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Constitutive Expression of Murine Sak-a Suppresses Cell Growth and Induces Multinucleation

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The murine *Sak* gene encodes two isoforms of a putative serine/threonine kinase, Sak-a and Sak-b, with a common N-terminal kinase domain and different C-terminal sequences. *Sak* is expressed primarily at sites where cell division is most active in adult and embryonic tissues (C. Fode, B. Motro, S. Yousefi, M. Heffernan, and J. W. Dennis, Proc. Natl. Acad. Sci. USA 91:6388–6392, 1994). In this study, we found that *Sak-a* transcripts were absent in growth-arrested NIH 3T3 cells, while in cycling cells, mRNA levels increased late in G_1 phase and remained elevated through S phase and mitosis before declining early in G_1. The half-life of hemagglutinin epitope-tagged Sak-a protein was determined to be ~2 to 3 h, and the protein was observed to be multiubiquitinated, a signal for rapid protein degradation. Overexpression of Sak-a protein inhibited colony-forming efficiency in CHO cells. Neither the Sak-b isoform nor Sak-a with a mutation in a strictly conserved residue in the kinase domain (Asp-154→Asn) conferred growth inhibition, suggesting that both the kinase domain and the C-terminal portion of Sak-a are functional regions of the protein. Sak-a overexpression did not induce a block in the cell cycle. However, expression of HA-Sak-a, but not HA-Sak-b, from a constitutive promoter for 48 h in CHO cells increased the incidence of multinucleated cells. Our results show that *Sak-a* transcript levels are controlled in a cell cycle-dependent manner and that this precise regulation is necessary for cell growth and the maintenance of nuclear integrity during cell division.

Progression through the eukaryotic cell cycle is controlled in part by a family of cyclin-dependent kinases (Cdks) in association with their regulatory partners, the cyclins (reviewed in reference 22). Recently, members of a family of serine/threonine kinases related to *Drosophila* Polo have also been implicated in regulating cell cycle progression. As with the Cdks, Polo-related proteins have been identified in evolutionary distant eukaryotic phyla and so far include *Drosophila* Polo protein (20), *Saccharomyces cerevisiae* Cdc5 (16), *Schizosaccharomyces pombe* plo1 (23), and four murine proteins, Snk (28), Fnk (4), Plk (3), and Sak (6). Analyses of mutant alleles of *polo*, *CDC5*, and *plo1* indicate that the gene products of these are required in the formation and function of the spindle apparatus (5, 20, 23, 25, 27). For example, monopolar spindles, branched microtubules, polyploidy, and overcondensed chromosomes were observed in *Drosophila* larval neuroblasts of homozygous $polo^1$ mutants (20, 29). In *S. cerevisiae* $cdc5^{ts}$ mutants, spindles did not form completely when the cells were shifted to the nonpermissive temperature prior to the first meiotic division. In cells shifted before the second meiotic division, spindles formed but did not elongate, while mitotically dividing cells arrested later in nuclear division (14, 25, 27). Mutations in the *plo1* gene were also reported to result in monopolar spindles and defects in septation, suggesting a role for plo1 kinase in regulating microtubular and microfilament reorganization during cell division (23).

The murine Sak (6) and Plk proteins (12) also appear to be essential for cell proliferation, as expression of antisense sequences in cell lines has been shown to block cell growth. *Plk* transcripts were observed to be at maximal level during mitosis, and Plk protein was specifically localized to the tubulin midbody separating dividing cells (10). However, constitutive expression of Plk induced quiescent cells to enter S phase, suggesting that perhaps Plk has an additional function or that the protein can substitute for a *polo*-related protein that acts at this earlier phase in the cell cycle (11). Murine *Snk* (28) and *Fnk* (4) are early growth response genes and examples of *polo*-related genes that are regulated in a manner distinct from that of *polo*, *CDC5*, and *Plk*.

In this study, we observed that *Sak-a* mRNA levels are periodically regulated in the cell cycle and that transcripts are absent in G_0-arrested fibroblast cells. We also present evidence that Sak-a protein has a short intracellular half-life and is conjugated to ubiquitin, a molecular tag which targets proteins for rapid proteolysis. In addition, overexpression of Sak-a was observed to inhibit cell growth in a manner that was dependent on the Sak-a specific C-terminal tail and a functional ATP-binding domain. Influenza virus hemagglutinin (HA)-tagged Sak-a overexpression in CHO cells resulted in multinucleation, which suggests that Sak-a may regulate a late mitotic event.

MATERIALS AND METHODS

Plasmid constructs. To generate HA-tagged forms of *Sak-a* and *Sak-b*, cDNAs were subcloned into the pAS1GAL vector in frame with the HA tag (vector gift from Stan Hollenberg, Fred Hutchinson Cancer Research Center, Seattle, Wash.). This was accomplished by using PCR to generate a *Bam*HI site in front of the ATG start codon of *Sak-a* and *Sak-b* for subcloning into the *Bam*HI site of pAS1GAL. The HA-tagged forms of *Sak* were then subcloned into the pCDNA1 expression vector (Invitrogen) behind the cytomegalovirus promoter, and the sequence was confirmed by dideoxy sequencing (pCD-HA-Sak-a and pCD-HA-Sak-b). The sequence at the NH_2 terminus of the HA-tagged forms of Sak reads MAYPYDVPDYASLGGHMAMEAPGIOM (the underlined region is the HA epitope; the boldface M is the start methionine of Sak). The untagged Sak construct with the complete 5′ untranslated region was designated pCD-5′-Sak-a. A point mutation was generated at nucleotide 651 of Sak-a (G to A), converting Asp-154 to Asn-154 (D154N) in the kinase subdomain 7 (i.e., KIA DFGLAT). The mutation was generated by using PCR oligonucleotide-directed mutagenesis as described by Landt et al. (19), and the sequence was verified by

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dideoxynucleotide sequencing [pCD-5′-Sak-a (D154N)]. The pCDNA1 vectors used in the colony formation assay contain a β-hygromycin resistance cassette and were generated as described previously (6).

To generate a glutathione *S*-transferase (GST) fusion protein, PCR was used to insert a *Bam*HI site in front of nucleotide 1712 of Sak-a and an *Eco*RI site following nucleotide 1988 for subcloning into the pGEX-2T vector. This GST–Sak-a construct produced a fusion protein containing amino acids 503 to 594 of Sak-a, a region of the protein which is not found in the Sak-b isoform.

Cell culture, cell cycle synchronization, and transient transfections. The NIH 3T3 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), and the CHO cell line was maintained in alpha minimal essential medium with 10% FCS. To arrest NIH 3T3 cells by contact inhibition, cells were seeded at approximately 30% confluency and fed daily with complete medium containing 10% FCS for 6 days. Alternatively, NIH 3T3 cells were growth arrested by washing cultures that were approximately 75% confluent and adding fresh medium containing 0.2% FCS to the cells for 48 h. The synchronous release of serum-starved cells into the cycle was achieved by trypsinization and replating cells at a lower density (1:4) in medium containing 10% FCS. To impose a mitotic block, growth-arrested NIH 3T3 cells were treated for 12 to 15 h following serum stimulation with 40 ng of nocodazole (Sigma) per ml for 5 h. After this time period, the nonadherent mitotic cells were collected. Mitotic cells were released synchronously into the cell cycle by washing the cells with phosphate-buffered saline (PBS) to remove the drug and replating the cells in fresh medium containing 10% FCS. For CHO cells, asynchronous cultures were treated with 100 ng of nocodazole per ml for 12 h to induce a mitotic block.

For the colony formation assay, 30 μg of each expression construct was linearized with *Cla*I and used to electroporate 6.5×10^6 cells in a volume of 800 μl, using a Bio-Rad Gene Pulser (500 μF, 0.25 V, 0.4-cm cuvette distance). Electroporated cells were plated into 10-cm-diameter dishes (80 μl per dish), and selection for drug-resistant colonies was started 48 h after transfection in medium containing 400 μg of hygromycin B (Calbiochem) per ml for 10 days. Hygromycin-resistant colonies were then stained in 0.06% methylene blue and 1.25% glutaraldehyde in PBS overnight prior to counting.

Transient transfections of CHO cells were done with 15 μl of Lipofectamine reagent (Gibco BRL) plus 3 μg of DNA in a total of 600 μl of Opti-MEM per 60-mm-diameter dish. The DNA-Lipofectamine mix was left on the cells for 5 h, 2 volumes of medium containing 20% FCS was added, and the culture was incubated for 48 h.

FACS analysis. To analyze the cell cycle stage of synchronized NIH 3T3 cells, the cells were scraped off the dishes with a rubber policeman and fixed in 95% ethanol, and then the nuclei were stained with propidium iodide (100 μg/ml) and RNase A (250 μg/ml) in PBS containing 1% FCS for 30 min at 37°C. DNA profiles were examined by fluorescence-activated cell sorting (FACS) using a FACStar processor, and the proportion of cells in each phase of the cell cycle was estimated by using the CellFIT cell cycle analysis program (Becton Dickinson).

CHO cells were cotransfected with 1 μg of pCMVCD20 (gift from Ed Harlow, Massachusetts General Hospital Cancer Center, Charlestown) and either 3 μg of pCDNA1 containing no cDNA insert or the *Sak-a* cDNA (pCD-5′-Sak-a). Cells were immunostained as described by van den Heuvel and Harlow (32). Briefly, cells were harvested and incubated with 20 μl of fluorescein isothiocyanate (FITC)-labeled anti-CD20 (Leu-16; Becton-Dickinson) for 20 min on ice. The cells were then washed with PBS–1% FCS prior to fixation in 70% ethanol. The fixed cells were stained with propidium iodide (10 μg/ml) and RNase A (250 μg/ml), and the DNA profile of the gated FITC-positive population was determined.

Immunofluorescence staining. Cells cotransfected with the pCMVCD20 expression construct and pCDHA-Sak-a, pCDHA-Sak-b, or pCDNA1 were trypsinized 24 h posttransfection and replated on acid-washed glass coverslips for an additional 24 h prior to staining. The coverslips were washed in PBS with 1% FCS and then inverted onto a slide with 20 μl of FITC-anti-CD20 antibody for 30 min on ice. The cells were then fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Nuclei were stained with 2 μM Hoechst 33258 (Pierce) in PBS for 5 min at room temperature.

RNA isolation and Northern (RNA) blot analysis. RNA was extracted from cell lines by using a guanidinium isothiocyanate procedure as described by Chirgwin et al. (1). Total RNA at 10 μg per lane was electrophoresed through a 1% formaldehyde gel and transferred to Gene Screen (Dupont) membrane. Hybridization was carried out at 65°C, using the Gene Screen buffer described by the manufacturer for Northern hybridization.

Generation and affinity purification of antisera. Polyclonal antibody to the Sak-a protein was generated in a rabbit, using a GST–Sak-a fusion protein as an immunogen. A total cell lysate prepared by sonication of an *Escherichia coli* strain expressing the GST-Sak fusion protein was clarified by centrifugation, and the supernatant was incubated with glutathione-coupled Sepharose beads (Sigma). Bound protein was eluted with 50 mM glutathione in 50 mM Tris-Cl (pH 8), which was subsequently removed by overnight dialysis in PBS. Rabbits were immunized with the purified antigen, using an immunization protocol previously described (13).

To affinity purify Sak-a-specific antibodies, affinity resins were prepared by conjugating purified GST and the GST-Sak fusion protein to cyanogen bromide-activated Sepharose 4B beads. Immune serum was first precleared of anti-GST antibodies by incubating the serum with GST beads overnight at 4°C. Protein not binding to GST-Sepharose was then incubated overnight at 4°C with GST–Sak-a-conjugated beads. The beads were poured into a column, and Sak-a-specific antibodies were eluted by using 50 mM glycine-Cl (pH 2.5), with aliquots being collected directly into 1/10 volume of 1 M Tris-Cl (pH 7.5). The eluted antibodies were concentrated in an Amicon 10 microconcentrator and resuspended in PBS. Antibody concentration was determined by using the bicinchoninic acid protein assay reagent (Pierce).

Western blotting (immunoblotting). Cells were lysed in radioimmunoprecipitation assay buffer (PBS with 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, and 1% sodium deoxycholate) containing 5 mM phenylmethanyl sulfonyl fluoride, 10 μg of leupeptin per ml, and 10 μg of aprotinin per ml for 30 min at 4°C. Lysates prepared from tissues were homogenized to dissociate the cells. Lysates were clarified by centrifugation and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), using prepoured Tris-glycine gels (Novex). Following electrophoresis, proteins were transferred to an Immobilon P transfer membrane (Millipore). For antiubiquitin immunoblotting, following the transfer of proteins to nitrocellulose, membranes were submerged in water and autoclaved for 15 min. Membranes were blocked in 10% bovine serum albumin and 0.1% Tween 20 in PBS for 1 h and then incubated with 1/10,000 dilution of antiubiquitin (Sigma). After washing, the membranes were incubated in 1/50,000 dilution of anti-rabbit-horseradish peroxidase conjugate (Amersham) and developed by using the Amersham ECL (enhanced chemiluminescence) kit. The blots were then stripped according to the instructions for the ECL kit and reblotted with 0.5 μg of affinity-purified anti-Sak per ml.

In vitro synthesis of Sak. Different forms of Sak were transcribed and translated in vitro from the expression constructs described above in the presence of 34 μCi (4 μl) of [^{35}S]methionine-cysteine (EXPRE^{35}S^{35}S protein labeling mix; New England Nuclear), using the coupled rabbit reticulocyte lysate system and T7 RNA polymerase (Promega).

Pulse-chase analysis. CHO cells were transiently transfected with the pCD-HA-Sak-a expression construct. Eighteen hours posttransfection, the cells were replated in eight 60-mm-diameter dishes, and 24 h posttransfection, the cells were incubated in methionine- and cysteine-free DMEM for 1 h before metabolic labeling. Cells in each 60-mm-diameter dish were labeled for 30 min with 240 μCi of ^{35}S-labeled methionine-cysteine (EXPRE^{35}S^{35}S labeling mix; New England Nuclear) in a 1-ml volume. Following the labeling reaction, the cells were washed twice with PBS and incubated in DMEM with 10% FCS and 15 mg of methionine per ml. At the indicated time points, cells were harvested and the cell pellets were lysed in radioimmunoprecipitation assay buffer. Equal amounts (counts per minute) of labeled lysate from each time point were immunoprecipitated with 1 μg of affinity-purified anti-Sak antibody. Immunoprecipitated proteins were run on SDS–8% polyacrylamide gels, subjected to fluorography using En^{3}Hance (Dupont), and analyzed by autoradiography. Quantitation of radioactivity was done with a PhosphorImager (Molecular Dynamics). A polyclonal anti-HA antibody (RPB101C; BabCo) was used at a dilution of 1/500 in immunoprecipitations.

RESULTS

***Sak-a* transcript levels are cell cycle regulated.** In adult and embryonic mouse tissues examined by RNA in situ hybridization, *Sak-a* transcripts were essentially absent from nondividing, terminally differentiated cells (6). To determine whether *Sak-a* expression is downregulated upon exit from the cell cycle, transcript levels were examined in NIH 3T3 cells over a 6-day period while the cells became contact inhibited (Fig. 1A). The distribution of cells in each phase of the cell cycle was monitored by flow cytometry. By day 6 of culture, the proportion of proliferating cells (S + G_2/M) decreased from 73.8 to 6.9%. By Northern analysis, the abundance of *Sak-a* mRNA was shown to decrease dramatically as contact-inhibited cells withdrew from the cell cycle (Fig. 1A). Similarly, cells made quiescent by serum starvation contained 95.6% noncycling cells and lacked detectable levels of *Sak-a* transcripts (i.e., time zero in Fig. 1B). Thus, *Sak-a* is not expressed at detectable levels in G_0 growth-arrested NIH 3T3 cells.

Sak-a transcript levels were also examined in serum-starved NIH 3T3 cells following release into the cell cycle in response to serum stimulation. *Sak-a* transcripts began to accumulate 10 h following the release from G_0, which corresponded to late G_1 phase of the cell cycle (Fig. 1B). As the cells progressed through S phase, *Sak-a* transcript levels remained elevated, and they were also maintained at high levels in cells arrested in

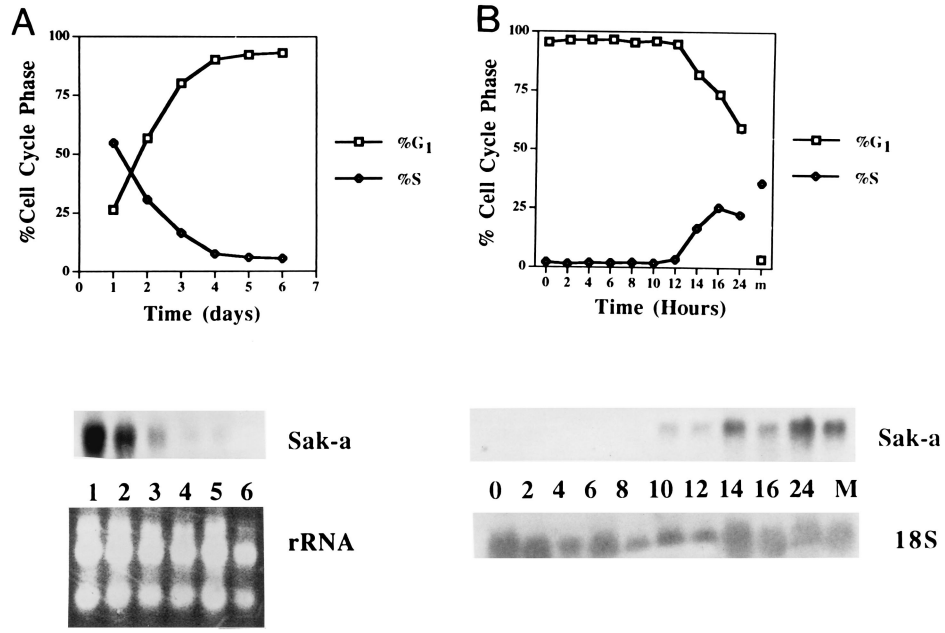

FIG. 1. Northern blot analysis of *Sak-a* transcripts in NIH 3T3 cells undergoing growth arrest by contact inhibition (A) or resuming growth in 10% FCS following 48 h of serum starvation (B). Total RNA was extracted, and cells were stained with propidium iodide for FACS analysis, daily, from dishes treated in parallel. Northern blot analysis was done with a *Sak-a* probe. Ethidium bromide staining of rRNA (A) and a probe for 18S rRNA (B) confirmed similar loading of the lanes.

mitosis by treatment with nocodazole (Fig. 2, initial time point).

These observations suggest that *Sak-a* transcripts may be regulated in a cell cycle-dependent manner or that *Sak-a* may be a late growth response gene which is expressed after 10 h of growth factor stimulation. To distinguish between these possibilities, *Sak-a* transcript levels were examined in NIH 3T3 cells following release from a nocodazole-induced mitotic block. Cells blocked in mitosis while cultured in the presence of serum proceed directly into G_1 phase without going through G_0 when nocodazole is removed. Following nocodazole treatment, high levels of *Sak-a* transcripts were detected in metaphase-arrested NIH 3T3 cells (Fig. 2, 0 h). Three hours following the release from the mitotic block, cells were in early G_1 phase and *Sak-a* message levels were much reduced. By mid-G_1 phase (Fig. 2, 6 h), *Sak-a* mRNA was no longer detected, indicating that transcript levels are periodically regulated in continuously cycling cells. The loss of *Sak-a* transcripts in mid-G_1 in this experiment correlates well with the absence of *Sak-a* mRNA during the mid-G_1 phase following release from serum starvation as shown in Fig. 1B. Nine hours postrelease, at a time when cells were beginning to enter S phase, *Sak-a* transcripts reappeared and levels remained elevated through the remainder of the cell cycle (Fig. 2). The observed accumulation of *Sak-a* transcripts beginning in late G_1 phase of the cell cycle, using two synchronization protocols (i.e., release from G_0 and M blockades), shows that expression is cell cycle regulated and suggests that Sak-a may function some time between late G_1 and the completion of mitosis.

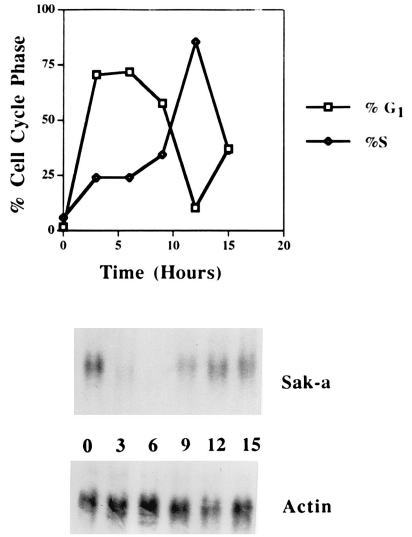

FIG. 2. *Sak-a* transcript accumulation is periodic in cells released from synchronization by a mitotic block. NIH 3T3 cells were growth arrested in medium containing 0.1% FCS for 48 h. After 12 h of serum stimulation, a metaphase block was imposed by using nocodazole. After 6 h, nonadherent mitotic cells were collected, washed with PBS, and replated in fresh medium containing 10% FCS. The cells were harvested 3, 6, 9, 12, and 15 h later for RNA extraction and flow cytometric analysis of DNA content. Northern blot analysis was performed with radiolabeled *Sak-a*, and comparable loading was confirmed with a β-actin probe.

Endogenous Sak-a protein levels are low in cell lines and mouse tissues. A polyclonal antibody was raised against a bacterially expressed GST fusion protein containing amino acids 503 to 594 of Sak-a. By Western blot analysis, the affinity-purified polyclonal antiserum recognized an HA epitope-tagged Sak-a protein in CHO cell lysates made 48 h following transient transfection of cells with the pCD-HA-Sak-a expression construct. The immune serum detected a ladder of immunoreactive bands ranging from 64 to 103 kDa in size (Fig. 3A, lane 1), which were specific since they were not seen in lysates prepared from CHO cells transfected with an empty expression vector (lane 2). The predominant band at 103 kDa had the predicted molecular weight for the HA-Sak-a protein. The

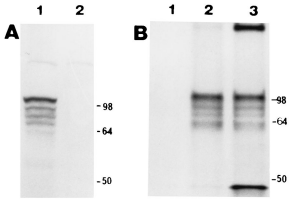

FIG. 3. Detection of HA-Sak-a protein by Western blotting and immunoprecipitation. (A) CHO cell lysates were prepared 48 h following transient transfection with expression pCD-HA-Sak-a vectors (lane 1) or pCDNA1, containing no cDNA insert (lane 2), and subjected to SDS-PAGE and Western blot analysis using affinity-purified anti-Sak-a antibodies. (B) CHO cells transiently transfected with the pCD-HA-Sak-a expression construct were metabolically labeled with [^{35}S]methionine-cysteine for 30 min. The labeled cells were lysed and immunoprecipitated with preimmune serum (lane 1) or affinity-purified anti-Sak antibodies (lanes 2 and 3). The immunoprecipitates in lane 2 were washed under higher stringency (0.65 M NaCl) than the immunoprecipitates in lane 3 (0.15 M NaCl). Numbers at the right are sizes of molecular weight markers in kilodaltons.

smaller immunoreactive proteins may be proteolytic degradation products of the full-length protein and/or posttranslationally modified forms of HA-Sak-a. A 103-kDa protein was also detected by anti-Sak-a immunoblot analysis in extracts from adult mouse testis and from the MDAY-D2 lymphoid cell line, which both express relatively high levels of *Sak-a* transcripts (data not shown). However, the anti-Sak-a antibodies did not detect endogenous Sak-a in CHO (lane 2), NIH-3T3, or Rat2 fibroblasts, although transcripts were detected by Northern analysis in these cell lines (data not shown). These observations suggest that the endogenous Sak-a protein is maintained at very low levels in most cell types, even when they are proliferating.

HA-Sak-a protein was immunoprecipitated from metabolically labeled CHO cells that had been transiently transfected with the pCD-HA-Sak-a expression vector. A ladder of labeled proteins ranging in size from 64 to 103 kDa was revealed, similar to the pattern of Sak-a immunoreactive proteins visualized by Western blot analysis. In addition, two proteins of approximately 190 and 43 kDa coimmunoprecipitated with HA-Sak-a under low-stringency (Fig. 3B, lane 3) but not high-stringency (lane 2) wash conditions. This result suggests that HA-Sak-a forms specific complexes with other cellular proteins, and their identification may help elucidate the regulatory pathway in which Sak functions.

HA-Sak-a has a short intracellular half-life, and multiubiquitinated forms of HA-Sak-a are detected in vitro and in vivo. Two observations suggested that Sak-a protein levels may be controlled by rapid turnover. First, an examination of the primary amino acid sequence of Sak-a revealed three potential PEST sequences (6), motifs common to proteins that have short intracellular half-lives (24). In addition, the immunoreactive protein bands of less than 103 kDa in Fig. 3 are consistent with rapid proteolytic turnover of Sak-a. Indeed, pulse-chase experiments using a 6-h chase period indicated that the HA-tagged Sak-a protein has a relatively short half-life of 2 to 3 h (Fig. 4). Because of the low levels of the endogenous Sak-a protein in cell lines, the half-life had to be measured following transient transfection of cells with a *Sak-a* expression vector. Although it is possible that this measurement does not accurately reflect the normal degradation kinetics for the protein, in a similar experiment, the half-life of an overexpressed histidine-tagged form of c-Jun compared well with that of the endogenous protein (31).

The HA-Sak-a protein was also observed to be short-lived following transcription and translation in vitro in a rabbit reticulocyte lysate (data not shown). Reticulocytes are rich in the components necessary for ubiquitin-mediated degradation (15). Ubiquitin, a 76-amino-acid protein, is conjugated in multiple copies to specific proteins and thereby targets them for rapid degradation by the 26S proteasome. The turnover of several growth and cell cycle regulators, including c-Jun (31) and cyclins (26), is regulated by ubiquitination. To determine whether Sak-a is subject to ubiquitination, HA-Sak-a was synthesized in reticulocyte lysates, immunoprecipitated with anti-Sak-a antibodies, and immunoblotted with a polyclonal antibody to ubiquitin. A ladder of high-molecular-mass (>180-kDa) antiubiquitin immunoreactive material was immunoprecipitated from translation reactions by using affinity-purified anti-Sak-a antibodies (Fig. 5A, lane 1) but not in control reactions in which the expression vector did not contain an insert (lane 2). Reprobing the Western blot with the anti-Sak-a antiserum verified that the 103-kDa HA-Sak-a protein was immunoprecipitated only from lysates primed with the vector containing the cDNA insert (lane 3). Ubiquitinated proteins are rapidly degraded, with an estimated half-life of 1 min (9), and therefore are expected to represent a very small proportion of the protein present in the cell, yet they carry many ubiquitin molecules per protein. As such, low-abundance, higher-molecular-weight forms of HA-Sak-a were detectable with the antiubiquitin antiserum but appeared to be below the level of detection for the anti-Sak-a antibody. High-molecular-weight, antiubiquitin-immunoreactive material was also observed in Sak-a immunoprecipitates from CHO cells that had been transiently transfected with an expression vector (Fig. 5B). These experiments show that HA-Sak-a is subject to ubiquitination both in vitro and in vivo and suggest that this proteolytic pathway may regulate Sak protein levels during the cell cycle.

Deregulated expression of Sak-a suppresses cell growth. To examine the effect of constitutive Sak-a expression on cell growth, constructs containing the *Sak-a* cDNA under the control of the cytomegalovirus promoter, together with a β-hygromycin resistance gene under the control of the β-actin promoter, were linearized and electroporated into CHO cells. Following 10 days of selection in hygromycin, macroscopic cell colonies were counted. The expression of Sak-a or HA-tagged

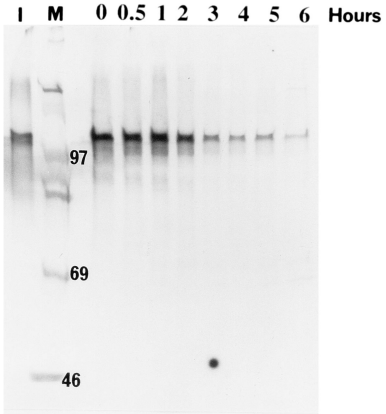

FIG. 4. Sak-a half-life. CHO cells were transiently transfected with the pCD-HA-Sak-a expression construct, labeled 24 h later with [^{35}S]methionine-cysteine for 30 min, and then chased with fresh medium for the times indicated. Equal amounts of labeled lysate (10^8 cpm) were immunoprecipitated with the affinity-purified anti-Sak-a antibody and analyzed by SDS-PAGE and autoradiography. In vitro-transcribed and -translated HA-Sak-a was also immunoprecipitated as a control (lane I). Lane M shows the positions of molecular weight markers in kilodaltons.

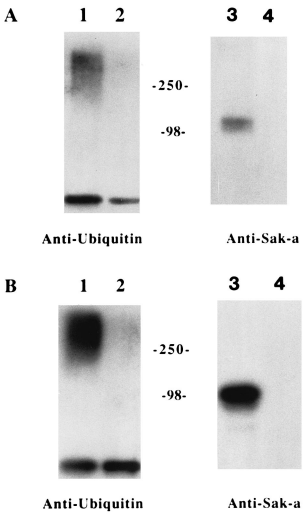

FIG. 5. HA-Sak-a is ubiquitinated in reticulocyte lysates and in CHO cells. (A) Expression constructs pCD-HA-Sak-a (lanes 1 and 3) or pCDNA1, without cDNA insert (lanes 2 and 4), were transcribed and translated in vitro in a rabbit reticulocyte lysate. After the reaction, the Sak-a protein was immunoprecipitated with anti-Sak-a antibodies and analyzed by Western blot analysis using either an antiubiquitin or affinity-purified anti-Sak-a antibody, as indicated. (B) CHO cells were transiently transfected with the pCD-HA-Sak-a expression construct (lanes 1 and 3) or an empty vector (lanes 2 and 4). The Sak-a protein was immunoprecipitated from CHO cells 48 h posttransfection and subjected to Western blot analysis using an antiubiquitin or anti-Sak-a antibody, as indicated. Numbers in the margin are sizes of molecular weight markers in kilodaltons.

Sak-a reduced the colony-forming efficiency of CHO cells ~25-fold compared with cells transfected with a vector expressing only the β-hygromycin resistance gene (Fig. 6). Similar results were obtained in NIH 3T3 cells, with a 10-fold suppression of colony formation by Sak-a (data not shown). Addition of an HA tag to the amino terminus of the Sak protein appeared to be neutral in this assay.

The Sak-a and Sak-b sequences are identical in the first 416 amino acids, which include the kinase domain, but differ in the C-terminal tails, comprising 509 and 48 amino acids, respectively. Compared with expression of HA-Sak-a, expression of the HA-tagged Sak-b isoform did not reduce colony-forming efficiency in CHO cells (Fig. 6). The ability of these vectors to direct the synthesis of HA-tagged Sak proteins to similar levels was confirmed by in vitro transcription and translation followed by immunoprecipitation with a polyclonal anti-HA antibody (data not shown). In addition, comparable expression levels of HA-Sak-a and HA-Sak-b proteins in CHO cells from very similar expression vectors lacking only the β-hygromycin resistance cassette are shown in Fig. 8C. This result suggests that the C-terminal tail of Sak-a contains an important functional domain which is not found in the Sak-b isoform. Although both *Sak-a* and *Sak-b* transcripts were detected in dividing cells of adult and embryonic tissues, *Sak-a* mRNA was much more abundant (6).

An inactivating mutation was made in the putative ATP-binding domain of Sak-a to study the effects in the colony formation assay. Mutations in a strictly conserved aspartate residue of kinase subdomain 7 have been shown to abolish the kinase activities of Cdc2, Cdk2, and Cdk3 (32) as well as Cdc5 kinase (16). As shown in Fig. 7, expression of a mutated form of Sak-a with the corresponding aspartate 154 residue mutated to asparagine (D154N) inhibited CHO colony formation less than 2-fold, compared with 25-fold for the wild-type protein, suggesting that Sak-a kinase activity was required for growth suppression in this assay. Western blotting with anti-Sak-a antibodies confirmed that wild-type and D154N forms of Sak-a proteins were expressed at similar levels in transfected CHO cells (data not shown). These results indicate that both the kinase domain and C terminus are functional domains of Sak.

Sak-a overexpression increases the incidence of abnormal nuclei. Sak-a overexpression suppressed CHO cell growth and therefore could cause the accumulation of cells in a specific phase of the cell cycle. Therefore, the effect of overexpressing Sak-a on cell cycle distribution was examined by cotransfecting CHO cells with a Sak-a and pCMVCD20 expression vector, using the cell surface CD20 molecule as a marker for transfected cells. The DNA content of transfected cells was analyzed by using flow cytometry and gating the $CD20^+$ cell population as previously described (32). Surprisingly, the cell cycle profiles of populations expressing Sak-a for 48 h did not differ significantly from the profiles of cells transfected with an empty expression construct in three independent experiments (Fig. 7A). To confirm that Sak-a-overexpressing cells were still cycling, as opposed to being blocked in all phases of the cell cycle, the transfected cells were treated with nocodazole at 36 h posttransfection to induce a mitotic block. FACS analysis after 12 h of nocodazole treatment confirmed that the majority of the Sak-a-expressing cells continued to cycle, as 67.0% of the population progressed to the mitotic block, while 82.7% mitotic cells were observed in cultures transfected with empty vector (Fig. 7B). However, the number of $CD20^+$ cells was reduced approximately twofold when the cells were cotransfected with Sak-a compared with the empty expression constructs. Taken together, these observations suggest that overexpression of Sak-a in CHO cells did not arrest growth in a specific phase of the cell cycle but rather resulted in a loss of viability over several cell divisions.

To further characterize the phenotype associated with Sak-a overexpression, cells cotransfected with pCMVCD20 along with Sak expression vectors were examined under the microscope after staining with FITC-conjugated anti-CD20 antibodies and with Hoechst 33258 dye to label nuclei. A significant increase in the number of cells with abnormal nuclear morphology was observed following 48 h of HA-Sak-a expression (Fig. 8A and B). Cells scored as abnormal were multinucle-

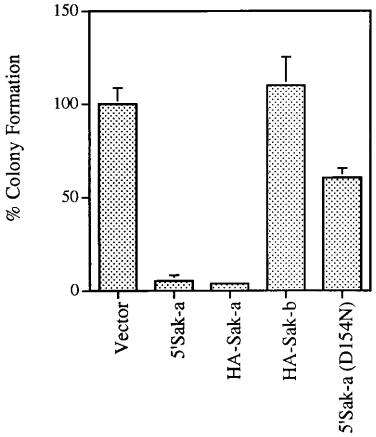

FIG. 6. Effects of expression of wild-type and mutant Sak proteins on the growth of CHO cells. CHO cells were transfected with the indicated *Cla*I-linearized plasmids, each containing a β-hygromycin resistance cassette. Following 10 days of selection in hygromycin B, macroscopic colonies were counted. Results are expressed as percentages of colony formation in cultures transfected with control construct lacking the *Sak* insert and are the means of two or more independent experiments.

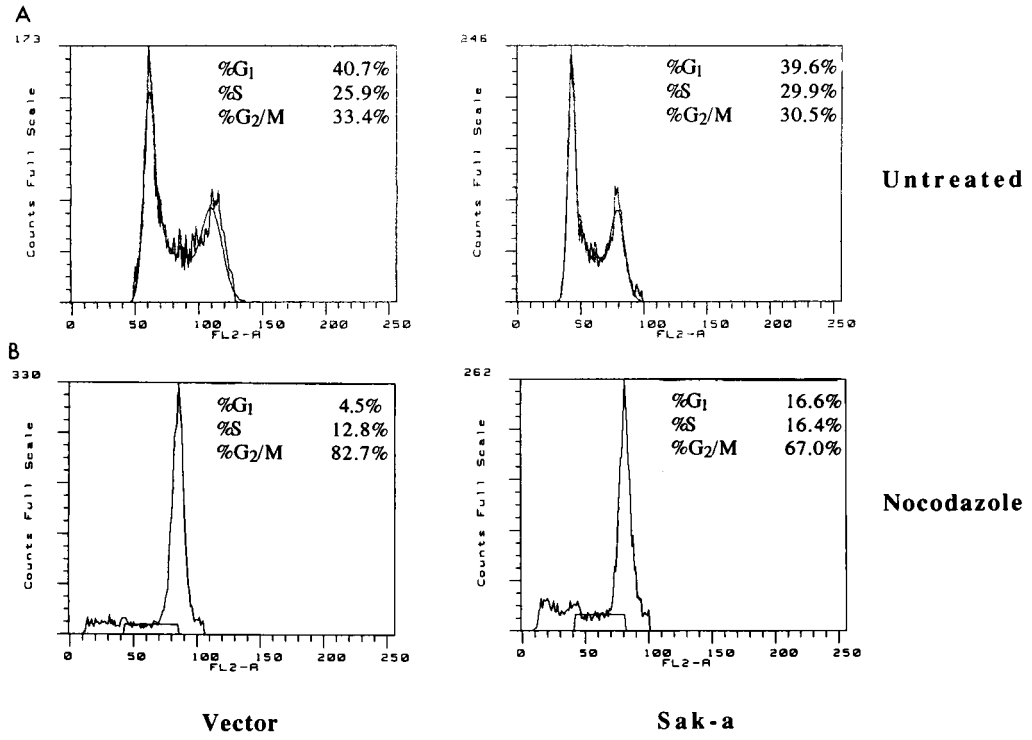

FIG. 7. Cell cycle distribution of CHO cells after 48 h of Sak-a overexpression. CHO cells were cotransfected with a pCMVCD20 expression vector and either the empty pCDNA1 vector (control) or pCD-5′-Sak-a. Cell cycle profiles of the $CD20^+$ cells were obtained by FACS analysis. (A) Representative experiment of three independent experiments; (B) experiment in which transfected cells were treated with nocodazole at 36 h posttransfection and then analyzed 12 h later.

ated, exhibiting three or more nuclear compartments. Overexpression of HA-Sak-a resulted in 18% ± 4.8% abnormal nuclei, compared with 5% ± 1.2% for cells expressing HA-Sak-b or 9% ± 0.2% for cells transfected with empty vector in a representative experiment. Consistent results were obtained in five independent experiments, with two- or threefold increases in the level of multinucleation following expression of Sak-a. Immunoprecipitation of HA-Sak-a and HA-Sak-b proteins from lysates of transfected cells with anti-HA antibodies revealed bands with sizes corresponding to the predicted molecular weights of the two Sak isoforms and showed that they were both expressed at comparable levels in CHO cells (Fig. 8C). The nuclear phenotype of HA-Sak-a overexpression is consistent with a Sak-a-induced disruption of karyokinesis and may reflect aberrant microtubular and/or microfilament function.

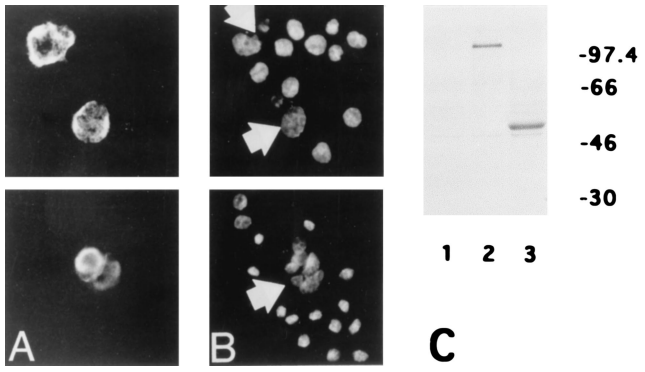

FIG. 8. Multinucleation in CHO cells overexpressing HA-Sak-a. CHO cells were cotransfected with the expression vectors pCMVCD20 and pCD-HA-Sak-a, pCD-HA-Sak-b, or pCDNA1 without insert. Transfected cells were plated on coverslips, and 48 h posttransfection, the cells were stained with FITC-anti-CD20 antibodies to identify transfected cells (A) and Hoechst dye to label nuclei (B). Transfected cells representing the multinucleated phenotype are indicated with arrows. Cells from a transfection with pCD-HA-Sak-a are shown. pCD-HA-Sak-b or expression vector containing no cDNA insert did not induce the multinucleated phenotype. (C) Transfected cells were metabolically labeled 24 h following transient transfection with [^{35}S]methionine for 1 h. The labeled cells were lysed and immunoprecipitated with polyclonal anti-HA antibodies, and the precipitates were subjected to SDS-PAGE and fluorography. Lane 1, pCDNA1 control; lane 2, pCD-HA-Sak-a; lane 3, pCD-HA-Sak-b. Positions of molecular weight markers are indicated in kilodaltons.

DISCUSSION

We have previously shown that *Sak-a* transcripts are restricted to dividing cells in mouse tissues, suggesting a potential role for the gene product in cell division (6). In this study, we show that *Sak-a* expression levels are regulated during the cell cycle, peaking in M phase, and that constitutive overexpression of Sak-a inhibits cell growth. CHO cells overexpressing Sak-a continue to progress through the cell cycle, but the accumulation of multinucleated cells indicates that this process is not completed normally. These results suggest a role for the Sak-a protein in regulating events occurring late in mitotic cell division.

The negative consequences of Sak-a overexpression on both colony formation and nuclear structure indicate that Sak-a levels must be precisely regulated in cycling cells. We characterized features of *Sak* mRNA and protein turnover which suggest that regulation occurs at multiple levels. In synchronized NIH 3T3 cells, *Sak-a* transcripts were first detected in late G_1 phase and remained elevated through S and M phases before declining early in G_1. In a similar manner, products of several *polo*-related genes have been shown to peak in either

level of expression or kinase activity during mitosis, consistent with a role of this family of protein kinases in the latter portion of the cell cycle (5, 10, 16, 18).

At the level of protein turnover, HA-Sak-a was determined to have a short half-life (2 to 3 h) in CHO cells. This finding suggests that as *Sak-a* transcript levels decline during G_1 phase, the Sak-a protein also disappears from dividing cells. Consistent with the short half-life of HA-Sak-a, we have observed that the protein is subject to multiubiquitination. Proteolysis by ubiquitin targeting has been conserved throughout eukaryotic evolution and is key to the regulation of a number of biological processes, including cell cycle progression (2, 15).

The HA-Sak-a and HA-Sak-b proteins showed similar degradation kinetics in vitro in the reticulocyte lysate system (5a), suggesting that the signals targeting Sak for degradation are located in the amino-terminal 416 amino acids common to both proteins. Several PEST motifs present in this region may contribute to Sak instability. However, sequences targeting proteins for ubiquitination are poorly defined and diverse, presumably reflecting the specificities of the multiple E_2 and E_3 ubiquitinating enzymes. Confirmation that Sak degradation is ubiquitin dependent will require additional evidence that turnover involves the E_1, E_2, and E_3 enzymes (15).

Suppression of colony formation was observed for HA-Sak-a but not Ha-Sak-b, suggesting that a unique functional domain is present in the carboxy terminus of Sak-a. However, the sequence of the nonkinase domain provided little information concerning function. The C-terminal tails of Sak-a and Sak-b do not contain the 29-amino-acid homology domain found in other members of the Polo subfamily (3), nor do they exhibit significant homology to proteins in GenBank. The Sak kinase domain shows 37 to 42% homology with those of other members of the *polo* family and is presently the most distantly related member.

An inactivating mutation in the ATP-binding domain of Sak-a neutralized growth-suppressing activity, suggesting that Sak-a functions as a protein kinase. However, we have been unable to demonstrate that the wild-type Sak-a protein has kinase activity, either by autophosphorylation or by using exogenous substrates such as casein, histone H1, or myelin basic protein. This observation suggests that substrate recognition of Sak-a may be very specific or that activating events, such as phosphorylation or association with accessory proteins, may be required. It is possible that the 43- and 190-kDa proteins observed to coimmunoprecipitate with HA-Sak-a could be either accessory proteins or potential substrates for Sak-a.

FACS analysis after 48 h of Sak-a overexpression showed that cell cycle progression was not affected at any particular stage, despite the suppression of cell growth in the colony formation assay. Furthermore, Sak-a-overexpressing cells responded normally to nocodazole, arresting in M phase of the cell cycle. This finding suggests that the Sak-a-overexpressing cells progressed into mitosis, where the mitotic spindle checkpoint functioned normally. However, a morphological examination of HA-Sak-a-overexpressing cells revealed that although the cells continue to progress through the cell cycle, nuclear abnormalities accumulate. Importantly, multinucleation was induced following the expression of the HA-Sak-a isoform but not the HA-Sak-b isoform, consistent with the different growth-suppressive activities of the two proteins in the colony formation assay. Since overexpression of Sak-a in CHO cells inhibits colony formation but does not block the cell cycle in the time frame of a single cell division, it appears as if the loss of viability is caused over multiple cell divisions as a result of disruption of nuclear integrity. Consistent with this view, the recovery of HA-Sak-a-overexpressing cells in the FACS experiments was reduced compared with controls. However, there was no evidence of programmed cell death, as characteristics of this process, such as chromosome condensation of DNA fragmentation, were not detected in Sak-a-overexpressing cells (5a). In addition, FACS analysis of the Sak-a-overexpressing cells did not reveal an increase in a subdiploid, apoptotic population of cells following Sak-a overexpression.

The phenotype of multinucleation observed in HA-Sak-a-overexpressing cells suggests that Sak-a may play a role in karyokinesis. Indeed, it was shown that agents which disrupt tubulin assembly and thus inhibit spindle function during mitosis induce nuclear abnormalities in CHO cells (21, 30). Correspondingly, we have observed that up to 58% of CHO cells are multinucleated when examined 12 h following their release from a nocodazole block, and these cells are phenotypically indistinguishable from HA-Sak-a-overexpressing cells (5a). Similarly, disrupting the expression of NuMA in fibroblast cell lines induced the formation of multiple micronuclei and has been postulated to reflect a requirement for this protein in spindle function (17) or maintenance of the integrity of the nuclear matrix (8). Disruption of microfilament function affecting the formation of the cleavage furrow during cytokinesis by overexpressing a mutant tropomyosin also caused multinucleation (33). Similar phenotypes were reported for members of the *polo* family: mutations in *CDC5*, *plo1*, and *polo*, as well as overexpression of *plo1*, have been shown to cause defects resulting in abnormal spindle function. In addition, the *plo1* gene product is required for the formation of the septum during cytokinesis in *S. pombe*, a process related to the formation of the contractile ring in mammalian cells (23). Furthermore, the tumor suppressor protein p53 was recently reported to exert a checkpoint function by regulating centrosome duplication in mouse embryonic fibroblasts (7). The lack of p53 was associated with a loss of cell viability and a multinuclear phenotype similar to that observed with Sak-a overexpression. It is therefore possible that Sak-a is involved in the regulation of mitotic structures.

In conclusion, our data suggest that Sak-a levels are regulated in a cell cycle-dependent manner and that this precise regulation is necessary for cell growth and the maintenance of nuclear integrity during cell division.

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