Identification of cellular mRNA targets for RNA-binding protein Sam68

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Received August 21, 2002; Revised and Accepted October 14, 2002

ABSTRACT

Sam68 (Src-associated in mitosis, 68 kDa), a nuclear RNA-binding protein, has been postulated to play a role in cell-growth control as a modulator of signal transduction and activation of RNA metabolism. Although Sam68 was demonstrated to bind to the UAAA sequences in synthetic oligoribonucleotides and poly(U) homopolymers in vitro, the legitimate cellular mRNA target remained unclear. By using the differential display and cDNA-representational difference analysis techniques, followed by reverse transcription polymerase chain reaction of RNAs co-immunoprecipitated with Sam68 from a HeLa cell lysate, we identified 10 mRNA species that bind in vivo to Sam68 in an RNA-binding-domain-dependent manner. Among them, the mRNA species for hnRNP A2/B1 and β-actin were found to bind prominently in vivo as well as in vitro, suggesting the possible involvement of Sam68 in the post-transcriptional regulation of these genes. Mapping of the Sam68-binding sequence revealed that Sam68 associates with these mRNAs through different nucleotide motifs, UAAA for hnRNP A2/B1 mRNA and UUUUUU for β-actin mRNA, and that both binding sequences must reside in a loop structure for recognition by Sam68. The results indicated that Sam68 recognizes both the UAAA motif and poly(U) sequences in vivo for binding to cellular target mRNAs.

INTRODUCTION

Sam68 (Src-associated in mitosis) is the 68 kDa protein that was originally identified as the only known substrate for Src-family tyrosine kinases during mitosis (1,2), and is a member of the STAR (signal transduction and activation of RNA metabolism) family (2), which was proposed to comprise mediator proteins connecting signal transduction pathways and RNA metabolism, based on the domain organization of the protein (3). Sam68 and related proteins contain a KH (hnRNP K homology) RNA-binding domain embedded in a larger domain of about 200 amino acids, which has been called the GSG (GRP33/Sam68/GLD-1) or SGQ (Sam68/GLD1/Quaking) domain. Proteins containing this domain include Artemia salina GRP33 (4), Caenorhabditis elegans GLD-1 (5), SF1 (6), T-STAR/SLM-2/Salpα (7–9), Drosophila Who/How (10,11), mouse Qk1 (12), Drosophila KEP1 and Sam50 (13) and Drosophila Qk1-related proteins (14), and share several properties, including RNA binding and self-association. Genetic analysis of them clearly demonstrated the essential role of their RNA-binding activities in their biological functions. As for Sam68, overexpression of a splice variant of it, Sam68ΔKH, which lacks a part of the KH-domain and the ability of RNA binding, in NIH 3T3 fibroblast cells inhibited cell-cycle progression (15), suggesting that specific RNA binding may be essential for cell-growth control.

In terms of the control of cellular functions through RNA metabolism, Sam68 has been reported to be associated and co-localized with various proteins involved in the regulation of RNA functions, such as RNA-dependent RNA polymerase 3D of poliovirus (16), Tap, RHA (17), splicing-associated factor YTS2-B (18) and scaffold attachment factor-B (SAF-B)/hnRNP A1 interacting protein (HAP) (19). In addition, Sam68 was shown to act as a functional homolog of Rev protein, and to mediate the cytoplasmic expression of RNAs containing Rev responsive element and constitutive transport element of type-D retrovirus (17,20).

Besides binding to RNA and its modulator proteins, Sam68 contains five proline-rich motifs and multiple potential tyrosine phosphorylation sites that are involved in the binding to proteins with Src homology 3 (SH3) and SH2 domains (21), including Src-family tyrosine kinases (22,23), Grb-2 (21), phospholipase Cγ1 (PLCγ-1) (21,24), ras-GTPase activating protein (ras-GAP) (25), Cbl (22,27) and Jak3 (22,26). These interactions support the potential role of Sam68 as a multifunctional adapter protein for tyrosine kinases as well as a modulator protein involved in gene expression at the post-transcriptional level.

To elucidate the role of Sam68 in cellular functions and its molecular mechanism, it is essential to identify cellular target mRNAs whose activity is regulated through the binding of Sam68. Although the UAAA motif has been identified in the preferential binding sequence of Sam68 by means of the SELEX (systemic evolution of ligands by exponential enrichment) technique (28), and the poly(U) homopolymer was shown to bind to Sam68 in vitro, legitimate cellular mRNA targets have remained unidentified. In this article, we report the identification of target mRNAs for Sam68. By using the differential display (29) and cDNA-representational difference analysis (cDNA-RDA) techniques (30), candidate
mRNA species that bind to Sam68 in vitro were identified. Their in vivo association with Sam68 was further verified by reverse transcription polymerase chain reaction (RT-PCR) of mRNA species in a cell extract that were co-immunoprecipitated with Sam68, resulting in the identification of 10 mRNA species as in vivo targets of Sam68. Among these mRNAs, those for hnRNP A2/B1 and β-actin were found to associate with Sam68 prominently. The following in vitro binding analysis revealed that Sam68 binds to these mRNAs through two different sequence motifs, UAAA for hnRNP A2/B1 mRNA and UUUUUU for β-actin mRNA. Since a point mutation in the KH-RNA-binding domain of Sam68 abolished both bindings, it was concluded that the two different sequence motifs were involved in the KH domain-dependent association of Sam68 with cellular target mRNAs. The identification of multiple recognition sequences in in vivo target mRNA species might indicate the possibility that Sam68 regulates the metabolism of target mRNAs in a different manner depending on the binding sequence.

MATERIALS AND METHODS

Construction of plasmids

The cDNAs for human Sam68 and Sam68ΔKH were prepared by PCR using a pair of primers (5′ primer, 5′-CCGCTAGC-CAGCGCGGGAAGCACC-3′; and 3′ primer, 5′-GGAATTCCTAAACGCTCATTGG-3′) based on the reported sequence and cDNA prepared from poly(A) RNA isolated from a human T cell line, Jurkat. The PCR products were cloned into the pCR2.1 vector (Invitrogen), and their nucleotide sequences were confirmed.

The cDNAs for Sam68 and Sam68ΔKH were digested with BamHI and EcoRI, and the resulting DNA fragments of 1185 and 1068 bp in length were cloned into the BamHI–EcoRI sites of the pGEX-2T vector to generate expression plasmids for the glutathione S-transferase (GST)-fused Sam68 (GST–SamΔN) and Sam68ΔKH (GST–SamΔNΔKH) proteins.

The expression plasmids for the hemagglutinin (HA) epitope-tagged Sam68 and Sam68ΔKH proteins (pCG-N/Sam and pCG-N/Sam68ΔKH) were constructed by inserting the Nhel–EcoRI cDNA fragments of the coding sequence of Sam68 and Sam68ΔKH into the XbaI–EcoRI sites of the pCG-N-Bl vector in frame.

The full-length cDNAs for hnRNP A2/B1 and β-actin mRNAs were amplified by RT–PCR using HeLa poly(A)+ RNA and a pair of PCR primers (hnRNP A2/B1: 5′ primer, 5′-GGAATTCCTAGCAGGGAGAAGCACC-3′; and 3′ primer, 5′-GGCATGCTTATTTATTTAATTAATCTAA-3′; β-actin: 5′ primer, 5′-GAGATCTGGCCATCAGCCCCGG-3′; and 3′ primer, 5′-GCTGAGAAGTGACCATTTATTCAG-3′), and then cloned into the pGEM-T Easy vector. Sub-fragments were prepared by PCR amplification with the respective primers, and then cloned into the pGEM-T Easy vector for transcription of the uniformly labeled RNA probes.

Preparation of GST fusion proteins

To prepare GST fusion proteins, Escherichia coli strain BL21 was transformed with the pGEX-2T based plasmid. The transformed cells were grown in 2× YT broth containing 50 μg/ml ampicillin at 30°C to a cell density of A600 = 0.6 ~ 0.8. The recombinant protein was induced by the addition of 0.1 mM isopropyl-1–β-D-galactopyranoside (IPTG), and incubation was continued for 16 h at 30°C. Cells were collected by centrifugation and the expressed protein was extracted.

Briefly, the cells were suspended in 1× PBS, 100 mM NaCl, 0.5% Triton X-100 and 5 mM DTT. After disruption by sonication, Triton X-100 was added to 1% and insoluble matter was removed by centrifugation at 5000 g for 30 min. About 2 mg of the cell lysate was diluted to 5 ml with GST-binding buffer (1× PBS, 100 mM NaCl, 1.0% Triton X-100, 5 mM DTT), and then mixed with 1 ml of a 50% slurry of glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech) equilibrated with GST-binding buffer. After 1 h gentle mixing at 4°C, the GST fusion protein immobilized on glutathione–Sepharose 4B beads was washed five times with 15 ml of the same buffer, and then suspended with 500 μl of the same buffer. Approximately 200 μg of the GST fusion protein was bound to 500 μl of glutathione–Sepharose 4B beads in a total 1 ml of GST-binding buffer.

Differential display and cDNA-representational difference analysis

Ten micrograms of GST fusion protein (GST–Sam68ΔN or GST–Sam68ΔNΔKH) on glutathione–Sepharose beads (25 μl) was mixed with 200 μg of HeLa poly(A)+ RNA in RNA-binding buffer (50 mM Tris–HCl, pH 7.4, 100 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 2.5% NP-40, 5 mM DTT) at 4°C for 30 min. After washing five times with the same buffer, the mRNA fraction associated with the GST fusion protein was recovered by phenol extraction and following ethanol precipitation. These mRNAs were subjected to differential display (29) and representational difference analysis (cDNA-RDA) (30).

For differential display, 0.2 μg of RNA associated with GST–Sam68ΔN or GST–Sam68ΔNΔKH was reverse transcribed with ReverTra Ace (TOYOBO, Japan) with one of four degenerate anchored oligo-dT primers (T12VG, T12VA, T12VC, containing 12 T nucleotides) and then cloned into the Easy vector for transcription of the uniformly labeled RNA probes. The cDNA-RDA was performed according to the published procedure (30) with a pair of RNA fractions associated with the GST–SamΔN and GST–SamΔNΔKH proteins. Double-stranded cDNA was prepared by reverse transcription of either RNA using an oligo-dT12–18 primer (Amersham Pharmacia Biotech). The nucleotide sequences of the synthetic oligonucleotides used for the cDNA-RDA were as follows:

- R-Bgl-24, 5′-AGCACTCTCAGCCTCCCATGCCCG-3′;
- R-Bgl-12, 5′-GGATCGGTTGCAAGTGGC-3′;
- J-Bgl-12, 5′-ACGACGTGACTTACCATGAACA-3′;
- N-Bgl-24, 5′-AGGCAACTCTGTCATCCGCCG-3′;
- J-Bgl-12, 5′-ACGACGTGACTTACCATGAACA-3′;
- N-Bgl-24, 5′-AGGCAACTCTGTCATCCGCCG-3′;
- J-Bgl-12, 5′-ACGACGTGACTTACCATGAACA-3′;
- N-Bgl-24, 5′-AGGCAACTCTGTCATCCGCCG-3′;
- J-Bgl-12, 5′-ACGACGTGACTTACCATGAACA-3′;
- N-Bgl-24, 5′-AGGCAACTCTGTCATCCGCCG-3′;
- J-Bgl-12, 5′-ACGACGTGACTTACCATGAACA-3′;
- N-Bgl-24, 5′-AGGCAACTCTGTCATCCGCCG-3′;
GGAA-3'; N-Bgl-12, 5'-GATCTTCCCTCG-3'. Two micrograms of double-stranded cDNA was digested with restriction enzyme DpnII, ligated to the R-Bgl-12/R-Bgl-24 adapter (R-Bgl-12, 5'-GATCTTGGGTGA-3'; R-Bgl-24, 5'-AGCA-CTCTCCAGCCTCCTCCAGA-3'), and then amplified by PCR with R-Bgl-24 as a primer to generate the 'representation'. The adapter was removed from 'representation' derived from RNA bound to GST–SamANAKH by DpnII digestion, and the resulting DNA was phenol-extracted and ethanol-purified to generate the 'driver'. The 'representation' prepared from GST–SamAN-bound RNA was gel purified and 2 μg of the purified 'representation' was ligated to an N-Bgl-12/N-Bgl-24 adapter (N-Bgl-12, 5'-GATCTTCCCTCG-3'; N-Bgl-24, 5'-AGCAACTCTGCTATCCCGAG GGAA-3') to generate the 'tester'. For the first subtractive hybridization, 70 ng of an N-Bgl-ligated tester was mixed with 4.5 μg of the driver (molar ratio, 1:65) and then amplified by PCR with the N-Bgl-24 primer, resulting in the first difference product (DP1). DP1 was PCR amplified with the J-Bgl-12 and J-Bgl-24 primers to convert both ends of the fragment to J-Bgl-12/J-Bgl-24 adapters, and then the processes of subtractive hybridization and selective amplification (using alternately the N-Bgl and J-Bgl adapters) were reiterated to generate DP2 and DP3. The molar ratios of the tester and driver in the reactions producing DP2 and DP3 were 1:560 and 1:66 000, respectively. The DP3 PCR products were cloned into the pGEM-T Easy vector and sequenced.

The resulting sequences were compared with the GenBank database using the BLAST search program.

**Preparation of RNAs associated with Sam68 protein**

**In vivo**

HeLa cells were grown in D-MEM (Dulbecco’s Modified Eagle Medium, Sigma) supplemented with 5% fetal calf serum. To isolate HeLa cells expressing HA–Sam68 or HA–Sam68ΔKH, 5 × 10⁵ HeLa cells were transfected with 3 μg of pCG-N/Sam68 or pCG-N/Sam68ΔKH with 0.5 μg of pSV-puro by using LipofectAmine (Invitrogen) and then selected for 2 weeks in the presence of 5 μg/ml of puromycin. The expression of HA-tagged Sam68 and Sam68ΔKH was confirmed by means of the western blotting procedure using 12CA5 anti-HA antibody (Boehringer Mannheim) and anti-Sam68 antibody (Santa Cruz Biotechnology). A total of 2 × 10⁶ of the transformant cells were collected and suspended in 400 μl of TNE buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40) containing a 1/10000 volume of a protease inhibitor cocktail (P-8340, Sigma), 5 mM DTT and 0.5 U/μl of RNaseOUT ribonuclease inhibitor (Invitrogen). After gentle mixing at 4°C for 30 min, the cell lysate was cleared by centrifugation and then pre-cleared with 30 μl of Protein G–Sepharose (Amersham Pharmacia Biotech). The lysate was incubated with 2 μg of 12CA5 anti-HA antibody at 4°C for 1 h under gentle mixing and then the antibody–protein complex was recovered by adding 100 μl of Protein G–Sepharose beads, followed by extensive washing (5 × 1 ml) with TNE buffer. The RNA species associated with the HA-tagged protein was obtained by phenol extraction of the immunoprecipitate and subjected to RT–PCR with specific primers. The sequences of oligonucleotide primers used for RT–PCR to detect the specific mRNA were as follows: for MMS2/EDAF-1, 5'-TTCCCTCTGAATTTTTGCC-3' and 5'-TAACTCTGTTATGCTCGC-3'; for DAP5/IRCP, 5'-AA- GGGAACAGAAAGAACC-3' and 5'-CCTGTTTATGCGC-3'; for nuleolarprotein-p40, 5'-CGCTCTCTCC-AGCTCAA-3' and 5'-CTTTGCTCTCCTGGTCC-3'; for PBP/PEA-8P, 5'-TCAGCGAGGACAGTCC-3' and 5'-ATACACACCTCGG- TAGCC-3'; for PAP/AXN, 5'-GGTGTCTCCTCAGCC-3' and 5'-CGCTCAATGGTTTCTCCA-3'; for dynein/ PIN, 5'-TCAGGGCGCTGGAGAATAA-3' and 5'-CCA-CGGGCCCCAGTTA-3'; for TCP1, 5'-ATGGCTTGCA- TTAATCC-3' and 5'-TTACCTTCCAAATTTGGC-3'; for CCT6A/HTR3, 5'-TTGGCTGTGTTGCGGTAG-3' and 5'-TGCAATTGCACCTTCAC-3'; for TAP1/2/OBD1, 5'-CAAAGCTGTGTAAGAC-3' and 5'-ATCCAGCGC-CACCAGT-3'; for CGI-90, 5'-CGAGGACTTCGGGCTG- CTCG-3' and 5'-CTGGCAGAAGGAGCCTCC-3'; for HSJ2, 5'-TACGATTTTGGGGTC-3' and 5'-GACATCCTTCT- CCTCCTC-3'; for ME-1, 5'-ATGGTGCTGAGCTTCA-3' and 5'-TCTTTTGGTTTGCGGT-3'; for β-actin, 5'- GCCTACAGTCCTGGAC-3' and 5'-TCTTCTCGATCTGTCG-3'; for NAD4, 5'-CCTCCGACCCCCCTAACA-3' and 5'-GGCGTCTGGTGTGCG-3'.

**In vitro RNA synthesis**

Uniformly ³²P-labeled RNA was synthesized using 1 μg of linearized pGEM-T Easy plasmid as a template in 1× transcription buffer (40 mM Tris–HCl, pH 8.0, 50 mM NaCl, 8 mM MgCl₂, 5 mM DTT), and 5 mM each of ATP, CTP and GTP, 1 mM UTP, 50 U Thermo T7 RNA polymerase (TOYOBO) and 20 μCi of [α-³²P]UTP. After 1 h incubation at 42°C, 5 U RNase-free DNase (TAKARA) was added, followed by incubation for an additional 15 min at 37°C. Unincorporated nucleotides were removed with a Micro Bio-Spin Column P-30 (Bio-Rad Laboratories).

**In vitro RNA-binding assay**

Two micrograms (~20 pmol) of GST fusion protein on glutathione–Sepharose beads (~5 μl) were incubated with 1 × 10⁵ c.p.m. (50 ~ 300 fmol) of ³²P-labeled RNA probe in 20 μl of 1× RNA-binding buffer (50 mM Tris–HCl, pH 7.4, 100 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 0.5% NP-40) containing 200 μg/ml yeast total RNA, 2 mM DTT and 0.5 U/μl RNaseOUT ribonuclease inhibitor for 20 min on ice. RNA binding was measured by counting the radioactivity retained on glutathione–Sepharose beads after extensive (500 μl × three times) washing with 1× RNA-binding buffer and then the relative retention as to the input radioactivity was calculated.

**Calculation of RNA secondary structures**

The secondary structures of RNA probes were determined with the ‘mfold’ program (version 3.1) (31).
RESULTS

Differential display and cDNA-representational difference analysis for selection of the candidate target RNAs for Sam68

To identify cellular mRNAs that can associate with Sam68, we first isolated an mRNA fraction specifically bound to Sam68 protein in vitro. Poly(A)+ RNA prepared from HeLa cells was incubated with glutathione–Sepharose 4B beads bearing immobilized GST protein fused with either N-terminus truncated Sam68 (GST–SamAN) or its natural mutant form lacking the KH-domain, Sam68AKH (GST–SamANAKH) (Fig. 1). After extensive washing, the mRNA fractions bound to the GST fusion protein were recovered by extraction of glutathione–Sepharose beads and then subjected to differential display and cDNA-RDA.

For differential display analysis, a pair of RNA fractions purified with either GST–SamAN or GST–SamANAKH was reverse transcribed using one of four degenerate anchored oligo-dT primers (T12VG, T12VA, T12VT, T12VC, where V may be G, A or C). The single-stranded cDNA was then PCR-amplified with the respective anchored oligo-dT primer and one of the 20 arbitrary random decamers in the presence of 32P-labeled dCTP. A pair of PCR products was resolved side by side on a denaturing polyacrylamide gel and only the cDNA band obtained with the mRNA bound to GST–SamAN was identified as a candidate Sam68 target. We selected 40 independent bands and re-amplified them with the same set of primers. These cDNAs were cloned and their nucleotide sequences were determined. Among them, 13 showed significant (>95%) homology to known genes, and nine exhibited limited homology to mitochondrial DNA when compared with the GenBank (EMBL) database. The rest of the cDNAs only matched the sequences reported as EST (expressed sequence tag), BAC (bacterial artificial chromosome) or YAC (yeast artificial chromosome) clones.

For cDNA-RDA, the mRNA fractions associated with GST–SamAN and GST–SamANAKH were used for the synthesis of the tester and driver cDNA, respectively (see Materials and Methods) and the final products (DP3) were cloned into the pGEM-T Easy vector. The nucleotide sequences of the cDNA inserts isolated from 70 independent colonies were determined and 23 clones were derived from the known 16 genes. The sequences of the inserts from 15 clones showed limited matching with the mitochondrial DNA, and the nucleotide sequences of 32 cDNA inserts were homologous to those of EST, BAC or YAC clones.

Therefore, 29 mRNA species were identified as in vitro targets of Sam68 (Table 1).

Detection of mRNAs associated with Sam68 in a HeLa cell lysate

To examine the in vivo association of these candidate mRNAs for Sam68, we performed RT–PCR analysis of the RNA fraction co-puriﬁed with Sam68 protein from a cell lysate. For this purpose, we established stably transformed HeLa cells expressing HA-tagged Sam68 (HA–Sam68) or Sam68AKH protein (HA–Sam68AKH). In these two cell lines, almost the same amount of HA-tagged recombinant proteins was expressed (data not shown). The cytoplasmic lysates of these cells were subjected to immunoprecipitation with 12CA5 anti-HA antibody and then the RNA fraction was isolated by phenol extraction of the immunoprecipitate. Between 73 and 3.4 μg of RNA was recovered from the cell lysates of 2 × 10⁶ HA–Sam68 and HA–Sam68AKH transformants, respectively, and an equal amount of RNA was used as the template for RT–PCR analysis with mRNA-specific primers. As a control, the primers for the mRNAs of β-actin and NADH dehydrogenase subunit 4 (NADH4) were included.

As shown in Figure 2A–I, the mRNAs for MMS2/EDAF-1, DAP3/IRCP, nucleolar protein-p40, hnRNP A2/B1, PAP/ANX5, dynein/PIN, TCP1, PBP/PEA-BP and CCT6A/HTR-3 were detected in the RNA fraction associated with HA–Sam68 (lane 2), but little, if any, in that associated with HA–Sam68AKH (lane 3). Unexpectedly, β-actin mRNA was co-puriﬁed with Sam68 but not with Sam68AKH protein (Fig. 2J). The reason why β-actin mRNA was not identiﬁed with the two in vitro selection procedures is unclear but it seems likely that the amount of the mRNA for β-actin was too great for differentiation by the PCR procedure because the PCR ampliﬁcation would quickly reach a plateau level and, in fact, one-tenth RNA was used for the PCR in Figure 2J, compared with the other PCRs in Figure 2, for detection in a linear range. Since the transformation of either HA–Sam construct did not affect the expression of the tested mRNAs (data not shown), it was concluded that the mRNAs listed above associate in vivo with Sam68 in a manner dependent on the KH-RNA-binding domain.

The mRNAs for Gs-α, SEC63L, 9G8/SFRS7, DRG1, Importin-β, TPT1, ENO1/MBP-1 and calmodulin were substantially detected in the RNA fraction associated with HA–Sam68AKH but much less than in that associated with HA–Sam68 (data not shown), showing the lower speciﬁcity of the Sam68 binding. On the contrary, a similar amount of mRNA was co-puriﬁed with either HA–Sam68 or HA–Sam68AKH in the case of the mRNAs for TPT1, PAGA/NKEFA, CAG-7/CGT-B3 (data not shown) and NADH4 (Fig. 2K). These mRNAs may bind to Sam68 in a KH-domain independent manner or non-speciﬁc binding to the affinity resin might have been detected. Little or no PCR fragment was detected in the Sam68-binding RNA fraction with the primers for TAPA-1/CD81, CGI-91, HSJ2 and ME-1 mRNAs (Fig. 2L–O), indicating that these mRNAs are not associated with Sam68 protein in vivo, although mRNAs for
incubated with GST-fused Sam68 (GST±Sam RNA was generated by using T7 RNA polymerase and then calculated. Since little retention of the RNA was observed the relative retention as to the input radioactivity was retained on the Sepharose beads after extensive washing and the RNA binding was measured by counting the radioactivity (lane 2) represented the KH-dependent binding to Sam68 protein. The results with the segmented RNA probes demonstrated that the mRNA species had strong Sam68-binding signals, while TCP1 mRNA associated with Sam68 preferentially through their 3'-UTRs. On the other hand, the mRNAs for MMS2/EDAF-1, dynein/PIN and CCT6A/HTR3 (Fig. 3A, F and I, respectively) bound to Sam68 preferentially through their 3'-UTRs, while TCP1 mRNA associated with Sam68 preferentially through their 3'-UTRs.

Table 1. Candidate mRNA targets of Sam68, isolated by the differential display and cDNA representational difference analysis methods

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<th>mRNAs bound to Sam68</th>
<th>Protein encoded</th>
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<tr>
<td>RT-differential display</td>
<td>Guanine nucleotide-binding protein alpha-subunit</td>
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<td>Gs-α</td>
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^nResults of RT-PCR are presented in this article.

these genes are abundantly expressed in HeLa cells and can bind to Sam68 protein in vitro as naked RNAs.

Location of the Sam68-binding sequence in the full-length mRNAs

The in vivo association of 10 mRNA species was confirmed in the above experiment. We then attempted to identify the regions of the mRNAs responsible for their Sam68 binding. For this purpose, we cloned the full-length cDNAs for these mRNAs, divided them into three parts, except for that of dynein/PIN, which was cut into halves, and then subcloned the parts downstream of the T7 promoter in the pGEM-T Easy vector for in vitro RNA synthesis. A uniformly 32P-labeled RNA was generated by using T7 RNA polymerase and then incubated with GST-fused Sam68 (GST–SamΔN or GST–SamΔNKH) protein on glutathione–Sepharose 4B beads. The RNA binding was measured by counting the radioactivity retained on the Sepharose beads after extensive washing and the relative retention as to the input radioactivity was calculated. Since little retention of the RNA was observed with GST–SamΔNKH protein (shown in the lane 3 in each panel) and with GST–SamΔN.G178D and GST–SamΔN.I178N proteins (data not shown), the RNA retention with GST–SamΔN protein immobilized on glutathione–Sepharose beads (lane 2) represented the KH-dependent RNA binding to Sam68 protein. The results with the segmented RNA probes demonstrated that the mRNAs encoding DAP3/IRCP, nucleolar protein-p40, hnRNP A2/B1, PAP/ANX5, PBP/PEA-BP and β-actin (Fig. 3B–E, H and J, respectively) bound to Sam68 preferentially through their 3'-UTRs. On the other hand, the mRNAs for MMS2/EDAF-1, dynein/PIN and CCT6A/HTR3 (Fig. 3A, F and I, respectively) bound to Sam68 via both the coding and 3'-non-coding sequences, while TCP1 mRNA associated with Sam68 mainly via the middle part of its coding sequence. Nevertheless, it is important to note that 9 of the 10 mRNA species retained the binding sequences in their 3'-UTRs, while many RNA species are known to harbor signals for protein binding and the regulation of its metabolism.

Sam68-binding sequence in the 3'-UTR of hnRNP A2/B1 mRNA

To identify the Sam68-binding sequences in cellular mRNAs in detail, we chose the hnRNP A2/B1 and β-actin mRNAs, because these mRNA species were found to associate with Sam68 protein prominently both in vivo (Fig. 2) and in vitro (Fig. 3). Especially Figure 2 shows that these two mRNA species gave the strongest signals, indicating that a substantial part of these mRNAs was associated with Sam68 in vivo. As the two mRNAs both retained strong Sam68-binding
sequences in their 3′-UTRs, we further dissected their 3′-UTRs and performed similar experiments to those above.

As shown in Figure 4, further dissection of 592 nt fragment C of hnRNP A2/B1 mRNA (A2/B1-C, nucleotides 1109–1693) revealed that a 116 nt fragment (A2/B1-C3c, nucleotides 1577–1693) at the 3′-end of the mRNA retained the Sam68-binding activity (Fig. 4). In the C3c fragment we found three UAAA sequences, the target sequence for Sam68 determined with the SELEX technique (28) (Fig. 5A). Since a single point mutation, from UAAA to UACA, in a probe oligoribonucleotide abolished the binding activity toward Sam68 in the SELEX experiment, we introduced the same point mutation into the UAAA sequences in the C3c fragment and also in the full-length hnRNP A2/B1 mRNA. The mutation of A to C at nucleotide 1623 (M1) dramatically reduced (>80%) the binding activity of the C3c fragment, whereas the other mutations, i.e. at nucleotides 1680 (M2) or 1691 (M3), or both nucleotide mutations (M4), little affected Sam68 binding (Fig. 5B). The same effect of the mutations in the context of full-length mRNA (Full-M1–4) was demonstrated. These results clearly showed that the 1621UAAA sequence of hnRNP A2/B1 mRNA is responsible for the binding to Sam68.

To answer the question of why Sam68 protein binds to 1621UAAA but not to 1678UAAA or 1689UAAA, the secondary structure of the C3c RNA fragment was analyzed by means of the ‘mfold’ program. As shown in Figure 5C, while the 1621UAAA-binding sequence was found in the single-stranded loop structure, 1678UAAA was found in the double-stranded stem structure and 1689UAAA was present as a 3′-ended unfixed structure. With the full-length hnRNP A2/B1 mRNA, a similar result was obtained (data not shown). These results strongly indicate that the binding sequence must be located in a loop as a single strand stretch with a fixed stem structure for the recognition by Sam68 protein.

Sam68-binding sequence in the 3′-UTR of β-actin mRNA

In Figure 3, fragment C, corresponding to the 3′-UTR of β-actin mRNA (βA-C, nucleotides 1166–1757), exhibited
strong binding activity toward Sam68. In order to locate a binding nucleotide sequence in the β-actin mRNA, we produced a series of deletion mutants from the βA-C fragment and were able to trim the binding fragment down to 106 nt (βA-C5c, nucleotides 1355–1461; Fig. 6). It is of note that the βA-C5c fragment does not overlap a nucleotide sequence called the ‘zip code’ (nucleotides 1170–1223, present in βA-C5a), which acts as a cis-acting element for the localization of β-actin mRNA at the periphery of fibroblast cells (32). Therefore, co-operative binding and functional interaction between Sam68 and the zip code-binding protein, ZBP-1 (33), on the 3′-UTR of β-actin mRNA could be expected. A 106 nt fragment, βA-C5c, maintains strong binding activity toward Sam68 but does not contain a UAAA sequence, which was identified as the Sam68-binding motif in the hnRNP A2/B1 mRNA (34), as well as by the SELEX procedure. The secondary structures of these deletion mutants were analyzed by means of a computer program. The secondary structures of the βA-C5c and d2 fragments shown in Figure 8 were found to share a stem–loop structure formed by a stretch of nucleotides, UUGUGUUUUUUAUAA (underlined U 6 form a loop, boxed in Fig. 8A and C), which was absent in the βA-d1 deletion mutant (Fig. 8B). Since analysis of the hnRNP A2/B1 mRNA demonstrated the nucleotides in a loop structure were a target for Sam68, a hexamer of U residues (nucleotides 1404–1409) in a loop of the βA-C5c and d2 fragments was focused on with respect to the Sam68 recognition. Subsequently, five uracil residues in the loop were changed by site-directed mutagenesis to a single cytosine residue to produce a deletion mutant, βA-M1 (Fig. 7B). As predicted, βA-M1 had lost the substantial Sam68-binding activity. A similar deletion was introduced into the βA-d2 probe (βA-M2 in Fig. 7B), and the affinity of Sam68 to this probe was reduced to the same level as in the case of βA-M1. These results strongly supported the hypothesis that the poly(U) stretch in the loop structure is essential for the association with Sam68.

However, deletion mutants that had lost the poly(U) stretch in the loop, including 3′-deletion mutants of βA-C5c, often showed low but substantial affinity to Sam68 (data not shown). Therefore, we further analyzed the secondary structures of these deletion mutants and noticed that a small stem structure consisting of the nucleotide sequence, ACAUUUGCUGU (underlined UUGU form a loop, boxed in Fig. 8C–E), was present in place of the original stem–loop structure (Fig. 8). To assess the role of this structure in Sam68 binding, we further deleted nucleotides 1396–1411 in the βA-M1 mutant to remove the new stem–loop (βA-M3, Fig. 7A). As shown in Figure 7B, this mutation totally abolished the binding activity of βA-M3 toward Sam68 protein.

Overall, we concluded that the stem–loop structure consisting of a stretch of U residues, U 6 as an authentic site and UUGU as a cryptic one, in the loop is essential for the RNA recognition by Sam68.

### Binding activities toward Sam68 with a point mutation in the KH-region

It has been reported that two loss-of-function point mutations (G178E and I184N) of Sam68 only abolished the binding to a synthetic oligomer containing the UAAA motif, i.e. not to poly(U) beads. This finding implied that the UAAA sequence is a relevant recognition sequence as to biological functions, whereas binding to homopolymeric ribonucleotides is just an indicator of non-specific RNA-binding potential. To determine the significance of poly(U) binding in the recognition of β-actin mRNA by Sam68, the binding activity of mutant proteins G178D and I184N, with the same properties in terms of RNA binding and self-association (34), toward the β-actin mRNA probe was analyzed.

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**Figure 4.** Identification of the Sam68-binding region in hnRNP A2/B1 mRNA. (A) Various DNA fragments derived from full-length hnRNP A2/B1 cDNA were subcloned to downstream of the T7 promoter in the pGEM-T EASY vector. (B) A 32P-labeled RNA probe, prepared with the truncated plasmid as a template, was incubated with GST–Sam6 protein immobilized on glutathione-Sepharose 4B beads. The binding activity of the RNA fragment was calculated as in Figure 3.
As shown in Figure 9, both mutant proteins lost the affinity to the βA-C5c probe as well as that to the hnRNP A2/B1-C3c fragment. It was thus suggested that the KH-domain is mainly involved in the recognition of the poly(U) stretch of β-actin mRNA, different from the apparent non-specific binding to poly(U) beads, in which the affinity was attributed to the proline-rich domain of Sam68. Therefore, we concluded that Sam68 binds to both a UAAA motif in hnRNP A2/B1 mRNA and a U6 stretch in β-actin mRNA in a KH domain-specific manner.

DISCUSSION

In this study, we identified cellular RNA targets for an RNA-binding protein, Sam68. Twenty-nine mRNA species were identified in vitro as candidate target RNA molecules for Sam68 by means of the differential display and cDNA-RDA techniques, and the in vivo association of 10 mRNA species with Sam68 was confirmed. Among them, the mRNAs of hnRNP A2/B1 and β-actin were found to associate with Sam68 prominently in vivo as well as in vitro. Mapping of the Sam68-binding nucleotide sequences in these two mRNAs revealed that two distinct sequence motifs, UAAA and a U6 stretch, were involved in the recognition of the mRNAs for hnRNP A2/B1 and β-actin, respectively, by Sam68.

Although both UAAA and the poly(U) stretch of RNA have been shown to bind to Sam68 protein in vitro, our finding in this study is the first demonstration of the recognition of these nucleotide sequences by Sam68 in the context of full-length cellular mRNA. This is of importance because not all RNA molecules with these nucleotide sequences can associate with Sam68, most probably due to their secondary structure in vitro and the binding of other RNA-binding proteins in vivo. Furthermore, the preferential presence of the Sam68-binding sequence in the 3′-UTR of target mRNAs including those for hnRNP A2/B1 and β-actin supports the possible involvement of these sequences in modulation of the RNA function, since the functional roles of the 3′-UTR in the regulation of RNA metabolism through association with RNA-binding proteins has been widely demonstrated.

The UAAA motif was originally identified as a Sam68-binding nucleotide sequence by means of the SELEX technique. Although the SELEX technique has been successfully utilized to determine the strongest RNA target sequence (35,36), a restriction is that short stretches of RNA are used as targets and the actual RNA molecules harboring the binding

![Figure 5. Mutation analysis of the Sam68-binding sequence in hnRNP A2/B1 mRNA.](image)
sequence remain unclear. Our results clearly confirmed the conclusion on SELEX analysis in the context of full-length mRNA. A substitution mutation of UAATA to UAACA on a full-length hnRNP A2/B1 mRNA abolished its binding capacity as to Sam68 (Fig. 5), and further mutational analysis of the 3′-UTR of hnRNP A2/B1 mRNA demonstrated that the only one of the three stretches of the UAATA sequence in the 3′-UTR was responsible for Sam68 binding as long as this sequence was present in a loop structure, indicating the importance of the nucleotide sequence in a loop structure for the recognition by Sam68. Involvement of the stem and loop structure has been also demonstrated in the nucleotide recognition by many other RNA-binding proteins. In the case of bacteriophage T4 DNA polymerase, it was shown that the stem structure in combination with the single-stranded flanking sequences provided substantial binding to the protein, although nucleotide sequence specificity was not required in the stem structure (36). It is thus possible that the stem domain next to the binding UAATA sequence can directly interact with Sam68 in a sequence-independent manner.

Protein binding to homopolymeric ribonucleotides has been occasionally used for the classification of RNA-binding proteins, and Sam68 exhibits preferential affinity to a poly(U) homopolymer. Recently, Lin et al. showed that two loss-of-function point mutations (G178E and I184N) in the KH-domain of Sam68 only abolished the binding to the UAATA motif containing a probe, i.e. not to poly(U) beads, and thus claimed that only the UAATA sequence, i.e. not the poly(U) sequence, is a relevant recognition sequence as to the biological function (28). In our experiment, however, similar loss-of-function mutations (35) abolished both the association with the UAATA target in the hnRNP A2/B1 mRNA fragment and that with the UAATA target in β-actin mRNA (Fig. 9), suggesting that the association of Sam68 with the UAATA stretch in β-actin mRNA is specific and distinct from the non-specific binding with a ribonucleotide homopolymer.

Therefore, it would be possible that, while Sam68 binds to a stretch of U ribonucleotides in β-actin mRNA in a specific manner, the accumulative association of a mutant protein with reduced affinity to the long U-stretch on homopolymer beads might have compensated for its defect in the association with a stretch of ribonucleotides.

Although we have demonstrated that Sam68 specifically binds to its target RNAs through the UAATA stretch as well as the UAATA motif, it remains unclear how Sam68 recognizes two distinct ribonucleotide sequences as a single protein. Since a polypeptide region rich in arginine–glycine (RG) repeats within the proline-rich domain has been suggested to be responsible for the poly(U) affinity of Sam68 (37), one may assume that combinational binding of the KH-domain and the RG-repeat could be responsible for the UAATA specificity. The presence of two different recognition sequences for Sam68 and the possible involvement of multiple protein motifs in Sam68 may predict association with distinct protein factors and thereby a distinct mechanism for the regulation of different target RNA molecules.

HnRNP A2 and B1 are the most abundant nuclear RNA-binding proteins and the major components of the hnRNP core complex, being presumably involved in pre-mRNA processing and the nuclear–cytoplasmic transport of several mRNAs (38,39). hnRNP B1 mRNA is a splicing variant of hnRNP A2 mRNA consisting of 2–5% of total A2/B1 transcripts (40), and overproduction of hnRNP B1 protein has been reported to be a useful diagnostic marker of cancer (41). In this respect, it is of note that neoplastic transformation of NIH 3T3 fibroblasts through functional inactivation of Sam68 has been reported (42). Thus, Sam68 might possibly function as a tumor suppressor in a manner closely related to post-transcriptional modulation of hnRNP B1 expression.

Recently, it was reported that the expression level of hnRNP B1 is dramatically decreased during the G2–M phase, whereas that of hnRNP A2 remains unchanged (43), suggesting distinct mechanisms for the post-transcriptional regulation of the two mRNA species during the cell cycle. Sam68 was originally identified as an M phase-specific target of Src tyrosine kinase and is also phosphorylated by Cdc2 kinase, which controls the progression of the G2–M phase in the cell cycle. Furthermore, we recently demonstrated that gene disruption of Sam68 in chicken DT40 cells resulted in cell-growth retardation due to elongation of the G2–M phase (44). These results strongly imply that Sam68 is involved in the post-transcriptional...
regulation of hnRNP A2/B1 mRNA in a cell-cycle-dependent manner.

As alternative splicing of pre-mRNA differentiates two mRNA species for hnRNP A2 and B1, Sam68 may play a role in mRNA splicing and/or the nuclear export of these mRNAs. Consistent with this possibility, Sam68 is co-localized with splicing-associated factors, YT521-B and SAF-B/HAP in nuclear dots, called Sam68 nuclear bodies (18,19). In addition, a Sam68-related protein, rSLM2 (rat ortholog of Salp-a), has been shown to influence the splicing patterns of CD44v5 and human tra2-b along with the association with various alternative splicing regulator proteins, such as SRp30c, YT521-B and SAF-B/HAP (45).

With respect to the nuclear export of alternatively spliced mRNA, Reddy et al. demonstrated that overexpression of Sam68 enhanced or even substituted for the function of the Rev protein of HIV-1 in the nuclear export of unspliced viral mRNA (20). However, as the function of Sam68 in the enhancement of Rev activity was inhibited by olomoucine, an inhibitor of Cdc2 kinase (20), the mechanism may be closely associated with mitotic control and could involve any post-transcriptional regulation of mRNAs other than nuclear export during mitosis, when the nuclear membrane is broken down.

Besides its possible function(s) in splicing and/or the nuclear export of mRNA, Sam68 may play another role in the regulation of β-actin mRNA. After serum or growth-factor stimulation, β-actin mRNA is localized close to the leading edge of fibroblasts (a region referred to as lamellae), the site of synthesis and active polymerization of β-actin, and it was also documented that Sam68 is co-localized with β-actin mRNA, peripherally redistributed by the serum stimulation (46). However, the cis-acting elements required for β-actin mRNA sorting have been identified as a 54-nt sequence in the 3'-UTR of β-actin mRNA (called the ‘zip code’), but it is upstream of the U6 target sequence for Sam68, and another 68 kDa protein (ZBP-1) was subsequently identified as the trans-acting factor directing the peripheral localization of β-actin mRNA (33). Therefore, simultaneous binding of Sam68 and ZBP-1 to β-actin mRNA and their distinct roles in regulation of the mRNA function may be postulated. In this sense, it is important to note that Sam68 loses its RNA-binding activity upon phosphorylation by various types of Src-family tyrosine kinases, which are often localized and activated by growth-factor signals at the periphery of a cell. Thus, Sam68 may play a role in masking and re-activation of translation of β-actin mRNA at the periphery, whereas ZBP-1 ensures the localization of β-actin mRNA to the leading edge of the cell. In fact, a sharp burst of translation has been demonstrated in the vicinity of the cytoplasmic membrane upon stimulation of an adherent protein, β1-integrin (47), although the identities of the mRNA species and proteins involved remained unclear. On the contrary, overexpression of a splicing variant of Sam68, in which part of KH is deleted, was reported to inhibit serum-stimulated progression to the S phase of the cell cycle. This phenomenon could be explained by the sustained initiation of translation involved in cell-growth progression after serum stimulation, probably including that of β-actin mRNA, through a mechanism in which the KH-minus Sam68 protein squelches the tyrosine kinase signaling pathway in an dominant-negative manner due to the remaining proline-rich...
A \( \beta A-C5c \)
\[ \Delta G = -49.0 \text{ Kcal} \]

B \( \beta A-d1 \)
\[ \Delta G = -45.3 \text{ Kcal} \]

C \( \beta A-d2 \)
\[ \Delta G = -44.6 \text{ Kcal} \]

D \( \beta A-M1 \)
\[ \Delta G = -51.7 \text{ Kcal} \]

E \( \beta A-M2 \)
\[ \Delta G = -45.5 \text{ Kcal} \]

F \( \beta A-M3 \)
\[ \Delta G = -43.5 \text{ Kcal} \]
Figure 9. Binding of Sam68 point-mutants to RNA probes for hnRNP A2/B1 and β-actin mRNAs. 32P-Labeled A2/B1 C3c and β-actin C5c RNA probes were incubated with the GST-fusion forms of two loss-of-function mutants of Sam68 protein, as well as those of SamαN and SamαNΔK, immobilized on glutathione-Sepharose 4B beads. The binding activity of the RNA fragment was calculated as in Figure 3.

and tyrosine-rich domains. The functional role of a KH-protein, hnRNP K, in translational masking has already been demonstrated in the differentiation-specific expression of the reticulocyte 15-lipoxygenase (LOX) gene, where the tyrosine phosphorylation of hnRNP K by Src kinase depresses the silencing of translation (48).

The restricted distribution of actin mRNA and protein should affect signal-mediated cytoskeletal organization, cell motility, polarity and morphology (32). Therefore, our finding that β-actin mRNA is an in vivo target of Sam68 may explain the accumulating evidence suggesting the involvement of Sam68 in the regulation of cell-cycle control. We recently demonstrated that a deficiency of Sam68 in chicken DT40 cells due to gene disruption led to elongation of the G2–M phase of the cell cycle (44). Since Cdc2 kinase activity, the key for G2–M progression, was not affected by the loss of function of Sam68 protein, as well as those of SamαN and SamαNΔK, immo-
mobilized on glutathione–Sepharose 4B beads. The binding activity of the RNA fragment was calculated as in Figure 3.

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In this report, we identified two mRNA species, one for hnRNP A2/B1 and the other for β-actin, as in vivo targets of Sam68. This finding should provide us with a crucial tool for elucidating the biological role of a STAR-family protein, Sam68, and its molecular mechanism, which will lead to a new concept of cell-growth control through modulation of RNA activity that may be tightly linked to the signal transduction machinery.

ACKNOWLEDGEMENTS

We thank Mr N. J. Halewood for checking the syntax and grammar. This study was supported by a grant from the Japanese Private School Foundation of the Ministry of Education, Culture, Sports, Science and Technology.


