growth, only 14% of mSKAP55RirGFP-transduced cells expressed GFP at levels of the original sorted cells (Fig. 7C). In contrast, 75% of control irGFP-transduced cells expressed GFP at levels of the original sorted cells. Given the polyclonality of the sorted cell populations, loss of GFP expression may have been due to outgrowth of contaminating, nontransduced cells or silencing of the viral promoter. To distinguish between these two possibilities, experiments were performed at the clonal level (Fig. 7D). Sorted irGFP^{high} or mSKAP55RirGFP^{high} cells were placed in agar culture. Independent clonal cell lines were established by removing developing colonies from agar and expanding these cells in liquid culture for 5 days. In six independent clones of mSKAP55RirGFP^{high} cells, GFP expression was heterogeneous with a significant proportion of cells that expressed low levels of GFP. In contrast, control irGFP^{high} clonal cell lines derived in the same manner could be maintained in liquid culture for at least 4 weeks without the emergence of low-expressing GFP cells (data not shown). Therefore, these clonal studies suggested that gene silencing rather than outgrowth of a low-expressing clone was the mechanism of loss of GFP expression in the mSKAP55RirGFP^{high} cells.

To examine more closely the growth characteristics of FDC-P1 cells expressing high levels of mSKAP55R, we sorted mSKAP55RirGFP-transduced cells to obtain populations of cells expressing high and low levels of mSKAP55RirGFP (mSKAP55RirGFP^{high} and mSKAP55RirGFP^{low} respectively) (Fig. 8A). The colony-forming ability of these sorted cells as well as unsorted control irGFP and parental FDC-P1 cells were examined in clonal cultures. Consistent with the relative loss of GFP expression after 5 days in liquid culture, individual mSKAP55RirGFP^{high} cells formed 50%–80% fewer colonies in agar than sorted mSKAP55RirGFP^{low} cells (Fig. 8B). The process of cell sorting did not affect colony formation because sorted mSKAP55RirGFP^{low} cells displayed a similar frequency of clonogenic cells compared with unsorted parental FDC-P1 cells. In addition, expression of GFP alone could not explain the reduction in clonogenic cells because control irGFP cells grew as well as parental cells. Despite the reduction in colony numbers, colony size did not significantly differ between mSKAP55RirGFP^{high} and control cells (data

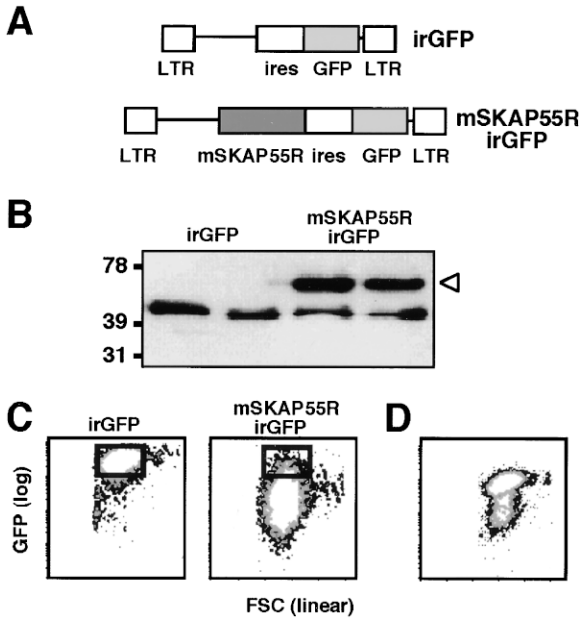

Figure 7. Enforced expression of mSKAP55R in FDC-P1 cells by transduction with a bicistronic retrovirus. **(A)** Schematic of the MSCV-based irGFP or mSKAP55RirGFP retroviral vectors. **(B)** Western blot of cell lysates from transduced FDC-P1 cells grown in culture for 5 days after selection of GFP-positive cells by FACS. Lysates were immunoprecipitated and immunoblotted with anti-FLAG antibody. The lower band in all lanes represents mouse immunoglobulin. **(C)** GFP expression of FDC-P1 cells transduced with control irGFP or mSKAP55RirGFP retrovirus. The gating used for isolation of mSKAP55RirGFP^{high} cells is shown boxed. **(D)** GFP expression of mSKAP55RirGFP^{high} cells after clonal growth of cells isolated from **(C)**. Note the heterogeneity of GFP expression despite the clonal origin.

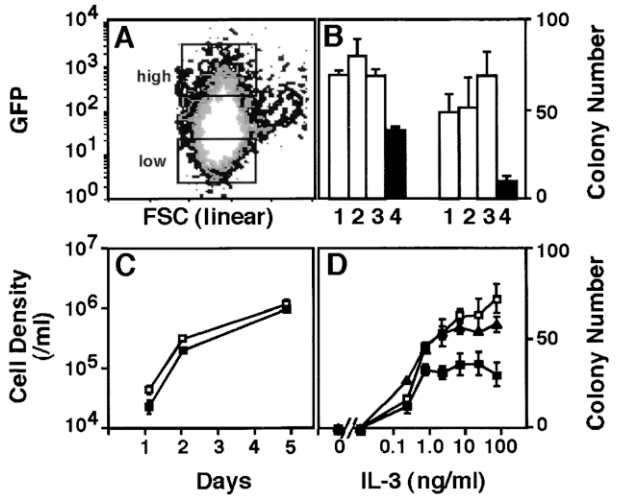

Figure 8. Enforced expression of mSKAP55R reduces colony formation of FDC-P1 cells. **(A)** Cells transduced with SKAP55RirGFP retrovirus were sorted by FACS into high0- and low-expressing populations on the basis of GFP expression. **(B)** FDC-P1 cells: parental (1), control MgirL22Y (irGFP) transduced (2), sorted mSKAP55RirGFP^{low} (3), or sorted mSKAP55RirGFP^{high} (4) were cultured in agar with IL-3 (100 ng/mL). Colonies were counted after 7 days. Mean and standard deviation of quadruplicate assays for two independent experiments are shown. Note the reduction in colonies formed by sorted mSKAP55RirGFP^{high} cells. **(C)** Sorted control irGFP^{high} (open squares) or mSKAP55RirGFP^{high} (closed squares) FDC-P1 cells were grown in liquid culture in the presence of IL-3. Cell counts of duplicate cultures were performed at 1, 2, and 5 days. **(D)** IL-3 dose response of sorted control irGFP^{high} (open squares), mSKAP55RirGFP^{high} (closed squares), or Y260FirGFP^{high} (closed triangles) FDC-P1 cells cultured in agar.

not shown). Furthermore, the growth curves of sorted cells in liquid culture were not significantly different (Fig. 8C). The rates of cell death in liquid culture with or without IL-3 also were not significantly different (data not shown). Cell cycle analysis revealed no consistent abnormality to explain the reduced colony growth of mSKAP55RirGFP^{high} cells (data not shown).

We then examined the effect of overexpression of the tyrosine260 mSKAP55R mutant Y260FirGFP on growth of FDC-P1 cells (Fig. 8D). In contrast to wild-type mSKAP55R, there was no effect on growth of FDC-P1 cells expressing high levels of Y260F. Thus, it was likely that the growth inhibitory effect of mSKAP55R required phosphorylation of tyrosine 260. The reduction in colonies by overexpression of mSKAP55R was not due to altered responsiveness to IL-3 because the IL-3 dose response of control, mSKAP55RirGFP^{high} cells, and Y260FirGFP^{high} were the same (Fig. 8D).

We also examined the effects of enforced expression of mSKAP55R on M1 cells. In light of the increased expression of mSKAP55R following exposure of M1 cells to IL-6 (Fig. 4), we were surprised to find no effect of enforced mSKAP55R expression on M1 cells (data not shown). In particular, sorted mSKAP55RirGFP^{high} M1 cells formed the same number of colonies in agar as controls. In addition, we could find no abnormality in the differentiation of mSKAP55RirGFP^{high} M1 cells in the presence of IL-6.

mSKAP55R inhibits growth of primary hematopoietic progenitors

The growth inhibitory effects on FDC-P1 but not M1 cells raised the possibility this may not be relevant to normal myeloid cell growth and differentiation. Therefore, we examined the effects of mSKAP55R enforced overexpression on the growth of primary bone marrow cells. Retroviral producer cell lines for mSKAP55RirGFP and Y260FirGFP were established using the MSCV-based retroviral construct shown in Figure 7A. A producer cell line for the control GFP vector (MGirL22Y) was kindly provided by A.W. Nienhuis. This control MSCV-based retroviral vector contains the GFP cDNA in the 5′ translational position. The viral titer of conditioned media from the control producer cell line was four-fold greater than the titers of mSKAP55RirGFP or Y260FirGFP producer cell lines estimated by the transfer of the GFP marker to NIH 3T3 cells.

Bone marrow harvested from 5-FU–treated mice was cocultivated with control MGirL22Y, mSKAP55RirGFP, or

Y260FirGFP producer cell lines. After 72 hours, cells were sorted on the basis of GFP expression (Fig. 9A). The percentage of transduced cells and the level of GFP expression were always greater using the control MGirL22Y producer (Fig. 9A). These observations can be explained in part by a higher titer and the 5′ translational position of the GFP cDNA. Sorted cells then were cultured in agar in the presence of IL-3, SCF, and erythropoietin, or macrophage colony-stimulating factor (M-CSF) alone. Consistent with the FDC-P1 data, growth of primary bone marrow colony-forming cells was inhibited by enforced expression of mSKAP55R but not the tyrosine mutant (Fig. 9B). In control cultures, transduced (GFP-positive) cells formed at least as many colonies as nontransduced (GFP-negative) cells. In some experiments, irGFP-transduced cells formed twice as many colonies as nontransduced, presumably reflecting the quiescent cell cycle status of the noninfected cells. In contrast, mSKAP55RirGFP-transduced cells formed approximately half the number of colonies compared with nontransduced cells (n = 4 experiments). The growth inhibitory effect required phosphorylation of tyrosine 260 because Y260FirGFP-transduced cells formed similar numbers of colonies compared with nontransduced cells.

Overexpression of mSKAP55R appeared to affect the growth of all colony types. The relative numbers of granulocytic, macrophage, or granulocyte/macrophage colonies formed by mSKAP55RirGFP-transduced cells was not significantly different than those formed by control cultures (data not shown). Furthermore, the effect of mSKAP55R was observed with cultures stimulated with M-CSF alone (Fig. 9C). Overall, these findings support the cell line data that overexpression of mSKAP55R inhibits myeloid cell growth by a phosphotyrosine-dependent mechanism.

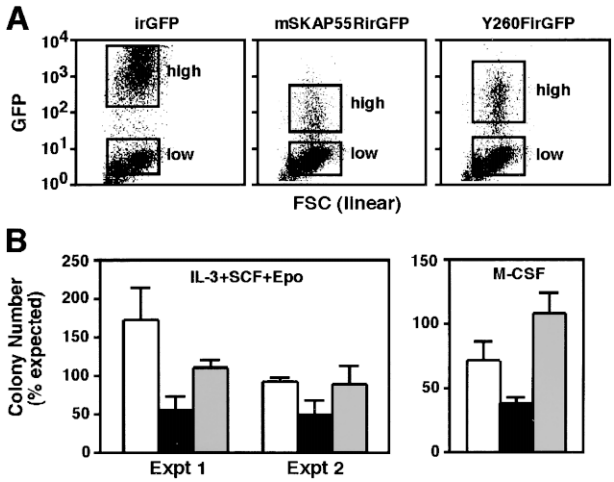

Figure 9. Enforced expression of mSKAP55R inhibits growth of primary hematopoietic progenitors. **(A)** GFP expression and sort gates used for isolation of bone marrow cells transduced with the control MgirL22Y (irGFP), mSKAP55RirGFP, or Y260FirGFP retrovirus. **(B)** Sorted GFP^{high} or GFP^{low} bone marrow cells transduced by the three different retrovirus were cultured in agar in the presence of IL-3, SCF, and erythropoietin (Epo), or M-CSF alone. Colonies were counted after 7 days. Colony number (percent expected) is expressed as the number of colonies formed by GFP^{high} cells divided by the number of colonies formed by GFP^{low} cells: control irGFP (open bars), mSKAP55RirGFP (black bars), and Y260FirGFP (gray bars). Mean and standard deviation of triplicate assays is shown.

Discussion

In this report, we describe the cloning of a murine homologue of hSKAP55R. We show that hematopoietic mRNA expression of mSKAP55R is predominant in myeloid and erythroid cells, whereas expression in T cells, the cells previously identified expressing hSKAP55R, is at least 10-fold lower. mSKAP55R mRNA rapidly increased during myeloid differentiation induced by cytokines and chemicals. Mutation of tyrosine 260, the putative site of phosphorylation by FYN, demonstrated that this tyrosine mediated the binding of FYN to mSKAP55R, presumably through the FYN SH2 domain. Finally, overexpression studies suggested that SKAP55R may play a role in growth arrest of myeloid cells, a cellular process intrinsically linked with differentiation.

Expression of hSKAP55R was reported to be more ubiquitous than demonstrated here for its murine homologue [22,33]. Compared to expression in bone marrow, mSKAP55R mRNA was barely detectable in thymus, muscle, heart, and brain. Given the different pattern of expression, it is possible that we have cloned a highly related homologue rather than the murine homologue of hSKAP55R. However, the high degree of amino acid identity in the functional domains (PH 97% and SH3 96%) and the conserved tyrosine at position 260 makes this possibility less likely. At this stage, we cannot explain the different expression patterns of human and mouse SKAP55R. It is of potential interest that human but not mouse SKAP55R contains a second classic src kinase family consensus motif at position 230.

The widespread hematopoietic expression, especially in myeloid and erythroid lineages demonstrated here, suggests that SKAP55R will be in many different receptor complexes. Candidate myeloid receptors would include the gp130-family of cytokine receptors in light of the rapid upregulation of mRNA expression of mSKAP55R following stimulation of M1 leukemic cells with IL-6. In addition, it is seems likely that SKAP55R will be a substrate for SRC PTK expressed in myeloid cells, such as LYN, HCK, and FGR [36]. hSKAP55R recently was reported to be associated with the immunoreceptor SHPS-1 in complex with a non-SRC PTK [37].

Here, we provide the first direct evidence that SKAP55R inhibited growth of myeloid cells. Overexpression studies using the EF-1α promoter did not yield any stable FDC-P1 transfectants expressing mSKAP55R. It is possible that clones expressing high levels of mSKAP55R were over-

grown by nonexpressing clones during puromycin selection. To study the effects of overexpression, we utilized the MSCV-based retroviral vector, thus allowing selection of transduced cells on the basis of GFP expression. This vector system has been used successfully to demonstrate the inhibitory effects on hematopoiesis of the GATA-2 transcription factor [38]. Cell sorting on the basis of GFP expression allows separation of transduced and nontransduced cells. mSKAP55R expression inhibited the growth of FDC-P1 cells and bone marrow colony-forming cells. One possible explanation is that there was a threshold level of SKAP55R above which cell growth was inhibited completely, whereas cells expressing lower levels of SKAP55R had normal growth characteristics. This hypothesis is consistent with three observations. First, parental FDC-P1 cells expressed readily detectable levels of endogenous SKAP55R mRNA. We cannot quantitative the level of SKAP55R protein as a result of enforced expression in transduced cells because we do not have an antibody that recognizes mSKAP55R. Second, using the retrovirus vector system, we were able to obtain stable cell lines expressing wild-type mSKAP55R. However, the level of expression (as assessed by GFP or FLAG Western blot) was at least a log greater with the mutant compared with wild-type mSKAP55R (Fig. 9 and data not shown). Finally, the size of the colonies formed by the transduced mSKAP55R cells was the same as the nontransduced cells. If overexpression of mSKAP55R had a more graduated effect, then we would have expected a range of colony sizes.

It was not clear how mSKAP55R mediated the growth inhibition. We could not find any abnormalities of cell survival based on dye exclusion after cell sorting. The cell cycle profile of sorted cells also was no different. Retroviral transduction followed by cell sorting provided limited numbers of mSKAP55RirGFP^{high} cells. An inducible promoter system may be a more appropriate method to examine more closely the growth effects of mSKAP55R. The lack of demonstrable effect on M1 cells may be explained partly by differences between M1 and FDC-P1 cells: M1 is a factor-independent line derived from a spontaneous myeloid leukemia, whereas FDC-P1 is a factor-dependent cell line derived from long-term culture of primary mouse bone marrow [32,39]. It is possible that the leukemic transforming events of M1 cells prevent the growth inhibitory effects of mSKAP55R. Together with the effects seen on primary bone marrow cells (growth suppression but no abnormality of differentiation), it appeared likely that enforced expression of mSKAP55R is sufficient to perturb the growth but not the differentiation of myeloid cells.

Phosphorylation studies of SKAP55 and SKAP55R have demonstrated differences in T cells. SKAP55, the highly related gene expressed exclusively in T cells, was reported to be constitutively phosphorylated and bound to FYN in quiescent T cells but not proliferating transformed T-cell lines such as Jurkat [40]. In contrast, hSKAP55R was neither constitutively phosphorylated nor associated with FYN in resting T cells [22]. These results were interpreted as suggesting that at least hSKAP55 may play a role in maintaining cellular quiescence. Our in vitro kinase assay demonstrated that mSKAP55R is constitutively phosphorylated under serum-stimulated conditions in SV40-transformed kidney cells (COS cells). The normal growth of primary bone marrow cells overexpressing the tyrosine 260 mutant (Figs. 8 and 9) suggested that the phosphorylation status of mSKAP55R may be important for its growth inhibitory effects. However, we could not exclude the possibility that mutation of Y260 had other effects on the mSKAP55R protein structure and function, such as protein stability.

Studies of SKAP55-interacting molecules suggest that SKAP55R may function to sequester other adaptor molecules important in positive growth signals [41–43]. Gene deletion studies of the adaptor protein SLP-76 suggest that SLP-76 mediates positive signals in T cells and platelets [41,44,45]. Another adaptor protein, FYB/SLAP-130, can bind to SLP-76 and inhibit the SLP-76–mediated IL-2 signaling in a phosphotyrosine-dependent [46,47]. The C-terminal SH3 domain of both SKAP55 and the SKAP55R proteins can bind to the proline-rich motif of FYB/SLAP-130 [20]. Thus, it is possible that SKAP55R inhibited positive growth signals by forming a complex with FYB/SLAP-130 and SLP-76. Another possible mechanism for the negative function of SKAP55R is recruitment of the tyrosine phosphatase SHP-1. SKAP55R and FYB/SLAP-130 recently were identified in a multiprotein complex with SHPS-1, an immunoreceptor that recruits the tyrosine phosphatase SHP-1 to the cell membrane [37]. In addition to identifying SKAP55R-interacting partners, analysis of the physiologic function of SKAP55R and its relative SKAP55 may be best elucidated by knockout experiments.

Acknowledgments

This work was supported in part by grants from the Anti-Cancer Council of Victoria, the National Health and Medical Research Council, Canberra, the Australian Government Cooperative Research Centres Program, the Bone Marrow Donor Institute, Melbourne, and NIH grant C-22556. We thank T. Gonda for the retroviral FDC-P1 cDNA library, P.Lock for the mouse FYN cDNA, C. McFarlane and T. Willson for the pEFBOS vectors, A.W. Neinhuis for the MgirLY22Y producer, and S. Anderson for cell sorting. We also thank H Zogos, N Seidel, W. Carter, and E. Viney for their excellent technical assistance.

References

1. Ward AC, Touw I, Yoshimura A (2000) The Jak-Stat pathway in normal and perturbed hematopoiesis. Blood 95:19
2. Corey SJ, Anderson SM (1999) Src-related protein tyrosine kinases in hematopoiesis. Blood 93:1
3. Koch CA, Anderson D, Moran MF, Ellis C, Pawson T (1991) SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science 252:668

4. Pawson T (1995) Protein modules and signalling networks. Nature 373:573
5. Songyang Z, Shoelson SE, Chaudhuri M, et al. (1993) SH2 domains recognize specific phosphopeptide sequences. Cell 72:767
6. Songyang Z, Gish G, Mbamalu G, Pawson T, Cantley LC (1995) A single point mutation switches the specificity of group III Src homology (SH) 2 domains to that of group I SH2 domains. J Biol Chem 270:26029-32
7. Bar-Sagi D, Rotin D, Batzer A, Mandiyan V, Schlessinger J (1993) SH3 domains direct cellular localization of signaling molecules. Cell 74:83
8. Ren R, Mayer BJ, Cicchetti P, Baltimore D (1993) Identification of a ten-amino acid proline-rich SH3 binding site. Science 259:1157
9. Eiseman E, Bolen JB (1992) Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases. Nature 355:78
10. Jouvin MH, Adamczewski M, Numerof R, Letourneur O, Valle A, Kinet JP (1994) Differential control of the tyrosine kinases Lyn and Syk by the two signaling chains of the high affinity immunoglobulin E receptor. J Biol Chem 269:5918
11. Stefanova I, Corcoran ML, Horak EM, Wahl LM, Bolen JB, Horak ID (1993) Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase p53/56lyn. J Biol Chem 268:20725
12. Wang AV, Scholl PR, Geha RS (1994) Physical and functional association of the high affinity immunoglobulin G receptor (Fc gamma RI) with the kinases Hck and Lyn. J Exp Med 180:1165
13. Bolen JB (1995) Protein tyrosine kinases in the initiation of antigen receptor signaling. Curr Opin Immunol 7:306
14. Appleby MW, Gross JA, Cooke MP, Levin SD, Qian X, Perlmutter RM (1992) Defective T cell receptor signaling in mice lacking the thymic isoform of p59fyn. Cell 70:751
15. Molina TJ, Kishihara K, Siderovski DP, et al. (1992) Profound block in thymocyte development in mice lacking p56lck. Nature 357:161
16. Stein PL, Lee HM, Rich S, Soriano P (1992) pp59fyn mutant mice display differential signaling in thymocytes and peripheral T cells. Cell 70:741
17. Chan VW, Meng F, Soriano P, DeFranco AL, Lowell CA (1997) Characterization of the B lymphocyte populations in Lyn-deficient mice and the role of Lyn in signal initiation and down-regulation. Immunity 7:69
18. Hibbs ML, Tarlinton DM, Armes J, et al. (1995) Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. Cell 83:301
19. Nishizumi H, Taniuchi I, Yamanashi Y, et al. (1995) Impaired proliferation of peripheral B cells and indication of autoimmune disease in lyn-deficient mice. Immunity 3:549
20. Liu J, Kang H, Raab M, Da Silva AJ, Kraeft S-K, Rudd CE (1998) FYB (FYN binding protein) serves as a binding partner for the lymphoid protein and FYN kinase substrate SKAP55 and a SKAP55-related protein in T cells. Proc Natl Acad Sci U S A 95:8779
21. Marie-Cardine A, Bruyns E, Eckerskorn C, Kirchgessner H, Meuer SC, Schraven B (1997) Molecular cloning of SKAP55, a novel protein that associates with the protein tyrosine kinase p59fyn in human T-lymphocytes. J Biol Chem 272:16077
22. Marie-Cardine A, Hendricks-Taylor LR, Boerth NJ, Zhao H, Schraven B, Koretzky GA (1998) Molecular interaction between the Fyn-associated protein SKAP55 and the SLP-76-associated phosphoprotein SLAP-130. J Biol Chem 273:25789
23. Rayner JR, Gonda TJ (1994) A simple and efficient procedure for generating stable expression libraries by cDNA cloning in a retroviral vector. Mol Cell Biol 14:880
24. Hilton DJ, Hilton AA, Raicevic A, et al. (1994) Cloning of a murine IL-11 receptor alpha-chain: requirement for gp130 for high affinity binding and signal transduction. EMBO J 13:4765
25. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403
26. Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci U S A 85:2444
27. Pearson WR (1990) Rapid and sensitive sequence comparison with FASTP and FASTA. Meth Enzymol 183:63
28. Curtis DJ, Robb L, Strasser A, Begley CG (1997) The CD2-scl transgene alters the phenotype and frequency of T-lymphomas in N-ras transgenic or p53 deficient mice. Oncogene 15:2975
29. Persons DA, Allay JA, Allay ER, et al. (1997) Retroviral-mediated transfer of the green fluorescent protein gene into murine hematopoietic cells facilitates scoring and selection of transduced progenitors in vitro and identification of genetically modified cells in vivo. Blood 90:1777
30. Curtis DJ, Hilton DJ, Roberts B, Murray L, Nicola N, Begley CG (1997) Recombinant soluble interleukin-11 (IL-11) receptor alpha-chain can act as an IL-11 antagonist. Blood 90:4403
31. Metcalf D (1984) Haemopoietic colony-stimulating factors. Amsterdam: Elsevier
32. Dexter TM, Garland J, Scott D, Scolnick E, Metcalf D (1980) Growth of factor-dependent hemopoietic precursor cell lines. J Exp Med 152:1036
33. Kouroku Y, Soyama A, Fujita E, Urase K, Tsukahara T, Momoi T (1998) RA70 is a src kinase-associated protein expressed ubiquitously. Biochem Biophys Res Commun 252:738
34. Tanigawa T, Elwood N, Metcalf D, et al. (1993) The SCL gene product is regulated by and differentially regulates cytokine responses during myeloid leukemic cell differentiation. Proc Natl Acad Sci U S A 90:7864
35. Tanigawa T, Nicola N, McArthur GA, Strasser A, Begley CG (1995) Differential regulation of macrophage differentiation in response to leukemia inhibitory factor/oncostatin-M/interleukin-6: the effect of enforced expression of the SCL transcription factor. Blood 85:379
36. Bolen JB, Rowley RB, Spana C, Tsygankov AY (1992) The Src family of tyrosine protein kinases in hemopoietic signal transduction. FASEB J 6:3403
37. Timms JF, Swanson KD, Marie-Cardine A, et al. (1999) SHPS-1 is a scaffold for assembling distinct adhesion-regulated multi- protein complexes in macrophages. Curr Biol 9:927
38. Persons DA, Allay JA, Allay ER, et al. (1999) Enforced expression of the GATA-2 transcription factor blocks normal hematopoiesis. Blood 93:488
39. Ichikawa Y (1969) Differentiation of a cell line of myeloid leukemia. J Cell Physiol 74:223
40. Schraven B, Marie-Cardine A, Koretzky G (1997) Molecular analysis of the fyn-complex: cloning of SKAP55 and SLAP-130, two novel adaptor proteins which associate with fyn and may participate in the regulation of T cell receptor-mediated signaling. Immunol Lett 57:165
41. Clements JL, Yang B, Ross-Barta SE, et al. (1998) Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development. Science 281:416
42. Clements JL, Koretzky GA (1999) Recent developments in lymphocyte activation: linking kinases to downstream signaling events. J Clin Invest 103:925
43. Marie-Cardine A, Schraven B (1999) Coupling the TCR to downstream signalling pathways: the role of cytoplasmic and transmembrane adaptor proteins. Cell Signal 11:705
44. Clements JL, Lee JR, Gross B, et al. (1999) Fetal hemorrhage and platelet dysfunction in SLP-76-deficient mice. J Clin Invest 103:19
45. Pivniouk V, Tsitsikov E, Swinton P, Rathbun G, Alt FW, Geha RS (1998) Impaired viability and profound block in thymocyte development in mice lacking the adaptor protein SLP-76. Cell 94:229
46. Musci MA, Hendricks-Taylor LR, Motto DG, et al. (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
47. Boerth NJ, Judd BA, Koretzky GA (2000) Functional association between SLAP-130 and SLP-76 in Jurkat T cells. J Biol Chem 275:5143