Specificity and Determinants of Sam68 RNA Binding

IMPLICATIONS FOR THE BIOLOGICAL FUNCTION OF K HOMOLOGY DOMAINS

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Sam68, a specific target of the Src tyrosine kinase in mitosis, possesses features common to RNA-binding proteins, including a K homology (KH) domain. To elucidate its biological function, we first set out to identify RNA species that bound to Sam68 with high affinity using in vitro selection. From a degenerate 40-mer pool, 15 RNA sequences were selected that bound to Sam68 with Kd values of 12–140 nM. The highest affinity RNA sequences (Kd = 12–40 nM) contained a UAAA motif; mutation to UACA abolished binding to Sam68. Binding of the highest affinity ligand, G8-5, was assessed to explore the role of different regions of Sam68 in RNA binding. The KH domain alone did not bind G8-5, but a fragment containing the KH domain and a region of homology within the Sam68 subgroup of KH-containing proteins was sufficient for G8-5 binding. Deletion of the KH domain or mutation of KH domain residues analogous to loss-of-function mutations in the human Fragile X syndrome gene product and the Caenorhabditis elegans tumor suppressor protein Gld-1 abolished G8-5 binding. Our results establish that a KH-domain-containing protein can bind RNA with specificity and high affinity and suggest that specific RNA binding is integral to the functions of some regulatory proteins in growth and development.

The tyrosine kinase activity of the Src proto-oncprotein is increased during mitosis by Cdc2-mediated Ser/Thr phosphorylation and by dephosphorylation of a regulatory phosphotyrosine residue (1–5). This is associated with increased tyrosine kinase activity and an increased ability of the Src SH2 (Src-homology 2) domain to bind tyrosine-phosphorylated ligands (6). The major target of Src in mitotic fibroblasts appears to be Sam68, which is phosphorylated by Src and which forms a stable complex with Src via interactions involving both the Src SH2 and SH3 (Src-homology 3) domains (7–9). Sam68 is a nuclear protein the sequence of which is similar to some RNA-binding proteins, particularly in that it includes a KH domain.

KH domains, first identified in heterogeneous nuclear ribonucleoprotein K, have been found in diverse proteins of prokaryotic and eukaryotic origin (10, 11). The structure of a KH domain from v-giglin, which contains 15 KH repeats, has been determined by nuclear magnetic resonance, revealing a ββαβαβ fold (12). Many KH-containing proteins have been shown to functionally interact with RNA in vivo and some have been shown to bind RNA in vitro. Examples include Escherichia coli NusA, which stabilizes transcriptional complexes at RNA hairpins to prevent termination (13); yeast Mrl1, a mediator of meiosis-specific RNA splicing (14); C. elegans Mex-3, which regulates anterior-posterior asymmetry during early development (15); Drosophila Bicaudal-C, which appears to control RNA localization during oogenesis and embryogenesis (16); and human Fmr1, the Fragile X mental retardation gene product (17). Functional lesions in gld-1 (18), frmr1 (19), bicaudal-C (16), and mex-3 (15) have been traced to substitutions of conserved amino acids within their KH domains. These substitutions result in growth or developmental defects, which are often more severe than the corresponding null phenotypes, suggesting that KH domains play essential roles in the functions of these proteins. However, with the exception of Fmr1 (see “Discussion”), the effects of these mutations on RNA binding have not previously been determined.

Sequence alignment shows that Sam68 belongs to a distinct “Sam68 subgroup” of KH-containing proteins, which includes the C. elegans tumor suppressor gene product Gld-1 (18), the mouse quaking gene product (20), and the mammalian splicing factors Zfm1/SM1 (21, 22). Subgroup members all share a single highly conserved KH domain and a region of homology of ~60 amino acids immediately upstream of the KH domain. We call this the SGQ (Sam68/Gld-U/Quaking homology) region.

The fact that Sam68 binds to ribonucleotide homopolymers in vitro with a preference for poly(U) (9) and poly(A) provides direct evidence that it can interact with RNA. If so, it is possible that it may interact with RNA in vivo and that Src may regulate gene expression at the post-transcriptional level via Sam68. This hypothesis could be related to the observation that the introduction of v-Src can alter cellular RNA splicing and/or transport (23). A first step in exploring this hypothesis is to determine whether Sam68 has high affinity, specific RNA ligands. We identified such ligands by in vitro selection and determined the RNA and protein determinants of specific RNA binding. Our results indicate that Sam68 indeed recognizes specific RNAs in a KH-dependent manner and, by sequence homology, suggest that the functional lesions in some related RNA binding proteins result from defects in specific RNA binding.

EXPERIMENTAL PROCEDURES

Plasmids—pcDNA3 HA-Sam68 was generated by subcloning a BamHI/EcoRI insert from pGEX-Sam68 into pcDNA3-HA, which encodes amino-terminally hemagglutinin (HA) epitope-tagged proteins when cloned into its BamHI site. Point mutations and deletions in mouse Sam68 cDNA were introduced by polymerase chain reaction using pcDNA3 HA-Sam68 as the template. Fragments of Sam68 were generated by polymerase chain reaction and subcloned into the BamHI

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1 The abbreviations used are: SH2 and SH3, Src-homology 2 and 3, respectively; KH, K homology; SGQ region, Sam68/Gld-1/Quaking homology region; HA, hemagglutinin; GST, glutathione S-transf erase.
2 Q. Lin, unpublished data.
3 Resnick, R. J., Taylor, S. J., Lin, Q., and Shalloway, D. (1997) Oncogene, in press.
with T7 RNA polymerase in 500 μM 6 GST-fusion proteins were performed as described (9). Expression of ethanol after addition of 10 μM oligonucleotides were analyzed for integrity and purity by 8% polyacrylamide gel electrophoresis. Labeled RNA was then used for solid phase RNA binding assays.

**In Vitro Transcription**—A double-stranded transcription template containing a T7 promoter followed by a degenerate 40-mer oligonucleotide was a generous gift from Dr. John Lis, Cornell University (25). The template DNA was digested with 50 units of RNase-free DNase I at 37 °C for 30 min. In the in vitro transcription, the template DNA was added to a reaction mixture containing 2 μg of DNA/60-mm dish. After 48 h, the cells were lysed with lysis buffer (40 mM HEPES, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 1 mM Na vanadate, 1 mM EDTA, 10 μg/ml aprotinin and 10 μg/ml leupeptin). The lysate was cleared by centrifugation at 14,000 rpm for 5 min. Anti-HA monoclonal antibody (12CA5) then was added to the lysate and incubated on ice for 30 min followed by incubation with 10 μl protein G-Sepharose beads (1:1 slurry) at 4 °C for 1 h with rocking. The beads were washed twice with lysis buffer and once with RNA binding buffer, then used for solid phase RNA binding assays.

**Selection of High Affinity RNA Ligands for Sam68**—We identified RNA sequences that bind to Sam68 with high affinity by using SELEX (27) with a degenerate 40-mer DNA pool.

**Regions of Sam68 Required for High Affinity RNA Binding**

**RESULTS**

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**Selection of High Affinity RNA Ligands for Sam68**—We identified RNA sequences that bind to Sam68 with high affinity by using SELEX (27) with a degenerate 40-mer DNA pool. Fifteen clones were selected in two separate experiments and sequenced (Fig. 1). The G8 RNA clones were selected with hexahistidine epitope-tagged (6xHis)-Sam68 after eight rounds of selection and amplification. The G7 RNA clones were selected with 6xHis-Sam68 for the first four rounds and then with GST-Sam68 for the next three rounds. All 15 independent sequences contain A/U-rich stretches of 6–16 bases (Fig. 1). In particular, UAAA and UUUA motifs were present in 14 of the 16 sequences.

The binding affinities of the selected RNA sequences to Sam68 were determined using gel-shift binding assays (Fig. 2A). Disassociation constants were estimated from the Sam68 concentrations at which half-maximal RNA was bound. The Kd values ranged from 12 to 40 nM. The highest affinity sequences (Kd~12–40 nM) all contained the UAAA motif (Fig. 2B). The lower affinity sequences (Kd~60 nM) all contained the UUUA motif, and the lowest affinity sequences (Kd~80–140 nM) contained an AAAA sequence. Since the G8-5 RNA sequence had the highest binding affinity (Kd~12 nM), we used it in subsequent experiments as a high affinity Sam68 ligand.

We compared the Sam68 binding affinities of poly(U) and E. coli tRNA to that of G5-6 by using these nonspecific ligands as competitors in the gel-shift assay (Fig. 5). By weight, poly(U) was almost 100× less effective and tRNA was even less effective than G5-6 in reducing the labeled G8-5 probe (cf. lanes 5, 8, and 10). Quantitative analysis (see “Experimental Procedures”) gives apparent binding constants at the G8-5 binding site of Kd~100 nM (assuming a 3′-overhang sequence with an average chain length of about 16 nucleotides) and Kd(TRNA)~1,400 nM. Thus, G8-5 binds Sam68 with much higher affinity than nonspecific ligands.

To determine whether the UAAA motif is involved in Sam68 binding, we made an A to C substitution in G8-5 lacking the 3′-primer (Δ3P) changing UAAA to UACA (A5C) (Fig. 4A). Gel-shift assays (Fig. 4B) showed that while the Δ3P control bound to Sam68 with high affinity, the single base mutation in the UAAA motif dramatically reduced binding, indicating that this motif is required (although not necessarily sufficient) for high affinity binding of G8-5 to Sam68.
binding by Sam68. Taken together, we conclude that the KH domain is required but not sufficient for specific, high affinity RNA binding.

We also tested the effects on RNA binding of point mutations within the KH domain analogous to the loss of function mutations in C. elegans Gld-1 and human Fmr1, G178E and I184N, respectively. These also abolished binding (lanes 6 and 7). However, a point mutation corresponding to loss of mouse Quaking function (E128G) did not affect G8-5 binding (lane 5). Also a double Pro → Ala mutation predicted to disrupt SH3 binding did not affect binding.

We have shown previously that Sam68 binds to poly(U) in preference to other polyribonucleotides (8). We therefore examined the effects of mutations on the ability of Sam68, transiently expressed in COS cells, to bind to poly(U) beads (data not shown). Fig. 6A summarizes the results. In contrast to the results obtained with G8-5, we found that the KH deletion mutant still retained partial poly(U) binding (∼30% of total bound), while the G178E and I184N mutants bound to poly(U) beads equally as well as wild type Sam68.

**DISCUSSION**

We demonstrated previously that Sam68 binds ribonucleotide homopolymers, with a preference for poly(U) (9). However, as it is unlikely that this is a physiological target, we used in vitro selection to determine whether Sam68 can bind specific RNA sequences with high affinity. Using SELEX, 15 RNA sequences were identified that bound to Sam68 with affinities as high as $K_d \sim 12$ nM. These affinities are in the same range as those reported for the binding of RNA to other RNA-binding proteins (28), consistent with the possibility that such binding may reflect physiologically significant interactions. Although no long consensus motif was identified, the 13 highest affinity RNA-binding sequences each contained a UAAA motif. Importantly, a single A to C mutation within this motif abolished binding of G8-5, the highest affinity RNA, to Sam68, implying that this motif is involved in high affinity binding, either directly or by contributing to a suitable structural conformation of the RNA. To our knowledge, this is the first demonstration of a KH domain-containing protein binding to RNA with both specificity and high affinity; it will be interesting to see if G8-5 is a high affinity target of other such proteins, particularly members of the Sam68 subgroup. In any event, the finding that Sam68 selects RNA ligands with recognizable sequence traits and that high affinity binding can be modulated at the single base level strongly suggests a capacity for specific RNA binding in vivo.

Although the UAAA motif present in the highest affinity binding RNAs was not extensive enough to allow data base searches for matching sequences, we have noticed that it does match the last four bases of the mammalian polyadenylation signal AUAAA (29). In fact, the whole poly(A) signal is contained within three of the selected sequences (G7-9, G8-4, G7-4, Fig. 2A), although not in G8-5. The presence of AU-rich stretches in all of the selected sequences also raises the possibility that Sam68 might interact with AU-rich elements in the 3′-untranslated regions of mRNAs for proto-oncoproteins and cytokines whose expression is regulated by controlling RNA stability. Indeed, four of the selected sequences (but not G8-5) include the AUUUA motif common to one type of AU-rich element (30). The potential for involvement of Sam68 in regulation of polyadenylation or RNA stability clearly warrants further investigation. Furthermore, the sequence information obtained from our SELEX experiments should prove invaluable in the design of probes or inhibitors of Sam68 function in vivo.
Investigating the other side of the RNA-protein interaction, we showed that the Sam68 KH domain was required but not sufficient for specific RNA binding. Although deletion of the KH domain abolished G8-5 binding, a fusion protein containing the KH domain (including the third α-helix, which is important for structural stability (12)) did not bind G8-5. However, high affinity binding to G8-5 was conferred when the fusion protein was extended to include the SGQ region. The observation that the Sam68 KH domain alone does not bind a high affinity, specific RNA ligand is consistent with previous demonstrations that the multiple KH domains in heterogeneous nuclear ribonucleoprotein K (three domains) and Fmr1 (two domains) appear to cooperate in binding to ribonucleotide homopolymers (31). Perhaps the SGQ region in Sam68 cooperates with the KH domain in a similar manner. It will be interesting to see if other single KH domain proteins, e.g. Mer1, have similar cooperative requirements.

Small deletions and point mutations within the Sam68 KH domain were sufficient to abolish specific RNA binding. It may be significant that deletion of residues 206–218 disrupts RNA binding. This corresponds structurally to the fourth loop of the KH domain (between β-strands 2 and 3), which is highly conserved in the Sam68 subgroup but is not conserved or not present in KH domains from other proteins (12).
could therefore be a determinant of RNA binding specificity for the Sam68 subgroup.

The KH domain residues that we chose to mutate were selected because they are analogous to residues whose mutation can seriously derange growth and development in model organisms or human patients (see Introduction). The biochemical basis of most of these effects has not been firmly established. One exception is the case of Fragile X syndrome, the most common form of hereditary mental retardation, where a connection to RNA binding has been suggested. The syndrome is usually caused by expansion of trinucleotide repeats in the untranslated region of the \( fmr1 \) gene resulting in loss of its expression (17). However, in one patient a severe form of the disease is associated with mutation of a conserved Ile residue in the second KH domain of Fmr1 (19), and mutation of this residue can diminish Fmr1 binding to poly U \( \text{in vitro} \) (31).

Consistent with this, we found that the analogous mutation in the Sam68 KH domain (I184N) abolishes binding to a specific, high affinity RNA ligand, G8-5. Notably, structural studies show that this mutation disrupts KH domain structure (12).

Two of the other point mutants were studied because they are analogous to loss-of-function mutations in \( gld-1 \) and \( quaking \), the biochemical basis of which is unknown. The \( gld-1 \) gene of \( C. \) elegans is a germ line-specific tumor suppressor gene that plays an essential role in oogenesis (18). Mutation of Gly227, the first Gly in the invariant GXXG KH domain motif, results in tumor formation, as in the null allele, indicating it is a loss of function mutation. Our results show that the analogous Sam68 mutation (G178E) abolishes binding to G8-5, suggesting that specific RNA binding is essential for Gld-1 tumor suppressor function. Similarly, loss of specific RNA binding could explain the inability of either Gly in the GXXG motif to disrupt cell fate during early development (15).
Interestingly, the mouse quaking gene provides a converse example. Mutations in this gene result in severe defects in myelination in the central nervous system during early development and an Asp to Gly mutation in its SGQ region causes embryonic lethality (20). However, the corresponding E128G mutation in Sam68 has no effect on high affinity RNA binding, suggesting that this mutation in Quaking may affect other essential functions. However, based on our proposal that SGQ may cooperate with KH in specific RNA binding, the possibility that this residue is important for binding of Quaking-specific RNA targets can certainly not be excluded.

In the absence of a more specific test, we and others have previously used binding to ribonucleotide homopolymer beads as a functional indication of RNA binding. However, our present results show that binding to poly(U) beads is unaffected by two loss-of-function point mutations (G178E and I184N), which completely abolish binding to the specific G8-5 target and is only partially diminished by deletion of the entire KH domain. We therefore believe that, whereas homopolymeric ribonucleotides are useful indicators of RNA binding potential, studies with specific RNAs are more relevant probes of biological function. Importantly the effects of functional mutations on high affinity RNA binding suggest that interaction between the RNA species selected by SELEX and Sam68 are not fortuitous but are of functional relevance.

Our results indicate that Sam68 is able to bind RNA with specificity and high affinity, raising the possibility that it plays a specific role in post-transcriptional regulation of RNA processing and gene expression in a way that could be affected by Src. Numerous biochemical and genetic studies provide examples of post-transcriptional control of gene expression. 1) Genetic studies in Drosophila have illustrated the central importance of alternative splicing in sex- and tissue-specific gene expression and indicate that regulation of splicing will play a fundamental role in controlling growth and developmental specific gene expression in eukaryotes (32, 33). 2) The essential Rev protein of human immunodeficiency virus type 1 mediates the nuclear export of incompletely spliced RNAs that encode viral structural proteins (34, 35). Normal host cell proteins may similarly control cellular nucleocytoplasmic RNA transport in uninfected cells. 3) The stability of some mRNAs encoding growth regulatory proteins such as Myc and Fos is regulated by the binding of specific RNA binding proteins to 3'-untranslated region AU-rich elements (30). 4) The translation of mRNAs can
be regulated by cytoplasmic polyadenylation; c-mos translation is specifically activated by polyadenylation in the context of meiotic maturation (36). 5) “Zipcode” sequences in 3′-untranslated regions of some mRNAs control their subcellular localization (37). For instance, β-actin mRNA is directed to the cell periphery following growth factor treatment (38, 39). 6) Translation is regulated by signal transduction pathways from cell surface receptors that result in phosphorylation of components of the translational machinery (40). These events have been shown, or are presumed, to be mediated by a diverse group of proteins that can interact with target RNAs under control of upstream regulatory proteins.

Because of its ability to specifically interact with RNA and with growth regulatory proteins such as Src, Sam68 is in a unique position to mediate post-transcriptional control of gene expression. Although its presence in the nucleus suggests a role for Sam68 in transcription or in the early steps of RNA processing, it is becoming clear that many apparently nuclear RNA binding proteins are able to shuttle between nucleus and cytoplasm by virtue of specific nuclear export and import sequences (41). The possibility that Sam68 may do the same is strengthened by the finding that Sam68 can interact with the RNA-dependent RNA polymerase of poliovirus and that Sam68 redistributes from the nucleus to cytoplasmic sites of viral replication a few hours after poliovirus infection of HeLa cells (42). Moreover, Sam68 could potentially interact with cytoplasmic RNAs during mitosis and these interactions might be modulated by binding to and phosphorylation by Src.

The high degree of sequence conservation between Sam68 and its relatives points to conservation of biochemical function and specific RNA binding. Since Gld-1 and Quaking play essential roles in regulation of growth and development in worms and mice, it is likely that Sam68 will play a key role in mammalian growth and/or developmental control. A role in growth control and cell cycle regulation is suggested by its association with a proto-oncoprotein, Src, specifically during mitosis. More direct evidence for a role of Sam68 in cell cycle control is provided by recent results showing that expression of a splice variant of Sam68 in which part of the KH domain is deleted inhibits serum-stimulated progression into S phase of the cell cycle (24). Binding of this splice variant to poly(U) is diminished (24), and our present studies predict that binding to specific RNAs will be abolished, suggesting that specific RNA binding is integral to the growth regulatory functions of Sam68. The next step will be to use the information gained from our studies and from genetic analyses to identify the biologically relevant RNA targets of Sam68 and its relatives.

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