J. Biochem. 124, 209-216 (1998)

Involvement of Stromal Membrane-Associated Protein (SMAP-1) in Erythropoietic Microenvironment[1]

Yo Sato, Hea Nam Hong, Nobuaki Yanai, and Masuo Obinata[2]

Department of Cell Biology, Institute of Development, Aging and Cancer, Tohoku University, Seiryomachi, Aobaku, Sendai 980-8575

Received for publication, March 5, 1998

Erythropoiesis is regulated by the hematopoietic microenvironment of the spleen and fetal liver in mice. We showed that established stromal cells of these organs selectively support erythropoiesis *in vitro*. To identify the cell surface molecule(s) on the stromal cells involved in erythropoiesis, we raised monoclonal antibodies against the stromal cells. Using one of these antibodies (11D), we cloned a new gene named *smap-1* (stroma membrane-associated protein-1). The SMAP-1 protein deduced from the nucleotide sequence of the cDNA was a newly identified membrane protein with direct repeats of the KKD/E units found in MAP1A and MAP1B, which is involved in the association with microtubules. By transfection of the anti-sense *smap-1* cDNA into the stromal cells, we showed that SMAP-1 may have a stimulatory effect on stroma-supported erythropoiesis. Its expression was detected in the yolk sac, fetal liver, spleen, and bone marrow, and was correlated with their erythropoietic activity.

Key words: erythropoiesis, hematopoietic microenvironment, monoclonal antibody, *smap-1*, stromal cell.

Hematopoietic stem cells and their progenitor cells can proliferate and differentiate in the microenvironments of bone marrow, spleen, and fetal liver. Erythropoiesis is first observed in the yolk sac and then switches in the fetal liver (*1, 2*). In the adult mouse, the spleen is usually considered to be responsible for the activity by which stem cells become predominantly committed to erythroid development (*3*). Thus, it is thought that the stromal cells of mouse spleen may provide a microenvironment adequate for erythropoietic cells. We previously established stroma cell lines (MSS cells) from newborn spleen. As expected, the stromal cells supported erythropoiesis *in vitro* (*4, 5*). Large erythroid colonies were formed from 13-day-old fetal liver erythroid progenitor cells in a semisolid medium containing erythropoietin (epo) in direct cell contact with the stromal cell layers (*4*). Stromal cells with similar activity were established from fetal liver (*6, 7*) and bone marrow (*8*), indicating that erythropoiesis may be regulated by stromal cells of hematopoietic organs. We reported that the production of SCF, a ligand for c-Kit, is essential for stroma-supported erythropoiesis (*7*). Recently, we demonstrated that the development of erythroid cells on stromal cells was inhibited by an anti-very late activation antigen-4 (VLA-4 integrin) antibody and by an antibody to vascular cell adhesion molecule-1 (VCAM-1), a ligand for VLA-4, but not by anti-VLA-5 antibody, although erythroid cells express both VLA-4 and VLA-5 (*9*). SCF and VCAM-1 therefore may be necessary, but may be molecules with insufficient ability to provide erythropoietic support for the stromal cells, because these two molecules are ubiquitously expressed in stromal cells which have no erythropoietic supporting ability. Thus, in this work, we searched for other molecules involved in the provision of erythropoietic support for spleen stromal cells by raising monoclonal antibodies, and a gene coding the protein recognized by one of the monoclonal antibodies was cloned by immunoscreening of a cDNA expression library. The protein encoded by the cloned cDNA was shown to encode a type II membrane protein.

MATERIALS AND METHODS

Preparation of Monoclonal Antibodies (MAb) against Membrane Proteins from MSS31 Cells—Erythroid supportive mouse spleen stromal cell line, MSS31 and MSS62 (*4*), were cultured in RITC80-7 (Kyokuto Pharmaceutical Industrial, Tokyo) supplemented with 2% FBS, 10 μg/ml bovine transferrin, 1 μg/ml insulin, and 10 ng/ml recombinant EGF (generously supplied by Wakunaga, Tokyo). To prepare membrane fraction, a monolayer culture of MSS31 cells was washed with PBS (Dulbecco's phosphate-buffered saline) and then lysed by the addition of a 10-fold volume of a hypotonic solution (2 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM $NaHCO_3$), and then allowed to stand for 15-20 min. After the cells had been removed with a cell scraper, they were homogenized with 10-15 strokes of a Dounce homogenizer on ice. After the nuclear fraction had been removed by centrifugation at 1,500 rpm for 10 min, the particulate fraction was recovered by centrifugation at 14,000 $\times g$ for 15 min. The pellet was applied to a stepwise sucrose

[1] This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.
[2] To whom correspondence should be addressed. Tel: +81-22-717-8483, Fax: +81-22-717-8488, E-mail: mobinata@idac.tohoku.ac.jp

density gradient (57, 42, and 20%), and then centrifuged at 52,000×g for 1 h. The membrane fraction was recovered from the layer formed between 20 and 42% sucrose, and then diluted with a 3-fold volume of 1 mM $NaHCO_3$. After centrifugation at 13,000×g for 15 min, the recovered pellet was suspended in PBS. Six-week-old Wistar rats were immunized by injecting the membrane fraction 3 times at 2-week intervals. After the last injection, immunized splenocytes were fused with mouse myeloma (NS-1) cells according to the method described by Yokoyama (*10*). The supernatants of growing hybridomas were subjected to the first screening by ELISA (enzyme-linked immunosorbent assay) using methanol-fixed MSS31, MSS62 (*4*), and BALB/3T3 fibroblast cells (cultured in DMEM supplemented with 10% FBS), as a negative control. Approximately 100 MSS31-positive, MSS62-positive, but BALB/3T3-negative hybridomas were selected from among approximately 4,000 hybridoma cells. After the second screening by immunostaining of adult spleen tissue slices, 3 hybridomas were selected after 2 successive clonings. To purify 11D MAb from culture supernatant, it was recovered by ammonium sulfate precipitation followed by affinity chromatography on Protein G-Sepharose (Pharmacia, Uppsala, Sweden).

Immunostaining with 11D MAb—To observe the expression of the antigen of 11D MAb in various hematopoietic tissues, yolk sacs, fetal livers, spleens, and whole embryos were prepared from C57BL/6 mice. To prepare anemic mice, 0.1 ml 0.8% phenylhydrazine hydrochloride per adult mouse was injected intramuscularly for 6 days. Two days after the final injection, anemic spleens were prepared for immunohistochemistry. All tissues were fixed in periodate-lysine-4% paraformaldehyde (PLP) fixative for 1 h at 4°C. The tissues were dehydrated in a graded ethanol series, and then embedded in paraffin. Deparaffinized sections were immersed in methanol containing 0.3% hydrogen peroxide for 30 min to inactivate endogenous peroxidase and then incubated with 3% skim milk in PBS to block nonspecific protein binding. The sections were then incubated with 11D rat MAb (anti-SMAP-1) for 1 h at room temperature, followed by rinsing with 0.02% Tween-20 containing PBS (T-PBS). Then, the sections were incubated with biotinylated anti-rat IgG (ZYMED, CA) for 1 h at room temperature. After rinsing with T-PBS, horseradish peroxidase (HRP) conjugated streptavidin (VECTOR Laboratories, Burlingame, CA) was applied for 1 h at room temperature. After a final wash with T-PBS, the presence of the antigen was revealed by incubation with 0.5 mg/ml DAB (3,3′-diaminobenzidine), and 0.03% H_2O_2 in PBS. All sections were counter-stained with methyl green.

Western Blotting—To prepare whole cell lysates of MSS62 stromal cells, murine erythroleukemia (MEL B8/3) cells, or adult tissues of C57BL/6J mice, the cells were lysed with 5 volumes of a lysis buffer comprising 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, and 1 mM PMSF, and homogenized with 10-15 strokes in a Dounce homogenizer on ice. The lysates were recovered by centrifugation at 12,000 rpm for 1 min. For *N*-glycanase digestion, 480 μg protein of a MSS62 cell lysate was diluted with phosphate buffer (pH 8.0) to 0.5 ml and 3 U of *N*-glycanase (Genzyme, Boston, MA) was added. After incubation at 37°C for 2 h, the reaction was stopped by the addition of the SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM DTT, and 10% glycerol). After boiling for 1 min, the protein fractions were applied to a 7.5% SDS-PAGE gel and then electrophoresed. The proteins were electrophoretically blotted onto nitrocellulose filters. The membranes were soaked in PBS containing 3% skim milk for 1 h and then incubated for 1 h with 0.5 μg/ml of 11D MAb. The filters were washed with T-PBS and incubated with HRP-conjugated goat IgG fraction against rat IgG for 1 h. Excess second antibodies were washed off with T-PBS and the proteins were visualized with ECL Western blotting detection reagents (Amersham, UK).

cDNA Cloning Using Expression cDNA Libraries of MEL Cells and Mouse Brain—The construction of the murine erythroleukemia (MEL) cell cDNA library in λgt11 was previously reported (*11*), and it contained 2×10^6 independent clones. The library was plated at 2×10^5 phage colonies on the $Y1090^{r-}$ bacterial strain. After incubation at 42°C for 3.5 h, 10 mM IPTG (β-D-thiogalactopyranoside)-impregnated nitrocellulose filters were overlaid on the agar plates for 3.5 h at 37°C, followed by further incubation of the IPTG-impregnated filters to obtain duplicates for 5 h at 37°C. The filters were rinsed with TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.02% Tween-20), followed by incubation with 5% skim milk in TBS-T for 30 min at room temperature. The filters were then incubated with MAb 11D (0.5 μg/ml) overnight at 4°C. After washing with TBS-T for 25 min, the filters were incubated with the HRP-conjugated goat IgG fraction against rat IgG overnight at 4°C, followed by washing with TBS-T for 15 min. Positive plaques were detected with ECL Western blotting detection reagents. A mouse (BALB/c) brain cDNA library in Uni-ZAP XR (STRATAGENE, La Jolla, CA), and a MEL cell cDNA library in λgt11 were used for further screening. A positive phage was subcloned into the Bluescript vector for restriction enzyme analysis and nucleotide sequencing. DNA sequencing was performed with an automatic sequencer (Pharmacia Biotech, Tokyo).

mRNA Detection by RT-PCR and Northern Blot Hybridization—To detect *smap-1* mRNA, RNAs were extracted from tissues and cell lines by means of the guanidine isocyanate method. Ten micrograms of total RNA was separated by agarose gel electrophoresis and then transferred to a nylon filter. The filter was hybridized with the ^{32}P-labeled *smap-1* cDNA probe and autoradiographed after washing. The intensities of the bands were measured with a Fuji image analyzer (BAS2000). To detect differentially spliced forms of *smap-1* mRNA from brain, MEL cells, and MSS62 cells, the RT-PCR method was used. Total RNAs were reverse-transcribed with MLV-reverse transcriptase and then the cDNAs were amplified with Taq DNA polymerase using two primers, 5′-CTGAGGGAGGAGGACAACAAGTAC-3′ and 3′-TGGACGATGGGACAGATGGCAATG-5′. The amplified dsDNAs were analyzed by agarose gel electrophoresis.

Transfection of SMAP-1 cDNA into MSS62 Stromal Cells—SMAP-1 cDNA (2.4 kb) was inserted into pcDNA3 (Invitrogen, San Diego, CA) at the *Bam*HI site under transcriptional control of the cytomegalovirus promoter and enhancer. The orientation of the cDNA as to the promoter was examined by digestion with *Hpa*I/*Hind*III. The plasmids containing sense and antisense cDNA were isolated with a Wizard column system (Promega, Madison, WI), and then digested with *Bgl*II. The linearized plasmid

DNA was dissolved in 0.25 M $CaCl_2$ and precipitated by the addition of the same volume of a buffer comprising 0.28 M NaCl, 1.48 mM Na_2HPO_4, and 50 mM HEPES (pH 7.05). The precipitated DNA (40 μg DNA/90 mm dish) was added to a culture of MSS62 cells (4×10^6 cells), followed by incubation for 16 h at 37°C. After washing with PBS, G418 (Sigma, St. Louis, MO) selection was started the next day with 400 μg/ml for the first week and 500 μg/ml for the second week. Ten independent clones for sense-plasmid transfectants and antisense-plasmid transfectants, respectively, were obtained. Four clones for each transfectant were used for further analysis.

Assaying of the Erythropoietic Stimulatory Activities of the Transfectants—Cocultivation of erythroid progenitor cells on stromal cells was performed as described previously (*4, 9*). For the stroma-dependent colony formation of transfected or non-transfected MSS62 cells, 2×10^4 feeder cells were seeded to form a monolayer in a 24-well-plate a day before cocultivation. Erythroid progenitors prepared from the livers of 13 day gestation ICR mice were suspended in Iscove's modified Dulbecco's modification of Eagle's medium (IMDM; Gibco, Grand Island, NY) containing 30% heat-inactivated FBS, 0.4% methylcellulose, 1% BSA, 100 μM 2-mercaptoethanol, and 0.1 U/ml Epo (recombinant erythropoietin; generously supplied by Kirin, Tokyo). After 4 days incubation, the cultures were directly stained with a 1/10 volume of a benzidine solution (12.9% acetic acid, 0.43% benzidine, and 4.3% hydrogen peroxide) to detect hemoglobin-producing cells.

RESULTS

Screening of Red-Pulp Specific Monoclonal Antibodies and Their Characterization—To obtain monoclonal antibodies against surface molecules of the mouse spleen stromal cell line, MSS31, crude membrane fractions of MSS31 cells were injected into rats. After two successive injections, the splenic cells from the immunized rats were hybridized with mouse myeloma (NS-1) cells. Approximately 2,000 antibody-producing hybrid cell clones were obtained, and then MSS31 and MSS62-positive, BALB 3T3-cell-negative clones were selected by ELISA assay. Using antibodies produced by 100 such clones, immunohistochemical screening of frozen sections of adult mouse spleen was performed. One of these monoclonal antibodies, 11D, specifically stained the red pulp of spleen in which erythropoiesis is predominant (see Fig. 4).

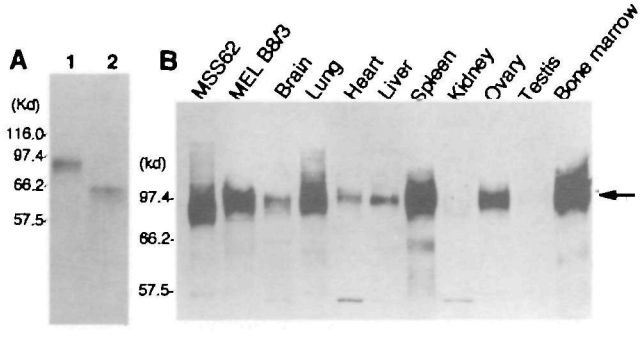

Fig. 1. **Expression of the protein recognized by 11D MAb.** (A) Glycanase-treated and -untreated membrane fractions of MSS62 cells were subjected to SDS-PAGE and Western blotting with 11D MAb. Lane 1, MSS62 membrane fraction; lane 2, glycanase-treated membrane fraction. On glycanase treatment, the 90 kDa protein became a 66 kDa protein. (B) Lysates of mouse tissues and cell lines were subjected to SDS-PAGE and Western blotting with 11D MAb. The arrow indicates the major broad band detected for many tissues and cells.

Analysis of Surface Molecules Recognized by the 11D Antibody—We then identified the surface molecule(s) recognized by the 11D antibody by Western blotting of cell lysates of MSS62 cells. 11D recognized a 90 kDa protein and when the proteins were pretreated with *N*-glycanase, the 90 kDa band shifted to the position of a 66 kDa protein, indicating that 11D recognized the protein moiety of the 90 kDa glycoprotein (Fig. 1A). Western blotting (Fig. 1B) showed that a broad band corresponding to 90 kDa was most abundant for hematopoietic organs such as spleen and bone marrow, rather abundant in lung, and ovary, but less abundant in testis, kidney, heart, and brain. Among the cell lines, expression was only detectable in mouse erythroleukemia (MEL) cells.

Cloning of the Gene Encoding the Protein Recognized by the 11D Antibody—Since 11D recognized the protein moiety of the 90 kDa glycoprotein and was expressed in MEL cells, whose cDNA expression library has previously been constructed (*11*), we tried to clone the gene encoding this protein from cDNA expression library of MEL cells by immunoscreening. Among 6×10^5 clones, we found one positive one. Because this clone did not contain the complete open reading frame (ORF) of the 5′-terminus, we rescreened the clones from the MEL cell cDNA library and a mouse brain cDNA library. We obtained a 2.4 kb overlapping clone from MEL cell cDNA library containing the complete ORF and named it *smap* (stromal membrane-associated protein)-*1*. The nucleotide sequence of *smap-1* cDNA from the MEL cell cDNA library comprised 2,318 bases and its ORF was 440 aa residues long (Fig. 2A).

The hydropathy profile of the protein (Fig. 2B) indicated that it contains a short hydrophobic region that may be a possible transmembrane region near the N-terminus, but it does not contain an N-terminal hydrophobic region indicating a signal peptide sequence. Thus, it may be a type II membrane protein whose C-terminus is extracellularly located. Within the extracellular region, 3 *N*-glycosylation sites (indicated in Fig. 2C) corresponding to its glycoprotein nature and 3 protein phosphorylation sites (dotted amino acids in Fig. 2A) were found.

Although the *smap-1* gene and its predicted protein did not show complete homology with any known proteins in gene databanks, we found a stretch of similarity with previously reported sequences. Between aa residues 139 and 193, we found KE (lysine and glutamine) rich sequences that exhibited homology with human microtubule-associated proteins [MAP1A (*12*) and MAP1B (*13*)], yeast centromere binding protein (CBF5p) (*12*), a possible vesicle-associated protein of Torpedo (Torp1) (*14*), and a malaria antigen protein (*15, 16*) (Fig. 2D). In addition, *smap-1* showed a stretch of similarity near and within the possible transmembrane domain (21-79 aa residues) with yeast GCS-1 protein (*17*), YIE4, GLO3, and GST1 (*18*), the homology region of which contained a Cys-Cys Zn-finger motif (Fig. 2E). We also found homology in the nucleotide sequence that had been reported in the databanks as pieces of unidentified sequences of *H. sapiens*, *M.*

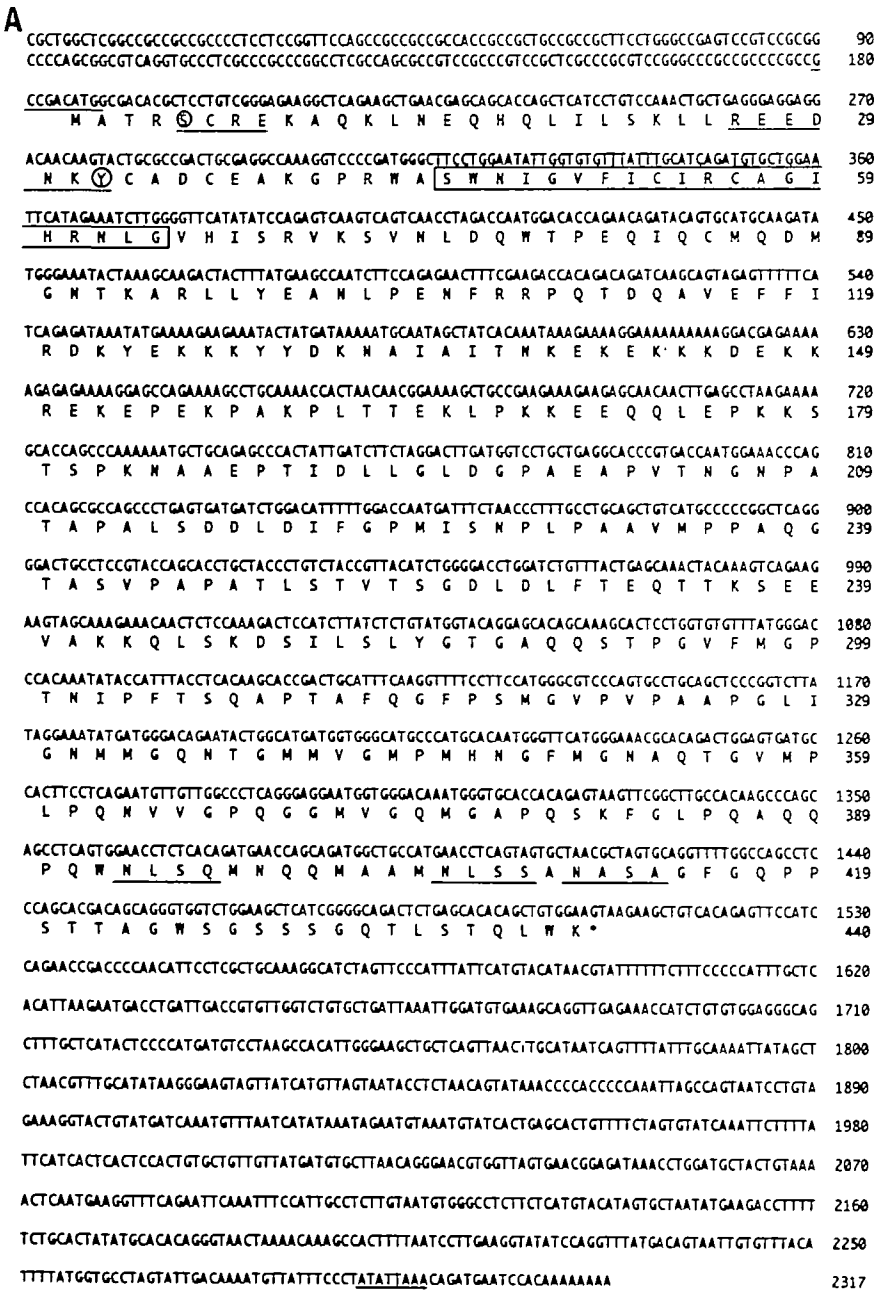

Fig. 2 (continued on next page)

muscles, and *C. elegans*, as described in the legend to Fig. 2.

smap-1 cDNA isolated from the mouse brain cDNA library comprised 2,398 bases and its ORF was 467 aa residues long, thus, brain cDNA for *smap-1* contains a 81 nucleotide insertion. We determined the form of mRNA in the MSS62 cell line by RT-PCR, and the result showed that the MEL cell type mRNA was expressed in MSS62 cells (data not shown). To show that the SMAP-1 protein deduced from the cloned cDNA is recognized by the 11D monoclonal antibody, a maltose binding protein (MBP)-SMAP-1 fusion protein was produced in *Escherichia coli* using a plasmid containing MBP fused to a cDNA region encoding possible extracellular region of the protein (aa residues 110 to 440). Detection by Western blotting of the *E. coli* lysate expressing the fusion protein with 11D antibody (data not shown) indicated that the SMAP-1 gene encodes the protein recognized by the 11D antibody. Thus, the SMAP-1 protein recognized by 11D is a type II membrane protein of which the C-terminal part is exposed on the outside of the cells.

Function of the smap-1 Gene Product in Stroma-Supported Erythropoiesis—To determine the function of SMAP-1 of stromal cells in erythropoiesis, we transfected the *smap-1* cDNA in the antisense orientation into MSS62 cells, that were shown to support large erythroid colony formation by epo-responsive fetal liver progenitor cells in the presence of epo (*4, 6*), and examined the effect of its

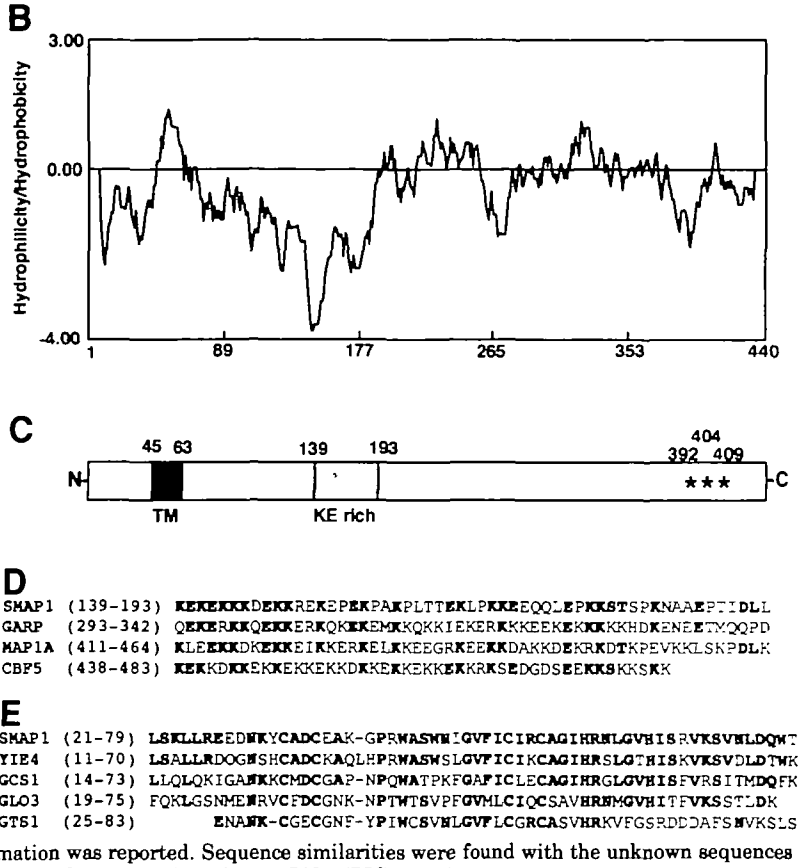

Fig. 2. **Structure of *smap-1* cDNA.** (A) Nucleotide sequence and deduced amino acid sequence of *smap-1*. Upstream, in-frame stop codons that do not exist prior to the initiator ATG and the Kozak consensus sequence located around ATG and the polyadenylation signals are all underlined. The position of translation termination is indicated by an asterisk. The transmembrane domain is boxed and 3 potential *N*-glycosylation sites are underlined within the ORF. Plausible phosphorylation sites are dotted underlined and phosphorylated amino acids are circled. (B) Hydrophobicity profile of the predicted amino acid sequence of SMAP-1. Regions above the line indicate relative hydrophilicity while those below the line indicate relative hydrophobicity. Notice that hydrophilic sequences have positive values in the hydrophilicity plot. (C) Diagram of the sequence elements in SMAP-1. The black box, TM, represents the sequence required for transmembrane. The KE-rich domain located between residues 139 and 193 is shaded, and *N*-glycosylation motifs located in residues 392, 404, and 409 are indicated by asterisks. (D) Alignment of the protein sequences that contain the KKE/D motif. Boldface letters indicate amino acids identical with SMAP-1 (139–193). (E) Alignment of the Zinc finger motifs identified in SMAP-1 and yeast zinc finger proteins. We also found homology in the nucleotide sequence that had been reported in the databanks as pieces of unidentified sequences of *H. sapiens* (HUMOREFc1K, HOMOREFV, EST05924, EST86936, EST02265, yp99c11r1, and a08003s), and *M. muscles* (MTEST 66), and these sequences showed high homology with *smap-1*. A mouse sequence (MTEST 66) was used as a germ cell-specific sequence tag, but no functional information was reported. Sequence similarities were found with the unknown sequences reported for *C. elegans* (CEL0411), *Arabidopsis tharlana* (ATTS3988, 235), and rice cDNA (RICR2884).

reduced level on the stroma-supported erythropoiesis *in vitro*. The levels of *smap-1* mRNA and SMAP-1 proteins were estimated by Northern and Western blotting, respectively (Fig. 3, A and B). The results revealed that 4 antisense cDNA transfectants showed significantly reduced expression (70–30% of the parental cell level), and the reduced expression of SMAP-1 significantly suppressed large erythroid colony formation (Fig. 3C).

Expression of SMAP-1 during Embryonic Development and in Hematopoietic Organs—Because of the functional importance of SMAP-1 in stroma-supported erythropoiesis, we examined its localized expression, using the 11D monoclonal antibody, in erythropoietic organs and in embryos during development (Fig. 4). In hematopoietic organs, SMAP-1 was detected in the endodermal cells and endothelial cell layers, but not in the blood cells of the yolk sac in 9.5 day embryos (Fig. 4C). In the fetal liver, its expression was observed in stromal cells at 10.5 days (Fig. 4, B and E) and the stained area increased until 14.5 days, when erythropoiesis is predominant (Fig. 4F), but it dramatically decreased at 18.5 days when erythropoiesis has ceased (Fig. 4G). At 18.5 days, the expressed stromal cells seemed to be eliminated with the accumulation of liver parenchyma cells and to be condensed around a vein (Fig. 4G). In the fetal liver, some hematopoietic cells seemed to be stained. The change in expression of *smap-1* mRNA during fetal liver development was measured by Northern blot hybridization. Its expression was high and gradually decreased thereafter (Fig. 5), indicating that the level of its expression is well correlated with the erythropoietic activity of the fetal liver. In adult spleen, the red pulp of stromal cell meshwork, and erythroid cells and some reticular cells in the germinal center of the white pulp were also stained, while staining was not observed in the most regions of the white pulp in which lymphopoiesis predominates (Fig. 4, H and I). In the anemic spleen, when anemia was induced by hemolysis with administration of phenylhydrazine, the expanded spleen red pulp regions were strongly stained (Fig. 4J).

Besides the hematopoietic organs, SMAP-1 expression was first seen in 9.5 day mouse embryos. Its expression was localized in both the myocardium and endocardium of the cardial wall (Fig. 4A) and in the limb buds (Fig. 4B), where it was detected only in the apical ectodermal ridge (AER) (Fig. 4D).

DISCUSSION

A hematopoietic inductive microenvironment adequate for the proliferation and differentiation of hematopoietic stem cells and their progenitor cells is thought to be created by stromal cells. However, little is known of how stromal cells create such conditions. We previously developed an *in vitro* erythropoietic microenvironment with stromal cells established from newborn spleen (*4*) and fetal liver (*6*), where the rapid expansion of the epo-reactive erythroid progenitors was promoted by the cell contact with the stromal cells. In the present work, we identified a new cell surface protein which is expressed in the red-pulp of spleen, anticipating its role in erythropoiesis, by raising monoclonal antibodies

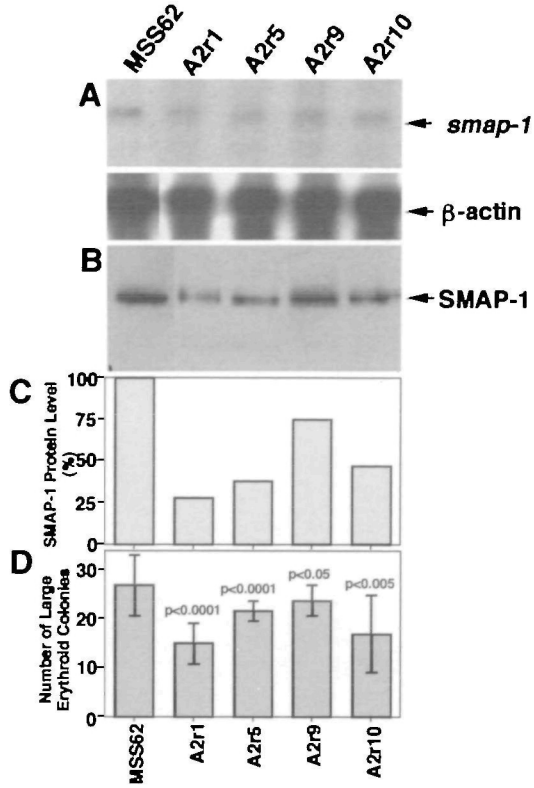

Fig. 3. **Expression of *smap-1* in MSS62 transfectants.** Ten microgram aliquots of total RNA (A) and cell lysates, 10 μg protein (B) of MSS62 cells transfected with *smap-1* in the antisense orientation (A2r1, A2r5, A2r9, and A2r10), and parental MSS62 cells were subjected to Northern blot (A) and Western blotting (B). *smap-1* indicates specific mRNA of *smap-1*, and beta-actin indicates the mRNA content in each lane. SMAP-1 indicates specific protein expression. The SMAP-1 protein level in each transfectant was estimated from the data shown in B. Large erythroid colony formation on each stromal transfectant was performed as described under "MATERIALS AND METHODS." Number of colonies stained positively with benzidine was determined after 4 days and their supporting activities were expressed relative to that of the parental cells (C).

against established spleen stromal cell line, and cloned its cDNA.

The protein, called SMAP-1, which was deduced from the ORF of the cDNA, is a type II membrane glycoprotein. SMAP-1 did not show complete homology with any previously known proteins and thus is a new protein, but we found two interesting regions of similarity with known proteins; direct repeats of KKD/E units were found between aa residues 139 and 193, which exhibit similarity with MAP1A (*19*) and MAP1B (*13*), yeast centromere binding protein (CBF5p) (*12*), an unknown Torpedo protein (Torp1) (*14*), and a malaria antigen protein (Fig. 2D). Two microtubule-binding proteins, MAP1A and MAP1B, contain as a similar domain (KKD/EX repeats), which in the case of MAP1B has been shown to be responsible for the interaction with microtubules both *in vivo* and *in vitro* (*13*). CBF5p is one of the major low-affinity centromere-binding proteins (*12*). This yeast protein contains a repeating KKD/E sequence domain near its C-terminus. The wild type protein was shown to bind to microtubules *in vitro*, whereas a C-terminal devoid of proteins lacking the $(KKD/E)_n$ domain does not. In addition, dividing yeast cells containing a C-terminal truncated CBF5 gene, producing CBF5p containing only three copies of the KKD/E repeat, are delayed with replicated genomes, at the G2/M phase of the cell cycle, while depletion of CFB5p arrests most cells at the G1/S phase: KKD/E repeat units may thus be essential for the function of CFB5p. A similar sequence domain has been found in Torp1, a possible vesicle-associated protein from *Torpedo californica* (*14*), and the malaria proteins, ABRA and p101. ABRA is located on the merozoite surface and within the parasitophorus vacuoles. The KKD/E repeats in Torp1 and ABRA may function in the interaction of these proteins with a membranous structure. Since a surface antigen protein was shown to be required for Plasmodium to home red blood cells, a similar adhesive interaction could occur between the stromal cells and erythroid progenitors.

SMAP-1 also showed a stretch of similarity near and within the possible transmembrane domain (21-79 aa residues) with yeast GCS-1 protein, GLO3 (*17*), and YIE4 and GST1 (*18*), the homology region of which contained a Cys-Cys Zn-finger motif (Fig. 2E). GCS-1, SPS18, and GLO3 constitute a family of novel Zn-finger proteins that mediate the transition from the stationary phase to cell proliferation. A point mutation within the finger motif produces a phenotype that mimics that of deletion of the GCS1, showing that the finger motif is essential for full Gcsp1 activity. The GTS1 gene was isolated as a homologue of the clock-affecting gene, *per*, of *Drosophila melanogaster*, and contains a Gly-Thr/Ser repeat, which is typical of the *per* gene and the finger motif. It affects the timing of budding and the cell size of yeast. The Cys-Cys Zn-finger domain of SMAP-1 similar to that of the yeast protein is also suggestive of protein-protein interaction.

To determine the functional activity of SMAP-1 in erythropoiesis, the antisense *smap-1* cDNA was transfected into MSS62 stromal cells, because the 11D monoclonal antibody and the polyclonal antibodies raised against the bacterially produced SMAP-1 protein were not inhibitory ones when added to the large erythroid colony formation assay, and because it is not possible to determine whether or not the transfection of SMAP-1 into non-supporting cells such as NIH3T3 cells converts them to supporting cells, since the erythroid-supporting activity of stromal cells may require SMAP-1 in combination with other molecules. Expression of the antisense gene in the transformants reduced the SMAP-1 proteins and also reduced the erythropoietic supporting activity. These results showed that SMAP-1 may be involved in the erythropoietic stimulatory activity of stromal cells.

Expression of SMAP-1 in erythropoietic organs was well correlated with the erythropoietic activity of the hematopoietic organs. In the yolk sac, SMAP-1 was detected in the blood vessel endothelial cells, but not in the blood cells (Fig. 4, A and B). Blood vessel formation was first seen in the wall of a yolk sac where the undifferentiated mesenchyme condenses to form angiogenetic clusters and the centers of these clusters form the blood cells, and the outsides of the clusters develop into blood vessel endothelial cells. Cell-to-cell interaction is expected to be important in the generation of erythropoietic cells in a blood island. In the fetal liver, expression of *smap-1* mRNA started to occur on day 11.5 and increased up to day 14.5 and then decreased, thus,

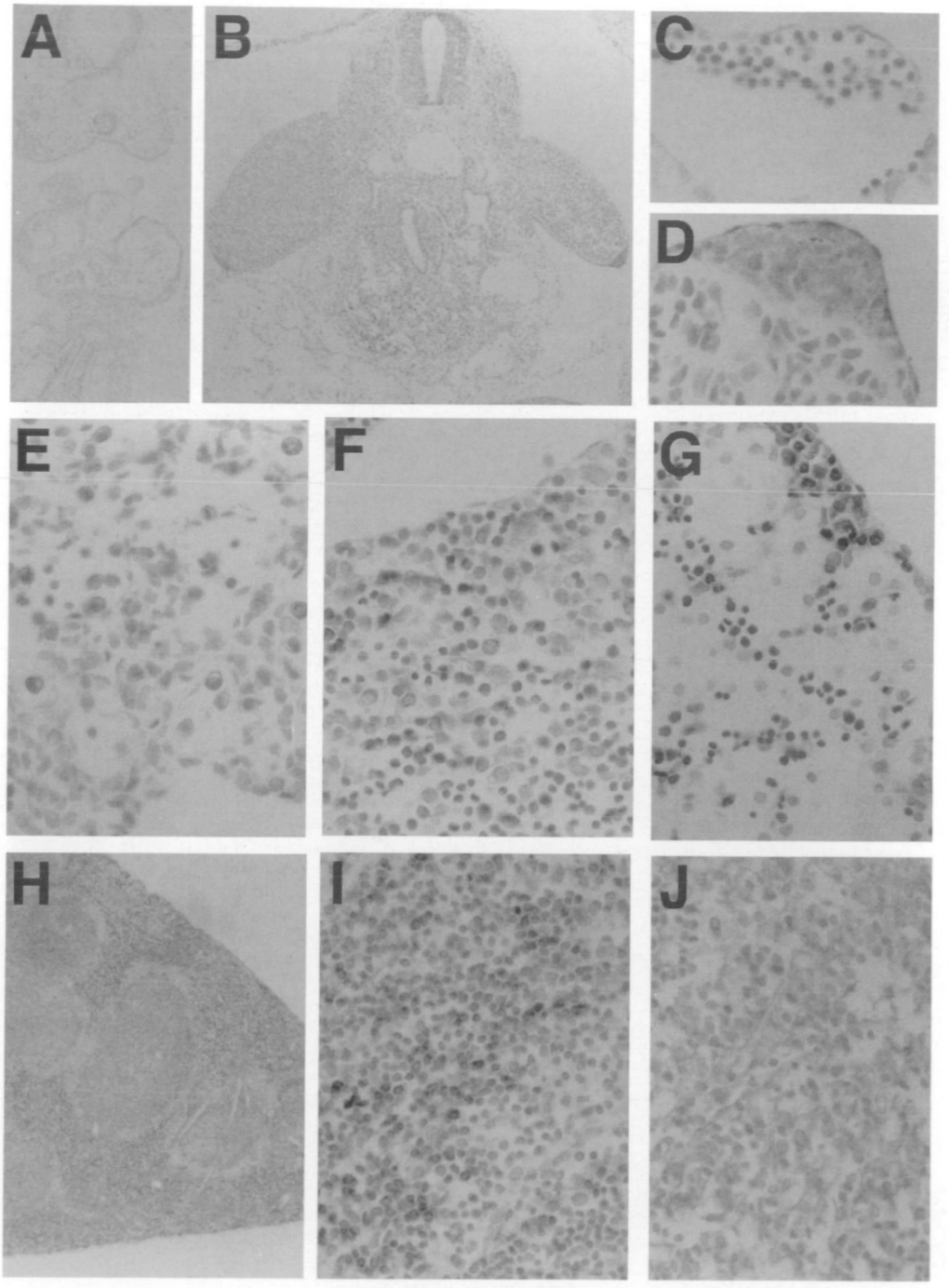

Fig. 4. **Immunohistochemical staining with 11D MAb.** Thin sections of 9.5-days embryo (A), a 10.5-day embryo (B), a yolk sac blood island of 9.5-day embryo (C), the apical ectodermal ridge of limb bud of a 10.5-day embryo (D), fetal livers (E: 10.5-days, F: 13.5-days, and G: 18.5-days), and adult spleen (H, I), and an anemic spleens (J) were, respectively, stained with 11D MAb. Magnification: A, B, and H, ×24; and C, D, E, F, G, I and J, ×192.

the level of SMAP-1 well correlated with the erythropoietic activity of the fetal liver. The number of stromal cells expressing SMAP-1 decreased depending on the expansion of hepatic parenchymal cells in the fetal liver on day 18. In the spleen, its expression is restricted to the red-pulp and the areas of its expression increased depending on the induction of erythropoiesis by hemolysis. In both fetal liver and spleen, it is not clear whether the stromal cell numbers increased or expression of SMAP-1 was induced in the preexisting stromal cells, but it seems that both may occur. It is possible that SMAP-1 is induced in stromal cells by the contact with erythroid cells and such cross-talk may be an interesting mechanism in stroma-supported erythropoiesis.

Considering the functional importance of SMAP-1 in stroma-supported erythropoiesis *in vitro*, SMAP-1 may be a key molecule that induce an erythropoietic microenvironment in hematopoietic organs.

In addition to the importance of SMAP-1 in the erythropoietic activity of hematopoietic organs, the restricted expression of SMAP-1 during embryonic development is intriguing. The regions of SMAP-1 expression were restricted to the limb bud, cardial wall, yolk sac, and liver in mouse embryos. Expression of SMAP-1 in the limb buds is quite interesting because it was detected only in the apical ectodermal ridge (AER), whose inductive effect upon the

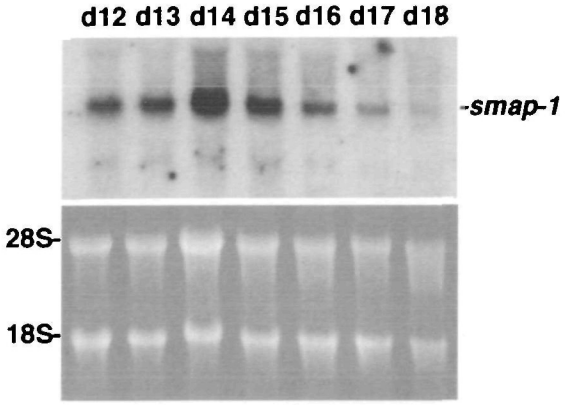

Fig. 5. **Northern blot analysis of *smap-1* mRNA expressed in fetal liver.** Ten micrograms of total RNA from fetal liver, from 12.5 days (d12) to 18.5 days (d18), was electrophoresed and hybridized with the *smap-1* probe. The lower panel shows ethidium bromide staining of total RNAs, and 18S and 28S indicate bands of ribosomal RNA.

underlying mesenchyme has been extensively studied (*20*). In the cardial wall, its expression was detected in both muscle cells and endothelial cells. The presumptive heart cells form a double-walled tube consisting of an inner endocardium and an outer epimyocardium, and fusion of the endocardial tubes occurs to form a single pumping chamber following movement. In the organization of these tissues, an inductive process is expected. Although we previously showed that MSS cell lines exhibit an endothelial nature forming microcapillaries in a collagen gel (*5*) and expression of SMAP-1 was detected in the endothelial cells of the yolk sac, cardial wall, and adult spleen, the blood vessels in other areas of the embryos did not show any expression (data not shown). Thus, SMAP-1 may be expressed at particular stages in endothelial cells in restricted organs rather than being ubiquitously expressed in endothelial cells. The restricted expression of SMAP-1 in these embryonic organs suggests that it may function in the induction of tissue formation during development, and that SMAP-1 may be a useful tool for analysis of the induction of tissue development.

REFERENCES

1. Metcalf, D. and Moore, M.A.S. (1971) Hematopoetic cells. *Front. Biol.* **24**, 172-271
2. Till, J.E. and McCulloch, E.A. (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiation Res.* **14**, 213-222
3. Wolf, N.S. and Trentin, J.J. (1967) Hematopoietic colony studies. V. Effect of hematopoietic organ stroma on differentiation of pluripotent stem cells. *J. Exp. Med.* **127**, 205-214
4. Yanai, N., Matsuya, Y., and Obinata, M. (1989) Spleen stromal cell lines selectively support erythroid colony formation. *Blood* **74**, 2391-2397
5. Yanai, N., Satoh, T., and Obinata, M. (1991) Endothelial cells create a hematopoietic inductive microenvironment preferential to erythropoiesis in the mouse spleen. *Cell Struct. Funct.* **16**, 87-93
6. Ohneda, O., Yanai, N., and Obinata, M. (1990) Microenvironment created by stromal cells is essential for rapid expansion of erythroid cells in mouse fetal liver. *Development* **110**, 379-384
7. Ohneda, O., Yanai, N., and Obinata, M. (1992) Combined action of c-Kit and erythropoietin on erythroid progenitor cells. *Development* **114**, 245-252
8. Kameoka, J., Yanai, N., and Obinata, M. (1995) Bone marrow stromal cells selectively stimulate the rapid expansion of lineage-restricted myeloid progenitors. *J. Cell. Physiol.* **164**, 55-64
9. Yanai, N., Sekine, C.N., Yagita, H., and Obinata, M. (1994) Roles for integrin VLA-4 in stroma-dependent erythropoiesis. *Blood* **83**, 2844-2850
10. Yokoyama, W.M. (1991) Production of monoclonal antibodies in *Current Protocols in Immunology* (Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M., and Strober, W., eds.) UNIT2.5, Greene Publishing Associates and Wiley-Interscience, New York
11. Shoji, W., Inoue, T., Yamamoto, T., and Obinata, M. (1995) MIDA1, a protein associated with Id, regulates cell growth. *J. Biol. Chem.* **270**, 24818-24825
12. Langkopf, A., Hammarback, J.A., Muller, R., Vallee, R.B., and Garner, C.C. (1992) Microtubule-associated proteins 1A and LC2. Two proteins encoded in one messenger RNA. *J. Biol. Chem.* **264**, 16561-16568
13. Noble, M., Lewis, S.A., and Cowan, N.J. (1989) The microtubule binding domain of microtubule-associated protein MAP1B contains a repeated sequence motif unrelated to that of MAP2 and Tau. *J. Cell. Biol.* **109**, 3367-3376
14. Ngsee, M.S., Lewis, A., and Cowan, N.J. (1989) Isolation and characterization of two homologous cDNA clones from *Torpedo* electromotor neurons. *DNA* **8**, 555-561
15. Weber, J.L., Lyon, J.A., Wolff, R.H., Hall, T., Lowell, G.H., and Chulay, J.D. (1988) Primary structure of a *Plasmodium falciparum* malaria antigen located at the merozoite surface and within the parasitophrus vacuole. *J. Biol. Chem.* **263**, 11421-11425
16. Triglia, T., Stahl, H.D., Crewther, P.E., Silva, A., Anders, R.F., and Kemp, D.J. (1988) Structure of a *Plasmodium falciparum* gene that encodes a glutamine-rich protein (GARP). *Mol. Biochem. Parasitol.* **31**, 199-201
17. Ireland, L.S., Johnston, J.C., Dredot, M.A., Dhillon, N., DeMaggio, A.J., Hoekstra, M.F., and Singer, R.A. (1994) A member of a novel family of yeast 'Zn-finger' proteins mediates the translation from stationary phase to cell proliferation. *EMBO J.* **13**, 3812-3821
18. Mitsui, K., Yagushi, S.-I., and Tsurugi, K. (1994) The GTS1 gene, which contains a Gly-Thr repeat, affects the timing of budding and cell size of the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **14**, 5569-5578
19. Jiang, W., Middleton, K., Yoon, H.-J., Fouquet, C., and Carbon, J. (1993) An essential yeast protein, CBF5p, binds in vitro to centromeres and microtubules. *Mol. Cell. Biol.* **13**, 4884-4893
20. Martin, G. (1995) Why thumbs are up. *Nature* **374**, 410-411