A Novel Secreted Form of Immune Suppressor Factor with High Homology to Vacuolar ATPases Identified by a Forward Genetic Approach of Functional Screening Based on Cell Proliferation*

Edgardo E. Tulin‡‡, Nobuhisa Onoda‡, Masatsugu Maeda‡, Masakazu Hasegawa‡, Tetsuya Nosaka‡, Hitoshi Nomura‡, Shigetaka Asano‡, and Toshio Kitamura‡‡

From the ‡Division of Hematopoietic Factors and the ¶Division of Molecular Therapy, Advanced Clinical Research Center, The Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan and §Chugai Research Institute for Molecular Medicine, 153-2 Nagai, Nihart, Ibaraki 300-4101, Japan

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In the search for stromal-derived growth factors, we have identified a novel secreted short form of immune suppressor factor (ISF) using a combination of a genetic approach and retrovirus-mediated functional screening. This protein, which we termed ShIF, was isolated based on its ability to support proliferation of a mutant clone S21, which was established from Ba/F3 cells that are usually interleukin-3-dependent but became dependent on a stroma cell line ST2 after chemical mutagenesis. ISF, a membrane protein harboring six transmembrane domains, was reported to have immunosuppressive functions. The coding region of ShIF started from the third transmembrane domain of ISF. Biochemical analysis demonstrated that ShIF was expressed in both the secreted and membrane-bound forms of 27-kDa protein, which was supposed to have an internal ATG present in the third transmembrane domain of ISF as a start codon. In addition to the full-length form of ISF, a major protein with a molecular size of 27 kDa was also expressed through the proteolytic process of ISF. ShIF resembles this naturally occurring short form of ISF (sISF). Deletion analysis of the major domains of ISF cDNA revealed that ShIF is an active functional domain of ISF with the capability to support proliferation of S21 cells. Enforced expression of ShIF in MS10 cells, bone marrow stroma cells that do not express endogenous ShIF or ISF, conferred on the cells an ability to support the growth of S21 cells as well as bone marrow cells. Interestingly, ShIF shows a high sequence homology to the C-terminal part of a 95-kDa yeast vacuolar H(+) ATPase subunit, Vph1p (39%), and a 116-kDa proton pump (VPP1) (54%) of the rat and bovine synapic vesicle. Therefore, it is possible that ShIF also acts as a proton pump and somehow prevents the cells from undergoing apoptosis.

Forward genetic screening in model organisms has revealed the molecular basis for diverse biological processes, including cell division in the yeast, Saccharomyces cerevisiae (1), programmed cell death in the nematode, Caenorhabditis elegans (2), and embryonic pattern formation in the fly, Drosophila melanogaster (3). Such screening uses a three-step procedure that entails: first, random mutagenesis of a large number of organisms; second, screening of the resulting mutants to identify those with a defect in the process of interest; and finally, identification of the mutations in specific genes that underlie the resultant phenotypes of interest. Thus, because of the ability of mutations to alter the function of a single product, the forward genetic screening is a powerful method in the identification of genes that regulate biological processes within the context of a complex cellular environment. We exploited this concept in a novel strategy to search for stroma-derived growth factors. The critical roles of bone marrow stroma cells in inducing proliferation and differentiation of hematopoietic stem cells are well recognized, and many stroma-derived growth factors have been identified and proved to play critical roles in hematopoiesis (4–7).

To search for unidentified stroma-derived factors, we designed a strategy in which a genetic approach was combined with retrovirus-mediated expression screening. We chemically mutagenized an interleukin-3 (IL-3)-dependent pro-B cell line Ba/F3, which does not proliferate on most stromal cell lines, and isolated mutant clones that were stroma cell-dependent. These mutant cells were then used as indicator cells in retrovirus-mediated expression cloning of stroma-derived factors that support the growth of the mutant Ba/F3 cells. One of the Ba/F3 mutants, S21, was able to grow on stromal cell line ST2 but not on MS10 cells. The introduction of a ST2-derived cDNA library into MS10 cells yielded identification of a cDNA having a product that supported the growth of S21 but not the parent Ba/F3 cells. This cDNA contained a putative open reading frame of 263 amino acids, a peptide named ShIF, which turned out to be the C-terminal part of a known protein, ISF (immune suppressor factor) (8). Interestingly, the ShIF cDNA expressed both soluble and membrane-bound forms of 27-kDa proteins, and deletion analysis of ISF revealed that ShIF corresponded to a functional domain of the full-length ISF protein. The findings of this paper suggest that ShIF, identified in this study, corresponds to a naturally occurring short form of ISF. Its growth-supporting activities on S21 cells as well as on bone marrow hematopoietic cells further suggest that ShIF is a growth stimulatory peptide involved in the regulation of cell growth.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) P15920.‡‡ To whom correspondence should be addressed: Div. of Hematopoietic Factors, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan. Tel.: 81-3-5449-5758; Fax: 81-3-5449-5453; E-mail: kitamura@ims.u-tokyo.ac.jp.

1 The abbreviations used are: IL, interleukin; ISF, immune suppressor factor; ShIF, secreted short form of immune suppressor factor; m, murine; GM-CSF, granulocyte/macrophage colony-stimulating factor; SCF, stem cell factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; EMS, ethyl methanesulfonate; RACE, rapid amplification of cDNA ends.
**EXPERIMENTAL PROCEDURES**

**Cytokines and Cell Lines—**Recombinant murine (m)LIF-3 was purified (9). Recombinant murine GM-CSF, m-IL-5, and m-SCF, and neutralizing antibodies (anti-macrophage-CSF, anti-SCF, and anti-IL-1) were obtained from R&D Systems (Minneapolis, MN). An anti-FLAG BioM2 antibody was purchased from Sigma. A retrovirus packaging cell line, BOSC23, for ecotropic retroviruses (10) was maintained in Dulbecco’s modified Eagle medium containing 10% (v/v) fetal calf serum (DMEM-10% FCS) and guanidine phosphoribosyltransferase selection reagents (GPT, Specialty Media, Lavellete, NJ). The cells were transfected into DMEM-10% FCS without GPT selection reagents 2 days before transfection. A murine pro-B cell line, Ba/F3, was transfected in RPMI 1640 medium containing 10% FCS in the presence of 1 ng/mL mIL-3. The mouse bone marrow-derived stromal cell lines ST2, PA6, and CF-1 were cultured in DMEM-F-12 containing 5% (v/v) FCS. MS10, another mouse bone marrow-derived stroma, was cultured in minimum essential medium (α-MEM, Life Technologies, Inc.) containing 10% FCS. COS7 cells were maintained in DMEM-10% FCS.

**Reagents—**Ethyl methanesulfonate (EMS) was purchased from Sigma. The following protease inhibitors were also purchased from Sigma: p-aminobenzoyl-Gly-Pro-d-Leu-p-Ala-hydroxyacid, captopril, bestatin, leupeptin, pepstatin A, and bacitracin. All of the protease inhibitors were dissolved in Me2SO as 10 mM stock solutions.

**Chemical Mutagenesis of Ba/F3 Cells—**Ba/F3 cells (1 × 10⁷) were chemically mutagenized with EMS at 10 μM for 2 h at 37 °C and 5% CO₂. To determine an appropriate dose and duration of EMS treatment, we constructed a dose-response curve over a time course period and monitored the percentage of surviving cells after a 24-h recovery period. The conditions resulting in 10–15% survival of 1 × 10⁷ cells. After 2 h of EMS treatment, the cells were recovered by centrifugation (1000 rpm, 5 min) and washed once with medium to remove residual EMS. The mutated genes were resuspended in a culture medium, allowed to recover for 24 h in the presence of mIL-3, collected by centrifugation (1000 rpm, 5 min), and resuspended in DMEM-F-12-10% FCS before seeding on stroma cell layers.

**Construction of Colonies of Clones ST2 Cells (5 × 10⁵) were cultured in 100-mm plates and grown to confluence. Mutated Ba/F3 cells were seeded at 1 × 10⁵ cells/plate, and the plates were incubated at 37 °C and 5% CO₂ in the absence of mIL-3. The medium was changed every 4 or 5 days. Only half of the medium was replaced with fresh medium, gently so as not to detach the cells from the stromal cells. Nonmutated cells were also cocultured with the stroma as a control. Colonies of mutant cells that proliferated on the stroma were isolated using cylindrical rings followed by trypsin-EDTA treatment and in some cases through direct pipetting using a thin tip. Individual clones were obtained by limiting dilution in 96-well plates, and 48 clones were isolated. All 48 clones were recultured on ST2 cells to confirm stroma-dependent proliferation of each clone, and clones in which proliferation was not inhibited by neutralizing antibodies to known cytokines were chosen for further analysis.

**Construction of a cDNA Library—Poly(A)⁺ RNA was prepared from a mouse bone marrow stroma cell line, ST2, using FASTTRACK (Invitrogen). A cDNA library was constructed using a cDNA synthesis kit (Superscript Plasmid System, Life Technologies, Inc.) according to the manufacturer’s recommendations, with some modifications (11, 12). Briefly, cDNA was synthesized using an oligo(dT) primer. (Superscript Plasmid System, Life Technologies, Inc.) according to the

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**Production of Retrovirus Stocks and Infection of MS10 Cells—**BOSC23 cells (2 × 10⁵) were seeded in 6-cm plates a day before transfection. DNAs derived from the subdivided library of ST2 cells were transfected using LipofectAMINE (Life Technologies, Inc.) to generate a high titer retrovirus stock. After 2 days, the culture supernatant was used to infect the MS10 cells, which did not support the growth of S21 mutants. About 1 × 10⁶ MS10 cells/well were seeded into 24-well plates the night before infection and incubated with 0.5 ml of virus stock representing each subdivided library for 4 h in the presence of 10 μg/ml polybrene (Sigma). Then, 0.5 ml fresh DMEM-10% FCS was added to the culture and the incubation continued. The medium containing the retrovirus was changed to fresh DMEM-10% FCS 24 h later. Three days after the library transduction, S21 cells were cocultured (1 × 10⁴ cells/well) on the transduced MS10 cells.

To monitor the infection efficiency of cDNA libraries, a PMX vector containing enhanced green fluorescent protein cDNA (CLONTECH) (PMX-GFP) (14) was used as a control. The PMX-GFP retroviruses prepared similarly were infected to MS10 cells, and the infection efficiency was analyzed by fluorescence-activated cell sorting (FACS).

**Nucleotide Sequencing—**The sequencing reaction was performed using a dRhodamine Terminator Cycle sequencing kit (Applied Biosystems), and the sequence was analyzed on an Applied Biosystems Prism 377 DNA sequencer.

**Cloning of the ISF Gene—**The full-length gene of the murine ISF was cloned from the ST2-derived cDNAs by 5’ RACE using the Marathon cDNA amplification kit (CLONTECH). A primer specific for ISF (5’-TCTTGCTGTTGGAGACAGGAA-C3’) was designed from the N-terminal end of C121, which was expected to amplify a region upstream of C121. GSP-1 was designed such that the product could overlap a 27-base pair sequence in C121 with a unique restriction site (AccI). Polymerase chain reaction was performed according to the recommended protocols of CLONTECH using the Advantage cDNA polymerase mix. One major band in the polymerase chain reaction product was detected corresponding to about 2.0 kilo-base pairs in size. The polymerase chain reaction product was subdivided directly into a TA vector (Invitrogen), sequenced, and identified as the 5’-end of ISF. A full-length cDNA was generated by ligation of C121 with the 5’ RACE product using the unique recombination site present in the overlap.

**Expression Constructs of FLAG-tagged cDNAs—**FLAG-tagged forms of ShIF and ISF cDNA were constructed at the C terminus by replacing their terminal codons with the FLAG epitope (MDYKDDDDK) through a NotI site. The resulting constructs were cloned into a mamalian expression vector pME18S (15), which is driven by the SRα promoter. cDNAs used in deletion experiments were generated by polymerase chain reaction using primers obtained from the ISF cDNA sequence. All deletion mutants were FLAG-tagged at the C terminus and cloned into pME18S. The sequences of these mutants were confirmed by sequencing.

**Immunoprecipitation—**To detect the secreted form of ShIF, COS7 cells (2 × 10⁵ cells/well) were seeded in 6-well plates a day before transfection and transfected with the FLAG-tagged pME-ShIF construct using the LipofectAMINE method (Life Technologies, Inc.). After 48 h of culture, the supernatant was collected and immunoprecipitated with anti-FLAG BioM2 affinity gel (Sigma) overnight at 4 °C. The immunoprecipitate was collected by centrifugation at 5000 rpm for 5 min and washed with lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.2 mM NaVO₄, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40) three times. The bound proteins were eluted by the sample buffer, applied to an SDS-polyacrylamide gel electrophoresis, and electroblotted onto a polyvinylidene difluoride membrane (Millipore). The blots were probed with anti-FLAG BioM2 antibody, incubated with horseradish peroxidase-conjugated complex, and subsequently developed with ECL Western blotting detection reagents (Amersham Pharmacia Biotech) and exposed to ECL Hyperfilm.

To detect membrane-bound ShIF, cells were lysed with lysis buffer and centrifuged to remove cellular debris. Cell lysates were immunoprecipitated with an anti-FLAG affinity gel, and detection of the FLAG fusion protein was performed as described above.

**Purification of ShIF Using anti-FLAG Affinity Gel—**One liter of culture supernatant produced from COS7 cells transfected with a FLAG-tagged construct of ShIF cDNA was applied to an anti-FLAG affinity gel column. Bound proteins were eluted from the column by competition with FLAG peptide (60 μg/ml) at 1 ml/min, and 2 ml fractions were collected. Fractions 10–16, which contained the purified FLAG fusion protein, were used for the bioassay.

**Immunofluorescence Microscopy—**COS7 cells were grown on coverslips for 1 day in the culture media and were transfected with FLAG-tagged ShIF or ISF constructs using LipofectAMINE reagent (Life Technologies, Inc.). After transfection, the cells were cultured for 2 days, washed twice with phosphate-buffered saline, and incubated with an anti-FLAG antibody (Sigma) for 2 h. The cells were washed again with phosphate-buffered saline and incubated with a Cy3-conjugated secondary antibody (Amersham Pharmacia Biotech) under dark conditions for 1 h. After washing with phosphate-buffered saline, cells were viewed under a fluorescence microscope.

**Cell Proliferation Assay—**Ba/F3 or S21 cells (1 × 10⁵/well) were cultured in 96-well plates (100 μl culture volume) in the presence of either the culture supernatant derived from ShIF-transfected COS7 cells or the purified ShIF protein for 72 h at 37 °C and 5% CO₂. Cell growth was determined using a standard assay kit (Cell Titer 96 Assay)
by Promega. To confirm the activity of ShIF, ShIF was removed from the supernatant by incubation with an anti-FLAG affinity gel for 2 h at 4 °C. The anti-FLAG-ShIF complex was collected by centrifugation for 10 min at 14,000 rpm, and the stripped supernatant was used for cell assay.

**Bone Marrow Cells Assay**—Stable cell lines of PA6 and MS10 cells expressing ShIF were generated by infecting PA6 and MS10 cells with virus supernatant derived from pMX-neo-ShIF-transfected packaging cells followed by selection with G418 (1.2 mg/ml).

Two 8-week-old mice (female, ICR strain) were used in the preparation of bone marrow cells using standard procedures. The unfractionated bone marrow cells (1 × 10^6 per well) were seeded on stromal layers that had been grown in 24-well plates for 48 h and were cultured for 1 week before photographs were taken.

**Protease Inhibitor Assay**—COS7 cells were plated (2 × 10^5/well) in 6-well plates 1 day before transfection and transfected with 1 μg of FLAG-tagged ShIF or ISF expression construct/well for 4 h using the LipofectAMINE (Life Technologies, Inc.) method. Then, the transfection solution was removed and was replaced with fresh DMEM-10% FCS. After 24 h, the medium was replaced with fresh medium supplemented with 10 and 100 μM of various protease inhibitors. Supernatants and cell lysates were collected after 48 h, immunoprecipitated with anti-FLAG, and applied to SDS-polyacrylamide gel electrophoresis, and FLAG fusion protein was detected by ECL Western blotting reagents as described.

**RESULTS**

**Isolation of Clone S21**—Culture of EMS-treated Ba/F3 cells on ST2 stromal cells took about 5–6 weeks before distinct colonies were visualized adhering to the stromal layer (Fig. 1).

In the case of nonmutated Ba/F3 cells (a negative control), the cells failed to survive after 6 days of coculture, and no visible colonies were found after 6 weeks. Of the 48 clones isolated, 8 clones failed to proliferate when they were recultured on ST2 stroma cells, and 19 clones responded to GM-CSF and proliferated, indicating that those mutants ectopically expressed the receptor for GM-CSF by mutagenesis. Among the remaining 21 clones, 4 clones (S6, S9, S21, and S24) that showed strong proliferation were challenged by the addition of neutralizing antibodies against macrophage-CSF, SCF, and IL-1. Of these 4 clones, only 1 clone (S24) was affected by antibody against mSCF (data not shown). Of the remaining 3 clones, S21 showed the best proliferative response and was subjected for further experiments.

**Expression Cloning of an ST2-derived Factor That Supports Growth of S21 Cells**—12,000 independent clones derived from an ST2 cDNA library were screened using subdivided pools. In the screening, the library was divided into small pools (120 clones/pool). Miniprep DNA was prepared from each pool, which was then used to transfect BOSC23 cells to obtain library-derived retroviruses. The resulting retroviruses were infected to MS10 cells that did not support the growth of S21 cells, and 2 days after infection S21 cells were seeded on the infected MS10 cells. After about 3–4 weeks of culture, one of the 96 screened pools (No. 2-21) was identified to support proliferation of S21 cells. A total of 148 single clones was picked from this pool for further screening.

Two single clones (2-21-C121 and 2-21-C126) were found to give a phenotype identical with that of the original pool 2-21. The mutant S21 cells attached and proliferated on the MS10 cell layer transduced with clone 2-21-C121 (Fig. 2a) and with clone 2-21-C126 (data not shown) but not on uninfected cells (Fig. 2b) or on cells infected with another clone (Fig. 2c). Parental Ba/F3 cells did not proliferate on MS10 cells transduced with the positive clone (Fig. 2d). The two positive clones (C121 and C126) turned out to include the same sequence.

**Primary Structure of ShIF**—The cDNA insert of clones C121 and C126 was 1.22 kilobase pairs long and contained an open reading frame encoding a polypeptide of 263 amino acids (Fig. 3A). The N-terminal 14 amino acid residues constituted a hydrophobic amino acid sequence typical of signal peptides with a predicted cleavage site between amino acids 20 and 21 (indicated by an arrowhead) (von Heijne method, PSORT II prediction). The initiator codon (ATG) was 75 bases from the 5′-end of the insert. No typical consensus sequence for translation initiation was found in the 5′-untranslated region. Hydrophobicity analysis for the reading frame predicted three membrane spanning segments (underlined).
We searched sequence data bases and found that our sequence matched to a C-terminal part of a protein named immune suppressor factor (ISF) (8). To confirm whether ISF was also expressed in ST2 cells, we cloned a full-length ISF gene by 5’RACE from cDNA derived from ST2 cells and obtained a full-length cDNA, which was identical with the published sequence (EMBL M31226). The full-length cDNA was 3010 base pairs long with a coding region of 2568 nucleotides (856 amino acids). There were at least six transmembrane regions in ISF, and ShIF contains three of these hydrophobic domains (Fig. 3B). ShIF showed 39% sequence identity with the 95-kDa yeast vacuolar H(+) -ATPase subunit, STV1 (16), 54% with human and rat, 53% with bovine, and 42% with the C. elegans 116-kDa synaptic vesicle proton pump (VPP1) (17) at the amino acid level.

**mRNA Expression**—To determine the expression of the mRNA corresponding to the C121 cDNA, Northern blot analysis was carried out using the C121 cDNA as a probe. Three transcripts were ubiquitously detected with sizes of about 3.0, 4.0, and 6.0 kilobases in all of the mouse tissues examined. Relatively higher expression levels were observed in kidney and liver, whereas lower levels of expression were observed in the spleen, testis, and skeletal muscle (Fig. 4A). Among various mouse stroma cell lines tested, only ST2 and ST0 cells ex-
pressed the transcripts hybridizing C121 cDNA, whereas the other mouse-derived cell lines including MS10 did not express them (Fig. 4B).

Expression of ShIF Protein—First, we asked whether ShIF was secreted into the culture media. To this end, we analyzed the supernatant and cell extract of COS7 cells transfected with a FLAG-tagged ShIF expression construct or a FLAG-tagged ISF expression construct. The FLAG-tagged ShIF protein was detected in the supernatant (Fig. 5, lane 3) as well as in the cell extract (Fig. 5, lane 4), suggesting that the membrane-integrated ShIF was shed into the supernatant. The predicted mature protein encoded by the ShIF cDNA consisted of 243 amino acids with a calculated molecular size of 27 kDa. The apparent molecular weight of the FLAG-tagged protein in the supernatant and cell extract, respectively, of ShIF-transfected COS7 cells; 5 and 6, supernatant and cell extract respectively, of ISF-transfected COS7 cells. Molecular size markers shown on the left are in kDa.

FIG. 4. Northern blot analysis of ShIF/ISF gene expression in various mouse tissues (A) and cell lines (B). The ShIF cDNA was labeled using a random primer labeling kit (Stratagene) and was used as a probe. A mouse multiple tissue Northern blot (MTN, CLONTECH No. 7762-1) and 1 µg of mRNA of each cell line were used. Blots were reprobed with β-actin and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as loading controls for the multiple tissue Northern blot and cell lines blot, respectively. MTSC, mouse thymic stroma cells; ST0, a subline of ST2 cells.

ISF is composed of a 400-amino acid extracellular domain, a 409-amino acid region spanning at least six transmembrane domains, and a short (47 amino acids) cytoplasmic tail (Fig. 4A). To examine the functional relevance of these domains, we generated a series of deletion mutants of ISF (Fig. 7A). DNAs were transiently transfected into COS7 cells in 6-well plates, and the supernatant and the cell extracts were examined for expression of the FLAG-tagged protein. As shown in Fig. 7B, all but for the ShIF deletion mutant (ΔShIF) produced the secreted form of the protein (left panel). When the cell extracts were examined (right panel), the membrane-bound FLAG-tagged products were detected, confirming that all of the constructs were efficiently transfected and expressed membrane-bound forms of the wild type and the mutant ISF. These results indicated that a ShIF-like molecule indeed existed as a naturally occurring form sISF of the molecule. Culture supernatants obtained from ShIF-transfected COS7 cells were able to support the growth of S21 but not the parent Ba/F3 cells (Fig. 8A). When ShIF was removed from the supernatant using anti-FLAG affinity gel, this activity was abolished, thereby confirming that the growth of the mutant cells depended on the presence of the soluble ShIF (data not shown). The growth-promoting activity of ShIF on S21 cells was also confirmed by an assay using purified recombinant ShIF (Fig. 8B).

Enforced expression of ShIF in MS10 cells, bone marrow stroma cells that do not express endogenous ISF, conferred the cells with an ability to support the growth of S21 cells (data not shown), which was more efficient than that of the culture supernatant of ShIF-transfected COS7 cells. This result indicated that the membrane-bound form ShIF was more potent than soluble ShIF in promoting proliferation of S21 cells.

To examine the role of ShIF/sISF in normal hematopoiesis, we cultured bone marrow cells on stroma cells MS5 and ST2 that expressed endogenous ShIF and stroma cells PA6 and MS10 that did not express endogenous ISF but were stably transduced with ShIF. After 1 week of culture, we found that stroma cells MS5 (Fig. 9A) and ST2 (Fig. 9B) that expressed...
FIG. 6. Subcellular localization of ShIF and ISF. COS7 cells were grown in tissue culture coverslips and transfected with FLAG-tagged pME-ShIF or pME-ISF construct. After 48 h, cells were washed with phosphate-buffered saline, incubated with anti-FLAG BioM2 antibody then with Cy3-conjugated secondary antibody. a, COS7 cells under epi-illumination showing immunofluorescent surface staining of ShIF. b, the same field of COS7 cells under visible light. c, COS7 cells under epi-illumination showing immunofluorescent surface staining of ISF. d, same field of COS7 cells under visible light. Control experiments using anti-FLAG alone (e) and secondary antibody alone (f) are also shown.

endogenous ISF as well as PA6 and MS10 stroma transduced with ShIF (Fig. 9, g and h, respectively), supported substantial proliferation of bone marrow cells. PA6 and MS10 cells that had not been transduced with ShIF or that had been transduced only with the empty vector (Fig. 9, c–f) did not support proliferation of bone marrow cells. These results suggested that ShIF/sISF promoted proliferation of normal hematopoietic cells as well.

FIG. 7. Expression analysis of ShIF and ISF. A, structures of deletion mutants of ISF. The predicted transmembrane domains (shaded boxes), the putative signal sequence (hatched box), and the position of the deleted areas (dashed lines) are indicated. B, Western blot analysis of the supernatants (left panel) and cell extracts (right panel) of COS7 cells transfected with the FLAG-tagged mutant DNA constructs. Immunoprecipitation-Western blotting was performed using anti-FLAG antibody. Lanes: 1, untransfected cells; 2, cells transfected with empty vector; 3–8, cells transfected with the following constructs: pME-ShIF (lane 3), pME-ISF (lane 4), pME–ΔShIF (lane 5), pME–SP+ShIF (lane 6), pME–SP+ShIF(TM) (lane 7), and pME–ΔED (lane 8).

Secretion of ShIF Is Due to Proteolytic Processing—To examine the regulation of ShIF/sISF secretion, we used a number of protease inhibitors and determined whether they inhibited the release of ShIF/sISF from the membrane. Addition of all the protease inhibitors tested blocked the release of ShIF/sISF into the medium (Fig. 10, A and C) in both ShIF- and ISF-transfected cells. On the other hand, the membrane-bound form remained unchanged in ShIF-transfected cells and mostly unchanged in ISF-transfected cells (Fig. 10B). In addition to the block of ShIF/sISF secretion, pepstatin A and leupeptin also blocked formation of sISF from ISF. Interestingly, a small amount of the full-length ISF was released into the supernatant, which was not blocked by any protease inhibitors (Fig. 10C). In ISF-transfected cells, expression of the membrane-bound ISF was either partially or completely inhibited by some of the inhibitors used (Fig. 10D). Even lower concentrations of the inhibitors (10 μM) still blocked the release of the soluble form of the protein except for bestatin (data not shown). Thus, it was indicated that the release of ShIF/sISF as well as formation of sISF from ISF was due to proteolytic processing.

DISCUSSION

In this paper, we have presented a novel approach of cloning cDNAs encoding stroma-derived proteins that would support proliferation of hematopoietic cells. The strategy consists of the following procedures: 1) mutagenesis of a factor-dependent cell line, Ba/F3; 2) establishment of stroma-dependent Ba/F3 mutants; 3) identification of supportive and nonsupportive stroma cell lines for each mutant; and 4) complementation cloning of the factor that stimulates proliferation of a mutant Ba/F3 clone. A unique feature of Ba/F3 cells has made this approach possible; they proliferate in response to a single cytokine, IL-3. In combination with EMS mutagenesis, theoretically all soluble growth factors derived from stroma cells can be identified by this screening. In addition, retrovirus-mediated expression...
cloning has made it possible to identify membrane-bound growth factors that require cell-cell contact to support cell growth. Using this strategy, we identified the ShIF protein from ST2 cells that supported the growth of Ba/F3 mutant S21 as well as bone marrow cells.

A database search demonstrated that the ShIF protein is a C-terminal part (263 amino acids) of ISF (856 amino acids) (8). The ISF protein was originally isolated from a T cell hybridoma using antibodies that bind proteins involved in modulating immune responses using in vitro translated products (8). It was reported that ISF suppressed a mixed lymphocyte reaction. We performed a similar assay using purified recombinant ShIF; however, we did not detect the inhibitory activities in mixed lymphocyte reaction (data not shown).

When we expressed ShIF in COS7 cells, both secreted and membrane-bound forms were detected. We then expressed ISF to examine whether the full-length mature protein could also be expressed, and interestingly what was released into the medium was mainly sISF, the short form of ISF. We performed a similar assay using purified recombinant ShIF; however, we did not detect the inhibitory activities in mixed lymphocyte reaction (data not shown).

When we expressed ShIF in COS7 cells, both secreted and membrane-bound forms were detected. We then expressed ISF to examine whether the full-length mature protein could also be expressed, and interestingly what was released into the medium was mainly sISF, the short form of ISF. Analysis of the cell extract obtained from the cells transfected with the ISF cDNA showed that in addition to the full-length mature protein, a smaller membrane-bound form (sISF) was present that corresponded to ShIF. Thus, a ShIF-like molecule, sISF, also exists as a naturally occurring protein. Experiments using protease inhibitors indicated that sISF was a proteolytic product of ISF. All protease inhibitors tested blocked the release of ShIF/sISF to the culture supernatant. The responsive protease and the proteolytic sites are still to be determined. Deletion analysis of the major domains of ISF indicated the importance of the ShIF/sISF domain of the molecule. In fact, MS10 cells expressing the ShIF-deletion mutant were not able to support the growth of S21 cells (data not shown).

Finally, the high homology of ShIF and ISF to vacuolar proton pumps suggested the possibility that these proteins are involved in coupling ATP hydrolysis to proton translocation thereby contributing to cell growth through yet unidentified mechanisms. It is also unclear at present how S21 became ST2 stroma-dependent by mutagenesis. In the complementation experiment presented here, the mutant cell was supposed to express a receptor for a stroma-derived factor. However, we do not know whether a receptor for proton pump exists. Increased proton export through pumps or ion exchange channels itself could be a mechanism by which these molecules protect cells from undergoing apoptosis (18). For instance, in hematopoietic

**FIG. 9.** Proliferation of mouse bone marrow cells on various stroma cells.
The bone marrow cells grew well on MS5 (a) and ST2 (b) cells but not on MS10 (c) and PA6 (d) cells. Transduction of ShIF (g and h) but not a control vector (e and f) conferred on MS10 (g) and PA6 (h) cells the ability to support the growth of bone marrow cells.

**FIG. 10.** Effect of protease inhibitors on expression of the secreted and the membrane-bound ShIF. COS7 cells were transfected with pME-ShIF (upper panels) and pME-ISF (lower panels) FLAG-tagged constructs and protease inhibitors were added 24 h after transfection. After 48 h of culture, supernatants (left panels) and cell extracts (right panels) were obtained and immunoprecipitated with anti-FLAG, and the FLAG-tagged protein was detected by Western blotting. Lanes: 1, untransfected cells; 2, cells transfected with empty vector; 3, pME-ShIF- or pME-ISF-transfected cells without any protease inhibitor; 4–9, pME-ShIF- or pME-ISF-transfected cells with protease inhibitors p-aminobenzoyl-Gly-Pro-o-Leu-o-Ala-hydroxamic acid (lane 4), captopril (lane 5), pepstatin A (lane 6), bestatin (lane 7), leupeptin (lane 8), and bacitracin (lane 9).
stem cells, GM-CSF delays apoptosis by cytoplasmic alkalinization through activation of the Na\(^+\)/H\(^+\) exchanger (19). The findings of the present study indicate that ShIF corresponds to a naturally occurring short form of ISF protein produced by the proteolytic machinery, suggesting that a proton pump-like molecule, ISF, plays a role in the regulation of cell proliferation.

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Edgardo E. Tulin, Nobuhisa Onoda, Masatsugu Maeda, Masakazu Hasegawa, Tetsuya Nosaka, Hitoshi Nomura, Shigetaka Asano and Toshio Kitamura

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