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Human protein Sam68 relocalization and interaction with poliovirus RNA polymerase in infected cells

(RNA replicase/two-hybrid system/virus−host interactions)

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ABSTRACT A HeLa cDNA expression library was screened for human polypeptides that interacted with the poliovirus RNA-dependent RNA polymerase, 3D, using the two-hybrid system in the yeast Saccharomyces cerevisiae. Sam68 (Src-associated in mitosis, 68 kDa) emerged as the human cDNA that, when fused to a transcriptional activation domain, gave the strongest 3D interaction signal with a LexA–3D hybrid protein. 3D polymerase and Sam68 coinmunoprecipitated from infected human cell lysates with antibodies that recognized either protein. Upon poliovirus infection, Sam68 relocalized from the nucleus to the cytoplasm, where poliovirus replication occurs. Sam68 was isolated from infected cell lysates with an antibody that recognizes poliovirus protein 2C, suggesting that it is found on poliovirus-induced membranes upon which viral RNA synthesis occurs. These data, in combination with the known RNA- and protein-binding properties of Sam68, make Sam68 a strong candidate for a host protein with a functional role in poliovirus replication.

A positive-sense virus of the family Picornaviridae, poliovirus has a 7440-nt RNA genome that encodes a single polypeptide. This 247-kDa polyprotein is cleaved to yield a small number of proteins, many of which have multiple functions. Poliovirus nonstructural proteins 2B, 2C, 3A, 3B, and 3D and protein precursors 2BC, 3AB, and 3CD have been implicated in RNA replication by virtue of their presence in RNA replication complexes, their biochemical properties, and the phenotypes of viruses with mutations in their coding regions (for review, see ref. 1). However, because a template-specific replicase has not yet been reconstituted in vitro from defined components, it is likely that host proteins are also involved in RNA replication. Several candidate host factors have been proposed (2–5). The importance of host proteins in genome replication of other RNA viruses has been demonstrated: translation elongation factors EF-Tu and EF-Ts, as well as ribosomal protein S1, are integral parts of the bacteriophage Qβ replicase complex (6), and a subunit of the translation initiation factor eIF-3 has been shown to be part of the brome mosaic virus template-specific replicase (7).

We have used the two-hybrid system in the yeast Saccharomyces cerevisiae to identify human polypeptides that can interact with the poliovirus RNA-dependent RNA polymerase, 3D (8, 9). A library of plasmids that contain HeLa cDNAs fused to sequences that encode a transcriptional activation domain was screened for those encoding polypeptides that interact with a LexA–3D hybrid protein (9). Because poliovirus RNA replicates well in HeLa cells, mRNAs that encode proteins important for replication should be represented in this HeLa cDNA library, which was provided by Roger Brent and Jenő Gyuris (Harvard University). We have identified cDNAs for several host proteins that interact with 3D polymerase, most notably a 68-kDa protein that associates with Src during mitosis, Sam68 (10, 11). Sam68 coinmunoprecipitates with 3D polymerase from infected cells, is found on poliovirus-induced membranes, and relocalizes dramatically during poliovirus infection. Sam68 is thus likely to be a host factor with a functional role in poliovirus replication.

MATERIALS AND METHODS

Yeast Strains and Plasmids. The yeast strain used in this study, EGY40 (MATa, his3, trpl, ura3, leu2) and yeast two-hybrid expression plasmids were from R. Brent and colleagues (Harvard University). The LexAop–lacZ reporter plasmid, pSH18-34, the LexA fusion plasmid vector, pLex(1-202)+PL, the activation-domain fusion vector, pJG4-5, and the LexA-bicoid negative-control plasmid, pRFH1, have been described (9, 12, 13). The HeLa cDNA library expressed from the pJG4-5 plasmid was constructed by Jenő Gyuris (9). The plasmid encoding Sam68, p62KL1, was from Frank McCormick (Onyx Pharmaceuticals).

Plasmid Constructions. The LexA-3D plasmid was constructed by excising the 3D polymerase coding sequence from pTST-3D (14) and inserting it in-frame into the EcoRI site of pLex(1-202)+PL. To create the plasmid encoding the B42–2B fusion protein, the 2B coding sequence was excised from a dicistronic plasmid (15) and inserted into a derivative of pJG4-5. The full-length coding sequence of Sam68 was transferred from p62KL1 into pLex(1-202)+PL to create the pLexp68 plasmid that contained the full-length coding sequence of Sam68 fused in-frame to LexA. A plasmid that encoded a fusion between the B42 transcriptional activation domain and full-length Sam68 was also constructed.

Antibodies. The polyclonal rabbit antibody that recognized Sam68 was obtained from Santa Cruz Biotechnology. This antibody is marketed as sc#333, to recognize p21<sup>src</sup> GTase-activating protein (GAP) associated protein p62; Sam68 was recently identified as its true antigen (10, 11). The monoclonal anti-3D antibody was raised by injection of mice with purified, bacterially expressed 3D polymerase, from J. Hansen and S. Schultz (University of Colorado, Boulder). Hybridoma subculture was performed by Kurt Christensen at the monoclonal antibody core facility, University of Colorado Cancer Center (Denver). The mouse monoclonal anti-2C and anti-2B antibodies were from Kurt Bienz and Denise Egger (University of Basel; ref. 16). The rabbit polyclonal antibody that recognized TATA-binding protein (TBP) was provided by J. Jaehning (University of Colorado Health Sciences Center, Denver). Total rabbit IgGs were purchased from Sigma. Affinity-purified mouse anti-myc antibody 9E10 (17) was provided by

Abbreviation: TBP, TATA-binding protein; SH, Src homology.

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M. Klymkowsky (University of Colorado, Boulder). Horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham (Arlington Heights, IL). Texas Red-conjugated donkey anti-rabbit IgG was purchased from Jackson ImmunoResearch (West Grove, PA).

**Transformation and Two-Hybrid Screening for 3D Polymerase-Interacting Proteins.** Yeast transformations into strain EGY40 [pSH18-34; pLexA-3D] were performed by using lithium acetate or electroporation (18, 19). HeLa cDNA library transformants were selected on minimal glucose plates that lacked uracil, histidine, and tryptophan and were transferred to plates that contained 2% galactose to induce expression of the B42 fusion proteins and 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-Gal) to identify colonies synthesizing β-galactosidase.

Screening ~1.1 × 10⁵ transformants revealed 63 library plasmids that produced blue colonies on plates containing galactose and X-Gal only in the presence of the LexA–3D polymerase hybrid protein. Fifty of these plasmids encoded ubiquitin-carboxyl extension protein (UBCEP) fusion proteins (20, 21). However, these colonies showed only low levels of β-galactosidase activity, and subsequent experiments suggested that the UBCEP–3D polymerase interaction was indirect. Eleven additional plasmids contained previously unidentified sequences (data not shown). Two colonies contained coding sequences for Sam68 (10, 11).

Plasmids encoding B42–HeLa polypeptide hybrid proteins were isolated from blue colonies and transformed into Escherichia coli strain HB101 (22). These library isolates were retransformed into yeast strains containing the LexA–3D plasmid, the LexA–bicoid plasmid, or the LexA vector to test them for specificity of transcriptional activation from the β-galactosidase reporter. β-Galactosidase assays were done as described by LeGrain and Rosbash (23).

**Immunoprecipitation.** HeLa cells were grown in spinner culture and plated before infection as described (24). Monolayers of ~4 × 10⁶ cells were infected at a multiplicity of infection (m.o.i.) of 100 plaque-forming units (pfu) per cell (25), harvested by scraping 5.5 hr after infection, and lysed on ice in 1 ml of a solution containing 1% Nonidet P-40, 160 mM NaCl, 1 mM MgCl₂, and 50 mM Tris-HCl pH 7.5 (IPB) supplemented with 1 mM sodium orthovanadate and protease inhibitors 1 mM phenylmethylsulfonyl fluoride, aprostin at 2 μg/ml, leupeptin at 0.5 μg/ml, and pepstatin at 0.7 μg/ml. Insoluble material was pelleted at 16,000 × g in a microcentrifuge at 4°C. Bovine serum albumin (5 mg/ml, final concentration) was added to the supernatant, which was then incubated with various amounts of antibody on ice for 1 hr. Antibody–antigen complexes were collected on magnetic beads conjugated to sheep anti-mouse IgG or sheep anti-rabbit IgG (Dynal).

**Immunoblotting.** Proteins were resolved by SDS/PAGE (26) and subsequently transferred to poly(vinylidene fluoride) membranes (Immobilon-P; Millipore, Bedford, MA). Immunoblots were probed with appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies before visualization by enhanced chemiluminescence (Amer sham). Immunoblots were stripped of antibodies by incubation in 100 mM 2-mercaptoethanol/2% SDS/62.5 mM Tris-HCl, pH 6.8 at 55°C for 1 hr.

**Immunofluorescence Microscopy.** HeLa cells were grown on coverslips, infected at an MOI of 100 pfu per cell, and fixed at different times after infection (25). Coverslips were incubated with primary antibody overnight at 4°C in the presence of bovine serum albumin at 3 mg/ml and then with a 1:100 dilution of Texas Red-conjugated secondary antibody (Jackson ImmunoResearch) for 1 hr.

**Polyovirus-Induced Membrane Isolation.** Monolayers of polyovirus-infected or uninfected HeLa cells were harvested and Dounce-homogenized in a solution containing 10 mM NaCl, 10 mM Tris-HCl, pH 8.0, and protease inhibitors. After removal of nuclei and intact cells by centrifugation at 2,000 × g in a Sorvall H-1000B rotor at 4°C for 5 min, the cytoplasmic extracts were adjusted to 160 mM NaCl and incubated overnight at 4°C in the presence of anti-2C antibody and bovine serum albumin at 5 mg/ml. Extracts were then fractionated on a discontinuous gradient containing 10%, 30%, and 45% sucrose layers. After centrifugation for 4 hr at 49,000 rpm at 4°C in a SW50 rotor (Beckman), the membrane fraction at the 30%–45% interface (m2) was collected (27, 28). Poliovirus-induced membranes were isolated from the m2 membrane fraction by using sheep anti-mouse-conjugated magnetic beads (Dynal) (A.S. and K.K., unpublished data).

## Results

**Selection of 3D Polymerase-Interacting Proteins with the Two-Hybrid System.** Before screening the HeLa cDNA library for peptides that interacted with the poliovirus polymerase, we confirmed that the LexA–3D hybrid protein was expressed and did not itself activate transcription from the β-galactosidase reporter. Immunoblotting with an anti-LexA antibody showed that a fusion protein of the correct molecular weight was expressed in yeast harboring the pLexA–3D plasmid (data not shown). The inability of LexA–3D to activate transcription on its own was demonstrated by the baseline β-galactosidase activity seen in strains that contained pLexA–3D and the B42 hybrid vector pJG4-5 (9; Table 1).

Two isolates from the library of human cDNAs fused to the transcriptional activation domain showed the largest amounts of β-galactosidase activity in the presence of LexA–3D. Both encoded C-terminal segments of the protein Sam68 (Src-associated in mitosis, 68 kDa) (10, 11). Although Sam68 was first identified in 1994 as a protein that is tyrosine-phosphorylated by Src during mitosis, a cDNA that encoded Sam68 was isolated 2 yr earlier (30, 31). Table 1 shows the β-galactosidase activities measured for strains containing full-length Sam68 and the original library isolates that contained only the C-terminal amino acids 253–443 and 285–443. In all three cases, the activity seen in strains containing the LexA–3D hybrid protein was two orders of magnitude greater than that seen in the presence of the LexA–bicoid fusion protein or LexA alone. Similarly, the 3D polymerase interaction signal was seen only in the presence of fusion proteins that contained

<table>
<thead>
<tr>
<th>Sam68(-253-443)</th>
<th>Sam68(-285-443)</th>
<th>Sam68(-1-443)</th>
<th>B2*</th>
<th>Vector</th>
</tr>
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<tbody>
<tr>
<td>900 ± 100</td>
<td>500 ± 100</td>
<td>600 ± 200</td>
<td>2 ± 1</td>
<td>ND</td>
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<tr>
<td>1 ± 0.1</td>
<td>1 ± 0.2</td>
<td>1 ± 0.5</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>5 ± 1</td>
<td>6 ± 3</td>
<td>3 ± 1</td>
<td>ND</td>
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- **β-Galactosidase activities were measured in Miller units for at least three individual transformants grown to OD₆₀₀ = 0.6–1.1 in liquid medium containing galactose (23, 29). Those amino acids from each protein included in the hybrid protein are shown in parentheses. ND, not determined.
- *B42 fusion to poliovirus protein B2.
both B42 transcriptional activation and Sam68 sequences; neither poliovirus 2B fused to B42 sequences nor B42 sequences alone gave a positive signal (Table 1). Thus, 3D and Sam68 fusion proteins interact specifically in the yeast nucleus.

3D Polymerase and Sam68 Interact in Poliovirus-Infected Cells. Immunoprecipitation experiments were done to test whether the 3D polymerase–Sam68 interaction occurred within infected human cells. Anti-3D antibody precipitated Sam68 from extracts of poliovirus-infected cells but not from extracts of uninfected cells (Fig. 1). A control monoclonal antibody did not precipitate Sam68 from either infected or uninfected cells (Fig. 1). To examine the specificity of the interaction between 3D polymerase and Sam68, extracts from cells labeled with [35S]methionine before infection were immunoprecipitated with anti-3D. Seven major bands that were not seen in anti-3D immunoprecipitates from uninfected cells coimmunoprecipitated with 3D polymerase from infected cells. One of these major bands migrated identically to Sam68 (data not shown). Thus, the coimmunoprecipitation of Sam68 with 3D polymerase does not reflect a wholesale precipitation of cellular proteins nonspecifically associated with 3D polymerase.

Immunoprecipitation experiments using the antibody that recognizes Sam68 confirmed the interaction between Sam68 and 3D polymerase in infected cells. When proteins precipitated by the antibody that recognizes Sam68 were probed with the anti-3D antibody, a number of proteins containing 3D polymerase sequences were identified (Fig. 2A). These proteins included 3D polymerase itself and its proteolytic precursors 3CD and 3ABCD, as well as some known products of aberrant processing (32, 33). No proteins were detected by the anti-3D antibody when Sam68 was immunoprecipitated from uninfected cells (data not shown).

To test the specificity of the coimmunoprecipitation of 3D polymerase and its precursors with Sam68, the blot in Fig. 2A was stripped and then probed with antibodies to other poliovirus proteins. Probing with anti-2B antibody showed the presence of poliovirus nonstructural proteins 2B, 2BC, and 2ABC in anti-Sam68 immunoprecipitates (Fig. 2B). The dilution series on the left of Fig. 2A and B allows a comparison of the efficiency of coimmunoprecipitation of the various poliovirus proteins with Sam68. The amounts of 3D and 3CD in the anti-Sam68 immunoprecipitate are equivalent to the amount of each protein found in ~5.0 and 14 µg of total cytoplasmic protein, respectively. In contrast, the amount of 2B coimmunoprecipitated with Sam68 is equivalent to the amount of this protein found in 0.45 µg of total cytoplasmic extract; 2BC, however, is coimmunoprecipitated with Sam68 almost as efficiently as 3D. Similarly, a dilution series probed with anti-2C antibody revealed that the amount of 2C coimmunoprecipitated with Sam68 was equivalent to that found in 1.8 µg of cytoplasmic extract (data not shown).

Thus, the coimmunoprecipitation data suggest that Sam68 interacts efficiently with 3D polymerase. Precipitation of viral proteins may reflect interactions of these proteins with either Sam68 or 3D polymerase. Tests of Sam68 interaction with viral proteins 2B, 2C, 3A, and 3AB in the two-hybrid system did not reveal any direct interactions detectable by this assay (data not shown). It would certainly not be surprising to find other protein–protein interactions within the viral replication complex, which may remain partially intact even following Nonidet P-40 treatment.

Intracellular Localization of Sam68 in Poliovirus-Infected Cells. Because Sam68 was reported to be predominantly...
nuclear (30) and we observed abundant Sam68 in nominally cytoplasmic extracts (Fig. 1), the intracellular localization of Sam68 in uninfected cells was tested. Furthermore, because poliovirus replication occurs in the cytoplasm, we were interested in whether the intracellular localization of Sam68 was altered by poliovirus infection. Fig. 3 shows the immunofluorescent staining of Sam68 in HeLa cells fixed at different times after infection. In agreement with previous work, the majority of Sam68 in uninfected cells was found in the nucleus; the nucleoli and the cytoplasm showed reduced amounts of the protein. Although Sam68 localization in cells fixed 1.5 hr after infection was similar to that in uninfected cells, by 3 hr after infection the intensity of Sam68 staining in the cytoplasm had increased (Fig. 3). By 4.5 hr after infection, the majority of Sam68 was found in the cytoplasm, with little remaining in the nucleus (Fig. 3).

To test whether another nuclear protein relocalized upon infection, we stained cells that had been fixed at different times after infection with an antibody that recognized human TBP. TBP was found in both the nucleus and cytoplasm of uninfected cells; the staining pattern of TBP did not alter over the course of poliovirus infection (Fig. 3). Similarly, previous work has shown that nuclear protein Lα, which is thought to be involved in poliovirus translation, relocalizes to the cytoplasm upon infection, while splicing factor SC-35 remains in the nucleus (34). Thus, the relocalization of Sam68 from the nucleus to the cytoplasm is not a characteristic of all nuclear proteins in infected cells.

Biochemical Localization of Sam68 in Infected Cells. Poliovirus induces the accumulation of membranous vesicles with a heterogeneous size distribution within the cytoplasm of infected cells; viral RNA synthesis occurs on the cytoplasmic surface of these poliovirus-induced membranes (35, 36). To test whether Sam68 was associated with poliovirus-induced membranes, cytoplasmic extracts from infected and uninfected cells were incubated with anti-2C antibody and fractionated on sucrose step gradients (27). Poliovirus protein 2C is found on the surface of virus-induced membranes (37) and therefore can be used as a tag for their isolation. A large fraction of virus-induced membranes, as monitored by the presence of poliovirus protein 2C and the incorporation of radiolabeled nucleotides, has been found between the 30% and 45% sucrose fractions (m2) of such gradients (27, A.S. and K.K., unpublished data). Antibody-bound material was isolated from m2 fractions by incubation with magnetic beads conjugated to an anti-mouse secondary antibody (Fig. 4A).

Immunoblotting of the proteins found in the immunosolubilized material showed that Sam68 was present in the 2C-containing material (Fig. 4B). Control material from uninfected cells isolated in the presence of anti-2C antibody, or isolated from infected cells in the absence of the anti-2C antibody, did not contain Sam68 (Fig. 4B). Immunoelectron microscopy further confirmed the localization of Sam68 to poliovirus-induced cytoplasmic membranes in infected cells (data not shown). Probing of anti-2C-associated material with the anti-3D antibody showed that this viral protein and its precursor 3CD were also present in the 2C-associated material (Fig. 4C). Similar to Sam68, 3D-containing polypeptides were not isolated from infected cell lysates by magnetic beads in the absence of anti-2C antibody (Fig. 4C). These results suggest that the Sam68-3D polymerase interaction detected by immunoprecipitation from detergent-lysed cells may also occur on poliovirus-induced membranes where viral RNA replication occurs. Thus, during poliovirus infection Sam68 is in the correct place at the proper time to have a functional role in viral RNA replication.

**DISCUSSION**

The two-hybrid system has been used in a number of laboratories to identify host proteins that interact with viral proteins. Goff and coworkers (38) identified cyclophilins A and B as host proteins that interact with the Gag protein of human immunodeficiency virus type 1. The immunosuppressive drug cyclosporin A, which has been shown to have antiviral activities, blocked the interaction of Gag with cyclophilins, suggesting that this interaction may be essential for viral propagation (38). The same group used the two-hybrid system to identify an interaction between the human immunodeficiency virus type 1 integrase protein (IN) and integrase interactor 1 (INI-1) (39). A glutathione S-transferase–INI-1 fusion protein stimulated the in vitro activity of recombinant IN, suggesting a potential function for INI-1 in the retroviral infectious cycle (39). A human protein, nucleoprotein interactor 1 (NPI-1), was found to interact with the influenza virus nucleoprotein (NP) by O'Neill and Palese (40) using the two-hybrid system. This predominantly nuclear protein both bound to NP in vitro and could be coimmunoprecipitated with NP from infected cells (40).

We have identified an interaction between the RNA-dependent RNA polymerase, 3D, of poliovirus and a human protein, Sam68. The experiments presented here strongly

**FIG. 3.** Intracellular localization of Sam68 and TBP before and after poliovirus infection. HeLa cells were grown on coverslips, infected with poliovirus, and fixed in −20°C methanol at the indicated times after infection. Fixed coverslips were incubated overnight with antibody that recognized either Sam68 or TBP. After incubation with a Texas-Red-conjugated secondary antibody, the proteins were visualized by fluorescence microscopy.
suggest that the interaction between host and viral proteins detected by the two-hybrid system reflects an interaction that occurs during the poliovirus infectious cycle. Whether the Sam68-3D interaction is direct or mediated by other molecules present in both yeast and human cells has not yet been demonstrated. However, it is clear that Sam68 is intimately associated with 3D present on membranes containing poliovirus replication complexes. Previously, a 67-kDa protein was purified from uninfected cells by Dasgupta and coworkers (2) and was shown to be required for purified 3D polymerase activity in vitro. It is not yet known whether this putative host factor is Sam68. However, the cytoplasmic localization of p67 in uninfected cells and its autophosphorylation activity (41) differ from Sam68, which is predominantly nuclear and displays no homology with the catalytic domain of known protein kinases (30).

Sam68 has a number of properties in addition to its membrane localization that could be useful during poliovirus RNA replication. A putative intracellular signaling molecule with both Src-homology (SH) 2- and SH3-binding domains, the role of Sam68 in the uninfected cell is likely to involve protein-protein interactions (10, 11). Both SH2- and SH3-binding domains were present in the C-terminal sequences selected for their interaction with 3D polymerase (Table 1; refs. 30, 42). Sam68 exhibits sequence similarity to a putative heteronuclear ribonucleoprotein protein (30) and displays both single- and double-stranded RNA-binding activity (10, 43), although its binding to heteropolymeric RNAs has not been tested. Taylor and coworkers (43) have found that the binding of the Src SH3 domain to Sam68 abrogates its binding to poly(U), suggesting the possibility for interplay between the protein-binding and RNA-binding domains of Sam68.

Immunoprecipitation experiments showed that Sam68 interacts with 3D polymerase, its precursor 3CD, and other 3D sequence-containing precursors and processing products in infected human cells (Fig. 2A). Because both 3D and 3CD function in RNA replication (44, 45) and 3CD is the processing protease for viral capsid proteins (28, 46), Sam68 might facilitate any of these functions. The RNA- and protein-binding properties of Sam68 suggest that it might interact both with poliovirus proteins and viral RNA, serving as an adaptor molecule during viral RNA replication.

We deeply appreciate the provision of plasmids and cDNA libraries for the two-hybrid system by Roger Brent and Jenö Gyoris. We especially thank Frank Luca for his guidance in preparing anti-3D monoclonal antibodies, Kurt Bienz and Denise Egger for providing anti-2B and anti-2C monoclonal antibodies, and Frank McCormick for the Sam68 cDNA clone. We thank John Doedens, Frank Luca, and Peter Sarnow for careful reading of the manuscript. A.E.M. is a predoctoral fellow and K.K. is an Assistant Investigator of the Howard Hughes Medical Institute. This work was also supported by National Institutes of Health Grant AI-25166. Hybridoma production and subculture was supported in part by National Cancer Institute Grant P30-CA46934.