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SFA-1, a Novel Cellular Gene Induced by Human T-Cell Leukemia Virus Type 1, Is a Member of the Transmembrane 4 Superfamily

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A novel cellular gene termed SFA-1 was isolated by differential hybridization of a cDNA library, using probes obtained from an adult T-cell leukemia cell line in comparison with probes obtained from normal $CD4^+$ T cells and the MOLT-4 cell line. The mRNA of the SFA-1 gene is approximately 1.6 kb in size and encodes a protein of 253 amino acids, containing four putative transmembrane domains, a number of cysteine residues, and one potential N-glycosylation site in a major hydrophilic region between the third and fourth transmembrane domains. Expression of the SFA-1 gene was either absent or present at a low level in lymphoid cells but was up-regulated after transformation by human T-cell leukemia virus type 1 and transactivated by Tax. SFA-1 was broadly expressed in many human cell types and conserved in different species. Computer-aided comparison showed that SFA-1 had significant sequence homology and common structural features with members of the transmembrane 4 superfamily. SFA-1 antigen was detected as a 29-kDa membrane protein by immunoblotting, using anti-SFA-1 monoclonal antibody.

Human T-cell leukemia virus type 1 (HTLV-1) is an exogenous human retrovirus closely linked with adult T-cell leukemia (ATL). HTLV-I has also been reported to be associated with myelopathy, alveolitis, arthropathy, Sjögren syndrome, and uveitis, which may result from immunologic alterations induced by HTLV-1 infection (11, 41, 43, 46, 58). The HTLV-1 genome encodes a 40-kDa protein, Tax, that functions as a transcriptional transactivator of the viral long terminal repeat and cellular genes. T-cell proliferation and immunologic alterations observed during HTLV-1 infection appear to be due to the effect of Tax on viral and cellular gene expression. Tax stimulates the expression of various cellular genes, including interleukin-2 (IL-2), IL-2 receptor α, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor β, transforming growth factor β, c-*fos*, c-*jun*, and vimentin (11, 24, 28, 34, 40, 52, 57). However, the mechanism of HTLV-1-induced disease still remains to be elucidated.

To examine the changes in $CD4^+$ T cells after HTLV-1 transformation, we have performed differential hybridization of a cDNA library, using probes obtained from an ATL cell line as well as probes obtained from normal $CD4^+$ T cells and the MOLT-4 cell line. By differential screening of this library, a new cDNA clone termed SFA-1 (stands for SF-HT-activated gene 1) was isolated and found to have striking homology with members of the transmembrane 4 superfamily, including CD9, CD37, CD53/OX-44, CD63/ME491, CD81/TAPA-1, CO-029, CD82/C33/R2, A15, SAS, Peripherin, Rom-1, Uroplakin Ia, TI 1, Sm23, and L6 (1, 2, 4, 6, 7, 9, 10, 13, 20, 21, 25, 27, 35, 37, 45, 54, 56, 59–61). In the present report, we describe the cloning and characterization of SFA-1, which was up-regulated by transformation with HTLV-1 and transactivated by Tax.

Human T-cell lines MOLT-4 and Jurkat; a human erythroleukemia cell line, K562; two human myelomonocytoid cell lines, HL-60 and U937; three human carcinoma cell lines, PANC-1 (pancreas), SW1116 (colon adenocarcinoma), and A549 (lung); and NIH 3T3 and COS-1 cells were obtained from the American Type Culture Collection (Rockville, Md.). Three human carcinoma cell lines, A172 (glioblastoma), Caki-1 (renal carcinoma), and Hep G2 (hepatocellular carcinoma), and a human skin fibroblast cell line, ULEH, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). JPX-9 and JPX/M cells (38) were kindly provided by K. Sugamura and M. Nakamura, Tohoku University, Sendai, Japan. SF-EB and HH-EB were Epstein-Barr virus-transformed B lymphoblastoid cell lines. HTLV-1-transformed T-cell lines MT-2, MT-4, and KN6-HT (23) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO BRL, Gaithersburg, Md.). SF-HT, a leukemic cell line established from an ATL patient, was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 0.5 U of human recombinant IL-2 (Takeda Chemical Industries, Osaka, Japan) per ml.

Peripheral blood mononuclear cells were isolated from healthy individuals by Ficoll-Conray gradient centrifugation. Normal T and B cells were separated by the sheep erythrocyte-rosetting method. Human monocytes were prepared by Nycodenz Monocyte (NYCOMED AS, Oslo, Norway) density centrifugation as described previously (16). $CD4^+$ T cells were enriched by treatment with OKT8 (CD8) (Ortho Diagnostic Systems, Raritan, N.J.), NKH1A (CD56) (Coulter, Hialeah, Fla.), Leu11b (CD16) (Becton Dickinson, Mountain View, Calif.), and low-toxic rabbit C (Cedarlane, Hornby, Ontario, Canada) as described previously (17). The $CD4^+$ T cells, which were cultured in RPMI 1640 medium with 10% fetal calf serum for 4 days after removal of phytohemagglutinin (PHA; GIBCO BRL) and IL-2, were used for the experiment. PHA- and IL-2-stimulated $CD4^+$ T cells were harvested at 24 and 12 h after the addition of 1 μg of PHA and 0.5 U of IL-2 per ml, respectively.

Total cellular RNA was extracted from the cells by the acid phenol-guanidinium isothiocyanate extraction method as described previously (19). poly$(A)^+$ RNA from SF-HT cells was selected by oligo(dT) cellulose chromatography and then used to contruct a cDNA library in the pcD-SRα expression vector system (55). Approximately 5×10^4 clones were screened by

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colony hybridization. For the subtractive probe preparation, single-stranded ^{32}P-labeled cDNA probes were prepared from 10 μg of poly(A)$^+$ RNA isolated from SF-HT cells, CD4$^+$ T cells, or MOLT-4 cells with random DNA hexamers (Takara Shuzo, Kyoto, Japan), using the avian myeloblastosis virus reverse transcriptase system (GIBCO BRL). Colonies that hybridized strongly only with the probe from SF-HT cells were isolated for further analysis. Northern (RNA) blot analysis was performed as described previously (15). The DNA probes used were a 0.9-kb *Bam*HI fragment of plasmid pcDSRαSFA-1 [nucleotides 595 to 3′ poly(A) tail in the SFA-1 gene] and a 0.4-kb human β-actin cDNA (Wako Pure Chemical Industries, Osaka, Japan). Nucleotide sequencing was performed by the dideoxynucleotide chain termination method (49). The two *Bam*HI DNA fragments (0.7 and 0.9 kb) from plasmid pcDSRαSFA-1 were cloned into the pUC18 vector. Nested deletions were generated in both directions by a deletion kit with exonuclease III-mung bean nuclease (Takara Shuzo). The full-length clones and subclones with overlapping deletions were sequenced in both directions with the AutoRead Sequencing kit (Pharmacia P-L Biochemicals Inc., Milwaukee, Wis.). Computer prediction of the nucleotide sequence and the secondary structure of the SFA-1 protein was performed with the Macvector system (International Biotechnologies, Inc., New Haven, Conn.) and DNASIS software (Hitachi Software Engineering Co., Ltd., Yokohama, Japan).

A recombinant plasmid pL2neoSRαIIISFA-1 was constructed by inserting the 2.5-kb *Sal*I fragment of pcDSRαSFA-1 into the *Sal*I site of the pL2neoSRαIII vector (55). NIH 3T3 cells were transfected with 10 μg of pL2neoSRαIIISFA-1 with Lipofectin (GIBCO BRL) and by following the instructions of the manufacturer. The stable transformants (NIH 3T3/pL2neoSRαIII SFA-1) were selected with 400 μg of G418 (GIBCO BRL) per ml, and the expression of SFA-1 was determined by Northern blot analysis or flow cytometric analysis. Monoclonal antibodies were produced by hybridoma technology as described previously (18). In brief, hybridomas were produced through the fusion of P3X63Ag8.653 cells with spleen cells from BALB/c mice immunized against the NIH 3T3/pL2neoSRαIIISFA-1 cells. The hybridoma culture supernatants which bound to NIH 3T3/pL2neoSRαIIISFA-1 cells but not to NIH 3T3/pL2neoSRαIII cells were screened. After cloning, the antibody-producing hybridomas were inoculated into BALB/c mice treated previously with pristane (Aldrich, Milwaukee, Wis.). The monoclonal antibody from the ascitic fluid was purified by affinity chromatography on a DEAE column. Immunoblotting and flow cytometric analysis were performed as described previously (18). HTLV-1 antiserum was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

The cDNA clones of RNA from an ATL cell line, SF-HT, were differentially screened as assessed by greater hybridization with the SF-HT cDNA probe than with normal CD4$^+$ T-cell and MOLT-4 cDNA probes. Thirty-two cDNA clones differentially hybridized to the SF-HT cDNA probe on the first screening of about 5×10^4 clones. Rescreening and additional Northern blot analysis yielded eight HTLV-1-induced cellular genes. Searches of the GenBank and EMBL databases, using the sequences of the eight clones, identified macrophage inflammatory protein-1α, transforming growth factor β, c-*jun*, vimentin, and four unknown genes. Of these, one clone, designated SFA-1, was analyzed further.

The expression of the SFA-1 gene in various hematopoietic and nonhematopoietic cell lines was evaluated by hybridizing the SFA-1 or β-actin probe with blots of lymphocytes or cell line RNAs (Fig. 1). The mRNA size of the SFA-1 gene was found to be approximately 1.6 kb. Expression of SFA-1 was increased by more than threefold in the ATL cell line SF-HT and in three HTLV-1-transformed T-cell lines (KN6-HT, MT-2, and MT-4) compared with the level seen in CD4$^+$ T cells. SFA-1 mRNA was either absent or present at low levels in T lymphocytes, B lymphocytes, and four other lymphoid cell lines—Jurkat, MOLT-4, SF-EB, and HH-EB—while monocytes, K562, and two myelomonocytoid cell lines (U937 and HL-60) showed significant expression of SFA-1. The gene was also induced in PHA-stimulated CD4$^+$ T cells but not by IL-2 stimulation. As shown in Fig. 1C, transcription of the SFA-1 gene was detected in various human nonhematopoietic cell lines: A172, Caki-1, PANC-1, Hep G2, SW1116, A549, and ULEH, though there were some differences in the level of expression. SFA-1 was also expressed in simian COS-1 cells.

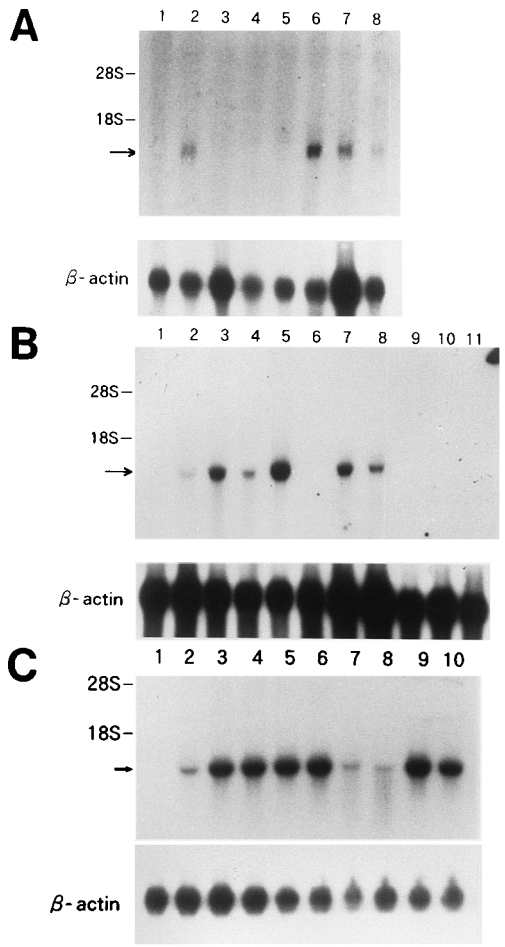

FIG. 1. (A) Expression of the SFA-1 gene in hematopoietic cells. Total RNA (10 μg) was size fractionated on formaldehyde agarose gels, transferred to charged nylon membranes, and hybridized with an SFA-1 or β-actin cDNA probe. RNA samples are as follows: normal CD4$^+$ T cells (lane 1), SF-HT cells (lane 2), normal B cells (lane 3), SF-EB cells (lane 4), HH-EB cells (lane 5), U937 cells (lane 6), K562 cells (lane 7), and HL-60 cells (lane 8). (B) Expression of the SFA-1 gene in monocytes and various T-cell lines. Lane 1, normal CD4$^+$ T cells; lane 2, SF-HT; lane 3, KN6-HT; lane 4, MT-2; lane 5, MT-4; lane 6, normal T cells; lane 7, monocytes; lane 8, PHA (1 μg/ml)-stimulated CD4$^+$ T cells; lane 9, IL-2 (0.5 U/ml)-stimulated CD4$^+$ T cells; lane 10, MOLT-4; lane 11, Jurkat. (C) Expression of the SFA-1 gene in nonhematopoietic cell lines. Lane 1, normal CD4$^+$ T cells; lane 2, SF-HT; lane 3, A172 (glioblastoma); lane 4, Caki-1 (renal carcinoma); lane 5, PANC-1 (pancreatic carcinoma); lane 6, Hep G2 (hepatocellular carcinoma); lane 7, SW1116 (colon cancer); lane 8, A549 (lung cancer); lane 9, ULEH (skin fibroblast); lane 10, COS-1.

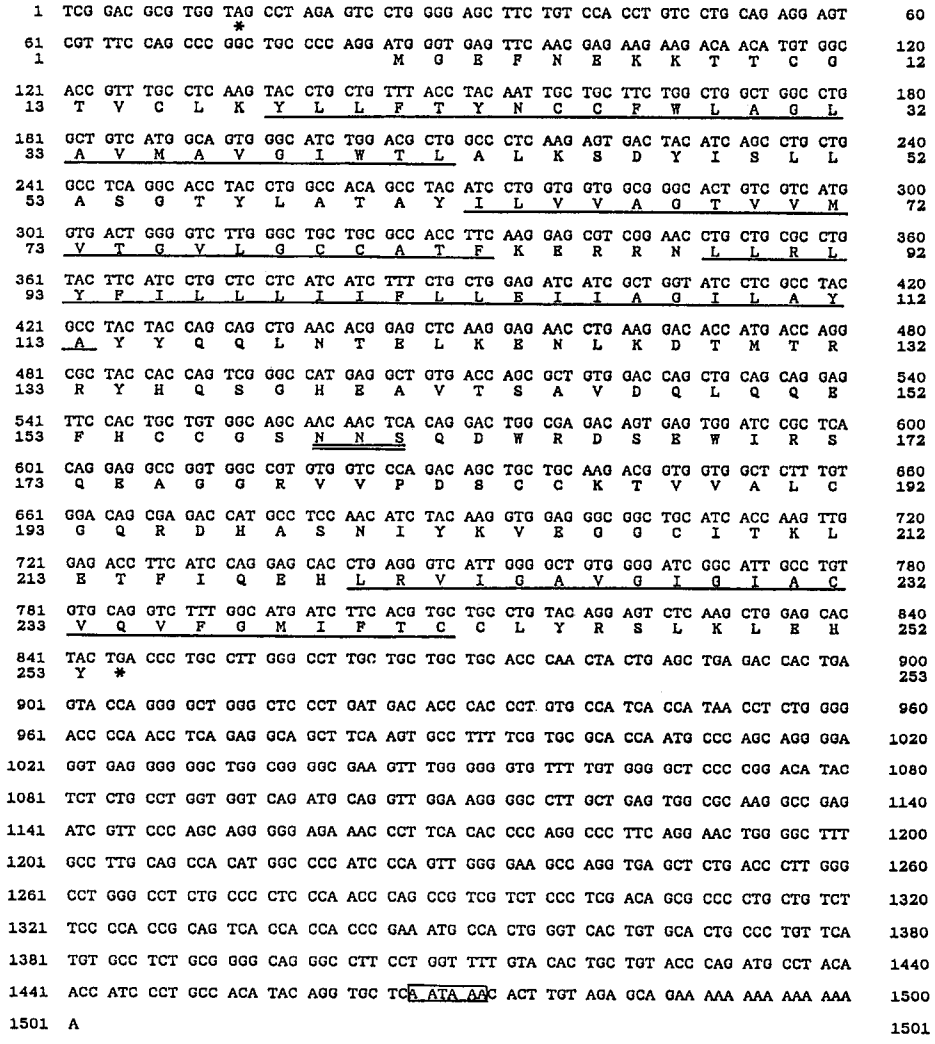

FIG. 2. Nucleotide and deduced amino acid sequences of the SFA-1 cDNA clone. The predicted amino acid sequence is shown below the nucleotide sequence, and the amino acid numbers start at the initiation methionine. The putative transmembrane domains are single underlined. A potential N-linked glycosylation site is double underlined. In-frame stop codons are indicated by asterisks. A potential polyadenylation signal, AATAAA, is boxed. For nucleotide sequence accession number information, see the text.

The complete nucleotide and deduced amino acid sequences of the SFA-1 gene are shown in Fig. 2. The SFA-1 gene (Fig. 2) is nearly full length, since it is close to the expected size. The 3′ untranslated region contains a potential polyadenylation signal, AATAAA. Translation probably starts from the ATG sequence at nucleotides 85 to 87 which conforms to a consensus translational initiation sequence (30). An in-frame stop codon at nucleotides 13 to 15 is also consistent with translational initiation at nucleotides 85 to 87. The SFA-1 gene has a 759-nucleotide open reading frame and encodes a protein consisting of 253 amino acids with a calculated molecular weight of 27,800. The Kyte-Doolittle hydrophilicity plot analysis (31) shows that this protein has four putative transmembrane domains, three consecutive ones at the amino-terminal site and one at the carboxyl-terminal site. One potential N-glycosylation site is located in a major hydrophilic region between the third and fourth transmembrane domains. Both the amino- and carboxyl-terminal domains are intracellular. This major extracellular hydrophilic region contains a number of cysteine residues probably involved in S-S bonding.

A search of the NR-AA protein database revealed significant homology of the SFA-1 protein with 15 recently described molecules. Similar values of identity and similarity were found in cross-comparisons with the human proteins CD9 (7), CD37 (9), CD53 (1, 2), CD63/ME491 (20), CD81/TAPA-1 (45), CO-029 (54), CD82/C33/R2 (13, 21), A15 (10), SAS (25), Peripherin (56), Rom-1 (4), Uroplakin Ia (61), TI 1 (27), and L6 (35), and with the following proteins in other species: mouse TAPA-1 (33), rat CD37 (9), OX-44 (rat CD53) (6), and the integral membrane protein of *Schistosoma mansoni*, Sm23 (13) (data not shown). Characteristic features of these proteins are comparable size (202 to 351 amino acids) and nearly identical profiles and spacing in the Kyte-Doolittle hydrophilicity plot. Three consecutive hydrophobic domains, probably transmembrane domains, are clustered at the amino-terminal site, followed by a single hydrophilic domain and a fourth potential transmembrane domain at the carboxyl-terminal site. The most pronounced similarity between these members is seen within the putative transmembrane regions. This alignment also holds true for the distribution and position of the cysteine residues. From 15 cysteines in SFA-1, three are conserved in all members except L6 and three are identical in at least 7 of the 16

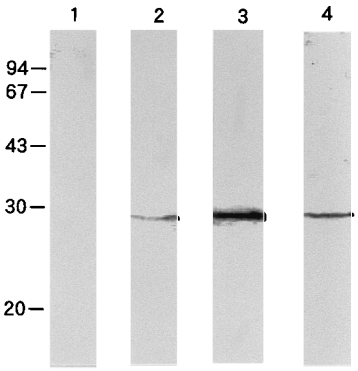

FIG. 3. Immunodetection of SFA-1 antigen in various cells. Cell lysates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblotting with SFA1.2B4 antibody. Lane 1, NIH 3T3 cells; lane 2, NIH 3T3/pL2neoSRαIIISFA-1 cells; lane 3, SF-HT cells; lane 4, MT-4 cells.

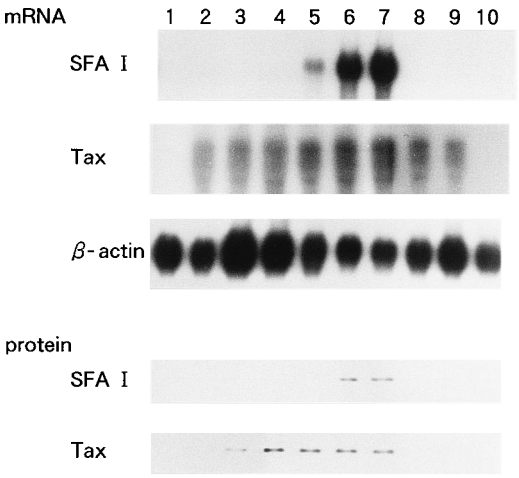

FIG. 4. Kinetics of induction of SFA-1 by Tax in JPX-9 cells. The gene expressions were performed by Northern blot analysis with the SFA-1, Tax, or β-actin probe. The protein levels of SFA-1 or Tax were examined by immunoblotting with SFA1.2B4 or HTLV-1 antiserum. The JPX-9 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 μM $CdCl_2$ and harvested at 0, 2, 4, 6, 8, 10, and 24 h after the addition of $CdCl_2$ (lanes 1 to 7, respectively). JPX/M cells were harvested at 10 h (lane 8) and 24 h (lane 9) after the addition of $CdCl_2$, while Jurkat cells (lane 10) were harvested at 24 h.

members of this family. Several short sequence motifs, such as the CCG at residues 155 to 157 in SFA-1, were also conserved. However, the major hydrophilic domain in each protein, which in SFA-1 is about 106 amino acids long, is more variable both in homology and in length. The amino acid variation and length difference in this region may reflect the interaction with different ligands or other proteins. Several potential N-glycosylation sites are also located in this region. These characteristics suggest that SFA-1 is another member of this transmembrane protein family.

To use transformant as an immunogen, we transfected the recombinant plasmid pL2neoSRαIIISFA-1 into NIH 3T3 cells. NIH 3T3 cells did not express any detectable levels of mRNA for SFA-1. The stable neomycin-resistant transformant (NIH 3T3/pL2neoSRαIIISFA-1) was isolated and expressed a significant level of SFA-1 mRNA (data not shown). After injection of the NIH 3T3/pL2neoSRαIIISFA-1 cells intraperitoneally twice, hybridomas were produced through the fusion of P3X63Ag8.653 cells with spleen cells from immunized BALB/c mice. One monoclonal antibody, designated SFA1.2B4, which bound to NIH 3T3/pL2neoSRαIIISFA-1 cells but not to NIH 3T3/pL2neoSRαIII cells, was obtained. We confirmed that this antibody reacted to SFA-1 by immunoblotting with histidine-tagged SFA-1 protein (data not shown). The surface expression of the SFA-1 antigen on various hematopoietic and nonhematopoietic cell lines was examined by flow cytometric analysis with SFA1.2B4. Strong expression of SFA-1 antigen was demonstrated on all four HTLV-1-transformed T-cell lines and PHA-stimulated $CD4^+$ T cells. SFA-1 antigen was expressed at low levels on $CD4^+$ T cells, T lymphocytes, B lymphocytes, granulocytes, and four other lymphoid cell lines—Jurkat, MOLT-4, SF-EB, and HH-EB—while monocytes, U937, and all human nonhematopoietic cell lines used in this study showed significant expression of SFA-1. These results are in good agreement with those obtained by Northern blot analysis (data not shown). By immunoblotting (Fig. 3), the SFA-1 antigen detected by SFA1.2B4 was a 29-kDa cell surface protein.

To analyze whether the SFA-1 gene is transactivated by Tax, we examined the kinetics of gene expression and protein levels of SFA-1 in JPX-9 cells. JPX-9 and JPX/M cells are transformants of Jurkat with the plasmid pMAXRHneo-1 containing the metallothionein promoter-driven Tax gene and with the mutant plasmid pMAXneo/M in which the frameshift mutation is introduced in the coding region of Tax gene and incapable of producing the functional Tax, respectively (38). Tax in JPX-9 cells is undetectable at the mRNA and protein levels without stimulation and can be induced by the addition of heavy-metal ions to the culture medium. As shown in Fig. 4, JPX-9 cells were harvested at the indicated times after the addition of 10 μM $CdCl_2$. Unstimulated JPX-9 cells did not express mRNA and protein of SFA-1 and Tax at detectable levels. The expression of mRNA and protein of SFA-1 increased after 6 to 8 h and after 8 to 10 h of stimulation, respectively, while mRNA and protein of Tax were detected after 2 and 4 h, respectively. Accordingly, activation of the SFA-1 gene was observed immediately (at least within several hours) after expression of the Tax protein. In contrast, SFA-1 expression was not induced by the addition of $CdCl_2$ in parental Jurkat cells and control JPX/M cells. This result indicated that SFA-1 was transactivated by Tax. It is, however, still unknown whether Tax transactivates the SFA-1 promoter directly, since the SFA-1 promoter has not been cloned. On the other hand, SFA-1 expression was not limited to Tax-containing cell lines, since SFA-1 was also expressed in non-Tax-containing cell lines such as K562 and U937. This finding suggested that SFA-1 expression was induced not only by Tax but also through other signalling pathways.

Although the biological functions of the transmembrane 4 superfamily are largely unknown, several studies of their function have been undertaken by using specific monoclonal antibodies (1, 3, 5, 8, 12, 14, 20–22, 26, 27, 29, 32, 36, 39, 44, 45, 47, 48, 50, 51, 54). These studies suggest a role for this superfamily in the regulation of cell development, proliferation, activation, and motility. CD9 regulated cell activation and aggregation through an association with β1 integrins (36, 48). Furthermore, CD9 regulated cell motility in a variety of cell lines (39). Anti-CD9 antibodies could enhance adhesion and proliferation of Schwann cells (3, 14). Three members of the family, CD37, CD53, and CD81/TAPA-1, may be also involved in the control of cell proliferation (20, 32, 45, 50). CD53/OX-44 and CD82/C33/R2 are also important in regulating signalling processes (1, 5, 22, 44, 47). CD82/C33/R2 is expressed on most hematopoietic and nonhematopoietic human cell lines, including

HTLV-1-positive T cell lines, and this expression is up-regulated in activated T cells (12, 13, 21). Anti-C33 antibodies inhibited HTLV-1-induced syncytium formation (12). SFA-1 has expression patterns similar to those of CD82/C33/R2 or CD9 and may therefore be a cell surface molecule involved in the process of HTLV-1-induced syncytium formation, cell proliferation, or motility, which in turn may affect transmission of HTLV-1 or growth and metastasis of the ATL cells. Further studies of the biological functions of the SFA-1 protein, using monoclonal antibodies, are now in progress.

Nucleotide sequence accession number. The nucleotide sequence data for SFA-1 appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D29963.

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