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Characterization of Sam68-like mammalian proteins SLM-1 and SLM-2: SLM-1 is a Src substrate during mitosis

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ABSTRACT **Sam68, the 68-kDa Src substrate associated during mitosis, is an RNA-binding protein with signaling properties that contains a GSG (GRP33, Sam68, GLD-1) domain. Here we report the cloning of two Sam68-like-mammalian proteins, SLM-1 and SLM-2. These proteins have an ≈70% sequence identity with Sam68 in their GSG domain. SLM-1 and SLM-2 have the characteristic Sam68 SH2 and SH3 domain binding sites. SLM-1 is an RNA-binding protein that is tyrosine phosphorylated by Src during mitosis. SLM-1 bound the SH2 and SH3 domains of $p59^{fyn}$, Grb-2, phospholipase Cγ-1 (PLCγ-1), and/or $p120^{rasGAP}$, suggesting it may function as a multifunctional adapter protein for Src during mitosis. SLM-2 is an RNA-binding protein that is not tyrosine phosphorylated by Src or $p59^{fyn}$. Moreover, SLM-2 did not associate with the SH3 domains of $p59^{fyn}$, Grb-2, PLCγ-1, or $p120^{rasGAP}$, suggesting that SLM-2 may not function as an adapter protein for these proteins. The identification of SLM-1 and SLM-2 demonstrates the presence of a Sam68/SLM family whose members have the potential to link signaling pathways with RNA metabolism.**

Sam68, the 68-kDa Src substrate associated during mitosis, is the only known substrate for Src-family tyrosine kinases during mitosis (1, 2). Sam68 associates with various SH3 and SH2 domain-containing signaling molecules, including Src-family tyrosine kinases (1–5), the adapter protein Grb-2 (5), and phospholipase Cγ-1 (PLCγ-1) (5, 6). More recently, Sam68 has been shown to associate with Nck (7), the poliovirus RNA-dependent RNA polymerase 3D, (8), Itk/Tsk (9), Grap (10), and Cbl and Jak3 in Hayai cells (11). These interactions support the potential role for Sam68 as a multifunctional adaptor protein for tyrosine kinases (5, 12). In addition to binding SH3 and SH2 domains, Sam68 is an RNA-binding protein. It has been shown to bind single-stranded RNA and single-stranded and double-stranded DNA as well as homopolymeric RNA *in vitro* (2, 13, 14). The RNA-binding activity of Sam68 is regulated by tyrosine phosphorylation, as the phosphorylation of Sam68 by the Src-family kinase $p59^{fyn}$ abolishes its homopolymeric RNA-binding activity (15).

Sam68 contains a KH domain, an RNA-binding motif that was originally identified in the heterogeneous nuclear ribonucleoprotein (hnRNP) K (16, 17). The Sam68 KH domain is embedded in a larger conserved domain of ≈200 amino acids called the GSG domain (GRP33, Sam68, GLD-1; refs. 18 and 19). This domain is also called STAR for signal transduction and activator of RNA (20). GSG domain-containing proteins include *Artemia salina* GRP33 (21), Sam68 (13), *Caenorhabditis elegans* GLD-1 (18), SF1 (22), *Drosophila* Who/How (23–25), *Xenopus* Xqua (26), mouse Qk1 (27), zebrafish Qk1 (28), *Drosophila* KEP1 and Sam50 (19), and *Drosophila* Qk1-related proteins (29). GSG domain-containing proteins share several properties, including RNA binding (13, 14, 19, 22, 26, 30) and self-association (14, 19, 31, 32).

Here we report the cloning of two mammalian GSG domain-containing proteins that have a high sequence identity with Sam68. SLM-1 and SLM-2 have a GSG domain, proline-rich motifs, arginine-glycine repeats, and a C-terminal tyrosine-rich region. SLM-1 is a substrate for Src during mitosis, suggesting it may be involved in some aspect of mitosis. The identification of SLM-1 and SLM-2 defines a Sam68/SLM family whose members have the potential to link signaling pathways to RNA metabolism.

MATERIALS AND METHODS

Cloning of SLM-1 and SLM-2. The cDNAs encoding SLM-1 and SLM-2 were obtained by screening a λ ZAP II mouse brain library (Stratagene) with a ^{32}P-labeled random-primed DNA fragment encompassing the entire insert of the human expressed sequence tag (EST) clone 530290 (GenBank accession no. AA083787). Clone 18-2 had an insert 1.9 kb in length and was the mouse 530290 cDNA. Seventeen other clones were obtained that were shorter than clone 18-2. Two other clones were obtained that were not identical to 530290. Clone 6-1 was a mouse Sam68 partial cDNA, and clone 1-1 had an insert of 1.3 kb with features of both Sam68 and 530290. Clones 1-1 and 18-2 were called SLM-1 and SLM-2, respectively. Clone 1-1 contained the entire SLM-1 coding sequence, and the full-length SLM-1 DNA sequence of 2.4 kb was obtained by sequencing several overlapping clones obtained by using clone 1-1 as a probe on a mouse brain λ ZAP II library. The cDNAs were sequenced on both strands with multiple overlapping reads by using an automated Applied Biosystems sequencer at the Sheldon Biotechnology Center, McGill University. The accession numbers of SLM-1 and SLM-2 are AF098796 and AF099092, respectively.

DNA Constructs. myc epitope-tagged SLM-1 and SLM-2 were generated by subcloning the entire *Eco*RI fragments of clones 1-1 and 18-2 into myc-Bluescript KS (Stratagene), respectively (5). GFP-SLM-1 and GFP-SLM-2 (GFP, green fluorescent protein) were generated by subcloning the *Eco*RI DNA fragment of clones 1-1 and 18-2 into pEGFP-C1. GFP-Sam68 was constructed by subcloning the *Eco*RI fragment of myc-Sam68f (5) into pEGFP-C1. Bluescript-fyn, myc-Sam68, HA-Sam68, and the plasmids encoding the SH3 and SH2 domains of $p59^{fyn}$, PLCγ1, $p120^{rasGAP}$, and Grb-2 are as described previously (5, 14). The purified glutathione *S*-transferase (GST) fusion proteins were covalently coupled to

Abbreviations: PLCγ-1, phospholipase Cγ-1; EST, expressed sequence tag; HA, hemagglutinin; GFP, green fluorescent protein; GST, glutathione *S*-transferase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF098796 and AF099092).

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Affi-Gel 10 (Bio-Rad) at concentrations of 2 mg/ml. The plasmid constructs were verified by dideoxynucleotide sequencing with Sequenase (United States Biochemical).

Northern Blot Analysis. The mouse and human multiple tissue Northern blot membranes were purchased from CLONTECH. A ^{32}P-labeled random-primed DNA fragment containing the entire insert of SLM-1 (clone 1-1) or SLM-2 (clone 18-2) was used to hybridize the membrane according to the manufacturer's protocol, using the ExpressHyb solution for 1 h at 68°C.

Protein Expression and Protein Analysis. Proteins were analyzed in HeLa cells by using the vaccinia virus T7 expression system as described (5). Immunoblotting and/or immunoprecipitations were performed with anti-myc 9E10, anti-hemagglutinin (HA), and anti-phosphotyrosine mixture containing 1:1000 PY20 and 1:2000 4G10. Anti-p59^{fyn} was kindly provided by André Veillette (McGill University), and anti-Sam68 antibody C20 (catalog no. sc333) and anti-Sam68 mouse monoclonal antibody 7-1 (catalog no. sc1238) were from Santa Cruz Biotechnology.

Transfection of v-Src-Transformed Cells. v-Src-transformed cells were plated 12 h before transfection, typically at a density of 10^5 cells per 35-mm well. Cells were transfected with DNA constructs encoding GFP alone, GFP-Sam68, GFP-SLM-1, and GFP-SLM-2 by using Lipofectamine Plus reagent (Life Technologies). Eight hours after transfection, nocodazole was added to at a final concentration of 40 ng/ml and incubated overnight. Before harvesting, cells were treated with pervanadate for 15 min and lysed, and the immunoprecipitated proteins were analyzed as described (5).

RNA-Binding Assays. Homopolymeric RNA-binding assays were performed with poly(A) (Sigma), poly(C) (Sigma), poly(G) (Sigma), and poly(U) (Pharmacia) covalently coupled to beads in lysis buffer supplemented with 2 mg/ml heparin as described (14, 15).

RESULTS

Identification of Two Sam68-Like Mammalian Proteins. To identify proteins related to Sam68, the public EST database was searched with the BLAST program (33) using the Sam68 amino acid sequence. The human EST 530290 was identified as a family member of Sam68. A mouse brain library was screened, using the EST 530290 as a DNA probe. Three different cDNAs were obtained: clone 6-2 was a mouse Sam68 cDNA, clone 1-1 contained a novel sequence, and clone 18-2 was the mouse homolog of EST 530290. The proteins encoded by clones 1-1 and 18-2 were very similar to Sam68 (Fig. 1) and were named SLM-1 and SLM-2, respectively, for Sam68-like mammalian proteins. The cDNAs had ORFs encoding proteins of 349 and 346 amino acids, respectively. The highest sequence identity between Sam68, SLM-1, and SLM-2 was in the GSG domain: 72% between Sam68 and SLM-1, 69% between Sam68 and SLM-2, and 80% between SLM-1 and SLM-2 (Fig. 1). The C termini of SLM-1 and SLM-2 contain numerous protein motifs, including proline-rich sequences, arginine-glycine repeats, and a tyrosine-rich region. Proline-rich motifs are known to be binding sites for SH3 and WW domain-containing proteins (34, 35). Arginine-glycine repeats are the site of arginine methylation (36). Tyrosines are potential sites of tyrosine phosphorylation, and phosphotyrosines can serve as attachment sites for SH2 and PTB domain-containing proteins (37). Thus, SLM-1 and SLM-2 are potential RNA-binding proteins, SH3, SH2, and WW domain-binding proteins, and potential substrates of arginine methylases and tyrosine kinases.

Northern blot analyses were performed to examine the tissue distribution of SLM-1 and SLM-2 transcripts. A 2.2-kb SLM-1 transcript was detected in mouse heart, brain, spleen, kidney, and testis (Fig. 2*A*, lanes 1–5). A 2.4-kb transcript was

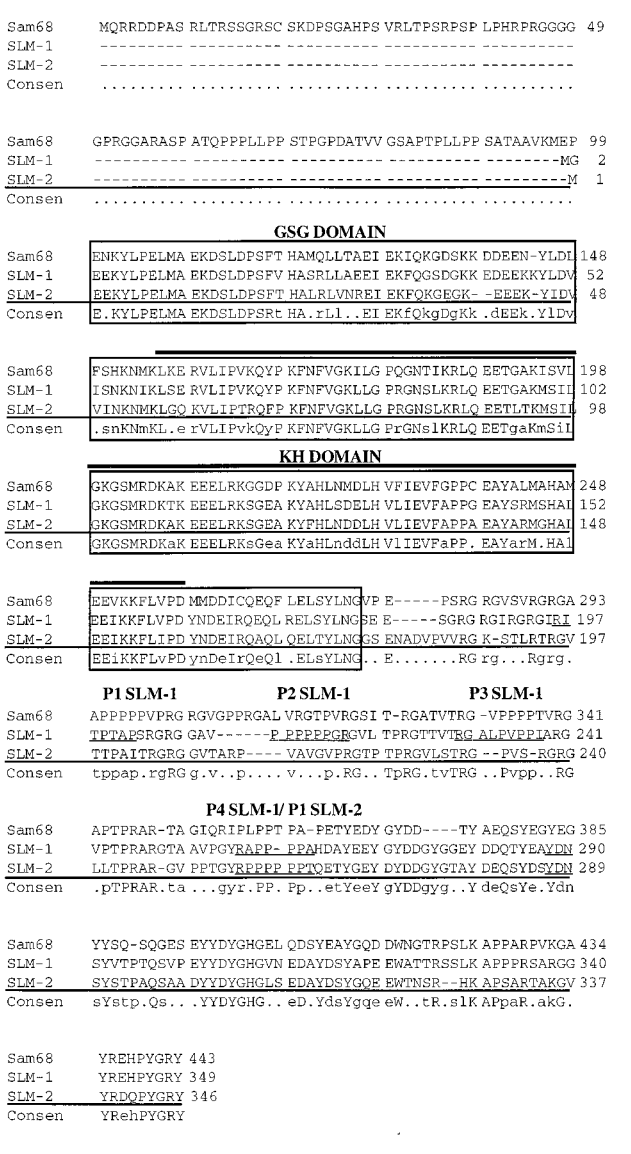

FIG. 1. Amino acid sequence comparison of mouse SLM-1, SLM-2, and Sam68. The mouse Sam68 sequence is shown on top in capital letters. The mouse SLM-1 and SLM-2 amino acid sequences are shown below. The GSG domain is boxed and the KH domain is marked by a line above the sequence. A hyphen in the sequence denotes a gap. The consensus is shown below and a capital letter denotes sequence identity among all three proteins, a lowercase letter represents sequence identity in two sequences, and a dot represents no sequence identity. The SLM-1 proline-rich sequences are labeled P1 to P4 and the proline motif of SLM-2 is labeled P1. The YDN sequence is underlined and denotes a Grb-2 SH2 domain-binding consensus sequence.

also observed in the brain (lane 2), and a smaller transcript of ≈2.0 kb was observed in the testis (lane 5). To expand the tissue distribution of SLM-1 transcripts, EST databases were searched with the SLM-1 DNA sequence. EST clones for SLM-1 were identified in libraries prepared from mouse mammary gland, kidney, heart, liver, lung, thymus, and T cells (data not shown). Northern blot analysis of SLM-2 transcripts demonstrated the presence of a ubiquitously expressed 2.5-kb mRNA that was most abundant in the human brain and skeletal muscle (Fig. 2*B*). An additional transcript of ≈3.3 kb was also observed in brain and skeletal muscle, suggesting that the *slm-2* gene may be alternatively spliced. SLM-2 EST clones were identified in libraries prepared from human retina, testis, and fetal liver and in human prostate, ovary, and lung tumors (data not shown). Taken together, these findings suggest that

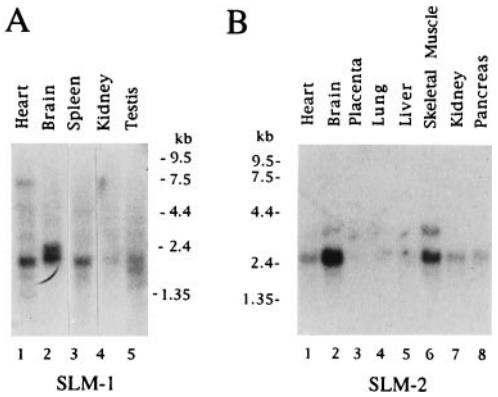

FIG. 2. Northern blot analysis of SLM-1 and SLM-2. (*A*) The mouse multiple tissue Northern blot membrane was hybridized with an SLM-1 DNA probe. (*B*) The human multiple tissue Northern blot membrane was hybridized, using EST 530290 as a DNA probe. Reprobing of the membranes with Sam68 and/or actin DNA probes showed equal loading.

the SLM-1 transcript is ubiquitously expressed and that the SLM-2 transcript is more restricted, with high levels in the brain and skeletal muscle.

SLM-1, but Not SLM-2, Is a Substrate of p59^{fyn}. We expressed SLM-1 and SLM-2 in HeLa cells with an N-terminal myc epitope sequence recognized by the monoclonal antibody 9E10 (38). We have shown in previous studies that an N-terminal myc epitope tag does not interfere with the signaling and RNA-binding properties of Sam68 (5, 14, 15). The plasmids expressing myc-SLM-1 and myc-SLM-2 were transfected into HeLa cells, and lysates of cells expressing these proteins were separated by SDS/PAGE and immunoblotted with anti-myc antibodies (Fig. 3). myc-SLM-1 protein migrated at ≈64 kDa (lane 11) and myc-SLM-2 migrated at ≈68 kDa (lane 12). The amino acid compositions of SLM-1 (349 amino acids), SLM-2 (346 amino acids), and Sam68 (443 amino acids) predict molecular masses in the 35- to 45-kDa range. The aberrant migration of Sam68, SLM-1, and SLM-2 on SDS/polyacrylamide gels is likely due to the presence of highly negatively charged C termini (Fig. 1). SLM-1 and SLM-2 contain 16 tyrosines in their C termini, and 15 are conserved with Sam68 (Fig. 1). Since Sam68 is a substrate of p59^{fyn} (5), we examined whether SLM-1 and SLM-2 were substrates of p59^{fyn}. SLM-1 and SLM-2 were co-expressed with p59^{fyn} in HeLa cells, and the cells were lysed and their contents were immunoprecipitated with control (IgG) or anti-myc antibodies. The bound proteins were separated by SDS/PAGE and immunoblotted with anti-phosphotyrosine antibodies. SLM-1 (lane 6) was readily tyrosine phosphorylated like Sam68 (lane 3). Surprisingly, SLM-2 was not tyrosine phosphorylated by p59^{fyn} (lanes 7–9). The absence of SLM-2 phosphorylation by p59^{fyn} suggests that SLM-2 is not a substrate for p59^{fyn} and/or Src kinases.

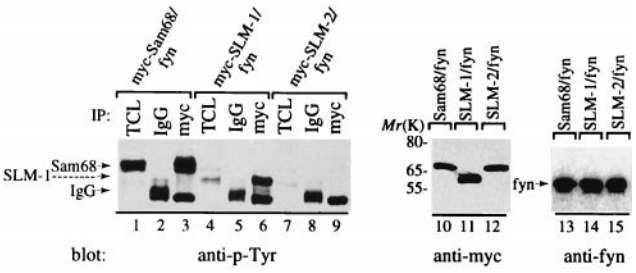

FIG. 3. SLM-1, but not SLM-2, is a substrate for the Src kinase p59^{fyn}. Sam68 (lanes 1–3), SLM-1 (lanes 4–6), and SLM-2 (lanes 7–9) were co-expressed with p59^{fyn} in HeLa cells. The cells were lysed and proteins were immunoprecipitated with IgG or anti-myc antibodies. The bound proteins were separated by SDS/PAGE and immunoblotted with anti-phosphotyrosine antibodies. The migration of Sam68, SLM-1, and the antibody heavy chain are shown. The total cell lysates were also immunoblotted with anti-myc (lanes 10–12) and anti-p59^{fyn} (lanes 13–15) antibodies to verify protein expression.

SLM-1 Is a Target for SH3 and SH2 Domain-Containing Proteins. SLM-1 contains four proline motifs named P1 to P4, and SLM-2 contains only one (Fig. 1). Proline motifs that serve as SH3 domain-binding sites have been classed as class I (RXXPXXP) and class II (PXXPXR; for reviews see refs. 34 and 37). SLM-1 P1 (RITPTAP) is the only proline motif that is in class I configuration. SLM-1 P2 (PPPPPPGR) is in a class II configuration. P3 (RGALPVPPI) and P4 (RAPPPPA) are not in a class I nor class II configuration. The SLM-2 proline motif (RPPPPPPT) is in a class I configuration. The SH3 and SH2 domain specificity of SLM-1 and SLM-2 was compared with that of Sam68 by performing GST "pull-down" assays. HeLa cells were transfected with myc-Sam68, myc-SLM-1, or myc-SLM-2. The cells were lysed, and the lysates were divided equally and incubated with GST alone, GST-fynSH3, GST-PLCγ-1SH3, or GST-GAPSH3 fusion proteins covalently coupled to beads. SLM-1 bound the SH3 domain of p59^{fyn} and PLCγ-1, but with lower relative affinities than Sam68 (Fig. 4*A*, compare lanes 3 and 4 with lanes 8 and 9). The Sam68 P3, P4, and P5 are known to associate with the SH3 domain of p59^{fyn} (5), and since these motifs are not fully conserved in SLM-1, this may account for the lower apparent affinity to the SH3 domain of p59^{fyn}. We have mapped Sam68 P4 as the binding site for the SH3 domain of PLCγ-1 (5), which is a class I motif (RFVPPPP). The increased spacing between the arginine and the first proline by one amino acid in SLM-1 (RGALPVPP)

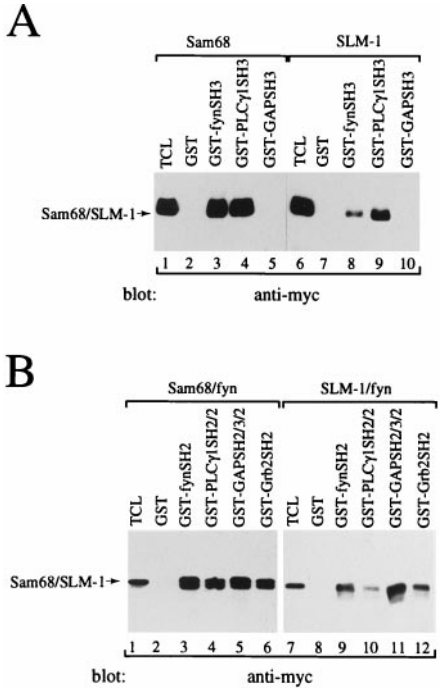

FIG. 4. SLM-1 associates with the p59^{fyn} and PLCγ-1 SH3 domains and the p59^{fyn}, PLCγ-1, GAP, and Grb2 SH2 domains. (*A*) myc-Sam68 and myc-SLM-1 were transfected in HeLa cells separately. The cell lysates were divided equally and incubated with affinity matrices containing GST alone, GST-fynSH3, GST-PLCγ-1SH3, or GST-GAPSH3. The bound proteins were identified by immunoblotting with anti-myc antibodies. The migration position of Sam68 and SLM-1 is shown. (*B*) myc-Sam68 and myc-SLM-1 were cotransfected with p59^{fyn}. The cell lysates were divided equally and incubated with affinity matrices containing GST alone, GST-fynSH2, GST-PLCγ-1SH2/2, GST-GAP2/3/2, or GST-Grb2SH2. The bound proteins were identified by immunoblotting with anti-myc antibodies.

may account for the lower apparent interaction observed with the SH3 domain of PLCγ-1. SLM-2 did not associate with the SH3 domains of $p59^{fyn}$, PLCγ-1, and $p120^{rasGAP}$ (data not shown). We have demonstrated that if the SH3 domain of $p59^{fyn}$ cannot associate with Sam68, Sam68 cannot become a substrate of $p59^{fyn}$ (5). Perhaps SLM-2 is unable to become tyrosine phosphorylated by $p59^{fyn}$ because there is no SH3 domain interaction between these proteins. The absence of PPXY motifs in SLM-1 and SLM-2 suggests that they are not targets for the WW domains of YAP (39), and indeed this was the case, as GST pull-down assays using the WW domains of YAP did not precipitate SLM-1 and SLM-2 (data not shown).

We next investigated whether tyrosine-phosphorylated SLM-1 could bind the SH2 domains of $p59^{fyn}$, PLCγ-1, $p120^{rasGAP}$, and Grb2. HeLa cells were cotransfected with $p59^{fyn}$ and myc-Sam68 or myc-SLM-1. The cells were lysed, and the lysates were divided equally and incubated with GST alone, GST-fynSH2, GST-PLCγ-1SH2/SH2, GST-GAPSH2/SH3/SH2, or GST-Grb2SH2 fusion proteins covalently coupled to beads. The bound myc epitope-tagged proteins were detected by immunoblotting with anti-myc antibodies. Tyrosine-phosphorylated SLM-1 bound the SH2 domains of $p59^{fyn}$, PLCγ-1, $p120^{rasGAP}$, and Grb2 *in vitro* (Fig. 4*B*). The SH2 domains of PLCγ-1 bound SLM-1 with a lower apparent affinity compared with Sam68 (Fig. 4*B*, compare lanes 4 and 10). The absence of SLM-2 tyrosine phosphorylation prevented us from verifying whether it was a docking protein for SH2 domain-containing proteins. We have shown previously that the SH2 domain of Grb2 directly binds Sam68, as determined by a blot overlay assay (5). Although the site of interaction was not mapped, it lies in the C-terminal tyrosine-rich domain of Sam68. Both SLM-1 and SLM-2 contain a Grb2 SH2 domain consensus binding site in their C termini that may serve as a site of the interaction with Grb2 (Fig. 1, underlined YXN).

SLM-1 and SLM-2 Are RNA-Binding Proteins That Heterodimerize with Sam68. The RNA-binding properties of SLM-1 and SLM-2 were investigated. HeLa cell lysates expressing myc-SLM-1 or myc-SLM-2 were incubated with homopolymeric RNA covalently coupled to beads. The bound proteins were visualized by immunoblotting with anti-myc antibodies. myc-SLM-1 bound both poly(A) and poly(U) beads (Fig. 5*A*, lanes 1–6). We next performed competition experiments to examine the relative affinities of these homopolymeric RNAs. Both soluble poly(U) and poly(A) homopolymeric RNA inhibited SLM-1 binding to poly(U) beads (Fig. 5*A*, lanes 7–16). Moreover, 100 μg of poly(G) competed slightly for poly(U) binding (lane 14). SLM-2 bound both poly(A) and poly(G) beads (Fig. 5*B*, lanes 1–6). The competition experiments demonstrated that both 10 and 100 μg of soluble poly(G) competed for the binding of SLM-2 to poly(G) beads (lanes 7–16). Poly(A) was unable to compete for the poly(G) binding, suggesting that SLM-2 binds poly(G) with a higher apparent affinity. These data demonstrate that SLM-1 and SLM-2 are RNA-binding proteins with different specificities for homopolymeric RNA.

We have recently demonstrated that Sam68 self-associates into homocomplexes (14). To determine whether Sam68 could associate into heterocomplexes with SLM-1 and SLM-2, we investigated whether SLM-1 and SLM-2 could co-immunoprecipitate Sam68 (Fig. 6). HeLa cells transfected with HA-Sam68 and myc-SLM-1 (lanes 7–9) or HA-Sam68 and myc-SLM-2 (lanes 10–12) were lysed and immunoprecipitated with control (IgG) or anti-myc antibodies. As controls, HA-Sam68 was expressed alone (lanes 1–3) or with myc-Sam68 (lanes 4–6). HA-Sam68 was observed in myc immunoprecipitates of cells cotransfected with myc-Sam68, myc-SLM-1, and myc-SLM-2 (lanes 6, 9, 12), demonstrating that SLM-1 and SLM-2 associated with Sam68. The association observed between SLM-2 and Sam68 was repeatedly weaker than that observed between Sam68 and SLM-1 or Sam68 with itself. The reason for this is unknown, but we suspect that the differences may lie in KH domain loop 1, where three of six amino acids are different in SLM-2 (PVKQYP Sam68/SLM-1; PTRQFP SLM-2). These data demonstrate that SLM-1 and SLM-2 can form heteromultimers with Sam68.

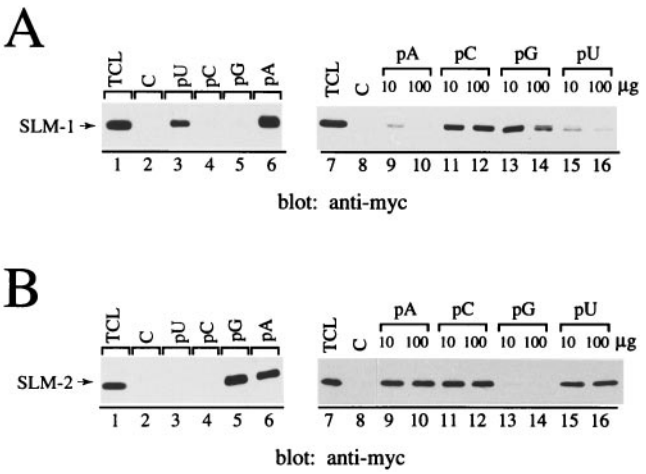

FIG. 5. SLM-1 and SLM-2 bind homopolymeric RNA with different specificities. HeLa cells transfected with myc-SLM-1 (*A*) or myc-SLM-2 (*B*) were lysed and incubated with poly(A), poly(C), poly(G), or poly(U) covalently coupled to beads in the absence of competitor (lanes 1–6) or in the presence of 10 or 100 μg of soluble homopolymeric RNA as indicated (lanes 7–16). The bound SLM-1 or SLM-2 was detected by immunoblotting with anti-myc antibodies. TCL, total cell lysate; C, control Sepharose beads.

SLM-1 Is a Target for Src During Mitosis. Because of the high sequence identity among the three proteins, anti-Sam68 antibodies were examined for their ability to recognize SLM-1 and SLM-2. We tested two known Sam68 antibodies; the mouse Sam68 monoclonal antibody (mAb) 7-1 and a rabbit anti-Sam68 polyclonal antibody (C20). The Sam68 mAb 7-1 was generated by immunizing BALB/c mice with a GST fusion protein containing the mouse Sam68 amino acids 331–443 (S.R. and A. S. Shaw, unpublished data) and was specific for Sam68 (data not shown). The second antibody was the rabbit polyclonal anti-Sam68 antibody that was generated against a peptide encompassing Sam68 amino acids 423–443 (2), and this antibody recognized Sam68, SLM-1, and SLM-2 (see Fig. 7). SLM-1 and SLM-2 share a 70–75% sequence identity with Sam68 in the last 20 amino acids, and this may explain the cross-reactivity observed with the anti-Sam68 C20 antibody.

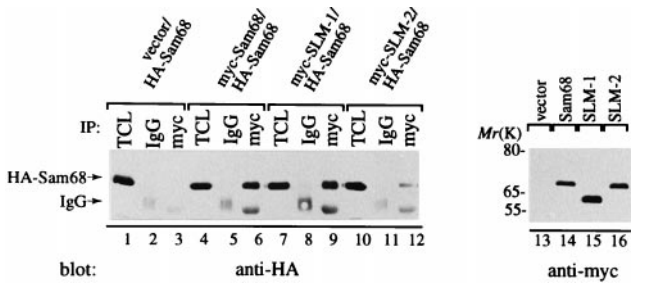

FIG. 6. SLM-1 and SLM-2 associate with Sam68. Vector alone, myc-SLM-1, myc-SLM-2, and myc-Sam68 were cotransfected with HA-Sam68 in HeLa cells as indicated. The transfected cells were lysed and the lysates were immunoprecipitated with IgG or anti-myc antibodies. The immunoprecipitated proteins (IP) were separated by SDS/PAGE and immunoblotted for the presence of HA-Sam68 with anti-HA antibodies. The migration positions of HA-Sam68 and the heavy chain of IgG are shown on the left. The expression of the myc-Sam68, -SLM-1, and -SLM-2 is shown to be equivalent (lanes 14–16).

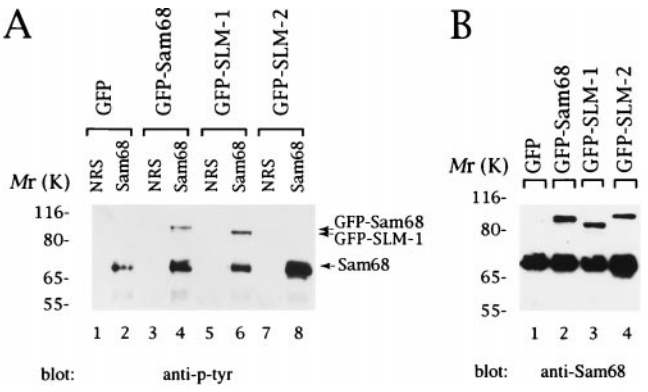

FIG. 7. SLM-1 is a substrate for Src during mitosis. (*A*) v-Src-transformed cells transfected with vector alone, GFP-Sam68, GFP-SLM-1, or GFP-SLM-2 were treated overnight with 40 ng/ml nocodazole. The cells were lysed and immunoprecipitated with normal rabbit serum (NRS; lanes 1, 3, 5, and 7) or the C20 anti-Sam68 antibody (lanes 2, 4, 6, and 8), which recognizes Sam68, SLM-1, and SLM-2. The immunoprecipitated proteins were separated by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine antibodies. (*B*) The expression of GFP-Sam68, GFP-SLM-1, and GFP-SLM-2 in *A* is shown by blotting with C20 anti-Sam68. The positions of molecular mass markers are shown on the left in kDa. The positions of GFP-Sam68, GFP-SLM-1, and endogenous Sam68 are shown.

Immunodepletion studies with HeLa cells using mAb 7-1 for five sequential immunoprecipitations reduced by ≈50% the 68-kDa band recognized by the anti-Sam68 C20 antibody (data not shown). As a control, anti-Sam68 C20 antibody immunoprecipitates completely immunodepleted the 68-kDa band recognized by C20. These findings imply that the anti-Sam68 C20 antibody may not be Sam68-specific but may recognize other proteins, including SLM-1, SLM-2, or unidentified SLMs.

To determine whether SLM-1 and SLM-2 were also substrates for Src during mitosis, v-Src-transformed cells transfected with plasmids encoding GFP alone, GFP-Sam68, GFP-SLM-1, and GFP-SLM-2 were synchronized in mitosis with nocodazole. These fusion proteins were expressed predominantly in the nucleus (data not shown) and migrated at ≈80–90 kDa (Fig. 7). Thus, their molecular masses allowed for an easy distinction from the endogenous Sam68/SLM proteins. v-Src-transformed cells were lysed and their proteins were immunoprecipitated with either normal rabbit serum or the anti-Sam68 antibody C20. The immunoprecipitated proteins were separated by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine antibodies. Anti-Sam68 antibody C20 immunoprecipitates contained tyrosine-phosphorylated GFP-Sam68 and GFP-SLM-1 in cells transfected with GFP-Sam68 and GFP-SLM-1, respectively (Fig. 7*A*, lanes 4 and 6). However, anti-Sam68 C20 antibody immunoprecipitates did not contain tyrosine-phosphorylated GFP-SLM-2 (Fig. 7*A*, lane 8). The anti-Sam68 C20 antibody immunoprecipitated GFP-SLM-2, as the reprobing of the membrane detected abundant levels of GFP-SLM-2 in lane 8 (data not shown), similar to that shown in lane 4 of Fig. 7*B*. The endogenous Sam68 protein observed in anti-Sam68 C20 immunoprecipitates was tyrosine-phosphorylated (Fig. 7*A*, lanes 2, 4, 6, and 8) as previously reported (1, 2). These data suggest that SLM-1, like Sam68, is a substrate for Src during mitosis.

DISCUSSION

SLM-1 and SLM-2 are two previously undescribed mammalian proteins that share the basic organization of Sam68: a GSG domain followed by proline motifs, arginine-glycine repeats, and a tyrosine-rich C terminus. SLM-1 and SLM-2 bound RNA with different specificities as determined with homopolymeric RNA. Both SLM-1 and SLM-2 associated with Sam68. This finding is not surprising, considering the GSG domain is necessary and sufficient for oligomerization (14, 19) and that SLM-1, SLM-2, and Sam68 share an ≈70% sequence identity in their GSG domains (Fig. 1). The presence of multiple proline motifs and a tyrosine-rich C terminus imply that SLM-1 and SLM-2 are most likely substrates of tyrosine kinases and bind SH2 and SH3 domain-containing proteins. Indeed, SLM-1 shared the same properties as Sam68: SLM-1 bound the SH3 domains of $p59^{fyn}$ and PLCγ-1, SLM-1 was tyrosine phosphorylated in mitosis by Src, SLM-1 was a substrate of $p59^{fyn}$, and SLM-1 bound the SH2 domains of $p59^{fyn}$, Grb2, $p120^{rasGAP}$, and PLCγ-1. Thus, SLM-1, like Sam68, may function as an adapter protein for Src kinases during mitosis. Although SLM-2 has a class I proline motif, it did not associate with the SH3 domains of $p59^{fyn}$, Grb-2, PLCγ-1, and $p120^{rasGAP}$. SLM-2 was not phosphorylated by Src or $p59^{fyn}$. The tyrosine kinase, if any, that phosphorylates SLM-2 remains unknown.

Since Sam68, SLM-1, and SLM-2 are predominantly nuclear, how can one envision their being substrates for tyrosine kinases? In the case of Sam68 and SLM-1, the nuclear membrane breakdown during mitosis is thought to be the mechanism by which Sam68 and SLM-1 associate and become tyrosine-phosphorylated by Src kinases (37, 40, 41). The predominant nuclear localization of SLM-2 and its absence of tyrosine-phosphorylation during mitosis suggest that SLM-2 may shuttle between the cytoplasm and the nucleus, as has been shown for other predominantly localized RNA-binding proteins (42). Alternatively, SLM-2 may be the substrate for nuclear tyrosine kinases such as abl.

We have demonstrated that other GSG family members self-associate into homomultimers, including KEP1, Sam50, Who/How, GLD-1, GRP33, and Qk1 (14, 19, 32). Moreover, it was demonstrated that *Xenopus* Xqua also self-associates (31). The high degree of sequence similarity between the GSG domains of SLM-1, SLM-2, and Sam68 suggest that the Sam68/SLM family members might form heteromultimers as well as homomultimers. Indeed, we have demonstrated that SLM-1 and SLM-2 co-immunoprecipitated with Sam68 in fibroblast cells. What might be the role of these heteromultimers? We speculate that these heteromultimers might diversify or dampen the signaling and the RNA-binding responses of these family members. Since the RNA-binding properties of SLM-2 are different from those of SLM-1 and Sam68, heteromultimers of SLM-2/SLM-1 or SLM-2/Sam68 may have distinct RNA-binding characteristics not observed with the homomultimers. As SLM-1 and Sam68 have similar homopolymeric RNA-binding properties, this suggests that hetero- and homomultimers of SLM-1/Sam68 might have similar RNA-binding properties. The exact RNA-binding properties of the homo- and heteromultimers will be known once specific high-affinity RNA targets for each of these complexes are identified. A high-affinity RNA motif, UAAA, has been identified for Sam68 by using SELEX (30). However, it remains to be determined whether this RNA sequence is a high-affinity target for Sam68/SLM homo- and heteromultimers. We have demonstrated that the RNA-binding and oligomerization properties of Sam68 are negatively regulated by the Src kinase $p59^{fyn}$ (14, 15). These findings suggest that signaling pathways that activate Src kinases may down-regulate the RNA-binding properties of Sam68. It will be necessary to determine whether homomultimers of SLM-1 and SLM-2 and heteromultimers are regulated by protein tyrosine phosphorylation.

Three other DNA sequences with different names have been deposited in GenBank that encode SLM-2. T-STAR (Venables and Eperon; accession no. AF069681) and SALP (Lee, Suh, and Burr; accession no. AF051321) map to chromosome

8 and represent the human SLM-2 protein. ÉTOILE (Vernet, Cowmeadow, and Artzt; accession no. AF079763) is identical to SLM-2 except for amino acid 160. The methionine chosen as the SLM-2 initiator by all groups is the one depicted in Fig. 1. The AUG encoding this methionine is located within a favorable Kozak consensus sequence (ACGAUGG; ref. 43). Moreover, the sequences 5′ to this AUG encode separate protein sequences in mice and humans, suggesting that this sequence may represent the 5′ untranslated region of SLM-2. Since there are no in-frame stop codons upstream of the designated initiator AUG, we cannot exclude the possibility that a larger ORF exists. The availability of specific SLM-2 antibodies will allow resolution of this issue.

During mitosis it is known that several signaling proteins such as Src (44–46), Raf-1 (47), and recently MKK (48) and MAP kinases (48, 49) are activated and may play a role in mitosis. By using anti-active MAP kinase antibodies, it was shown that active MAP kinases localize to the kinetochores and may be involved in mitotic entry and progression (48, 49). The identification of additional Src substrates during mitosis such as SLM-1 may help clarify the signaling pathways that regulate MAP kinase activation. In addition to serving as a substrate for Src during mitosis, Sam68 is a known substrate for the mitotic serine/threonine kinase Cdc2 (50). Since SLM-1 and SLM-2 contain several potential Cdc2 minimal consensus phosphorylation sites (S/TP), they may also serve as substrates for Cdc2.

The identification of SLM-1 and SLM-2 defines a previously unrecognized protein family. The Sam68/SLM protein family may link tyrosine kinase signaling cascades with some aspect of RNA metabolism. The tyrosine phosphorylation of SLM-1 and Sam68 during mitosis by Src suggest that one role for these proteins may be to function as multifunctional adapter proteins during mitosis.

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