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The Ras-Specific Exchange Factors Mouse Sos1 (mSos1) and mSos2 Are Regulated Differently: mSos2 Contains Ubiquitination Signals Absent in mSos1

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We have compared aspects of the mouse *sos1* (m*sos1*) and m*sos2* genes, which encode widely expressed, closely related Ras-specific exchange factors. Although an m*sos1* plasmid did not induce phenotypic changes in NIH 3T3 cells, addition of a 15-codon myristoylation signal to its 5′ end enabled the resulting plasmid, myr-*sos1*, to induce approximately one-half as many foci of transformed cells as a v-H-*ras* control. By contrast, an isogenic myr-*sos2* plasmid, which was made by fusing the first 102 codons from myr-*sos1* at homologous sequences to an intact m*sos2* cDNA, did not induce focal transformation directly, although it could form foci in cooperation with c-H-*ras*. Pulse-chase experiments indicated that the half-life of Sos1 in NIH 3T3 cells was greater than 18 h, while that of Sos2 was less than 3 h. While in vitro-translated Sos1 was stable in a rabbit reticulocyte lysate, Sos2 was degraded in the lysate, as were each of two reciprocal chimeric Sos1-Sos2 proteins, albeit at a slower rate. In the lysate, Sos2 and the two chimeric proteins could be stabilized by ATPγS. Unlike Sos1, Sos2 was specifically immunoprecipitated by antiubiquitin antibodies. In a myristoylated version, the chimeric gene encoding Sos2 at its C terminus made a stable protein in NIH 3T3 cells and induced focal transformation almost as efficiently as myr-m*sos1*, while the myristoylated protein encoded by the other chimera was unstable and defective in the transformation assay. We conclude that mSos2 is much less stable than mSos1 and is degraded by a ubiquitin-dependent process. A second mSos2 degradation signal, mapped to the C terminus in the reticulocyte lysate, does not seem to function under the growth conditions of the NIH 3T3 cells.

The *ras* genes encode GTPases that transduce a variety of extracellular signals (26, 29). The pleiotropic downstream effects of the Ras proteins include alteration of the plasma membrane, cytoskeleton, and gene expression through activation of Raf-mitogen-activated protein kinase, Rac, and Rho pathways among other intracellular signaling systems (23, 29, 30, 40). The components of the signaling cascade that lies upstream from Ras (6) are similar in nematodes, flies, and mammals. The upstream molecules include membrane-spanning proteins such as receptor tyrosine kinases and the transferrin receptor, SH2- and SH3-containing adapter proteins such as Sem-5 in *Caenorhabditis elegans* (14), Drk in *Drosophila* (36), and Shc and Grb2 (43) in mammals, and Ras-specific guanine nucleotide release/exchange factors such as Sos (4, 9, 25, 48) and guanine nucleotide-releasing factor (GRF) (8, 31, 47, 50).

The Ras proteins function as membrane-associated intracellular switches that are active when bound to GTP and inactive when bound to GDP (3). Sos and GRF activate Ras by converting it from inactive GDP-Ras to active GTP-Ras through catalyzing the dissociation of GDP from Ras, which is rapidly rebound with GTP. The Ras signal is inactivated by hydrolysis of GTP-Ras to GDP-Ras. This inactivating step is enzymatically regulated by GTPase-activating proteins (GAPs) such as Ras-GAP and neurofibromin, the protein product of the gene that is mutated in type 1 neurofibromatosis.

While the Ras proteins have a long half-life and are therefore regulated in large part by cycling between their GDP- and GTP-bound forms, other proteins possess much shorter half-lives and are tightly regulated by their rate of synthesis and/or degradation. One regulatory system that contributes to the steady-state level of proteins is the ubiquitin-dependent protein degradation pathway (12). Ubiquitin is an evolutionarily conserved 76-amino-acid protein that, following its covalent linkage to lysine residues on certain proteins, targets the proteins for degradation, which is accomplished via the proteasome, a complex that contains several proteolytic enzymatic activities. The ubiquitin system has been implicated in growth control and other biologic processes, including cyclins (19, 27, 37, 46), proto-oncoproteins (11, 13, 34, 49), the functional maturation of NF-κB (38), and the processing of antigens for presentation by class I-restricted molecules (32, 42).

In the Ras pathway, one difference between *sos* in flies and mammals is that flies contain a single *sos* gene, while mammals contain two, *sos1* and *sos2* (2, 4, 9, 17). Analysis of cDNAs encoding the human Sos (hSos) and mouse Sos (mSos) proteins has found that the predicted mSos1 and hSos1 amino acids are approximately 98% identical, as are mSos2 and hSos2, while hSos1 and hSos2 are approximately 70% identical. Northern blotting of mouse RNA has shown that m*sos1* and m*sos2* are expressed widely and at similar levels (4).

In spite of these similarities between Sos1 and Sos2, we now report that m*sos1* is much more active biologically than m*sos2*, that these differences seem to result primarily from mSos2 protein having a half-life much shorter than that of mSos1, and that in vitro analysis shows that mSos2 contains ubiquitination signals that are not present in mSos1.

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MATERIALS AND METHODS

Plasmid constructions. The genes encoding mSos1 and mSos2 were constructed from previously described cDNAs (4). A unique *Xho*I site downstream of the mSos1-coding sequence was generated by digestion of a unique *Kpn*I site, blunting with T4 polymerase, and insertion of an *Xho*I linker. A unique *Sac*II site is present a few bases upstream of codon 18 of the mSos1 open reading frame; this codon is a methionine codon with a good Kozak sequence (24). This is reported to be the initiation methionine codon for h*sos1* and h*sos2*. These unique *Sac*II and *Xho*I sites were used for transfer of the *sos* genes. A *Mun*I site present in the 5′ end in both cDNAs (but not at completely homologous sites) was used to supply the mSos2 protein with an mSos1 N terminus; subsequently, a double-stranded 11-mer with *Mun*I overhangs was cloned into the *Mun*I site in the mSos2 fusion protein to supply the missing 5 amino acids. This mSos2 protein thus consists of N-terminal 87 amino acids from mSos1 (amino acid 18 to 105 of mSos1 [4]; in the hSos proteins, this region is 76% identical), followed by 1,245 amino acids of mSos2 sequence. A natural *Xho*I site is present in the mSos2 3′ region. A fragment encoding the Src myristoylation site (16) was generated by PCR amplification of a plasmid carrying the myristoylation sequence with a sense primer with a *Sac*II site (underlined) and myristoylation sequences (bold) (5′at gcggccgcggccacc**atggg**3′) and an antisense primer carrying myristoylation sequence (bold) and m*sos1* sequence (italics) including the N-terminal codon 18 down to a *Pvu*II site (underlined) located in codons 22 and 23 (5′*ggcagctgctgcg cctgcat***ccggcgctggctgg**3′). The *Sac*II-*Pvu*II-digested fragment was cloned into the 5′ m*sos1* sequences, where it results in an N-terminal extension upstream of the mSos1 starting methionine (originally codon 18) of 15 amino acids. The cloned fragment was sequenced and transferred to the m*sos2* hybrid gene.

A sequence encoding the hemagglutinin HA1 epitope (18, 33) was transferred to m*sos1* by amplifying the extreme 3′ coding region with a sense primer encoding a natural *Nsi*I site (underlined) and sequences surrounding it (5′*tccatgcatag agatggac*3′) and an antisense primer encoding m*sos1* sequence (italics), the epitope and a stop codon (bold), and an *Xho*I site (underlined) (5′cgcctcgag**tca caagctagcgtaatctggaacatcgtatgggtaaagctt***ggaagaatgggcattctccagca*3′). The *Nsi*I-*Xho*I-digested PCR fragment was cloned into similarly cut m*sos1*. A KT3 epitope (28) was transferred to the C terminus of m*sos2*, using a natural *Bsp*EI site (underlined) with the sense primer 5′*gcctccggaacactttat3*′ and the antisense primer 5′cgcctcgag**tcatgtttctggttctggtggtggtgtaagctt***ttgaggagtttctgcattttcc*3′, carrying C-terminal m*sos2* sequences (italics), sequences coding for the epitope and a stop codon (bold), as well as an *Xho*I site. The *Bsp*EI-*Xho*I-digested PCR fragment was cloned into similarly cut m*sos2*.

Recombinants between myr-m*sos1* (with HA1 tag) and myr-m*sos2* (with KT3 tag) genes were made by PCR, using primers inserting a *Kpn*I site by changing one base in codon 1063 of the myr-mSos1 protein (no coding change) and three bases in codons 1061 and 1062 in myr-mSos2, changing the coding of codon 1062 from S to T, which is the amino acid present in mSos1. The primers used for amplification of N-terminal m*sos1* sequences were 5′acccgccgcggccacca3′ (sense, with *Sac*II site underlined) and 5′tctcatggtacctggtcttgg3′ (antisense, with *Kpn*I site underlined and mismatch base in italics). The primers for amplification of N-terminal m*sos2* sequences utilized the same sense primer as the m*sos1* sequence together with 5′gaggtggtaccatggcggccagc3′ (antisense, with underlined *Kpn*I site and mismatch base in italics). The primers for amplification of C-terminal m*sos1* were 5′gaccaggtaccatgagacatccc3′ (sense, with underlined *Kpn*I site and mismatch in italics) and 5′ggacatgactgaccctcgag3′ (antisense, with underlined *Xho*I site). The primer for amplification of C-terminal m*sos2* (5′gccat ggtaccacctcaggcacg3′; sense, with underlined *Kpn*I site and mismatches in italics) was used with the same antisense primer as for m*sos1* 3′-terminal amplification. The recombinants were constructed in three fragment ligations using *Sac*II-*Kpn*I-cut 5′ m*sos* fragments, *Kpn*I-*Xho*I-cut 3′ m*sos* fragments, and *Sac*II-*Xho*I-cut expression vector.

For all in vivo expression studies, a derivative of pBW1423 with the histidinol selectable marker *hisD* was used. The *hisD* gene was obtained from pSV2HisD (20) by *Sfi*I and *Dra*I digestion and replaced the sequences between *Sfi*I and *Nru*I, carrying *neo*. For in vitro transcription studies, the *Sac*II-*Xho*I-carrying *sos* fragments were cloned into *Sac*II-*Xho*I pBluescript vectors.

Cell culture and DNA transfection. The procedures have been described elsewhere (51). NIH 3T3 clone 7 cells were grown in 10% fetal bovine serum and Dulbecco's medium. Transfections were carried out as described previously (51) except that selection was with histidinol.

Immunological procedures. Pulse-chase experiments were carried out as described previously (7), incubating the cells for 15 min with 300 μCi of [^{35}S]methionine per ml and 2% dialyzed fetal calf serum and chasing with nonradiolabeled medium. Equal numbers of trichloroacetic acid-precipitable counts from cell extracts were immunoprecipitated with Sos1 and Sos2 peptide antibodies SC-257 and SC-258, respectively (Santa Cruz Biotechnology, Inc.), and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In preliminary experiments, it was determined, by immunoprecipitation of in vitro-translated Sos1 and Sos2 proteins with the respective peptide antibodies, that the antibodies were specific for their designated proteins and that they bound similar amounts of protein.

Subcellular fractionation was performed as described previously (5), with minor modifications: in buffer A, NaF, Na_3VO_4, *p*-nitrophenylphosphate, phenyl arsine oxide, trypsin inhibitor, benzamidine, and phenylmethylsulfonyl fluoride were omitted, and centrifugation was at 55,000 rpm. Protein concentrations were determined by the Bradford method (Bio-Rad), using bovine serum albumin as the standard. Fractions were analyzed by Western blotting with anti-Sos1 antibodies SC-257 (Santa Cruz Biotechnology), monoclonal antibody from the 12CA5 cell line against the hemagglutin HA1 epitope (18), and monoclonal H-Ras antibody from the 146 3E4 hybridoma (Quality Biotec, Camden, N.J.). In Fig. 2, 10 μg was used for the Western blots; in Fig. 6, 5×10^6 cpm was used per lane.

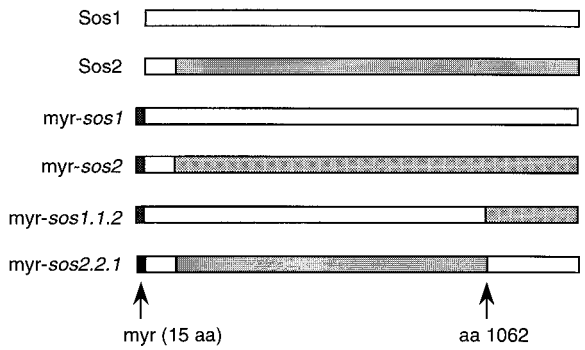

FIG. 1. Structures of Sos-coding sequences in plasmid constructions. Sos1 is 1,319 amino acids. As described in the text, Sos2 is a fusion between 87 N-terminal amino acids of Sos1 and 1,245 amino acids of Sos2. The 15-amino-acid myristoylation signal (myr) is derived from Src. myr-Sos1.1.2 and myr-Sos2.2.1 encode chimeric proteins; the *Kpn*I site used in making the chimeras is located at amino acid 1062.

In vitro studies of mSos with rabbit reticulocyte extracts and WGE. For the experiments shown in Fig. 4 and 5, two coupled transcription-translation kits (Promega), in which translation is programmed by either a rabbit reticulocyte lysate (RRL) or a wheat germ extract (WGE), were used, with T3 polymerase. The coupled reaction was run in the presence of [^{35}S]methionine for 90 or 120 min at 30°C for the RRL or the WGE, respectively, according to the manufacturer's instructions, at which time 2 mM methionine was added. Protein stability was studied by incubating the reaction mix at 37°C, in some instances in the presence of ATPγS (4 mM). Samples were analyzed by SDS-PAGE, either directly or after immunoprecipitation with ubiquitin antibody 13-1600 (Zymed) or U-5379 (Sigma), under conditions where the antibodies were limiting. The anti-p21^{Waf} antibody SC-817 (Santa Cruz Biotechnology) was used as a control antibody.

RESULTS

Addition of the Src N terminus to mSos1 targets the protein to the membrane. Although transforming activity has not been demonstrated for a wild-type mammalian *sos* gene, whose encoded protein is predominantly cytoplasmic, a *sos1* gene fused to sequences encoding a membrane-targeting signal has been shown to induce focal transformation of mouse NIH 3T3 cells either directly (1) or in cooperation with c-*ras* (41). To initiate our study of m*sos*, we constructed a retroviral expression plasmid that would encode an analogous membrane-targeted mSos1 protein by fusing the first 15 codons from the *src* gene (which encode a myristoylation signal) immediately upstream from the N-terminal coding sequences of an m*sos*1 cDNA that encodes the full-length mSos1 protein (Fig. 1). A sequence encoding a hemagglutinin (HA1) epitope tag was added in frame to the 3′ end of the m*sos*1 coding sequences. This plasmid was designated myr-*sos1*.

To verify that addition of the Src myristoylation signal leads to an mSos1 protein that is predominantly membrane associated, extracts of NIH 3T3 cells that had been transfected with myr-m*sos*1 and selected in histidinol were separated by centrifugation into membrane and cytosolic fractions and Western blotted with anti-Sos or anti-HA1 antibodies (Fig. 2). p21Ras, which is predominantly membrane associated, was used as a control for the lack of contamination of the cytosolic fraction with membrane components. As reported previously for this

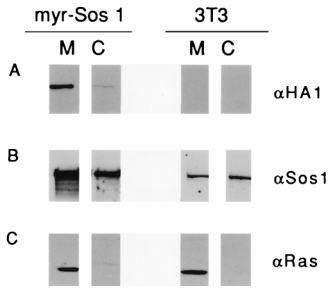

FIG. 2. Membrane association of myr-mSos1. NIH 3T3 cells transfected with HA1-tagged myr-mSos1 were fractionated into membrane (M) and cytoplasmic (C) fractions as described in Materials and Methods and Western blotted with antibodies against Sos1, HA1, and Ras (αHA1, αSos1, and αRas).

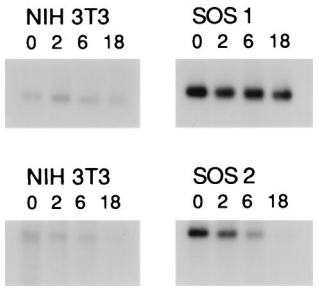

FIG. 3. Pulse-chase analysis of mSos1 and mSos2. NIH 3T3 cells transfected with myr-m*sos1* (SOS 1) and myr-m*sos2* (SOS 2) were pulsed with [^{35}S]methionine for 15 min, chased with nonradiolabeled methionine for the number of hours indicated, immunoprecipitated with anti-Sos1 (upper panels) or anti-Sos2 (lower panels) antibodies, and processed for SDS-PAGE.

procedure, similar amounts of the endogenous Sos1 protein were found in both fractions. By contrast, the vast majority of myr-Sos1 protein was found in the membrane fraction, as seen when the Western blots were probed for the exogenously added myr-Sos1 protein by using the epitope tag.

The membrane-targeting signal to mSos1 renders it directly transforming, while mSos2 fused to the same signal is transformation deficient. Since the extreme 5′ end of m*sos2* has proved difficult to clone, a membrane-targeted mSos2 protein was engineered by fusing the first 102 codons from myr-*sos1* (encoding the 15 Src amino acids and the 87 N-terminal amino acids of mSos1) to an m*sos2* cDNA at a homologous site. This plasmid, which was isogenic with myr-*sos1* (except for having a KT3 tag), was designated myr-*sos2* (Fig. 1). Eight of the ten amino acids of mSos1 and mSos2 that flank the site of recombination between mSos1 and mSos2 are identical. Control plasmids *sos1* and *sos2* were also constructed; they were the same as myr-*sos1* and myr-*sos2*, respectively, except that they lacked the membrane-targeting signal.

The biological activity of the constructs was examined by testing their ability to induce focal transformation of NIH 3T3 cells. As expected, neither *sos1* nor *sos2* was active in this assay. By contrast, myr-*sos1* induced focal transformation; its efficiency of focus formation was approximately one-half of that of a mutationally activated v-H-*ras* gene, which served as the positive control (Table 1). Myr-*sos1* plasmids lacking a C-terminal tag had transforming activities that were similar to that of the myr-*sos1* plasmid with the C-terminal HA1 tag (data not shown). In contrast to myr-*sos1*, the myr-*sos2* plasmid unexpectedly did not possess any focus-forming activity. Therefore, although addition of the membrane-targeting signal had rendered *sos1* directly transforming, the same signal fused to *sos2* was not sufficient to increase its activity above background in this assay.

The half-life of mSos2 is shorter than that of mSos1. Since the myr-*sos1* and myr-*sos2* plasmids were isogenic and the first 86 codons of the two clones were identical, differences in their protein products, rather than level of RNA or efficiency of translation, were likely to account for the strikingly higher biological activity of the myr-*sos1* plasmid than of myr-*sos2*. One possibility was that the half-life of the myr-*sos2*-encoded protein is shorter than of the myr-*sos1*-encoded protein. To examine the stability of each of the encoded proteins, mass cultures of cells containing the myr-*sos1* and myr-*sos2* plasmids were selected by growing them in medium with high concentrations of histidinol, and the mSos proteins were metabolically labeled and subjected to pulse-chase analysis (Fig. 3). The pulse-chase analysis of myr-mSos1 indicated that its half-life was more than 18 h. By contrast, the half-life of the myr-mSos2 protein was less than 3 h. Results obtained with overnight labeling as well as pulse-chase analysis of parental NIH 3T3 cells indicated that the stability of each of the endogenous Sos1 and Sos2 proteins was similar to those found for the ectopically expressed myristoylated versions (Fig. 3 and data not shown). We conclude that myr-mSos2 and endogenous Sos2 proteins are much less stable than their Sos1 counterparts.

Membrane-targeted mSos2 cooperates with Ras. Since the myr-*sos2* plasmid by itself did not transform the NIH 3T3 cells, its activity was tested in a less stringent bioassay that had been used previously to demonstrate a biological activity for membrane-targeted mSos1 (41). Cells that had been transfected with myr-*sos2* and selected with histidinol were transfected with c-H-*ras* and examined for focal transformation. As shown in Table 2, while under these conditions the c-H-*ras* plasmid did not induce foci in the parental NIH 3T3 cells, it did induce many foci in the myr-*sos2*-transfected cell line. Thus, myr-*sos2* cooperated with c-H-*ras* to induce focal transformation.

TABLE 1. Transforming activities and in vivo half-lives of transfected Sos proteins

Plasmid (with *hisD*)	Relative focus formation[a]	Sos half-life in vivo (h)
Vector	0.00	
v-H-*ras*	1.00	
c-H-*ras*	0.00	
sos1	0.00	
sos2	0.00	
Myr-*sos1*	0.50	>18
Myr-*sos2*	0.00	3
Myr-*sos1.1.2*	0.37	>18
Myr-*sos2.2.1*	0.00	4

[a] v-H-*ras* induced 700 foci/1,000 histidinol-resistant colonies.

TABLE 2. Increased susceptibility to transformation of myr-m*sos2*-transfected cells

Transfecting plasmid[a]	No. of foci/1,000 G418 colonies in NIH 3T3 cells	
	myr-*sos2*	Parental
Vector	4	0
c-H-*ras*	47	0
v-H-*ras*		165

[a] Each contained *neo*, giving resistance to G418.

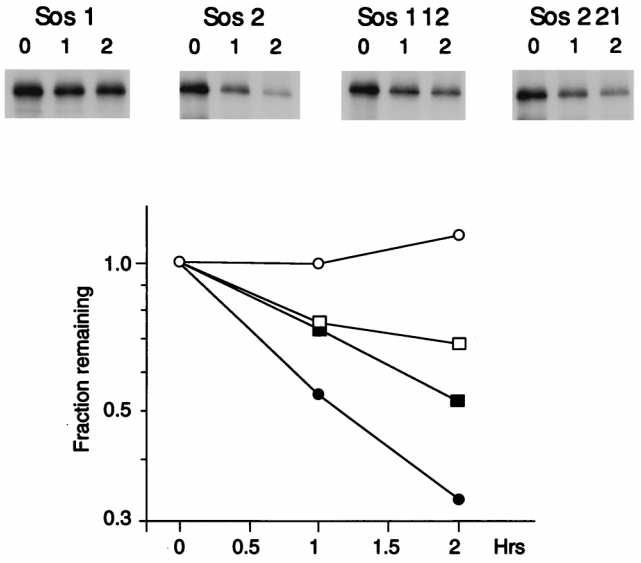

FIG. 4. In vitro degradation of mSos proteins. Sos proteins were synthesized in the presence of [^{35}S]methionine in a coupled transcription-translation kit. The proteins were further incubated in the presence of an excess of unlabeled methionine, during which time samples were removed and processed for SDS-PAGE. Sos protein was quantitated by counting the gel on a β-scanner. Data are presented as fraction of protein remaining (log scale) as a function of time. ○, mSos1; ●, mSos2; □, myr-mSos1.1.2; ■, myr-mSos2.2.1.

In vitro-translated mSos2 is unstable in rabbit reticulocyte extracts, in contrast to mSos1. One possible explanation for the shorter half-life of mSos2 might be that it contains ubiquitin-dependent degradation signals that are not present in mSos1. To test this possibility, we synthesized ^{35}S-labeled mSos1 and mSos2 in vitro and tested their relative stabilities in an RRL, which is used commonly as the source of an active ubiquitin system (13). Incubation of mSos2 in the RRL after addition of unlabeled methionine led to progressive degradation of the protein (Fig. 4). Under the same conditions, mSos1 was completely stable.

The in vitro instability of mSos2 is linked to the ubiquitin system. These results were compatible with the hypothesis that the degradation of mSos2 arose because it was sensitive to the ubiquitin system in the reticulocyte extract, in contrast to mSos1. If this were the case, a nonhydrolyzable ATP analog such as ATPγS would prevent degradation of ubiquitinated proteins by the proteasome, although the analog does not prevent their ubiquitination (12). Thus, if the degradation of mSos2 is mediated by the ubiquitin system, addition of ATPγS to the reticulocyte extract after synthesis of radiolabeled Sos proteins should inhibit ubiquitin-dependent degradation but not its ubiquitination. Indeed, incubation of mSos2 in the presence of ATPγS completely inhibited its degradation by the reticulocyte extract (Fig. 5A) and led to the appearance with time of a more slowly migrating band. The more slowly migrating band was not seen with Sos1, and ATPγS had no effect on Sos1 (ATPγS data shown only for Sos2).

Another feature of some proteins that are degraded in a ubiquitin-dependent manner, such as p53 in the presence of the human papillomavirus type 16 E6 protein, is that they are efficiently ubiquitinated and degraded in RRL but not in a WGE (45). When Sos2 was synthesized and incubated in a WGE, it remained stable (Fig. 5C), in contrast to the results obtained in the RRL.

The foregoing results suggested that Sos2 would be efficiently ubiquitinated in RRL but not in WGE, while Sos1 would not be ubiquitinated in either. To examine this possibility, Sos1 and Sos2 proteins synthesized in RRL or WGE were immunoprecipitated with an antiubiquitin antibody (Fig. 5B and D). Sos2 protein made in RRL was efficiently precipitated by the antiubiquitin antibody. Since the antiubiquitin antibodies used for immunoprecipitation are known to have greater affinity for polyubiquitinated protein than for monoubiquitinated protein (39), it was not surprising that the more slowly migrating band that was barely detectable in the input lysate precipitated even more efficiently than the main input band; some immunoreactive species that migrated even more slowly than this upper band were also seen. We interpret the more slowly migrating band(s) to represent polyubiquitinated Sos2

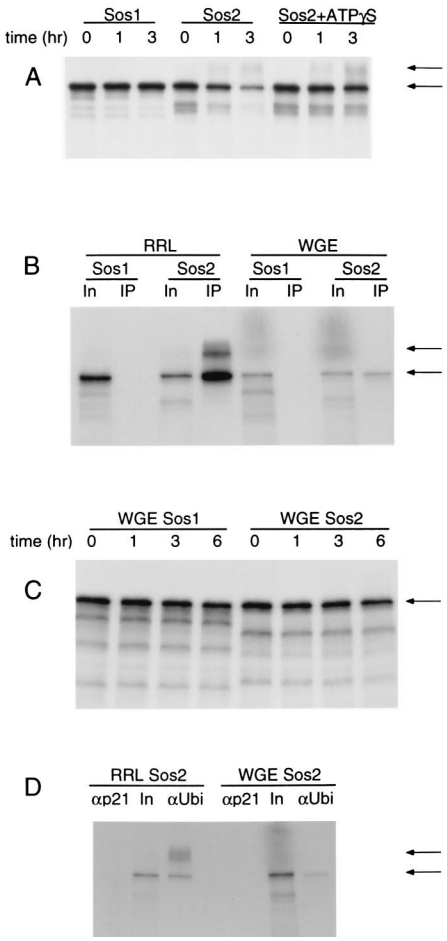

FIG. 5. Characterization of in vitro-translated Sos1 and Sos2 proteins. (A) Effect of ATPγS on stability of mSos2 proteins made in RRL. ATPγS was added where indicated to a final concentration of 4 mM. Samples were collected at the indicated times and processed for SDS-PAGE and autoradiography. (B) Immunoprecipitation of mSos proteins made in RRL or WGE by antiubiquitin antibody U-5379 (Sigma). The input lane (In) shows 1/200 of the input material used in the immunoprecipitations (IP). (C) Stability of mSos proteins in WGE. Samples were collected at the indicated times and processed for SDS-PAGE and autoradiography. (D) Specificity of antiubiquitin antibody. mSos2 proteins made either in RRL or WGE were immunoprecipitated with an antiubiquitin antibody (αUbi) or a control antibody (anti-p21^{Waf} [αp21]). The input lane (In) shows 1/100 of the input material used in the immunoprecipitations. Different Sos2 protein preparations were used in panels B and D. In the panel with the single arrow (C), it represents the Sos migration rate of approximately 170 kDa. In the panels with two arrows (A, B, and D), the Sos migration rate is represented by the lower arrow; that of the main slower-migrating band, which is approximately 230 kDa, is represented by the upper arrow.

protein. Unlike the RRL-synthesized Sos2, the Sos2 protein made in WGE was much less efficiently precipitated by the antibody (Fig. 5B). Under the same conditions, no Sos1 was immunoprecipitated. To rule out the possibility that the Sos2 immunoprecipitation represents a nonspecific result, the Sos2 made in RRL or WGE was immunoprecipitated with an irrelevant antibody (anti-$p21^{Waf}$), with negative results (Fig. 5D).

The results from Sos2 synthesized in the RRL lysate suggest that most Sos2 made in RRL quickly becomes monoubiquitinated and is subsequently polyubiquitinated and degraded. By contrast, Sos2 made in WGE may be monoubiquitinated very inefficiently, is not detectably polyubiquitinated, and remains stable. Sos1 is not detectably ubiquitinated in RRL or WGE.

In vitro analysis of chimeric mSos proteins indicates that mSos2 contains N-terminal and C-terminal sites for degradation. In a preliminary effort to map the region of mSos2 that was subject to ubiquitin-dependent degradation, two chimeric plasmids were constructed. One, designated *sos1.1.2*, was composed of m*sos1* from its 5′ end through the region encoding the catalytic domain fused to the region of m*sos2* encoding the C terminus downstream from the catalytic domain (Fig. 1). The other, designated *sos2.2.1*, encoded the reciprocal chimeric protein (Fig. 1).

The chimeric proteins were subjected to in vitro analysis in RRL. Instead of the stability of one chimera resembling that of mSos1 and the stability of the other resembling that of mSos2, each chimera, when incubated in the RRL, was found to be less stable than mSos1 but more stable than mSos2, stable in the presence of ATPγS, and immunoprecipitated by antiubiquitin antibodies (Fig. 4 and data not shown).

Biological activity of the mSos chimeras. To test the biological activity of the two chimeric m*sos* genes described above, the Src myristoylation signal was fused in frame to the 5′ ends of their coding sequences, and they were placed in the retroviral vector used earlier for myr-*sos1* and myr-*sos2*. The reciprocal chimeras were designated myr-*sos1.1.2* and myr-*sos2.2.1* (Fig. 1). When their biological activities were tested, myr-*sos2.2.1* was unable to induce focal transformation of NIH 3T3 cells, as might have been expected from the relative instability of mSos2.2.1 in the reticulocyte extracts (Table 1). In spite of mSos1.1.2 being less stable than mSos1 in the reticulocyte extract, myr-*sos1.1.2* induced focal transformation with an efficiency that was almost as high as that of myr-*sos1* (Table 1).

These contrasting biological results obtained with myr-*sos2.2.1* and myr-*sos1.1.2* suggested that the in vivo stabilities of their encoded proteins might be quite different. To determine whether this was the case, histidinol-selected cells that had been transfected with myr-*sos2.2.1* or myr-*sos1.1.2* were subjected to pulse-chase analysis. As predicted from its high biological activity, the half-life of myr-*sos1.1.2* was approximately 18 h (Table 1). However, the half-life of the protein encoded by the nontransforming myr-*sos2.2.1* was similar to that of myr-mSos2 (Table 1).

To rule out the possibility that the myr-Sos2.2.1 protein is less transforming because it was not associated with the membrane, a subcellular fractionation experiment similar to that performed for myr-Sos1 in Fig. 2 was carried out for a cell line selected for its high expression of myr-Sos2.2.1 (Fig. 6). Although endogenous Sos1 was cytosolic, the vast majority of myr-Sos2.2.1 was in the membrane fraction, similar to findings for myr-Sos1.

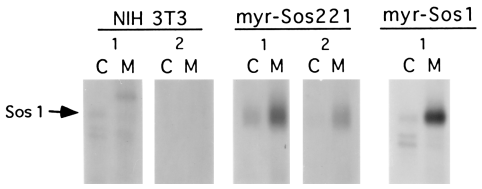

FIG. 6. Membrane association of myr-mSos2.2.1. NIH 3T3 cells transfected with HA1-tagged myr-mSos1 and myr-Sos2.2.1 were fractionated into membrane (M) and cytoplasmic (C) fractions as described in Materials and Methods and immunoprecipitated with antibodies against Sos1 (lanes 1) and HA1 (lanes 2).

DISCUSSION

Our comparison of isogenic versions of m*sos1* and m*sos2* has found that m*sos1* possessed much greater biological activity than m*sos2* and that this difference was correlated with mSos2 protein having a half-life much shorter than that of mSos1. These observations point to an important difference in the regulation of the two proteins that has not been noted previously. In vitro analysis of the mSos1 and mSos2 proteins suggested that mSos2 is sensitive to ubiquitin-dependent degradation, while mSos1 is not ubiquitinated under these conditions and remains stable. This qualitative difference between mSos1 and mSos2 appears to account for the shorter half-life of mSos2 in vivo and for its lower biological activity.

Previous comparisons between the two *sos* genes and their encoded proteins have identified qualitatively similar characteristics, many of which also appear to be quantitatively similar. At the RNA level, the two *sos* genes have been reported to be widely expressed, at similar levels, in most tissues and in cell lines, including BALB/3T3 (4). Their encoded proteins also have similar structural organizations (2). These shared features include an N-terminal region that contains Dbl and pleckstrin homology domains, a central region with homology to the catalytic domains of other Ras-specific exchange factors, and a C-terminal region that is rich in prolines. The two proteins also possess similar biochemical activities, with their central regions having a Ras-specific guanine nucleotide exchange activity and their C termini binding the Grb2 adapter protein. The exchange activities of the two proteins appear to be quantitatively similar (18a), while the apparent binding affinity of hSos2 for Grb2 is reported to be even greater than that of hSos1 (52). In spite of these similarities, our results argue that under the growth conditions of our cultured cells, mSos1 is quantitatively more important than mSos2, although the evolutionary conservation of mSos2 suggests that it may serve a critical role in other situations, which remain to be defined.

The biological activity of the m*sos1* plasmid carrying the membrane-targeting sequence was considerably higher than that reported previously for membrane-targeted *sos1*. While the myr-*sos1* plasmid induced focal transformation approximately one-half as efficiently as the v-H-*ras* control, the comparable *sos*1 plasmids were reported in one instance to induce less than 5% as many foci as an activated *ras* gene (1) or to induce foci only when cotransfected with c-*ras* (41). One potentially important difference is that the *sos1* gene was targeted to the membrane via its C terminus, using a CAAX box, while the myristoylation signal used here for the m*sos1* gene is located at the N terminus of the encoded protein. In addition, differences in plasmids, *sos* genes, and transfection techniques could all be contributing factors. Regardless of the explanation underlying these differences in biological activity, the sensitivity of our transformation assay permits us to conclude that myr-m*sos1* induced foci 2 orders of magnitude more efficiently than myr-m*sos2*.

The precise members of the ubiquitin system that are involved in Sos2 have not been identified. However, at least some of the components may be absent from WGE, since we

found that, in contrast to rabbit reticulocyte extracts, Sos2 was stable when made in WGE. This result is similar to what has been reported for human papillomavirus type 16 E6-dependent degradation of p53. In that system, a ubiquitin ligase, E6-AP, present in rabbit reticulocytes but absent from wheat germ represents the critical reticulocyte-specific component (15, 44, 45).

In vitro analysis of the chimeric Sos proteins suggested that mSos2 may contain more than one signal for ubiquitin-dependent degradation. In the RRL, we found the degradation rate for the chimeric mSos proteins that contained only the N- or C-terminal mSos2 signal for degradation was slower than for mSos2, which suggests that the signals act independently of each other. Most proteins that are substrates for ubiquitin-dependent degradation appear to have only one such signal, although more than one lysine residue at the site may serve as a potential anchor for the covalent link with ubiquitin (49). However, the yeast MATα2 transcription regulatory factor has been reported to have two ubiquitination signals (21). For MATα2, distinct ubiquitin-conjugating enzymes have been reported to be required for ubiquitination of each signal (10).

The Ras-specific exchange factor in *Saccharomyces cerevisiae*, CDC25, has been reported to have a short half-life, which resulted from a sequence (RSSLNSLGN) that is similar to a type A cyclin destruction box (CDB; consensus sequence RXALGXIXN) (22, 35). In mitotic cyclins, the CDB has been shown to be required for ubiquitin-dependent degradation (19). Although in cyclins the CDB functions in a cell cycle-dependent manner, the CDB in CDC25 was found to be active throughout the cell cycle. The N terminus of mSos2 contains two sequences with partial CDB homology that differ somewhat from that of mSos1 (RQYLRELNM in mSos2 versus RQYIRELNL in mSos1; RYVLPRLML in mSos2 versus QYVLPRLLL in mSos1); their possible functional significance will be evaluated as the mSos2 ubiquitination signals are mapped more precisely.

An intriguing feature of mSos2 is that although in vitro examination identified separate N-terminal and C-terminal signals for degradation, in vivo analysis of the chimeric proteins suggests that only the N-terminal signal is functioning in NIH 3T3 cells under the conditions tested. Thus, distinct in vivo factors must regulate the N-terminal and C-terminal signals. The factors that override the C-terminal degradation signal in vivo remain to be determined. Posttranslational modification, such as phosphorylation, or protein-protein interaction might account for the difference. One or both of these features are believed to account for the cell cycle-dependent stability and degradation of several cell cycle regulators, including S-phase and M-phase cyclins and the cyclin-dependent kinase inhibitor p27. It also remains possible that a putatively important component present in the RRL is either not active in the NIH 3T3 cells or not readily accessible to the Sos protein in the cells. Elucidation of these possibilities should provide molecular insight into the components that regulate Sos2 and their contribution to the strength of Sos-dependent Ras activation.

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