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KH Domain Integrity Is Required for Wild-Type Localization of Sam68

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The protein Sam68 (Src-associated in mitosis, 68 kDa) has been found to bind to SH2 and to SH3 domain-containing proteins and to RNA. Although its protein–protein interactions implicate Sam68 in cell signaling, the significance of its RNA binding remains obscure. In most cells, Sam68 shows diffuse nucleoplasmic staining. Upon treatment with transcription inhibitors, however, Sam68 localized into punctate nuclear structures. Mutant forms of mouse Sam68 were overexpressed in human cells to test the importance of the KH domain, which is required for RNA binding, in the intracellular localization of Sam68. A small deletion within the KH domain (Δ206–218) or point mutation I184N had no effect upon the localization of overexpressed Sam68. Sam68 that contained a deletion of the entire KH domain (ΔKH, Δ157–256) or point mutation G178E, however, localized to distinct nuclear spots. Furthermore, ΔKH Sam68, unlike wild-type Sam68 and several other mutant Sam68 proteins, did not relocalize upon poliovirus infection and caused the normally cytoplasmic viral polymerase to localize to the nuclear spots. Thus both ongoing transcription and an intact KH domain are crucial determinants of the dynamic intracellular localization of Sam68. © 1998 Academic Press

INTRODUCTION

The major mitotic substrate for tyrosine phosphorylation in src-transformed fibroblasts, Sam68 (Src-associated in mitosis, 68 kDa), has been found to bind many SH2- (Src homology 2) and SH3-domain proteins including tyrosine kinases Src, Fyn, and Lyn; phospholipase C-γ; and GRB-2 [1–4]. Although the properties of the proteins with which it associates have implicated Sam68 in cell signaling, no cellular function for Sam68 itself has yet been elucidated. In addition to the C-terminal domains responsible for these protein–protein interactions, Sam68 has a central KH (hnRNP K homology) domain, also found in many presumptive nucleic acid-binding proteins [5]. Although no specific cellular RNA ligand for Sam68 has been identified, Sam68 has been shown to bind RNA *in vitro* [3, 6]. *In vitro* selection of RNA ligands has shown that Sam68 can bind to certain RNA sequences with high affinity and specificity [7]. N-terminal to the KH domain of Sam68 is a sequence sometimes termed the SGQ domain, which is conserved among a subfamily of KH proteins including Sam68, the *Caenorhabditis elegans* protein Gld1 (defective in germline development), the mouse protein Qk1 (quaking1), and the human splicing factor SF1 [8–10]. Expression of glutathione *S*-transferase fusion proteins has demonstrated that, together, the SGQ and the KH domains are sufficient for *in vitro* binding to a SELEX-derived RNA ligand [8].

Several experiments have pointed to the possibility of interplay between the protein- and RNA-binding properties of Sam68. Binding of Sam68 to poly(U) beads is reduced in the presence of an SH3 domain-containing peptide [6] suggesting that, although different regions of Sam68 are responsible for these two functions, binding of a ligand to the proline-rich SH3-binding domain can allosterically affect RNA binding by Sam68. Coexpression of Sam68 with Fyn has been shown to abrogate the binding of purified Sam68 to RNA, presumably due to Fyn-mediated tyrosine phosphorylation of Sam68 [11]. Because phosphorylation occurs at the C-terminus of Sam68 and is required for its binding to SH2 domains, this result also indicates the potential for coordinate regulation of the RNA- and protein-binding functions of Sam68. In addition, Sam68 has been shown to self-associate *in vivo*, and this protein–protein interaction is dependent upon the presence of RNA [12].

Previous work revealed that Sam68 interacts via its C-terminal domain with the poliovirus RNA-dependent RNA polymerase, 3Dpol, as a fusion protein both in yeast and in poliovirus-infected cells [13]. The presence of Sam68 on the cytoplasmic membranes on which polioviral RNA replication occurs suggested that Sam68 might be involved in this process. Normally, Sam68 is predominantly nuclear. However, upon infection with poliovirus, Sam68 dramatically relocalized to the cyto-

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plasm, indicating a connection between viral processes and Sam68 localization [13].

This paper describes experiments to investigate the role of the KH domain and any Src-dependent properties of Sam68 in its cellular localization. The relocalization of Sam68 during poliovirus infection is used as one of the few available tools to study the dynamics of Sam68, with the goal of understanding the dynamics of Sam68 in uninfected cells as well.

MATERIALS AND METHODS

Plasmids. The construction of pcDNA3-based plasmids for the overexpression of mutant forms of Sam68 has been described previously [8]. The green fluorescent protein (GFP)-expressing plasmid allowed the constitutive expression of GFP from a CMV promoter in the pCDM7 vector [14]. The specific plasmid used (psyngfpfs64lt; E. C. Park and B. Seed, Massachusetts General Hospital, personal communication) expressed a mutant form of GFP containing mutations F64L and S65T, which increase the intrinsic fluorescence of GFP [15].

Chemical reagents. Cycloheximide was diluted to 20 μg/ml from a 1 mg/ml aqueous stock solution. Incubation of HeLa cells for 1 h in the presence of [^{35}S]methionine and 20 μg/ml cycloheximide reduced 10% trichloroacetic acid-precipitable counts to 5.8 $\pm$ 0.5% of those observed in the absence of the drug, indicating an efficient inhibition of translation. Actinomycin D (Act. D; Boehringer-Mannheim) was diluted to either 1 or 5 μg/ml from a stock solution of 2.5 mg/ml in ethanol. DRB (5,6-dichlorobenzimidazole riboside, Sigma) was diluted to 20 μM from a stock solution of 10 mM in ethanol. Incubation of HeLa cells in the presence of [^{3}H]uridine and either 1 μg/ml Act. D or 20 μM DRB reduced the incorporation of trichloroacetic acid-precipitable counts into total RNA to 8.2 $\pm$ 0.2 or 42 $\pm$ 2% of those seen in the absence of the drug, respectively.

Cell culture and media. HeLa cells were grown at 37°C in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum (GIBCO-BRL). Wild-type (src+/+; src8S) and mutant (src−/−; src1S) mouse fibroblast cell lines were provided by P. Soriano (Fred Hutchinson Cancer Research Center) [16] and monolayers were grown in DME supplemented with 10% calf serum. Cells were plated on coverslips at least 18 h prior to transfection, infection, or treatment with inhibitors. Viral infections were initiated by incubation with type 1 (Mahoney) poliovirus in phosphate-buffered saline containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS+) at the indicated multiplicity of infection (m.o.i.). After 30 min of adsorption, virus was removed, and cells were rinsed with PBS+ and then incubated with DME supplemented with 10% calf serum for the indicated time.

Transient transfections. HeLa cells were transfected with pcDNA3-based plasmids using Lipofectamine (GIBCO-BRL) liposome-mediated transfection according to the manufacturer's recommendations. Briefly, 2.5 μg plasmid DNA and 24 μl liposomes were preincubated in 500 μl OptiMEM (GIBCO-BRL) for 20 min at room temperature. Following the addition of 2 ml OptiMEM, the transfection mix was transferred to HeLa cell monolayers in 60-mm dishes. After 5 h of incubation of cells with the transfection mix containing liposomes, DNA, and OptiMEM, an equal volume of DME supplemented with 20% calf serum was added and cells were incubated at 37°C overnight. Reactions were scaled down for experiments performed in 6- and 12-well dishes.

Antibodies. The anti-Sam68 polyclonal rabbit antiserum was purchased from Santa Cruz Biotechnology (sc#333) and used at a dilution of 1:500. The anti-SC35 monoclonal mouse antibody was provided by T. Maniatis (Harvard University) [17] as a tissue-culture supernatant and was used at a dilution of 1:200. The production of the anti-3D polymerase monoclonal mouse antibody was previously described [13]; the antibody was used as a tissue culture supernatant at a dilution of 1:20. The anti-hemagglutanin (HA) monoclonal antibody was purchased from Berkeley Antibody Co. and used at dilutions of 1:1000 for immunofluorescence assays and 1:2000 for immunoblot analysis. Texas red-conjugated anti-rabbit IgG, Texas red-conjugated anti-mouse IgG, fluorescein dichorotriazine (DTAF)-conjugated anti-mouse IgG, and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibodies (Jackson ImmunoResearch) were used at dilutions of 1:100.

Poliovirus RNA transfection. Full-length poliovirus RNA was generated by digestion of T7pGEMpolio [18] with *Eco*RI and transcription with T7 RNA polymerase (Promega). Half of the transcription mix was transfected into 80% confluent src+/+ and src−/− cell monolayers on coverslips in 60-mm plates using DEAE–dextran [18]. Cells were fixed for immunofluorescence microscopy at 6 h post-transfection.

Indirect immunofluorescence microscopy. Cells on coverslips were fixed by incubation in methanol at −20°C for 10 min. Antibody incubations were performed at the dilutions indicated above in PBS containing 3 mg/ml bovine serum albumin (Sigma). Coverslips were mounted with Vectashield (Vector Laboratories, Inc.) and viewed using an Olympus system microscope Model BX60. Photographs were taken with a PM-C35DX camera as part of an automatic photomicrographic system, Model PM20 (Olympus). Prints were converted into computer files using a Studioscan IIsi (Agfa-Gevaert, NV) scanner in combination with Fotolook 2.07.2 and Adobe Photoshop 3.0 Macintosh software.

Immunoblot analysis. HeLa cells were transfected with the indicated plasmid (1.5 μg/60 mm plate), harvested at 27 h after transfection, and lysed on ice with occasional vortexing in 1 ml of a solution containing 1% NP-40, 150 mM NaCl, 0.1% SDS, 50 mM Tris–HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin. Insoluble material was pelleted at 16,000*g* in a microcentrifuge at 4°C.

Soluble proteins (40 μg) were resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis [19] and subsequently transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore). Immunoblots were pretreated with PBS supplemented with 2% BSA and probed with the anti-HA antibody. Following incubation with a 1:10,000 dilution of a sheep anti-mouse IgG horseradish peroxidase-conjugated secondary antibody, HA-tagged Sam68 was visualized using enhanced chemiluminescence techniques (Amersham).

Fluorescence-activated cell sorting (FACS). HeLa cells were grown on 60-mm plates and cotransfected, in triplicate, with 12.5 μg of a pcDNA3-based construct and 2.5 μg of the GFP-expression plasmid. Twenty-four hours posttransfection, cells were infected with poliovirus (m.o.i. 50) and incubated at 37°C for 5 h. Cells were detached from the plates by treatment with PBS containing 50 mM EDTA. Cells were sorted with a Becton Dickinson FACSTAR fluorescence-activated cell sorter operated by Paul Fallon (Stanford University). Ten thousand cells that were above an empirically determined fluorescence gate of 200 units, defining the population of cells that expressed GFP, were collected. Sorted cells were resuspended in PBS+, virus was released by three freeze–thaw cycles, cytoplasmic extracts were prepared, and viral yield was determined by plaque assay.

RESULTS

Sam68 Localization in src Knockout Cells

We have shown that poliovirus infection of human HeLa cells results in redistribution of nuclear Sam68 to the cytoplasm, the site of viral RNA replication [13].

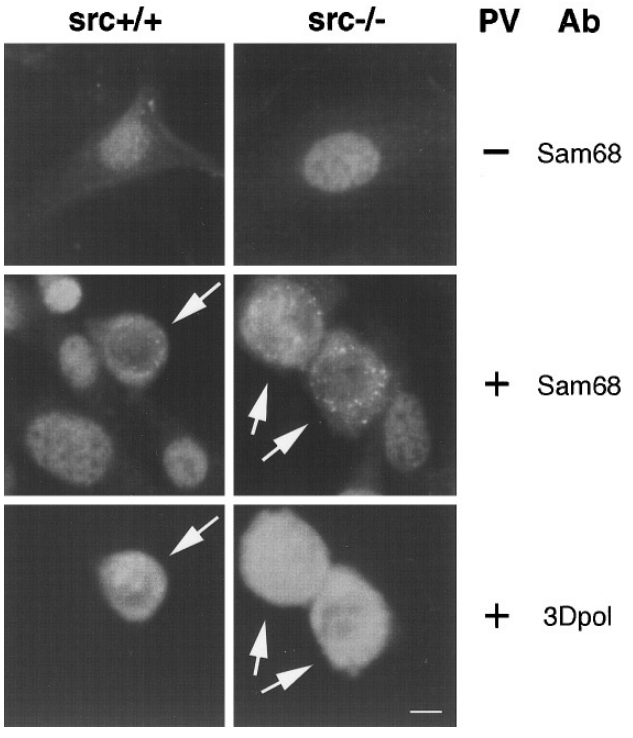

FIG. 1. Sam68 localization in the absence of the Src tyrosine kinase. Wild-type (src+/+) and src knockout (src −/−) mouse fibroblasts were or were not transfected with full-length poliovirus RNA. Six hours after transfection cells were fixed and prepared for immunofluorescence microscopy. Sam68 was detected using a rabbit polyclonal anti-Sam68 antiserum followed by a Texas red-conjugated anti-rabbit IgG antibody. 3D polymerase was detected using a mouse monoclonal anti-3Dpol tissue-culture supernatant followed by a DTAF-conjugated anti-mouse IgG secondary antibody. Transfected cells are indicated with arrows.

Sam68 is known to associate physically with the cytoplasmic tyrosine kinase Src in mitotic cells [1, 3]. To determine whether interaction with Src was required for poliovirus-induced redistribution of Sam68, we transfected fibroblasts derived from wild-type (src+/+) and src knockout (src−/−) mice with poliovirus RNA (Fig. 1). Sam68 relocalized to the cytoplasm in both cell lines, indicating that Src is not required for poliovirus-induced cytoplasmic localization of Sam68. However, it should be noted that src−/− cells do express Fyn, a closely related Src family kinases that can also interact with Sam68 [2, 20, 21].

Distribution of Sam68 within the Nucleus Is Affected by Transcriptional Status

The relocalization of Sam68 from the nucleus to the cytoplasm during poliovirus infection [13] suggests that, although predominantly nuclear at steady state, Sam68 might shuttle between the nucleus and the cytoplasm; only a small fraction of cellular Sam68 is cytoplasmically localized in uninfected cells [22]. Both hnRNP A1 and hnRNP K have been shown to shuttle in a transcription-dependent fashion [23–25]. To test whether Sam68 might shuttle by a similar pathway, we tested its localization in the presence of transcription inhibitors. In cells that were not treated with an inhibitor, Sam68 was predominantly nucleoplasmic with the exclusion of nucleoli (Fig. 2, control), as has been reported previously [13, 26]. In the presence of transcription inhibitors actinomycin D or DRB (5,6-dichlorobenzimidazole riboside), however, Sam68 concentrated into heterogeneous spots within the nucleus (Fig. 2).

At the concentrations used in this experiment, treatment with actinomycin D has been shown to inhibit all cellular transcription whereas treatment with DRB has been shown to inhibit transcription by RNA polymerases I and II [27, 28]. Tests of [^{3}H]uridine incorporation into HeLa cells in the presence of actinomycin D and DRB in the present study were consistent with this interpretation (Materials and Methods). The nuclear spots that contained Sam68 formed in the presence of actinomycin D or DRB were fewer in number and did not colocalize with previously described nuclear "speckles" stained for the splicing factor SC35 (Fig. 2) [17, 29]. Thus, if the spots reflect a default or storage compartment for Sam68 within the nucleus in the absence of ongoing RNA synthesis, it is not the compartment that contains SC35 and associated splicing factors.

Although these experiments did not reveal a transcription-dependent pathway for nucleocytoplasmic shuttling of Sam68, they demonstrated that diffuse nucleoplasmic distribution of Sam68 requires ongoing transcription. A requirement for ongoing transcription could reflect a need either for a short-lived protein or for nascent RNA. The absence of highly concentrated nuclear spots of Sam68 in cells that were treated with the translation inhibitor cycloheximide showed that the spots visible in Act. D- or DRB-treated cells were caused by the lack of nascent RNA, rather than a short-lived protein (Fig. 2). These results suggest that Sam68 may be directly involved in transcription or early events in RNA processing.

We were interested in determining whether the subnuclear structures we observed after incubation with transcription inhibitors were dynamic or static, representing either a steady-state localization of Sam68 that was still able to move through the cell or the trapping of Sam68 in a subnuclear compartment. To test these possibilities, HeLa cells were infected with poliovirus at a low multiplicity of infection and, after adsorption of the virus, either treated with actinomycin D or not (Fig. 3). At a concentration of 5 μg/ml, actinomycin D does not inhibit poliovirus replication [30], but causes Sam68 to relocalize into the punctate structures within 30 min of treatment (data not shown). At 4.5 h after

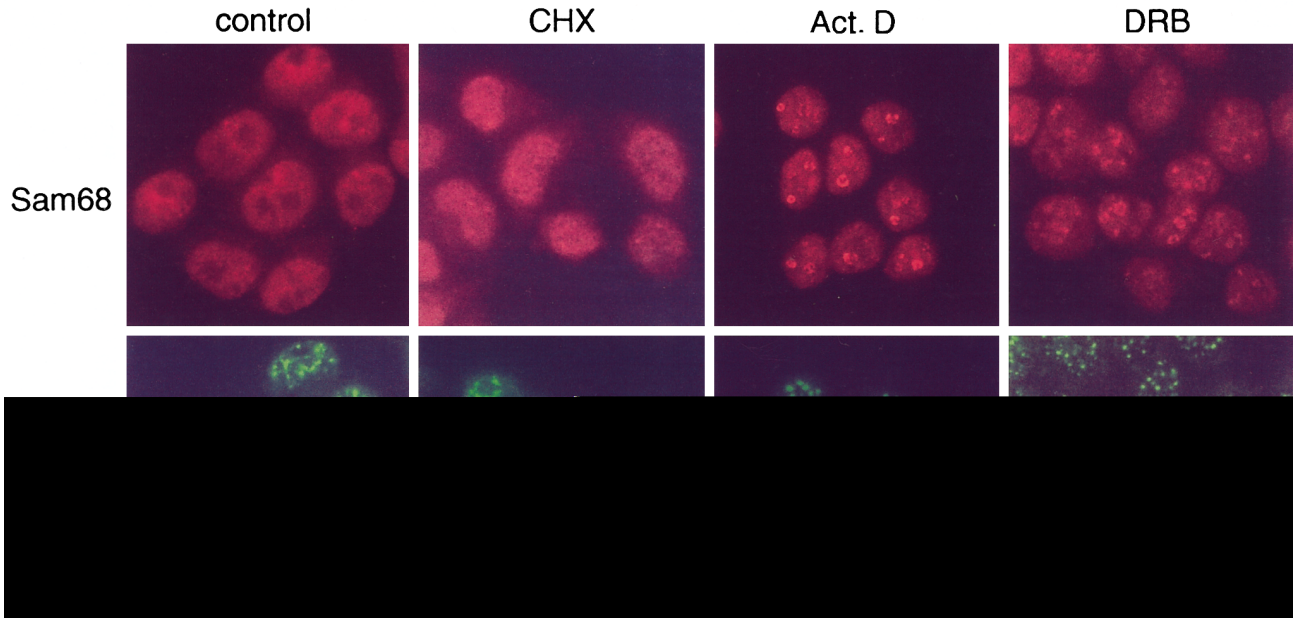

FIG. 2. Effect of transcription inhibitors on Sam68 localization. HeLa cells grown on coverslips were treated for 2 h at 37°C with ethanol (control; 1%), cycloheximide (20 μg/ml), actinomycin D (1 μg/ml), or DRB (20 μM); fixed in methanol at −20°C; and prepared for immunofluorescence microscopy. Sam68 was detected with a polyclonal rabbit antiserum and a Texas red-conjugated anti-rabbit IgG secondary antibody. Splicing factor SC35 was detected with a monoclonal anti-SC35 antibody (T. Maniatis, Harvard University) and a DTAF-conjugated anti-mouse IgG secondary antibody.

infection, cells were fixed and stained for Sam68, to test for its localization, and for the poliovirus polymerase 3Dpol, to identify infected cells. The relocalization of Sam68 from the nucleus to the cytoplasm was seen in infected cells whether or not cells were treated with actinomycin D (Fig. 3, arrows). No punctate staining was seen in the nucleus of the infected cell treated with actinomycin D, although such staining was seen in the surrounding uninfected cells. These data support the possibility that the nuclear spots seen on treatment with inhibitors of cellular transcription are dynamic structures.

The Effect of Mutations in the KH Domain on the Localization of Sam68

To investigate the potential importance of RNA binding by Sam68 in its localization, we determined the localization of previously characterized mouse Sam68 proteins that contain mutations known to disrupt its interaction with RNA. Mutant forms of mouse Sam68 containing an N-terminal HA epitope tag [8] were expressed in HeLa cells under the control of a constitutive CMV promoter [31, 32]. Mouse and human Sam68 proteins are greater than 95% identical in sequence [2]. However, before testing the localization of mutant forms of mouse Sam68 in HeLa cells, we wanted to determine whether overexpressed mouse Sam68 behaved similarly to endogenous human Sam68. To this end, we tested overexpressed wild-type mouse Sam68 for the two phenotypes described above: formation of nuclear spots on inhibition of cellular transcription and relocalization to the cytoplasm in poliovirus-infected cells (Fig. 4).

The anti-Sam68 antibody was used to determine the localization of either endogenous human Sam68 (Fig. 4, left) or the overexpressed mouse Sam68 (Fig. 4, right). Shorter exposures (Fig. 4, right) allowed the identification of mouse Sam68 in transfected cells in the absence of visible signal from endogenous human Sam68. In untreated control cells, overexpressed mouse Sam68 showed general nucleoplasmic staining with the exclusion of nucleoli as seen for the endogenous human Sam68 (Fig. 4). Treatment with actinomycin D also led to the formation of nuclear spots by overexpressed mouse Sam68 (Fig. 4). Finally, overexpressed mouse Sam68 also relocalized to the cytoplasm during poliovirus infection of transfected cells (Fig. 4). Therefore, overexpressed mouse Sam68 appeared to behave identically to endogenous human Sam68 and could be used to study the effect of mutations in Sam68 on its localization in HeLa cells.

Four mutations in the KH region of Sam68 were tested for an effect on localization (Fig. 5). Two point mutations were based on mutant forms of other KH proteins with known phenotypes. A mutation of a conserved Ile to an Asn in a KH domain of the human

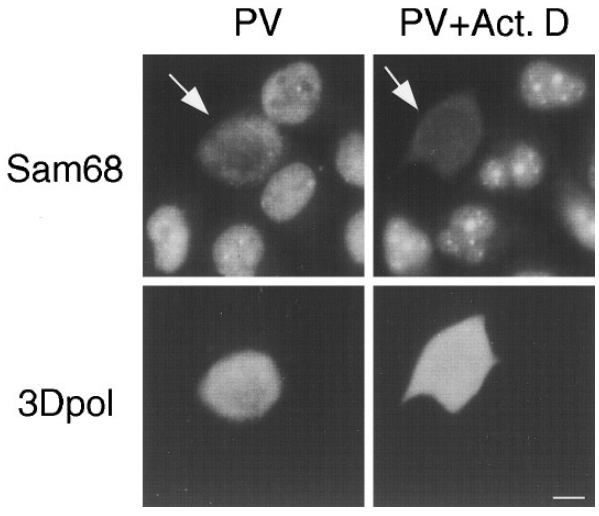

FIG. 3. Effect of actinomycin D on Sam68 relocalization during viral infection HeLa cells grown on coverslips were infected with Type 1 (Mahoney) poliovirus at m.o.i. 0.1 plaque-forming units per cell. After 30 min adsorption, virus was removed and cells incubated in the absence or presence of actinomycin D (5 μg/ml). At 4.5 h postinfection, cells were fixed and stained with an anti-Sam68 polyclonal antiserum and a monoclonal antibody that recognizes the poliovirus polymerase (3Dpol). Proteins were visualized by indirect immunofluorescence microscopy following incubation with Texas red-conjugated anti-rabbit IgG and DTAF-conjugated anti-mouse IgG secondary antibodies. Arrows indicate poliovirus-infected cells. Bar, $\approx$10 μm.

FMR1 gene was identified in a patient with severe fragile-X syndrome [33]. This mutation and analogous mutations in a KH domain of hnRNP K were found to disrupt binding to homopolymeric RNA *in vitro* [34]. In the context of a vigilin KH domain, an analogous mutation disrupted the proper folding of the entire KH domain when examined by nuclear magnetic resonance spectroscopy [5]. The *GLD-1* gene in *C. elegans* is a tumor suppressor gene that is specific for the female germ line; point mutation of an absolutely conserved Gly of the KH domain to a Glu causes germ-line tumor formation [10, 35]. The cognate mutations in mouse Sam68 are I184N and G178E, as shown in Fig. 5. These mutations have been shown to affect the binding of Sam68 to a SELEX-derived RNA ligand for Sam68 but not to poly(U) [8]. Two larger mutations were also introduced into mouse Sam68: a deletion of the entire KH domain (ΔKH) and a deletion of a smaller segment within the KH domain (Δloop4; Fig. 5). These residues deleted in Δloop4 correspond to the fourth loop in the structure of the vigilin KH domain but are specific to the SGQ subfamily of KH proteins [5]. Although both mutant proteins failed to bind the SELEX-derived Sam68 ligand, the ΔKH mutant Sam68 was also impaired in binding to poly(U) RNA [8].

To determine the intracellular localization of mutant Sam68 proteins, HeLa cells that overexpressed each mutant Sam68 protein were stained with anti-HA and anti-Sam68 antibodies (Fig. 6). The anti-HA antibody, which specifically recognized the tagged mouse Sam68 proteins, identified the transfected cells (anti-HA; Fig. 6). With the anti-Sam68 antiserum, short exposures were sufficient to detect the overexpressed, but not the endogenous Sam68 (anti-Sam68; Fig. 6). The specificity of each of these antibodies for overexpressed Sam68 was demonstrated by the minimal staining of cells transfected with the control vector (Fig. 6).

Two of the mutant proteins, I184N and Δloop4, showed expression patterns similar to wild-type mouse Sam68 (Fig. 6). The two other mutant Sam68 proteins displayed staining patterns that differed from those of wild-type Sam68 (Fig. 6). The G178E mutant Sam68 localized to distinct patches within the nucleus (Fig.

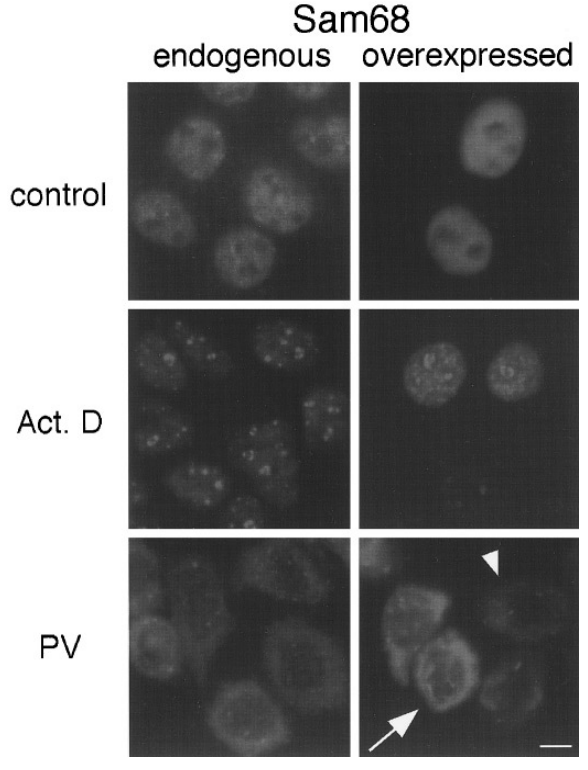

FIG. 4. Localization of endogenous human Sam68 and overexpressed mouse Sam68. HeLa cells were or were not transfected with the pcDNA3 Sam68-expressing plasmid and were incubated overnight at 37°C. Nontransfected and transfected cells were either fixed without further treatment or fixed after incubation with actinomycin D (5 μg/ml) for 2 h or after infection with poliovirus at an m.o.i. of 100 plaque-forming units per cell for 4.5 h. All coverslips were fixed in −20°C methanol. Sam68 was visualized by staining with Sam68 antiserum and a Texas red-conjugated anti-rabbit IgG antibody. Decreased exposures allowed the selective visualization of overexpressed Sam68 in the control and Act. D-treated samples. Increased exposure times necessary for visualization of cytoplasmic Sam68 in the poliovirus-infected cells resulted in the visualization of both overexpressed Sam68 in transfected cells (arrow) and endogenous Sam68 in nontransfected cells (arrowhead). Bar, $\approx$10 μm.

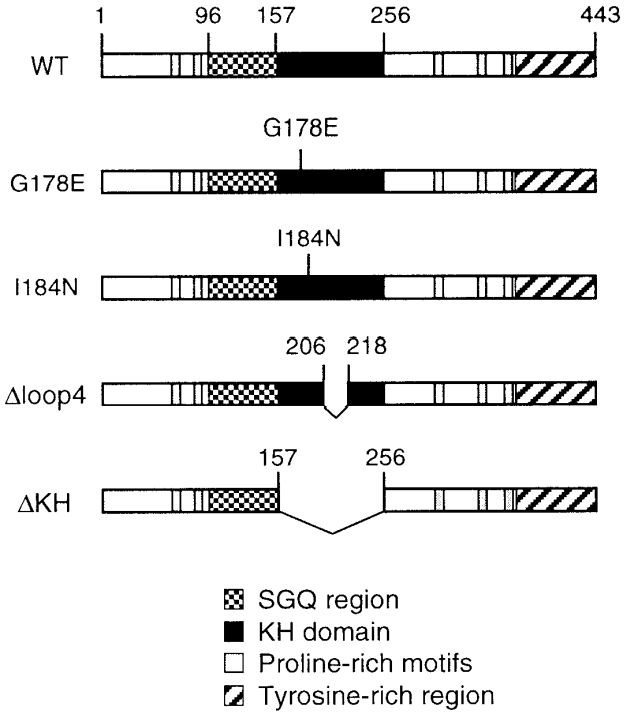

FIG. 5. Sam68 sequence motifs. Primary sequence motifs in Sam68 are shown. The SGQ region contains sequences conserved among the Sam68, Gld-1, Qk-1, and SF-1 subfamily of KH proteins. The KH domain contains residues conserved among all KH proteins [5]. The C-terminal proline-rich motifs have been implicated in binding to SH3-domain proteins [2] and the tyrosine-rich region is thought to be responsible for binding to SH2 domains [11]. Mutations introduced into the mouse Sam68-expressing plasmid are shown.

6). Sam68 that bore the large ΔKH deletion displayed punctate nuclear staining, often at the edge of the nucleus (Fig. 6). The KH region of Sam68 therefore appears to be required for a diffuse nucleoplasmic distribution.

To ensure that punctate staining of G178E and ΔKH mutant Sam68 proteins did not result simply from increased or decreased expression relative to the wild type, I184N, and Δloop4 mutant Sam68 proteins, which showed diffuse nuclear staining, transfected cell lysates were immunoblotted and probed with an anti-HA antibody. Immunofluorescence assays of cells transfected in parallel revealed that the transfection efficiencies were similar for all the plasmids (data not shown). Neither the G178E nor the ΔKH mutant Sam68 proteins showed significantly different expression levels from HA-tagged wild-type Sam68 (Fig. 7). The only mutant protein that appeared to be expressed to a slightly lower level was Δloop4, which showed wild-type localization (Figs. 6 and 7). Thus, the observed differences in intracellular localization of G178E and ΔKH mutant Sam68 proteins could not be attributed to differences in expression levels.

To determine whether the relocalization of Sam68 during poliovirus infection was affected by mutations in the KH domain, HeLa cells were transfected with Sam68-encoding DNA and infected with poliovirus. Cells were fixed at 4 h after infection and stained with anti-Sam68 antiserum to identify cells that overexpressed mouse Sam68 proteins. Cells were also stained with anti-3Dpol to confirm that all cells were infected and to determine the intracellular localization of this viral Sam68-interacting protein (Fig. 8). The anti-3Dpol antibody did not recognize any proteins in uninfected cells (Fig. 8A). By 4 h postinfection, wild-type Sam68 protein as well as mutant Sam68 proteins Δloop4 (Fig. 8B), I184N, and G178E (data not shown) showed greater cytoplasmic staining than in uninfected cells, showing that they could relocalize during poliovirus infection.

The punctate nuclear staining pattern seen for the ΔKH Sam68 deletion variant, however, was seen in infected as well as uninfected cells and redistribution to the cytoplasm was not observed in response to poliovirus infection (Figs. 8A and 8B). Thus, the ΔKH Sam68 protein is unable to relocalize during poliovirus infection. One possible explanation of this effect is that KH domain function is essential for the mechanism of Sam68 relocalization during poliovirus infection.

Alternatively, the failure of ΔKH Sam68 to relocalize during poliovirus infection might be due to the inhibition of viral replication by the overexpression of ΔKH Sam68. The anti-3Dpol staining of ΔKH-transfected cells revealed that these cells could support viral translation. To determine whether any later step in viral replication was inhibited by the ΔKH mutant Sam68 and to quantify the production of virus, it was necessary to separate transfected and untransfected cells following infection.

To identify transfected cells, Sam68-expressing plasmids were cotransfected with GFP-encoding plasmids. The expression of GFP allowed transfected cells to be sorted in a FACS (Fig. 9A). When HeLa cells were transfected with the pcDNA3 vector plasmid alone, analysis of the cells by FACS revealed a relatively high level of autofluorescence (Fig. 9A). However, cotransfection of the pcDNA3 vector with the GFP-expressing plasmid led to a second population of cells (Fig. 9A), presumably those that expressed GFP. To ensure that expression of GFP could be used as a marker for Sam68 expression, immunofluorescence microscopy of cells cotransfected with Sam68- and GFP-expressing plasmids was performed. Almost all cells that expressed GFP also stained brightly with the anti-Sam68 antibody, indicating a high cotransfection efficiency (data not shown).

To test for effects of Sam68 overexpression on poliovirus replication, HeLa monolayers were transfected with the GFP-expressing plasmid and the

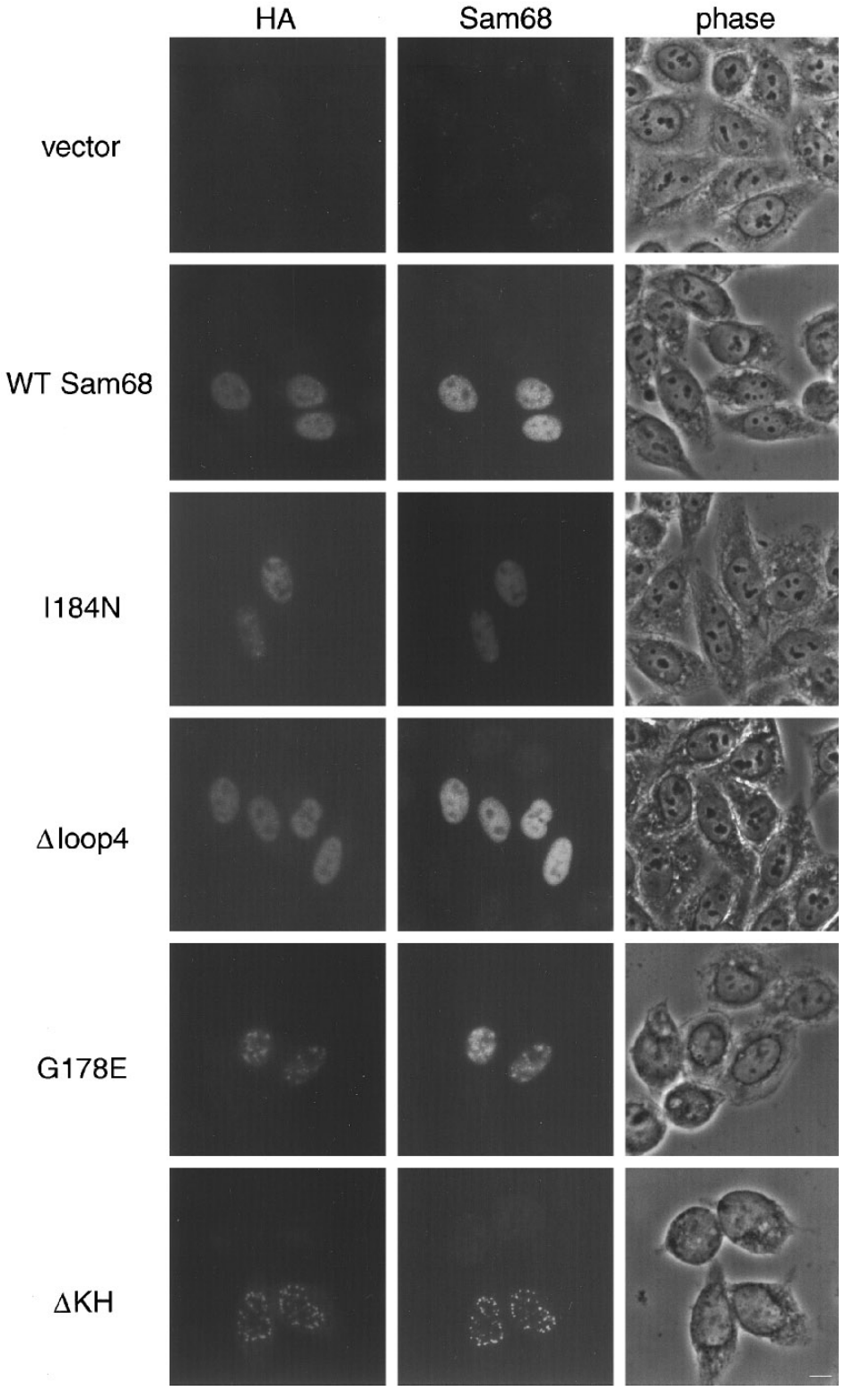

FIG. 6. Localization of mutant Sam68 proteins. HeLa cells were transfected with the pcDNA3 expression vector, the HA-epitope-tagged wild-type Sam68 expression plasmid (WT Sam68), or plasmids that express HA-tagged Sam68 containing the I184N mutation, a deletion of amino acids 206–218 (Δloop4), the G178E mutation, or the deletion of amino acids 157–256 (ΔKH). At 28.5 h posttransfection, cells were fixed in methanol and stained with an anti-HA monoclonal antibody and the Sam68 antiserum. Following incubation with Texas red-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG antibodies, cells were mounted and visualized by indirect immunofluorescence microscopy. Images prepared by phase microscopy identify both nontransfected and transfected cells in each field. Bar, ≈10 μm.

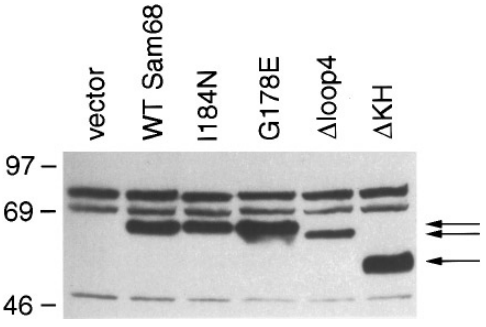

FIG. 7. Expression of mutant Sam68 proteins. HeLa cells were transfected with the pcDNA3 expression vector, the HA epitope-tagged wild-type Sam68 expression plasmid (WT Sam68), or plasmids that express HA-tagged Sam68 containing the I184N mutation, the G178E mutation, a deletion of amino acids 206–218 (Δloop4), or the deletion of amino acids 157–256 (ΔKH). At 27 h posttransfection, cells were harvested and lysed, and cellular proteins were displayed by SDS–PAGE and immunoblotted with an anti-HA antibody. Epitope-tagged Sam68 was detected using enhanced chemiluminescence techniques. Arrows indicate overexpressed Sam68 proteins. The migration of molecular weight markers (kDa) is noted at left.

pcDNA3 vector alone, the plasmid that expressed wild-type mouse Sam68, or the plasmid that expressed the ΔKH mutant Sam68. Twenty-four hours posttransfection, cells were infected with poliovirus at m.o.i. 50 and incubated for 5 h at 37°C. Cells were detached from the substratum and GFP-expressing cells were collected by FACS. Sorted cells were lysed and virus was titered by plaque assay (Fig. 9B). No difference in virus production was observed in the presence of ΔKH mutant or wild-type Sam68 (Fig. 9B). Thus, expression of the ΔKH mutant form of Sam68 did not inhibit poliovirus replication. The lack of relocalization of ΔKH Sam68 must therefore reflect a requirement for an intact KH domain in the relocalization of Sam68 during viral infection.

Costaining with an anti-3Dpol antibody revealed that, in addition to their normal cytoplasmic location, 3D-containing sequences also localized to the nuclear spots formed by the ΔKH Sam68 protein (Fig. 8B). This signal was not due to bleed through of the strong anti-Sam68 FITC signal or to the cross-reactivity of either the anti-3Dpol antibody or the Texas red-conjugated anti-mouse IgG secondary antibody with Sam68, because both the 3Dpol signal and the Sam68 signal were dependent on the presence of the appropriate primary antibody (data not shown). This colocalization of 3Dpol and Sam68 staining in infected cells demonstrates that 3Dpol or other 3D-containing viral peptides can enter the nucleus during infection and, more importantly, that the ΔKH mutant Sam68 is not completely malfolded, retaining the ability to interact with at least one of the ligands with which wild-type Sam68 interacts.

DISCUSSION

Studies of src-transformed fibroblasts revealed Sam68 as a major mitotic binding partner of the Src tyrosine kinase [1, 3]. Subsequent work has shown that Sam68 interacts with other Src family kinases including Fyn [2, 21]. The functional redundance of these kinases in mammalian cells was suggested by the viability of mice homozygous for a disruption in any one of the Src, Fyn, or Yes genes and the perinatal death of src/yes or src/fyn double mutant mice [20]. The intracellular localization of Sam68 in the presence and absence of poliovirus replication was the same in wild-type and src knockout cells (Fig. 1), demonstrating that Src itself is not required for the distribution of Sam68 observed in cultured cells. It is possible, however, that the interaction of Sam68 with a Src family tyrosine kinase plays a role in its localization and that this role is performed by either Fyn or Yes in the src knockout cells.

The nucleoplasmic localization of Sam68 and its dependence on ongoing transcription suggest that Sam68 might be involved either in RNA transcription or in the processing and transport of newly synthesized RNAs. Subcellular localization of several other nucleic acid-interacting proteins implicated in transcription, RNA processing, and RNA transport has been shown to be influenced by transcriptional status. RNA polymerase II and the splicing factor SC35 were colocalized to distinct spots within the nucleus when transcription was inhibited [29]. The lack of Sam68 and SC35 colocalization during treatment with Act. D or DRB (Fig. 2), however, shows that Sam68 localization is not closely coordinated with at least one component of the splicing machinery. A situation similar to that observed for Sam68 is that of the yeast RNA-binding protein Npl3p, which has been implicated in both RNA export and protein import [36–38]. Npl3p was shown to localize to distinct spots in the nucleus upon treatment with the transcription inhibitor thiolutin [37]. Sam68 relocalized to the cytoplasm during poliovirus infection, even when actinomycin D was present from the beginning of infection (Fig. 3). This result demonstrated that the spots of Sam68 seen in Act. D-treated cells are dynamic structures and do not merely represent irreversible aggregation of Sam68.

The overexpression of forms of Sam68 with mutations in the KH domain demonstrated the importance of this domain in subnuclear distribution of Sam68 (Fig. 6). Whereas one point mutation, I184N, and a small deletion, Δloop4, did not have major effects on Sam68 localization, another point mutation in an absolutely conserved residue, G178E, and the deletion of the entire KH sequence significantly altered Sam68 localization (Fig. 6). The I184N and Δloop4 mutations disrupted binding to a SELEX-derived RNA ligand of

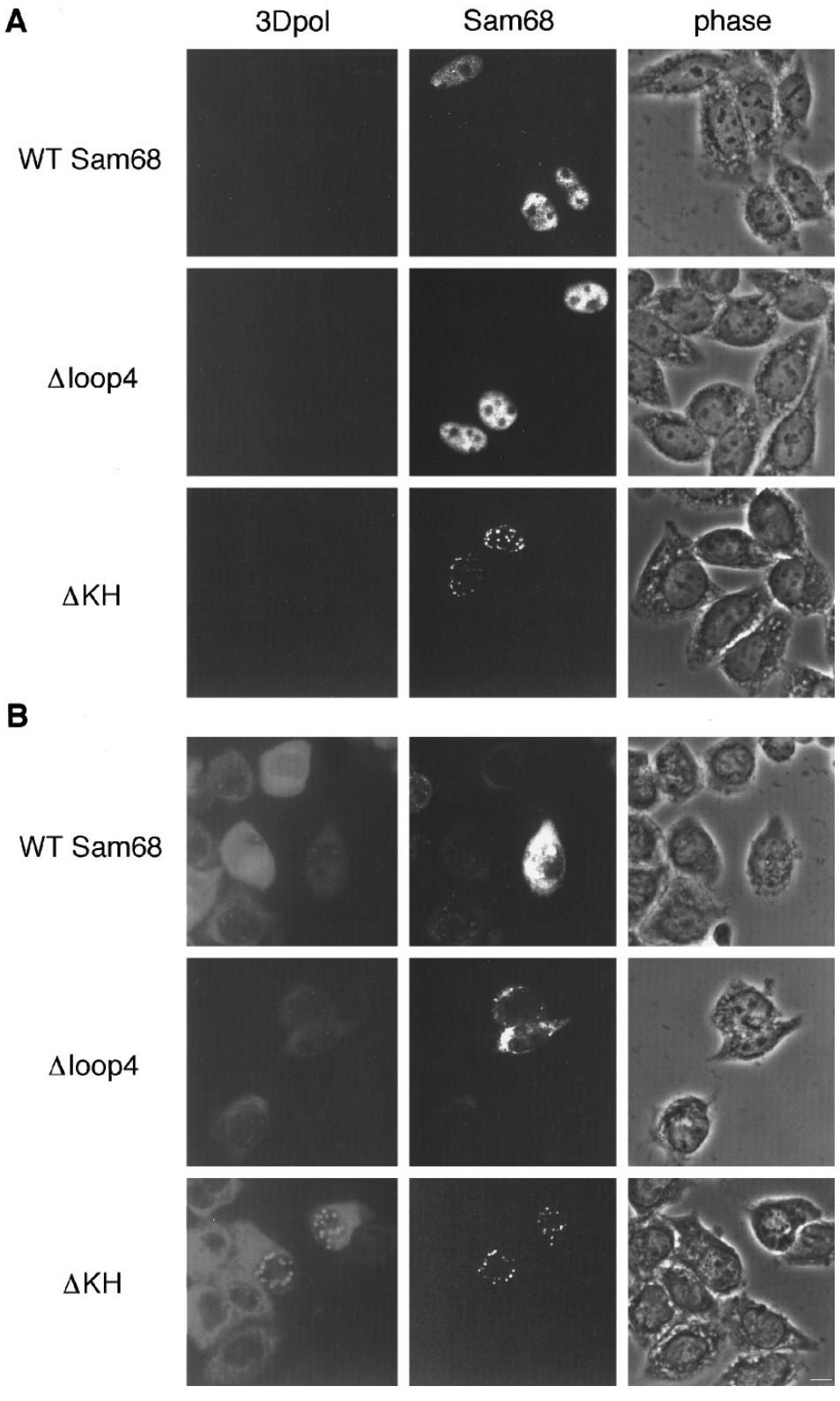

FIG. 8. Localization of mutant forms of Sam68 in poliovirus-infected cells. HeLa cells were transfected with plasmids that express either wild-type Sam68 or the Δloop4 or ΔKH deletion mutant Sam68 proteins. Twenty-four hours after transfection, cells were or were not infected with poliovirus at m.o.i. 100 plaque-forming units per cell. At 4 h postinfection, cells were fixed and stained with the anti-Sam68 antiserum and the anti-3Dpol antibody, followed by Texas red-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG antibodies. Immunofluorescence microscopy was used to identify transfected and infected cells; phase microscopy was used to identify all cells in each field. Bar, ≈10 μm.

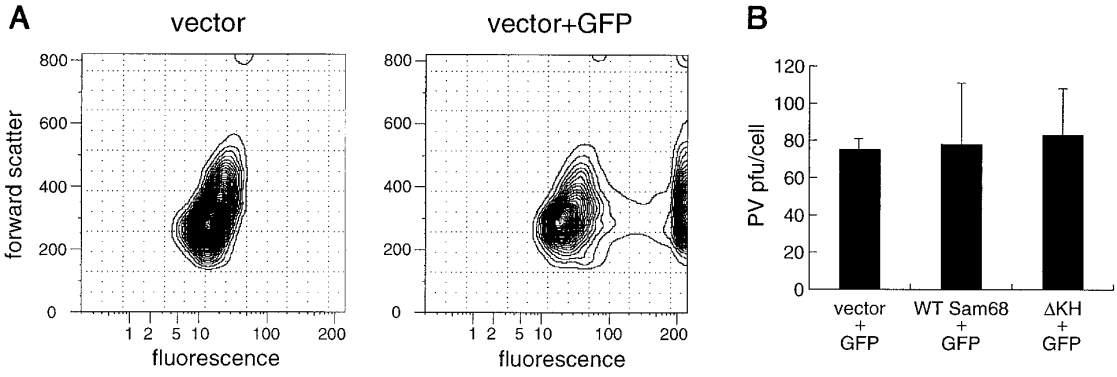

FIG. 9. Viral replication in cells overexpressing wild-type and mutant Sam68. (A) HeLa cell monolayers were transfected with 5 μg of pcDNA3 vector or 2.5 μg of pcDNA3 and 2.5 μg of a GFP-expressing plasmid using liposome-mediated transfection. After 24 h cells were detached from the plate and analyzed on a cell sorter for forward scatter, reflecting the size of cells, and fluorescence to detect cells that expressed GFP. Background fluorescence from HeLa cells in the absence of GFP expression was seen between 3 and 100 fluorescence units on this arbitrary scale, whereas a population of GFP-expressing cells was seen at >150 units. (B) HeLa cells on 60-mm plates were cotransfected with 12.5 μg Sam68 overexpressing plasmid and 2.5 μg GFP-expressing plasmid. Twenty-four hours after transfection, cells were infected with poliovirus and incubated at 37°C for 5 h. Cells were detached from the plate and a fluorescence-activated cell sorter was used to collect the population of cells that expressed GFP. The FACS profiles resembled those in (A) and cells that displayed a fluorescence level of 200 and higher were collected. The transfection efficiency, defined as the percentage of cells that displayed fluorescence greater than 200, was between 4 and 7%. Virus was released from sorted cells by three freeze–thaw cycles and quantitated by plaque assay. The experiment was performed in triplicate and error bars represent one standard deviation from the mean.

Sam68, but not to poly(U) [8]. The point mutation G178E also disrupted binding to the selected Sam68 ligand but not to poly(U) RNA, and it significantly altered Sam68 localization (Fig. 6). Whether this result correlates with a more pronounced defect in RNA binding is not known; the RNA-binding assays performed previously were not designed to detect subtle quantitative differences.

The most dramatic effect on Sam68 localization (Fig. 6) and on Sam68 binding to RNA *in vitro* [8] was seen in the ΔKH mutant. The deletion of the KH domain resulted in punctate Sam68 staining, frequently seen near the nuclear rim (Fig. 6 and data not shown). Unlike the other mutants, the ΔKH Sam68 failed to bind both the SELEX-derived RNA ligand and the poly(U) *in vitro* [8]. The concentration of Sam68 in subnuclear structures was reminiscent of the distribution seen in the presence of transcription inhibitors (Fig. 2). In combination, these results raise the possibility that the binding of Sam68 to RNA is essential for its general nucleoplasmic distribution. If either the RNA ligand of Sam68 (nascent RNA) or its KH domain (which is required for RNA binding) is removed, this distribution is lost. Interestingly, both the KH domain of Sam68 and cellular RNA have been shown to be required for the self-association of Sam68 [12]. Our results could therefore reflect the importance of Sam68–Sam68 interactions, in addition to Sam68 RNA binding, for its cellular localization.

In spite of the similarity of Sam68 distribution in cells treated with transcription inhibitors and in cells expressing ΔKH Sam68, a major difference between these results lies in how the subnuclear structures respond to poliovirus infection. The spots caused by Act. D treatment dispersed during viral infection (Fig. 3), whereas those containing the ΔKH mutant Sam68 did not (Fig. 8). A trivial explanation for the failure of the ΔKH mutant Sam68 protein to function properly in cytoplasmic relocalization or in the *in vitro* RNA binding assays would be that the ΔKH mutant Sam68 protein was completely malfolded. We do not think that this is the case for several reasons. First, the ΔKH mutant Sam68 protein was folded sufficiently well to bind to poliovirus polypeptides containing 3Dpol epitopes and to cause its relocalization into the nuclear spots (Fig. 8). The ΔKH mutant Sam68 protein also accumulated to similar steady-state concentrations as wild-type Sam68 and other mutant Sam68 proteins in transfected cells, arguing that folding problems, if any, did not result in increased susceptibility to intracellular proteases (Fig. 7). In addition, natural isoforms of Sam68 that contain deletions within the KH domain (residues 170–208) have been identified [39] and shown to be specifically expressed at growth arrest in mouse fibroblast lines. Finally, when overexpressed, this natural ΔKH isoform was found to have a slight effect on entry into S phase and thus is likely to function within transfected cells [39].

There are at least three possible explanations for the relocalization of Sam68 during poliovirus infection and the effect of the ΔKH deletion in Sam68 on its relocalization. (i) Viral infection activates a signaling pathway for the export of Sam68 from the nucleus, and the ΔKH Sam68 mutant does not respond to this pathway. (ii)

Sam68 normally shuttles between the nucleus and cytoplasm, but becomes trapped in the cytoplasm of poliovirus-infected cells due to the overexpression of a viral protein or RNA to which it binds. The ΔKH mutant Sam68 does not bind to this cytoplasmic ligand. (iii) Sam68 normally shuttles, but becomes trapped in the cytoplasm of poliovirus-infected cells because nuclear import is blocked in poliovirus infection, and the ΔKH mutant Sam68 either does not shuttle or evades the blockage of nuclear import. A possible block to nuclear import could also explain the cytoplasmic relocalization of other cellular proteins such as the La autoantigen [40] and nucleolin (S. Waggoner and P. Sarnow, Stanford University, personal communication) during poliovirus infection. Experiments are ongoing to distinguish between these possibilities.

The experiments described here have demonstrated an effect of both nascent RNA and the KH domain of Sam68 on subnuclear localization of this putative signaling protein. Inhibitors of cellular transcription caused Sam68 to accumulate in punctate structures within the nucleus. However, poliovirus infection, itself a generous source of nascent RNA, could disrupt these structures and cause relocalization of Sam68 into the cell cytoplasm either in the presence or in the absence of actinomycin D. Destruction of the ability of Sam68 to bind to RNA by deletion of the KH domain also caused Sam68 to localize to punctate subnuclear structures. These, however, could not be disrupted by poliovirus infection. Taken together our results indicate a dynamic role of Sam68 in transcription or early events in RNA processing that might involve nucleocytoplasmic shutting. The punctate staining pattern of Sam68 observed in the absence of transcription and in a severe Sam68 mutant might represent a default or storage compartment from which Sam68 might be rapidly recruited to fulfill this role. Further studies of the RNA-binding, protein-binding, and intracellular localization properties of mutant and wild-type Sam68 proteins will help elucidate its many suggested roles in intracellular signaling, RNA processing, and viral infection.

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