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Molecular Cloning of a Novel Mitogen-Inducible Nuclear Protein with a Ran GTPase-Activating Domain That Affects Cell Cycle Progression

MASAKAZU HATTORI,[1] NORIYUKI TSUKAMOTO,[1] M. S. A. NUR-E-KAMAL,[2] BONNEE RUBINFELD,[3] KAZUHIRO IWAI,[1] HIROSHI KUBOTA,[1] HIROSHI MARUTA,[2] and NAGAHIRO MINATO[1]*

Department of Immunology and Cell Biology, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan[1]; Ludwig Institute for Cancer Research, Melbourne, Victoria 3050, Australia[2]; and Onyx Pharmaceuticals, Richmond, California 94806[3]

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We have cloned a novel cDNA (*Spa-1*) which is little expressed in the quiescent state but induced in the interleukin 2-stimulated cycling state of an interleukin 2-responsive murine lymphoid cell line by differential hybridization. *Spa-1* mRNA (3.5 kb) was induced in normal lymphocytes following various types of mitogenic stimulation. In normal organs it is preferentially expressed in both fetal and adult lymphohematopoietic tissues. A *Spa-1*-encoded protein of 68 kDa is localized mostly in the nucleus. Its N-terminal domain is highly homologous to a human Rap1 GTPase-activating protein (GAP), and a fusion protein of this domain (SpanN) indeed exhibited GAP activity for Rap1/Rsr1 but not for Ras or Rho in vitro. Unlike the human Rap1 GAP, however, SpanN also exhibited GAP activity for Ran, so far the only known Ras-related GTPase in the nucleus. In the presence of serum, stable *Spa-1* cDNA transfectants of NIH 3T3 cells (NIH/Spa-1) hardly overexpressed Spa-1 (p68), and they grew as normally as did the parental cells. When NIH/Spa-1 cells were serum starved to be arrested in the G_1/G_0 phase of the cell cycle, however, they, unlike the control cells, exhibited progressive Spa-1 p68 accumulation, and following the addition of serum they showed cell death resembling mitotic catastrophes of the S phase during cell cycle progression. The results indicate that the novel nuclear protein Spa-1, with a potentially active Ran GAP domain, severely hampers the mitogen-induced cell cycle progression when abnormally and/or prematurely expressed. Functions of the Spa-1 protein and its regulation are discussed in the context of its possible interaction with the Ran/RCC-1 system, which is involved in the coordinated nuclear functions, including cell division.

Most of the immunocompetent lymphocytes recirculate in the body through lymphoid tissues as resting cells for variable periods, sometimes even years long, until they adventitiously encounter the corresponding antigens. The antigen-bound receptors trigger a number of intracellular processes, including the production of autocrine/paracrine mitogenic cytokines and their receptors, which induce clonal expansion as well as various effector functions to manifest overt immune responses. Specific antigen receptors transduce the signal through receptor-coupled tyrosine kinases of the Src family and the ZAP-70/Syk family (22, 23, 43). They initiate cascades of intracellular signal transduction, including phosphatidylinositol-PLCγ-induced phosphatidylinositol turnover, leading to mobilization of Ca^{2+} and the protein kinase C (PKC)-dependent pathway (7) as well as to activation of Ras and successive kinase cascades, including Raf1 and mitogen-activated protein (MAP) kinase (32, 42). These pathways mediate the signal into the nuclei, leading to activation of primary-response nuclear proto-oncogenes, such as c-*rel* (NF-κB), c-*fos*, c-*jun*, *fra-1*, c-*myb*, and c-*myc*, by phosphorylation (18, 19). The primary response is rapid and largely independent of new protein synthesis (18). Most of these nuclear proto-oncogenes are transcriptional factors (19), and they activate a number of secondary response genes required for such diverse functions as cellular differentiation, cell cycle progression, and possibly even cell death (18).

In terms of T-cell proliferation, for instance, stimulation of the antigen receptor leads to the synthesis of both the cyclins and the serine/threonine kinases necessary for G_1 progression (12). Such "committed" T cells, however, rarely enter the proliferative cycle (S phase) before they receive a second signal by cytokines represented by interleukin 2 (IL-2) (12). IL-2 not only promotes further induction of the cyclins mentioned above, but it also newly induces D cyclins (1). Most recently it was shown that IL-2 stimulation led to the inactivation of a $p27^{Kip1}$-like Cdk inhibitor still present in the committed T cells and thus to the release of Cdk2 from the inhibition, possibly through cyclin D induction (12). The results unveiled a mechanism of the second signal (IL-2) to drive committed T cells into progression from G_1 to S phase. However, it still remains to be clarified whether the rest of the proliferative cycle progresses autonomously once the Cdk inhibitor is down-regulated.

We have previously reported a continuous lymphoid cell line whose proliferation is reversibly regulated by IL-2: the cells remain largely quiescent in the absence of IL-2 for weeks, whereas in its presence they continue to proliferate (26). By differential hybridization, we have isolated several new genes that were transcriptionally induced by the cytokine when the cells entered into the cycling state. In this paper, we report one of these novel genes, *Spa-1*, whose transcription is induced in normal lymphocytes following receptor-mediated mitogenic stimulation. The protein encoded by *Spa-1* is a nuclear protein whose N-terminal domain is highly homologous to a human

* Corresponding author. Mailing address: Department of Immunology and Cell Biology, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan. Phone: 075-753-4659. Fax: 075-753-4347.

Rap1 GTPase-activating protein (Rap1 GAP) (41). A fusion protein of this domain exhibits GAP activity in vitro not only for Rap1/Rsr1 but also for nuclear Ran. Both Rap1 and Ran GTPases are involved in the regulation of cellular proliferation. Rap1 (Krev-1) is one of the Ras-related GTPases associated with Golgi membranes, and it was reported to counteract the mitogenic function of Ras (21), at least partly because Rap1 can interact with Ras GAPs and Raf in a competitive manner (13, 15). Ran (TC4) is a new subfamily of Ras-related GTPases ubiquitously present in the nuclei which is partly in association with RCC-1 (regulator of chromosomal condensation) (5). RCC-1 acts as a guanine nucleotide exchange factor (GEF) specific for Ran (6). A number of recent pieces of evidence revealed that Ran protein is involved in a variety of nuclear functions, such as nuclear transport and cell division (29, 30, 39). In analogy with the Ras system, the presence of Ran GAP, which might act as either a negative regulator or effector of the Ran or both has been speculated upon (9). Most recently a Ran GAP of 65 kDa has indeed been purified to homogeneity from human HeLa cells (4).

Herein, we describe the basic features of a Spa-1, a nuclear protein of 68 kDa, which has a potential GAP domain for both Rap1 and Ran. Our results from *Spa-1* cDNA transfection experiments suggest that the Spa-1 protein plays a significant role in the control of cell cycle progression. Possible interaction of Spa-1 with the Ran/RCC-1 system will be discussed in the context of control of cell cycle progression initiated by the receptor-mediated external signals.

MATERIALS AND METHODS

Cell lines and cell culture. An IL-2-responsive lymphoid cell line (LFD-14) established from murine fetal liver as described before (16) was maintained in RPMI 1640 containing 10% fetal calf serum (CS), 5×10^{-5} M 2-mercaptoethanol, and 100 U of recombinant human IL-2 per ml. Other murine cell lines, β2M1, LFD14, TGM7B.3 (T lineage), 70Z/3, P3U1 (B lineage), and FDC-P2 (myeloid), were also maintained in culture. NIH 3T3 fibroblasts were cultured in Dulbecco modified Eagle medium with 5% CS unless otherwise indicated. A CD3/T-cell receptor $(TCR)^+$ T-cell hybridoma (2.4H.6) as well as its $CD3/TCR^-$ subclone (2.4H.34) was stimulated in the culture with solid-phase anti-CD3 antibody (10 μg/ml). 2.4H.6 cells exhibited transient growth arrest at G_1 as a result of anti-CD3 stimulation, although unlike most T-cell hybridomas they did not show apoptosis. Normal spleen cells from BALB/c mice were stimulated in vitro with concanavalin A (ConA) (2 μg/ml) for various periods.

Differential hybridization, cDNA cloning, and DNA sequencing. *Spa-1* cDNA was cloned by differential hybridization between LFD14 cells starved of IL-2 for 3 weeks $(LFD14^-)$ and LFD14 cells restimulated with IL-2 for 20 h $(LFD14^+)$. A cDNA library was constructed by using $poly(A)^+$ RNA prepared from $LFD14^+$ cells in a CDM8 cloning vector (2). [α-^{32}P]dCTP-labeled cDNA probes were synthesized to approximately 5×10^9 cpm/μg from $poly(A)^+$ RNAs prepared from $LFD14^-$ and $LFD14^+$ cells. Duplicate filters of the cDNA library were hybridized with each of the cDNA probes described above at 10^6 cpm/ml in hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5× Denhardt's solution, 50 μg of salmon sperm DNA per ml, 50 mM sodium phosphate, 0.1% sodium dodecyl sulfate [SDS]) at 65°C overnight. Filters were washed with 0.1× SSC–0.1% SDS at 65°C before autoradiography. A cDNA (*Spa-1*) selectively detected by the $LFD14^+$ probe was isolated, subcloned into pBluescript phagemids (Stratagene), and sequenced on both strands by the dideoxynucleotide method by using subclones generated by exonuclease III digestion.

Northern (RNA) blot analysis. $Poly(A)^+$ RNAs were prepared from various normal murine organs, the spleen cells stimulated with ConA, and T-cell hybridomas stimulated with anti-CD3 antibody. Two micrograms of each was treated with 1% formaldehyde, electrophoresed on 1% agarose, transferred to nylon membranes (Amersham), and hybridized with [α-^{32}P]dCTP-labeled probes. The probes were as follows: Spa-1 probe, a full-length *Spa-1* cDNA; tumor necrosis factor beta (TNFβ) probe, a full-length mouse TNFβ cDNA (27); NFκB probe, a PCR product obtained by using sense primer (5′ TCTAGAATTCGGATATGTGTGTGAAG) and antisense (5′ TCTAGAATTCTTTCTGAACCTTGTCACA); and mouse β-actin probe, a PCR product obtained by using sense (5′ TCGTGCGTGACATCAAAGAG) and antisense (5′ TGGACAGTGAGGCCAGGATG) primers.

MAbs. To produce truncated SpanN (see below) fusion protein, a *Bam*HI-*Eco*RI fragment (670 bp) of *Spa-1* cDNA, which encoded the N-terminal human Rap1 GAP-homologous domain, was prepared by PCR and subcloned into *Bam*HI-*Eco*RI sites of a pGEX-1 vector (Pharmacia), which allowed overproduction of SpanN as a glutathione *S*-transferase (GST) fusion protein in *Escherichia coli* by IPTG (isopropyl-β-D-thiogalactopyranoside) induction. Truncated SpanC (see below) was produced as a murine dihydrofolate reductase (DHFR) fusion protein in *E. coli* by subcloning a *Pst*I fragment (nucleotides 1757 to 2177) of *Spa-1* cDNA into the *Bgl*II site of a pQE13 (QIAGEN) vector. The fusion proteins were purified from *E. coli* extracts by using a glutathione-Sepharose column (Pharmacia) and an Ni-NTA column (QIAGEN), respectively. By immunizing Armenian hamsters with the GST-SpanN and DHFR-SpanC fusion proteins, anti-SpanN and anti-SpanC hamster monoclonal antibodies (MAbs), F6 and H10, respectively, were established by cell fusion with P3U1 myeloma cells. The specificity of these MAbs was confirmed by enzyme-linked immunosorbent assay and immunoblot analysis using pairs of fusion partners (GST or DHFR) and fusion proteins. Monoclonal anti-$p34^{cdc2}$ antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, Calif.

Immunoblotting and immunoprecipitation. Cells were lysed with lysis buffer (0.1% Nonidet P-40, 250 mM NaCl, 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.6], antipain, leupepsin, chymostatin, trypsin inhibitor). Ten micrograms of total cell lysate was separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to Immobilon membranes, blotted with each MAb, and visualized with ^{125}I-labeled protein A (Amersham). In some experiments, total cell lysates were first immunoprecipitated with 2 μg of F6 or unrelated hamster antibody followed by protein A-Sepharose 4B beads (Pharmacia), and the precipitates were analyzed by immunoblotting with F6 or H10 antibody as described above.

Immunostaining. β2M1(T-lineage) cells attached to glass slides were fixed with 3.7% formaldehyde–phosphate-buffered saline (PBS), permeabilized with 0.25% Triton X-100–PBS, blocked with 3% bovine serum albumin (BSA), and then incubated with 10 μg of normal hamster immunoglobulin G per ml and purified H10 or F6 MAb followed by fluorescein isothiocyanate-conjugated goat anti-hamster antibody. To stain DNA, cells were pulsed with 10 μM bromodeoxyuridine 20 min before harvest, fixed as described above, treated with 4M HCl, and then incubated with fluorescein isothiocyanate-conjugated anti-bromodeoxyuridine antibody (Boehringer Mannheim).

GAP assay. *E. coli* expression vectors (pGEX, related vector pGEX-2TH or pGEX-3X) were used to overproduce the following proteins as GST fusion proteins (34, 35): murine SpanN (residues 1 to 209; see Fig. 3), human Rap1 GAP (residues 75 to 663), human c-HaRas protein (residues 1 to 189), a Glu-63 mutant of human Rap1A protein (residues 1 to 184), yeast Rsr1 protein (residues 1 to 272), human Ran protein (residues 1 to 216), human RhoA protein (residues 1 to 193), and chimeric Rap1(1-80)/c-HaRas (residues 81 to 189) protein. Glu-63 Rap 1A was used because its GST fusion protein was highly soluble in bacterial extract, while GST–wild-type Rap 1A was barely soluble. These GST fusion proteins were purified in a single step on a GSH-agarose affinity column (34, 35). The hydrolysis of [γ-^{32}P]GTP (50 nM) bound to each GTPase as a GST fusion protein was determined by incubating the GTPases at 25 or 37°C for various periods in the presence or absence of either SpanN or Rap1 GAP preparation (35 μg/ml) in 100 μl of buffer containing 50 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, 2.5 mM EDTA, 3 mM ATP, 1 mM dithiothreitol, and 0.5 mg of BSA per ml. The unhydrolyzed [γ-^{32}P]GTP was precipitated with charcoal, and the radioactivity of the γ-$^{32}P_i$ in the supernatant was measured as described previously (28). Since free GTP or GDP does not rebind to G proteins under the assay conditions, the changes in the rate of γ-^{32}P release were not due to changes in the rate of GTP-GDP exchange on G proteins. GAP activity was expressed as a stimulation index (fold), 0-fold meaning no stimulation, 1.0-fold meaning 100% stimulation (twice the intrinsic activity), 0.2-fold meaning 20% stimulation, 15.5-fold, meaning 1,550% stimulation, and so on.

DNA transfection. A *Bgl*I-*Dra*I fragment of *Spa-1* cDNA lacking most of the 5′ untranslated region was subcloned into a pcDL-SRα296 expression vector, since in vitro translation analysis indicated that the 5′ UT region was very inhibitory for the translation (unpublished observation). NIH 3T3 cells were transfected with either a purified plasmid (pSRαSpa-1) or the control vector (pcDL-SRα296) along with the pSV2-Neo plasmid by using Lipofectin (GIBCO BRL, Gaithersburg, Md.). G418-resistant clones were selected, isolated by using cloning cylinders, and screened for the expression of truncated *Spa-1* mRNA (2.5 kb) by Northern blot analysis.

Cell cycle analysis. Analysis of the cell cycle was performed with a CellFit program of the FACScan system (Becton Dickinson) by using a CycleTEST kit.

Nucleotide sequence accession number. The EMBL and GenBank accession no. of *Spa-1* cDNA is D11374.

RESULTS

Molecular cloning of *Spa-1* cDNA inducible by mitogenic stimulation in lymphocytes. A murine lymphoid cell line, LFD14, continuously proliferated in the presence of IL-2, while in its absence these cells remained largely quiescent but still viable for weeks. The IL-2-starved LFD14 cells reinitiated the proliferation as a result of the addition of IL-2 after some lag period (Fig. 1A). By the differential hybridization between

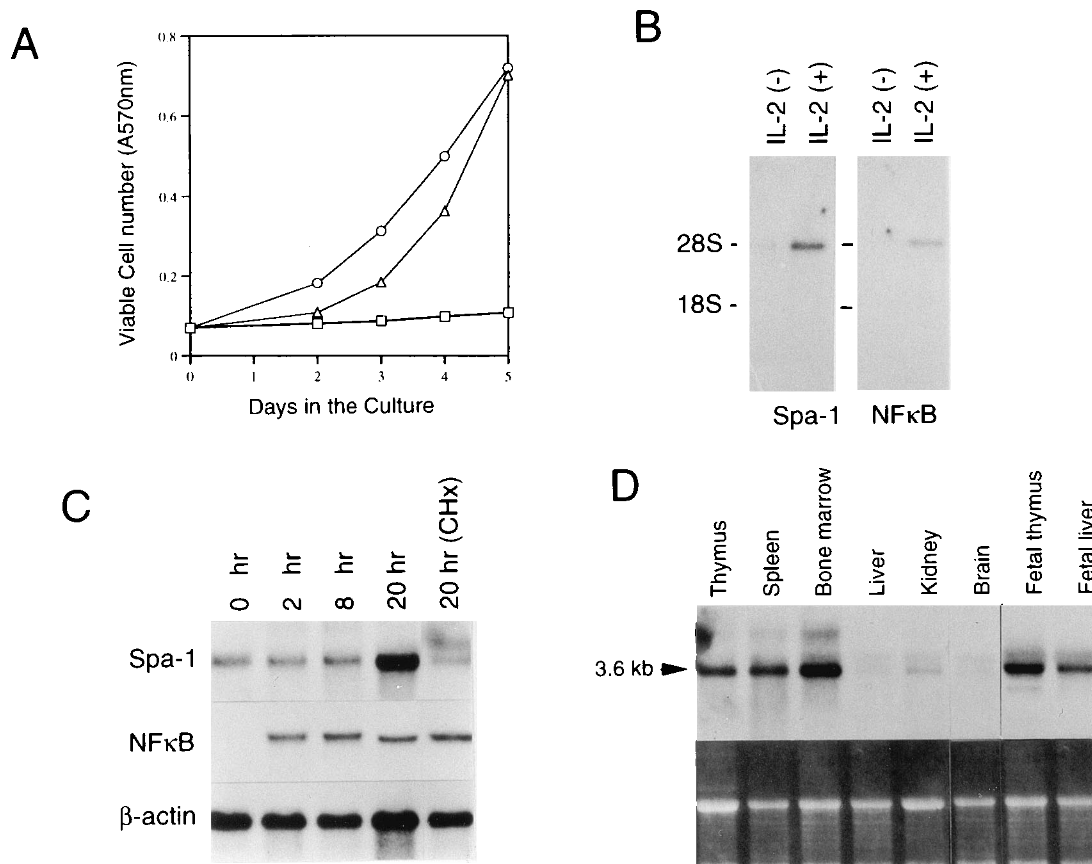

FIG. 1. Isolation and expression of Spa-1 cDNA. (A) Growth curve of LFD14 cells. LFD14$^-$ cells (starved of IL-2 for 3 weeks) were cultured in the absence (□) or presence (△) of 100 U of recombinant IL-2 per ml. LFD14$^+$ cells that had been maintained in the continuous presence of IL-2 were also cultured in the presence of 100 U of IL-2 per ml (○). Viable cell numbers were assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (31). (B) Induction of *Spa-1* mRNA by IL-2 in LFD14$^-$ cells. RNAs were extracted from LFD14$^-$ cells before and 20 h after the addition of IL-2 and purified by using oligo(dT) columns. Two micrograms of each poly(A)$^+$ RNA was electrophoresed. The same filter was hybridized sequentially with Spa-1 and NFκB probes. After each hybridization, the filter was washed twice with boiling 0.1× SSC–0.1% SDS and it was confirmed that previously hybridized probe was completely stripped. (C) Kinetics of *Spa-1* mRNA expression in normal splenic cells following stimulation with 2 μg of ConA per ml in the absence or presence of cycloheximide (CHx, 10 μg/ml). For comparison, the Northern blotting pattern of NFκB with the same filter, as well as that of β-actin, is also shown. (D) Expression of *Spa-1* mRNA in various tissues of normal mice. RNAs were extracted from adult tissues (thymus, spleen, bone marrow, liver, kidney, and brain) and fetal lymphohematopoietic tissues (thymus and liver of the 18th gestational day) and purified by using oligo(dT) columns. Northern blot analysis was performed with the Spa-1 probe. 28S rRNA of each sample is shown at the bottom.

the IL-2-starved quiescent LFD14 cells and those restimulated with IL-2 for 20 h, a cDNA clone of 3.5 kb called *Spa-1* that was preferentially expressed in the latter was isolated (Fig. 1B). A single mRNA of the same size was weakly expressed in normal splenic lymphocytes, but it was strongly induced following ConA stimulation (Fig. 1C). The *Spa-1* gene induction took place quite late, compared with that of the primary response genes, such as the NFκB gene, and it was completely inhibited by cycloheximide (Fig. 1C). Cross-linking of the antigen receptors of normal lymphocytes with anti-CD3 or anti-immunoglobulin M antibodies caused essentially the same results (data not shown). Among normal mouse tissues, *Spa-1* mRNA was preferentially expressed in lymphohematopoietic tissues, including those of fetuses, with little expression in other tissues, such as brain, liver, kidney (Fig. 1D), heart, lung, and testis tissues (data not shown).

Expression of *Spa-1* mRNA is associated with lymphocyte proliferation rather than activation. Mitogenic stimulants such as IL-2 and ConA induce not only proliferation but also cellular activation and/or differentiation in normal lymphocytes. To distinguish proliferation and activation, T hybridoma cells were examined, and they showed transient growth arrest as a

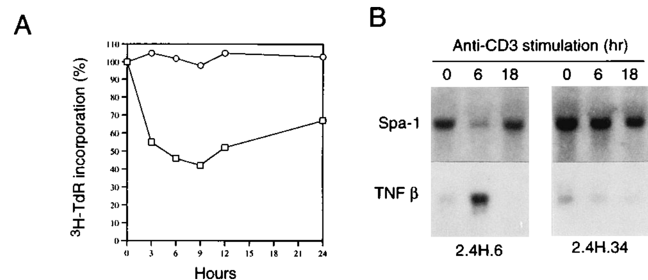

FIG. 2. Relation between *Spa-1* mRNA expression and the proliferation of T hybridoma cells. (A) DNA synthesis of CD3/TCR$^+$ T-cell hybridoma (2.4H.6; □) and its CD3/TCR$^-$ subclone (2.4H.34; ○) in the presence of solid-phase anti-CD3 antibody (10 μg/ml). Cells were pulsed with [^{3}H]thymidine (TdR) for 1 h at the indicated time, and the uptake was normalized to make that of cells in the absence of antibody equal 100%. (B) Transient reduction of *Spa-1* mRNA expression by anti-CD3 stimulation in T-cell hybridoma. RNAs were extracted from 2.4H.6 and 2.4H.34 hybridoma cells before and at 6 and 18 h after stimulation with anti-CD3 antibody. Ten micrograms of each total RNA was electrophoresed and hybridized with ^{32}P-labeled Spa-1 and TNFβ probes as described in the legend to Fig. 1.

result of stimulation with anti-CD3 antibody, simultaneously producing cytokines (3). As shown in Fig. 2A and B, the amount of *Spa-1* mRNA, which was abundantly expressed in cycling T hybridoma cells, was transiently reduced upon the inhibition of DNA synthesis by anti-CD3 antibody, while it returned to close to the original level as the inhibition was gradually released. On the contrary, TNFβ mRNA was clearly induced by the same stimulation. The effects were specific for the CD3/TCR complex, as no such changes were induced by the same antibody in a CD3/TCR-negative subclone (2.4H.34). Thus, the level of *Spa-1* mRNA expression appears to be associated with the proliferation rather than the activation induced by the antigen receptor stimulation.

***Spa-1* is a novel cDNA with a human Rap1 GAP homology domain.** The *Spa-1* cDNA is 3,518 bp long, and it contains a long open reading frame (ORF) (positions 1199 to 3280), encoding 693 amino acids, in addition to very short ORFs in the 5′ region (Fig. 3). The ATG at position 1199 is most likely the initiation codon, as judged by in vitro translation analysis (unpublished observation). There is no apparent leader sequence or possible N-glycosylation site in the ORF, but a number of possible serine/threonine phosphorylation sites, including those of PKC and MAP kinase, have been noted. A computer search has revealed that the N-terminal portion (residues 1 to 190; termed SpanN) is highly homologous to a human Rap1 GAP (41) (43% identical amino acids; Fig. 4), while the remaining C-terminal portion (residues 191 to 693; termed SpanC) showed no homology to any reported sequence. Interestingly, the region of SpanN containing residues 54 to 92 is also homologous to human tuberin (residues 1593 to 1631), whose dysfunction is associated with tuberous sclerosis, a human hereditary disease (10).

***Spa-1* encodes a 68-kDa mitogen-inducible nuclear protein in vivo.** In order to identify the protein encoded by the *Spa-1* gene in vivo, MAbs F6 and H10 against the GST-SpanN and DHFR-SpanC fusion proteins, respectively, were made. Immunoprecipitation of the LFD14 cell lysate with F6 followed by blotting with F6 or H10 indicated that a single 68-kDa protein (p68) contains both SpanN and SpanC epitopes (Fig. 5A). By immunoblotting both antibodies were reacted with the p68 in the lysates of various lymphohematopoietic cell lines (Fig. 5B). Like *Spa-1* mRNA synthesis (Fig. 1C), p68 production was slowly induced by stimulation of normal resting spleen cells with ConA, with comparable kinetics, as long as 24 h after the stimulation (Fig. 5C), at the end of which time significant DNA synthesis began to be detected (data not shown). Analysis using fractionated cell lysates further indicated that p68 was localized mostly in the nuclei (Fig. 5D). Immunostaining with fluorescent antibodies confirmed the results presented above. Thus, the antigen was localized mostly in the nuclei, except for the nucleoli in interphase cells (Fig. 6). In metaphase cells, however, condensed chromosomes were totally spared from the immunostaining, with possible faint cytoplasmic staining (Fig. 6, arrowhead).

GST-SpanN fusion protein exhibits GAP activity for Ran in addition to Rap1 and Rsr1. Since SpanN is highly homologous to a human Rap1 GAP, we examined possible GAP activity of GST-SpanN for a series of small GTPases. Like the Rap1 GAP, the SpanN fusion protein indeed activated both Rap1 and Rsr1 GTPases but neither Ras or Rho (Table 1). Interestingly, however, GST-SpanN had no effect on a Rap1(1–80)/Ras(81–189) chimeric GTPase, which was still activated by human Rap1 GAP, implying a functional difference between SpanN and the human Rap1 GAP. Furthermore, both Rap1 and Rap1 GAP are located in the membrane (20, 37), while Spa-1 is present mostly in the nuclei. We therefore examined

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GTCCTGCATGCAGCTGCCCCAGGAGCTCCTGTGTCCTTGAGGCCCATCTGAACAGCCCCC   60
TCCTCTGCAGTGCAGAAACCACTGAAGCCTCAGCCTTCTGGGTGGGCACCAAGGACCCGT  120
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CGGAGGTGTGGGGAGCCCTCGGCGGGCATGGCCCCTGCACCTACCGATGACCTCTTTGCC  240
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 H  G  R  G  E  A  I  T  L  R  L  D  G  A  P  G  Q  A  V  G
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 E  V  V  A  R  L  Q  L  V  S  R  G  C  E  T  R  E  L  A  L
GCCCAGAGATGGCCAAGGTCGCCTGGGCTTCGAGGTGGATGCAGAAGGCTTCATCACGCA 2280
 P  R  D  G  Q  G  R  L  G  F  E  V  D  A  E  G  F  I  T  H
CGTGGAGCGCTTCACGTTTGCGGAGACCACGGGGCTTCGGCCTGGAGCTCGTTTGCTGCG 2340
 V  E  R  F  T  F  A  E  T  T  G  L  R  P  G  A  R  L  L  R
AGTCTGCGGCCAGACGCTGCCCAAGCTGGGTCCCGAAACTGCTGCCCAGATGCTGCGCTC 2400
 V  C  G  Q  T  L  P  K  L  G  P  E  T  A  A  Q  M  L  R  S
TGCGCCCAAGGTCTGCGTCACGGTCCTACCCCCAGACGAGAGCGGCCGGCCGCGAAGGAG 2460
 A  P  K  V  C  V  T  V  L  P  P  D  E  S  G  R  P  R  R  S
CTTTTCGGAGCTCTATATGCTCTCTCTGAAGGAACCCAGCCGGCGGGGGGGCCCAGAGCC 2520
 F  S  E  L  Y  M  L  S  L  K  E  P  S  R  R  G  G  P  E  P
AGTACAGGATGAAACTGGGAAGTTGGTCATATTGCCTCCCACCAAGCAGCTGCTACATTT 2580
 V  Q  D  E  T  G  K  L  V  I  L  P  P  T  K  Q  L  L  H  F
TTGCCTGAAAGACAGCAGCAGTCCTCCGGGGCCTGGGGATCTGACTGAGGAGAGGACAGA 2640
 C  L  K  D  S  S  S  P  P  G  P  G  D  L  T  E  E  R  T  E
GTTCCTGCGCACGCACAACTCCCTGTCATCTGGAAGCTCCCTGTCCGATGAGGCTCCAGT 2700
 F  L  R  T  H  N  S  L  S  S  G  S  S  L  S  D  E  A  P  V
CCTGCCCAACACCACTCCAGACCTCCTCCTTGTCACCACTGCCAACCCATCTGCACCTGG 2760
 L  P  N  T  T  P  D  L  L  L  V  T  T  A  N  P  S  A  P  G
TACTGACAGAGAAACACCCCCTTCCCAGGACCAGTCAGGAAGCCCCAGTAGCCATGAAGA 2820
 T  D  R  E  T  P  P  S  Q  D  Q  S  G  S  P  S  S  H  E  D
CACCAGTGACTCAGGCCCAGAACTGAGGGCCTCCATCCTGCCCAGAACCTTGTCTCTGCG 2880
 T  S  D  S  G  P  E  L  R  A  S  I  L  P  R  T  L  S  L  R
GAATTCCATCAGTAAGATTATGTCGGAAGCTGGCAGTGAGACCCTGGAGGATGAGTGGCA 2940
 N  S  I  S  K  I  M  S  E  A  G  S  E  T  L  E  D  E  W  Q
GTCCATCTCAGAGATCGCCTCCACTTGCAACACAATTCTGGAGTCACTGTCCCGGGAGGG 3000
 S  I  S  E  I  A  S  T  C  N  T  I  L  E  S  L  S  R  E  G
ACAACCCATCTCAGAGAGCGGAGACCCCAAGGAAGCTTTAAAGTGTGATTCTGAGCCAGA 3060
 Q  P  I  S  E  S  G  D  P  K  E  A  L  K  C  D  S  E  P  E
ACCCGGGAGCCTGTCAGAAAAGGTCTCTCACCTAGAGTCCATGCTCTGGAAGCTCCAGGA 3120
 P  G  S  L  S  E  K  V  S  H  L  E  S  M  L  W  K  L  Q  E
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 D  L  Q  R  E  K  A  D  R  A  A  L  E  E  E  V  R  S  L  R
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CTCTAAGCATCTGGGTGCACCCACTACTGACCTGGCCTGAGTTCCAATCTGAATCTGGAC 3300
 S  K  H  L  G  A  P  T  T  D  L  A  * 693
CTGCTTGGAACTGCCTGGCCCCTCAGAGCAACTGGGTCATACTAGTGCCCTTCCTCAGGA 3360
CTTCTTCCCTGCGCTGAGGCGCGTCTTAGCACTGCCCCCTCTTCCCAGCCCATTTGGTGG 3420
TAATGCCTGTCCCTGTTTGTAAATATCCTGTAAAGAAAAGGAGACATCAGAGTTTAAAAA 3480
AAAGAAACAACAAGAAGAAGCAAAAAAAAAAAAAAAA   3518
```

FIG. 3. Complete nucleotide sequence of *Spa-1* cDNA and deduced amino acid sequence of the longest ORF. The cDNA was originally cloned from an IL-2-stimulated LFD14 line as described in Materials and Methods, and it was recloned later from three different cDNA libraries, with identical resulting sequences. The 5′ UT region was quite long (1.2 kb), and it was loaded with as many as 8 ATG codons in a manner similar to that of many proto-oncogenes.

the GAP activity of GST-SpanN for Ran, so far the only known small nuclear GTPase (5). As shown in Table 1, SpanN activated Ran GTPase, while both human Rap1 GAP and other known GAPs (NF-1 and Rho GAP) failed to do so (8).

Spa-1 (p68) overexpression in NIH 3T3 fibroblasts at $G_{1/0}$ as a result of serum starvation causes cell death at S phase after serum restimulation. To gain functional insights, we investigated further the effects of Spa-1 overexpression in NIH 3T3 cells, which normally express far less Spa-1 protein, if any, than lymphoid cells. Since the 5′ UT region appears to be quite

```
mSPA-1     MYNNQEAGAA FMQFLTLLGD VVRLKGFESY RAQLDTKTDS TGTHSLYTTY QDHEIMFHVS TMLPYTPNNQ   70
           .....*...* *..**..**. .*.*..*... *..**..... ***.*.*... ...******* *.****....
hRap1GAP   LFSTNEESPA FVEFLEFLGQ KVKLQDFKGF RGGLDVTHGQ TGTESVYCNF RNKEIMFHVS TKLPYTEGDA  279

mSPA-1     QQLLRKRHIG NDIVTIVFQE PGSKPFCPTT IRSHFQHVFL VVRAHAPCTP HTSYRVAVSR TQDTPAFGPA  140
           ***.****** ****..***.  ...**.*.. *.*.*.*... **.*...... ...*.*.*.. ..*.*.***.
hRap1GAP   QQLQRKRHIG NDIVAVVFQD -ENTPFVPDM IASNFLHAYV VVQAEGGGPD GPLYKVSVTA RDDVPFFGPP  348

mSPA-1     LPEGGGPFAA NADFRAFLLA KALNGEQAAG HARQFHAMAT RTRQQYLQDL 190
           **. ...*.. ...*..***. *..*.*.*.. .*..*..... ***...*..*
hRap1GAP   LPD-PAVFRK GPEFQEFLLT KLINAEYACY KAEKFAKLEE RTRAALLETL 397
```

FIG. 4. Homology matching between murine (m) Spa-1 and human (h) Rap1 GAP. Identical residues are indicated with stars, and conservative changes are indicated with dots.

inhibitory for translation (unpublished observation), NIH 3T3 cells were stably transfected with *Spa-1* cDNA deleted of most of 5′ UT region as described in Materials and Methods (NIH/Spa-1). In the presence of 5% serum, NIH/Spa-1 cells grew as normally as the control cells transfected with a vector alone (NIH/SRα). When the cells were serum starved for 48 h and then restimulated with 20% serum, however, the majority of NIH/Spa-1 cells went into crisis and died within 24 h, while the NIH/SRα cells progressed into a normal growth cycle (Fig. 7A). A similar, but less drastic, effect was observed when cells were starved for only 24 h. The cell death was characterized by the rounding up of cells and by nuclear condensation, i.e., its features resembled those of so-called mitotic catastrophes (Fig. 7B). Cell cycle analysis revealed that NIH/Spa-1 cells

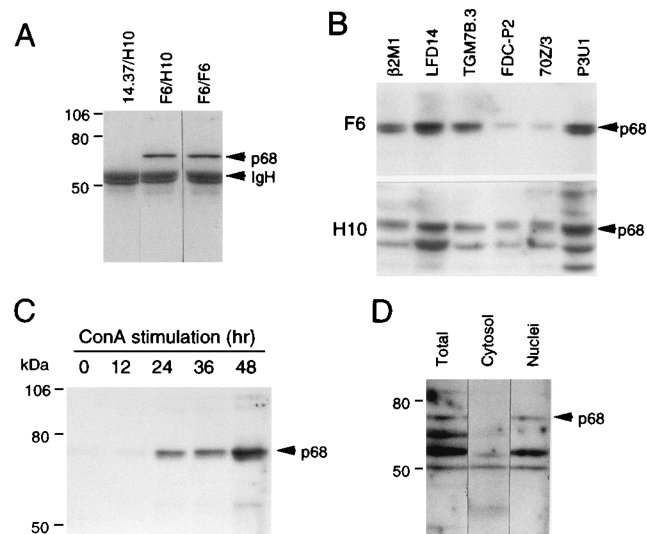

FIG. 5. Detection of Spa-1 protein in vivo with two distinct MAbs, F6 (anti-SpanN) and H10 (anti-SpanC). (A) Presence of both epitopes on the same 68-kDa molecule (p68). LFD14 cells were lysed with lysis buffer (0.1% Nonidet P-40, 250 mM NaCl, 50 mM HEPES [pH 7.6], protease inhibitors). Fifty micrograms of total cell lysate was first immunoprecipitated with F6 or unrelated hamster MAb (14.37) followed by protein A-Sepharose 4B beads. The precipitates were analyzed by immunoblotting with F6 or H10 antibody and visualized with ^{125}I-labeled protein A. IgH, immunoglobulin heavy chain. (B) Detection of p68 Spa-1 protein in the lysates of various cell lines, including murine T lymphoid lines (β2M1, LFD14, and TGM7B.3), a myeloid line (FDC-P2), and B lymphoid lines (70Z/3 and P3U1) by immunoblotting. Arrows indicate the protein (p68) commonly detected by both MAbs. (C) Immunoblotting analysis of Spa-1 protein in normal spleen cells with F6 before and at various times after stimulation with ConA. (D) Localization of p68 Spa-1 protein in the nuclei. LFD14 cells (10^7) were swollen in the presence of 0.5% Nonidet P-40, and the nuclei were separated from the cytoplasm by centrifugation. Fifty micrograms of protein extracted from nucleus and cytosol fractions was then analyzed by immunoblotting with H10 antibody.

were indeed arrested at the G_1/G_0 phase of the cell cycle by serum starvation, as were the control cells, and that the death of NIH/Spa-1 cells occurred during S phase after the addition of serum (Fig. 8A). Rather unexpectedly, NIH/Spa-1 cells continuously maintained in the regular serum-containing medium exhibited as little p68 as did the control cells (Fig. 8B) in spite of the expression of truncated *Spa-1* mRNA (data not shown). However, NIH/Spa-1 cells showed abundant accumulation of p68 when they were serum starved for 48 h, in sharp contrast to the similarly starved NIH/SRα cells (Fig. 8B). As shown in Fig. 7A, a small fraction (at most 10%) of the serum-starved NIH/Spa-1 cells survived after the addition of serum and grew normally. We collected the apparently viable adherent NIH/Spa-1 cells at various times after the addition of serum and examined their levels of expression of Spa-1 p68. At 24 h, when the majority of the cells had already died off, the Spa-1 p68 level in the minor fraction of viable cells was becoming much lower, while the $p34^{cdc2}$ level was greatly elevated (Fig. 8C). The results suggested that those cells which either happened to accumulate less Spa-1 p68 at G_1/G_0 or managed to down-regulate its expression after the serum stimulation could be spared from cell death, supporting the theory that the abnormal overexpression of Spa-1 p68 at G_1/G_0 was primarily responsible for the cell death at S phase after the mitogenic stimulation.

DISCUSSION

We have cloned a novel cDNA (*Spa-1*) that is induced in lymphocytes following mitogenic stimulation with various agents, such as IL-2 and ConA (Fig. 1) as well as antibodies for the antigen receptors (unpublished observation). The effects of these stimulants on lymphocytes are usually pleiotropic, inducing not only proliferation but also a number of activation or differentiation processes, which are not easily dissected from each other. In this respect T-cell hybridomas exhibit a rather unique feature, i.e., they show growth arrest in response to CD3/TCR-stimulation, while activation processes such as lymphokine production are still induced (3). When cycling T hybridoma cells were stimulated with anti-CD3 antibody, *Spa-1* mRNA expression was reduced as their proliferation was arrested, while TNFβ mRNA was induced (Fig. 2). This result suggests that the mRNA expression is associated with proliferation rather than with a cellular activation process, and we tentatively call this gene *Spa-1* (signal-induced proliferation associated gene 1). *Spa-1* mRNA was expressed in most of the lymphohematopoietic cell lines that we examined as well as in some nonlymphoid lines, such as embryonic carcinomas (unpublished observation), while in the case of normal tissues significant expression was detected only in those that were lymphohematopoietic. It is not clear at present whether the selective tissue expression of *Spa-1* mRNA reflects the regen-

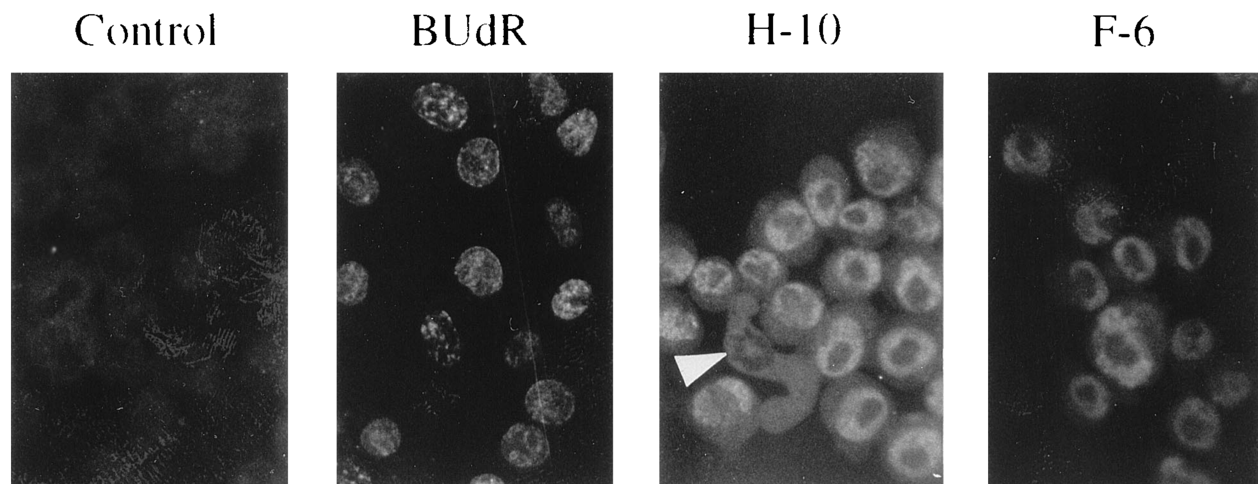

FIG. 6. Immunostaining of β2M1 (T-cell line). β2M1 cells were attached to slide glasses, fixed with 3% formaldehyde–PBS, permeabilized with 0.1% Nonidet P-40–PBS, blocked with 3% BSA, and incubated with 10 μg of normal hamster immunoglobulin G per ml (control), H-10 antibody, or F-6 antibody, and then they were incubated with fluorescein isothiocyanate-conjugated goat anti-hamster immunoglobulin G. To stain the DNA, β2M1 cells were pulsed with 10 μM bromodeoxyuridine (BUdR) for 20 min prior to harvest, fixed and permeabilized as described above, treated with 4 N HCl, and incubated with fluorescein isothiocyanate-conjugated anti-bromodeoxyuridine. Note that condensed chromosomes in the metaphase cells were spared from staining (arrowhead). Also, the intensity of staining varied significantly from cell to cell.

erative nature of the lymphohematopoietic tissues or is indeed tissue specific, in which case expression in some nonlymphohematopoietic cell lines might be associated with continuous in vitro propagation, for instance. In support of the possibility of lymphohematopoietic tissue specificity, little *Spa-1* mRNA was detected in testis tissue also.

Full-length *Spa-1* cDNA is 3,518 bp long. There were a number of very short ORFs in the 5′ region (around 1 kb), but only one long ORF, which could encode 693 amino acids, was observed (positions 1199 to 3280). In vitro transcription and translation analysis using 5′ region-deleted plasmids strongly suggested that the ATG at position 1199 is most likely an initiation codon, although it does not fit well with Kozak's rule (data not shown). Long 5′ UT regions loaded with a number of ATG codons were reported to be present in the cDNAs of many proto-oncogenes and some cytokine genes, and it was suggested that they provide one of the regulatory mechanisms for the expression of these proteins, which requires very tight control (25). Our unpublished result indeed indicated that the 5′ UT was quite inhibitory for translation in vitro. By two independent MAbs against SpanN and SpanC fusion proteins, Spa-1 protein was identified in vivo as a protein with an apparent molecular mass of 68 kDa (p68). Production of p68 was induced in normal lymphocytes by ConA stimulation, with kinetics comparable to that of *Spa-1* mRNA synthesis: significant p68 production took as long as 24 h after the stimulation with ConA, i.e., it was achieved at the time corresponding to the entry into S phase rather than the time corresponding to the probable G_0-to-G_1 transition (unpublished data). Immunoprecipitation of fractionated cell lysates and immunostaining revealed that the p68 was located mostly in the nuclei. Interestingly, in vitro transcription and translation of *Spa-1* cDNA produced somewhat larger proteins, with molecular masses of around 80 kDa, which were reactive with both H10 and F6 MAbs (unpublished observation) , implying posttranslational processing in vivo.

TABLE 1. Selective activation of Rap1/Rsr1 and Ran GTPases by SpanN[a]

GTPases	Intrinsic GTPase activity (turnover no./min)	Stimulation (fold) by:	
		SpanN	Rap1 GAP
Rsr1	0.001	18.4	7.1
Rap1 (Glu-63)	0.0015	6.2	10.3
HaRas	0.022	0.3	0
RhoA	0.060	0.6	0
Ran	0.003	15.5	1.2
Rap (1–80)/Ras (81–189)	0.020	0.2	4.1

[a] Hydrolysis of [γ-^{32}P]GTP bound to each GTPase (50 nM) was measured at 37°C as described in Materials and Methods. The intrinsic GTPase activity was determined in the absence of any GAP, while the activation of each GTPase was determined in the presence of either GST-SpanN or GST-Rap1 GAP fusion protein (35 μg/ml). All GTPases used were GST-fusion proteins (see Materials and Methods). The GAP assay was performed under conditions that gave a linear relationship between the GAP concentrations and their activities in the presence of Mg^{2+}, where only a single round of GTP-GDP exchange was allowed. GAP activity was expressed as a stimulation index (fold), in which 0-fold means no stimulation over the intrinsic activity, 1.0-fold means 100% stimulation (twice that of the intrinsic activity), 0.2-fold means 20% stimulation, 15.5-fold means 1,550% stimulation, and so on.

One of the most notable features of Spa-1 is the presence of a human Rap1 GAP homology region (SpanN), which corresponds to a part of the catalytic domain of the Rap1 GAP (40). Present results indicate that the SpanN fusion protein indeed exhibits GAP activity in vitro for Rap1 and Rsr1 but not for Ras and Rho GTPases, as is the case with the human Rap1 GAP (Table 1). However, it is very unlikely that Spa-1 is simply a murine counterpart of the human Rap1 GAP. First of all, the C-terminal half of Spa-1 has no sequence homology with the rest of the human Rap1 GAP. In terms of GAP activity for Rap1, the result obtained with a Rap1/Ras chimeric GTPase implies that even their modes of action are distinct from each other (Table 1). The subcellular localization of the two proteins is also distinct, Rap1 GAP being localized in the membrane (37), while Spa-1 appears mostly in the nuclei (Fig. 5D and 6). Finally, SpanN, but not the human Rap1 GAP, exhibited GAP activity for nuclear Ran. Recently, a human Ran GAP of 65 kDa (Ran GAP1) was purified to homogeneity

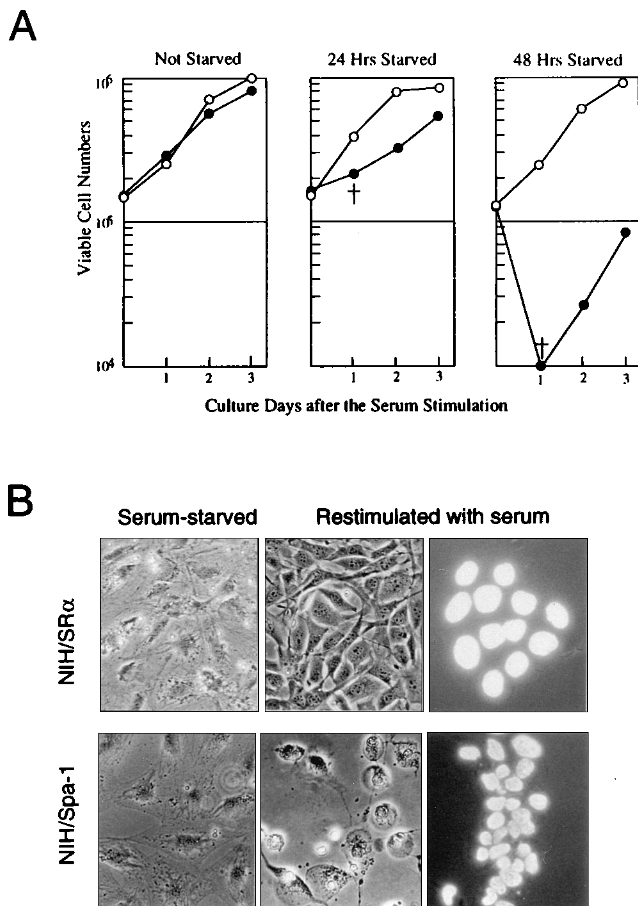

FIG. 7. Cell death caused by mitogenic stimulation after the cycle block at $G_{1/0}$ in NIH 3T3 cells stably transfected with *Spa-1* cDNA (NIH/Spa-1). (A) NIH/Spa-1 (●) and control NIH/SRα (○) (NIH 3T3 transfected with a vector alone) cells were cultured in 5% CS-medium for 24 h until they became nearly confluent (2×10^5 cells per 35-mm-diameter dish), and then they were shifted to 0.5% CS-medium for 0, 24, or 48 h and then to 20% CS-medium. At various days after the addition of 20% serum, viable cell numbers were counted. †, dying cells were easily observed under the microscopy (see below). (B) Phase-contrast microscopy of the culture wells before (left) and 18 h after (middle and right) the addition of 20% CS for NIH/SRα and NIH/Spa-1 cells, which had been starved of serum for 48 h. To visualize the nuclei, both floating and adherent cells of each culture were collected, attached to glass slides, and stained with Hoechst 33427 (right). Note the small condensed nuclei in the NIH/Spa-1 cells (lower right).

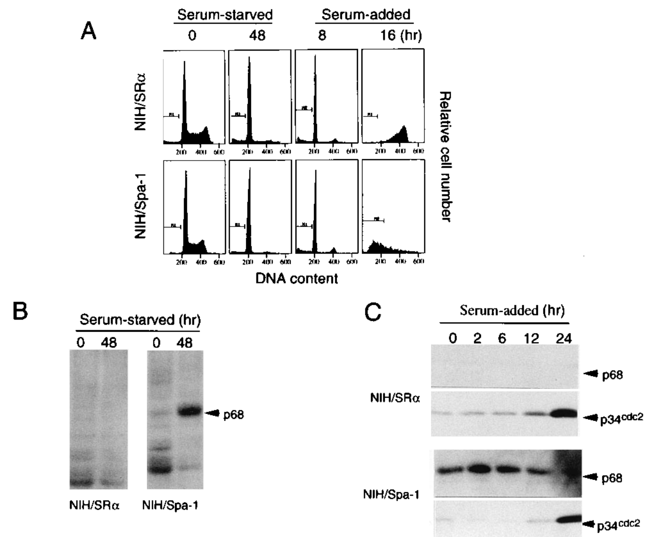

FIG. 8. Disturbed cell cycle progression after G_1 arrest of NIH/Spa-1. (A) NIH/SRα cells and NIH/Spa-1 cells that had been serum-starved for 48 h were analyzed for cell cycle progression at various times after the addition of 20% CS. Both viable adherent cells and floating dying cells were pooled for the analysis. The bar represents degraded DNA. (B) Accumulation of p68 Spa-1 protein in NIH/Spa-1 cells but not in NIH/SRα cells during serum starvation. (C) Immunoblotting analysis with F6 and anti-p34^{cdc2} of serum-starved NIH/Spa-1 and NIH/SRα cells before and after the addition of 20% CS. Only adherent viable cells were collected at various times after the addition of 20% CS, lysed, adjusted to the same protein concentration (2 mg/ml), and 10 μl of each lysate applied to SDS-PAGE gels for immunoblotting. Although not shown, significant DNA synthesis was observed to begin at around 12 h after the addition of serum.

from HeLa cells (4). Although the primary structure has not been reported yet, it is interesting to note that our anti-SpanN and anti-SpanC antibodies both cross-reacted with a 65-kDa protein in a HeLa cell lysate (unpublished observation). It is thus possible that Spa-1 represents one of the Ran GAPs in mice. In this respect, it is worth noting that the immunostaining pattern of the Spa-1 protein (Fig. 6) is very similar to that of Ran (39). Unfortunately, however, both bacterially produced recombinant full-length Spa-1 and the Spa-1 expressed in Sf9 cells by a recombinant Spa-1-expressing baculovirus were completely insoluble (unpublished observation), and the exact nature of the GAP activity of a complete Spa-1 protein in vivo still remains to be clarified.

Since Spa-1 expression appeared to be associated primarily with cellular proliferation, we directly examined the effects of Spa-1 overexpression on cell growth by stable cDNA transfection of NIH 3T3 cells, which expressed endogenous Spa-1 only marginally, if at all. In spite of the expression of truncated *Spa-1* mRNA, which is expected to show higher efficiency in translation, the stable transfectants (NIH/Spa-1) did not overexpress p68 when propagated in the presence of serum, and they grew as normally as the control cells did. During passage in the culture, however, it was noticed that they went into massive crisis rapidly when kept in a confluent state. We therefore examined the p68 expression during the cell cycle arrest more directly by serum starvation. We found that the serum-starved NIH/Spa-1 cells, unlike similarly arrested control NIH/SRα cells, expressed abundant p68, although cell viability during the period of arrest did not differ significantly. The phenotypical difference became evident only when the starved cells were restimulated with serum. Following serum restimulation, NIH/Spa-1 cells showed massive cell death during S phase. The morphological features resembled those of so-called mitotic catastrophes, which have been reported to be induced by overexpression of various cell cycle-related factors, such as cyclin A/B and cdc2 (17), p53 and E2F (44), and c-myc (11).

Overexpression of p68 in serum-starved NIH/Spa-1 cells was rather unexpected. A likely explanation may be that the abundance of p68 is regulated at the posttranslational level in cycling cells, for instance, by protein degradation, in addition to regulation at the transcriptional level (Fig. 1), and that such a regulatory mechanism is progressively lost as the cells are arrested in $G_{1/0}$, which in turn allows the protein derived from transfected cDNA to be accumulated. Although such overexpressed Spa-1 protein does not seem to affect cell viability in $G_{1/0}$, once cells are restimulated by the mitogenic stimulation (serum), the abnormally or prematurely overexpressed Spa-1 seriously disturbs the succeeding cell cycle progression at S phase. A recent study using a mutated Ran deficient in GTP hydrolysis indicates that Ran-GTP in the nuclei provides an

inhibitory burden for cell division, possibly by acting on preMPF (38), which is compatible with the original finding by Nishimoto et al. that functional loss of RCC-1 (Ran GEF) results in premature chromosomal condensation (33, 36). Also, Ran-GTP has been implicated as a critical factor for coupling of completion of DNA synthesis and mitosis in a *Xenopus* system (24). Assuming that nuclear Spa-1 can function as a Ran GAP in vivo, Spa-1 protein prematurely overexpressed before S phase would be expected to functionally mimic the loss of function of RCC-1 and reduce the size of the nuclear Ran-GTP pool, leading to the uncoupling of DNA synthesis and mitosis and thus to the premature onset of mitotic events. It has also been suggested that Ran-GTP plays important roles in nuclear transport (29, 30), and abnormal Spa-1 expression may affect this function as well. If Spa-1 acts as a Ran GAP in vivo, constitutive overexpression of Spa-1 in cycling cells above a certain threshold should be essentially lethal, and the present NIH/Spa-1 clones might have derived from cells that somehow happened to express the protein at regulatable levels. In support of this notion, the efficiency in obtaining stable *Spa-1* transfectants among NIH 3T3 cells has been very poor (unpublished observation).

In any case, the present results indicate that Spa-1 can severely disturb the cell cycle progression when abnormally expressed in NIH 3T3 cells, although Spa-1 per se may not necessarily be a major physiological regulator in the fibroblasts. It is possible that distinct gene products with similar functions exist in nonlymphoid cells. The assumption that Spa-1 might function as a mitogen-inducible nuclear Ran GAP is intriguing, because both Ran and RCC-1 are constitutively expressed proteins and little is known about their functional regulation. Further analysis should provide an insight into the molecular linkage between possibly lineage-specific signal-transducing systems and ubiquitous cell cycle regulatory machinery such as the Ran/RCC-1 system in the control of proliferation by external stimuli.

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