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In Vivo and In Vitro Association of hsc70 with Polyomavirus Capsid Proteins

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Members of the 70-kDa family of cellular stress proteins assist in protein folding by preventing inappropriate intra- and intermolecular interactions during normal protein synthesis and transport and when cells are exposed to a variety of environmental stresses. During infection of A31 mouse fibroblasts with polyomavirus, the constitutive form of hsp70, hsc70, coimmunoprecipitated with all three viral capsid proteins (VP1, VP2, and VP3). In addition, the subcellular location of hsc70 changed from cytoplasmic to nuclear late in polyomavirus infection, coincident with the nuclear localization of the viral capsid proteins. VP1 and VP2 expressed in Sf9 insect cells with recombinant baculovirus vectors also coimmunoprecipitated with an hsp70-like protein, and VP1 expressed in *Escherichia coli* coimmunoprecipitated with the hsp70 homolog DnaK. Capsid proteins expressed by in vitro translation coimmunoprecipitated with the hsc70 protein present in the reticulocyte translation extract. Therefore, the polyomavirus capsid proteins associate with hsc70 during virus infection as well as in recombinant protein expression systems. This association may play a role in preventing the premature assembly of capsids in the cytosol and/or in facilitating the nuclear transport of capsid protein complexes.

DNA viruses normally assemble in the nuclei of infected cells. For papovaviruses, the specific subcellular site of assembly appears to be regulated in part by the properties of the major capsid proteins. For example, when expressed in insect cells with recombinant baculovirus vectors, the major capsid protein of polyomavirus, VP1, and the L1 protein of human papillomavirus type 11 assemble into capsid structures only after transport to the nucleus (9, 27, 35). This specificity of intranuclear capsid assembly suggests that there are cellular mechanisms which prevent inappropriate cytoplasmic assembly as well as those which promote appropriate nuclear packaging of the viral genome. Previously, the differential availability of calcium in different cellular compartments was proposed as a regulator for polyomavirus capsid formation in the nucleus (27). Inappropriate assembly may be inhibited by cellular stress or chaperone proteins.

Stress proteins, particularly those of the hsp70 family, are transcriptionally induced by and associate with viral proteins. Infection with adenovirus (45), vaccinia virus (40), cytomegalovirus (6), and simian virus 40 (SV40) and polyomavirus (17) induce mRNAs for hsp70 family proteins. Early viral genes, including E1A from adenovirus (45) and the large T antigens of SV40 (43) and polyomavirus (18), transactivate the hsp70 promoter in vitro. The adenovirus E1A protein binds to hsp70 (44), and complexes between SV40 large T antigen and hsp70 have been detected in transformed cells (39). Complexes between hsp70 and capsid proteins during viral infection have been detected for Sindbis virus (11), vesicular stomatitis virus (11), adenovirus type 5 (23), and poliovirus and coxsackievirus (24). hsc70, the constitutively expressed cognate of hsp70, has also been detected in mature virions of RNA viruses, including rabies virus, vesicular stomatitis virus, Newcastle disease virus, influenza A virus (36), and canine distemper virus (31).

The major known functions of hsp70-like proteins are to maintain protein homeostasis during protein synthesis and transport and to be a response to protein damage caused by chemical and physical agents (14). hsc70 transiently binds many nascent polypeptides on polysomes and unfolded proteins destined to be translocated across subcellular membranes. Its function is thought to be complemented by the inducible hsp70 protein for protection against damaged proteins induced by stress. The amino terminus of hsp70 binds adenine nucleotides, particularly ADP, and the carboxy terminus binds protein substrates. Proteins bound to ADP-hsp70 are released by the binding of Mg-ATP to ADP-hsp70 (15). Thus, the addition of ATP to hsp70-bound complexes results in complex dissociation (32). ATP-dependent release may explain the energy requirement of translumenal protein transport into mitochondria, peroxisomes, and the endoplasmic reticulum (12). Transport into these organelles is accomplished by cytosolic hsp70-like proteins transferring polypeptides to lumenal hsp70-like proteins. hsp70 and hsc70 are also required for nuclear translocation of karyophilic proteins bearing nuclear localization signals, such as SV40 large T antigen, nucleoplasmin, and histone H1 (16, 41, 46). In contrast to organellar transport which involves transfer to secondary proteins, the mechanism of hsp70-mediated nuclear localization is unclear. Finally, hsp70-like proteins (e.g., BiP) are thought to facilitate the assembly of multimeric proteins such as immunoglobulins by binding nascent polypeptides, preventing their aggregation, and maintaining them in an assembly-competent conformation (14).

Because of the diverse functions of hsp70-like proteins and their increased expression and association with viral proteins during viral infections, it is likely that hsp70 assists in aspects of virion assembly as a cellular chaperone protein. In this study,

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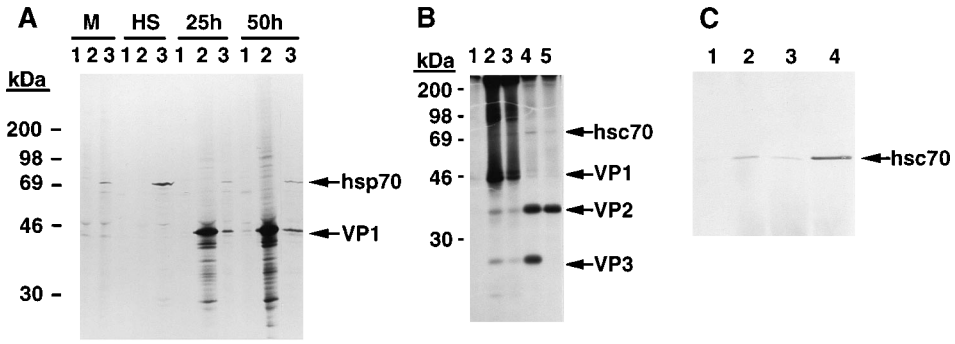

FIG. 1. hsc70 associates with polyomavirus capsid proteins during virus infection. Virus-infected A31 cells were ^{35}S labeled for 2 to 3 h prior to lysis at the indicated times postinfection. (A) Lysates immunoprecipitated with preimmune rabbit serum (lanes 1), anti-VP1 antibody I58 (lanes 2), and polyclonal rabbit anti-hsp70 antibody (lanes 3). (B) Lysates immunoprecipitated with preimmune rabbit serum (lane 1), anti-VP1 antibodies I58 and Fd9 (lanes 2 and 3, respectively), anti-VP2/VP3 antibody R4 (lane 4), and anti-VP2 antibody R3 (lane 5). (C) Immunoblot with anti-hsp70/hsc70 antibody SPA820 of lysates immunoprecipitated with preimmune rabbit antiserum (lane 1), anti-VP1 antibody I58 (lane 2), anti-VP2/VP3 antibody R4 (lane 3), and anti-hsc70 antibody SP815 (lane 4). Lanes M, mock-infected cells; lanes HS, cells exposed to heat shock.

we investigated the possible role of hsp70 proteins during polyomavirus infection.

MATERIALS AND METHODS

Cells and virus infection. For infection of A31 (BALB/c 3T3) mouse fibroblasts with the wild-type murine polyomavirus, virus stocks of strain NG59RA were sonicated for 1 min, incubated at 45°C for 15 min, and centrifuged at 1,200 × *g* for 5 min to pellet debris. Virus supernatants were diluted in adsorption buffer (phosphate-buffered saline [PBS] supplemented with 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 2% calf serum) prior to infection. The same buffer was used for mock infections. Infections were performed for 1 h at a multiplicity of infection of 5 to 10. Heat shock was performed at 43°C for 30 min prior to cell harvest.

Preparation of recombinant proteins. Recombinant VP1 and VP2 were expressed in Sf9 insect cells with the baculovirus vectors AcNPV-VP1 and AcNPV-VP2 (8, 27). Lysates were harvested 45 h after infection. VP1 was expressed in *Escherichia coli* and purified as previously described (19).

^{35}S labeling of cells. At 54 h postinfection (unless otherwise indicated), cells were washed with PBS and incubated for 30 min in Dulbecco minimal essential medium lacking methionine and cysteine (GIBCO Laboratories, Grand Island, N.Y.) and supplemented with 10% dialyzed calf serum. One hundred microcuries of [^{35}S]methionine-cysteine (1,175 Ci/mmol; Exprelabel; Amersham, Arlington Heights, Ill.) per 100-mm-diameter dish was then added for 2 to 3 h. Cells were harvested on ice in 0.5 ml of RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Nonidet P-40 [NP-40], 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) with 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.) as a protease inhibitor. For pulse-chase experiments, cells were labeled for 5 min with [^{35}S]methionine-cysteine, washed three times with PBS, and incubated in Dulbecco minimal essential medium supplemented with 0.27 mg of methionine per ml and 0.5 mg of cysteine (10-fold excess) per ml.

Preparation of cell lysates. After ^{35}S labeling, cells were harvested by the addition of 500 μl of RIPA buffer (50 mM Tris-HCl [pH 7.2], 0.1% SDS, 1% sodium deoxycholate, 1% NP-40, 150 mM NaCl) containing 40 U of DNase I per ml to each 100-mm-diameter culture plate and incubation on ice for 20 min. Lysates were spun in a microcentrifuge at 12,000 × *g* for 10 min at 4°C. Supernatants were either used fresh or frozen at −20°C.

Immunoprecipitation. The antibodies used for immunoblots and immunoprecipitations were rabbit polyclonal anti-polyomavirus VP1 (I58) (8, 27, 29); mouse monoclonal anti-VP1 (Fd9) (7); an anti-peptide antibody raised against the common carboxy-terminal 24 residues of polyomavirus VP2 and VP3, designated R4 (7, 20); and an anti-peptide antibody raised against amino acids 91 to 115 of VP2, a domain not shared by VP3, designated R3 (7). The anti-heat shock protein antibodies were SPA810 (mouse monoclonal anti-hsp70), SPA815 (rat monoclonal anti-hsc70), SPA820 (mouse monoclonal antibody raised against a common epitope of hsp70 and hsc70), and SPA804 (rabbit anti-hsp60; Stressgen, Victoria, Canada). A polyclonal rabbit anti-hsp70 recognizing both hsp70 and hsc70 was kindly provided by P. Sarnow (University of Colorado School of Medicine). AXL623 is a polyclonal rabbit antibody recognizing the *E. coli* DnaK protein (Accurate Chemical and Scientific Corp, Westbury, N.Y.).

Aliquots of each cell lysate were precleared with protein A- or protein G-Sepharose beads and incubated with 2 μl of antibody at 4°C for 1 to 18 h on a continuous rocker. Fifty microliters of a 1:1 dilution of protein A- or protein G-Sepharose beads was added for 0.5 to 2 h. Beads were washed with RIPA buffer three times and no salt buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.0], 1% NP-40) once or alternatively once with buffer A (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, 1% NP-40), once with 2 M NaCl, twice with RIPA buffer, and once with water. Proteins were eluted by boiling in SDS sample buffer and subjected to SDS–10 to 15% polyacrylamide gel electrophoresis (PAGE). Gels were fixed as previously described (7). For immunoblots, gels were transferred to polyvinylidene difluoride paper (Millipore Corp., Bedford, Mass.) by using a semidry apparatus (Hoefer Scientific Instruments, San Francisco, Calif.), blocked at room temperature for 30 min with blotting buffer (100 mM Tris-HCl [pH 8.0], 1.5 M NaCl, 0.5% polyoxyethylene [20] sorbitan monolaurate) containing 3% albumin, and incubated with antibodies diluted 1:1,000 in blotting buffer. Alkaline phosphatase-linked secondary antibodies (anti-mouse or anti-rabbit) were used, and immunoblots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma). The biotinylated antibodies used for enhanced chemiluminescence blots were prepared as previously described (7).

Immunofluorescence. Cell fixation and staining were performed by previously described procedures (13). A31 cells were plated on glass coverslips on 100-mm-diameter dishes and infected as described above. At 30 h postinfection, cells were washed with PBS three times, fixed with fresh 4% paraformaldehyde for 10 min, and treated for 1 min with 1% NP-40. Cells were then dehydrated with successive washes of cold ethanol at 50% (3 min), 100% (5 min), and 50% (3 min). Cells were incubated with primary antibody for 30 min, washed three times with PBS, and incubated with the secondary fluorescein isothiocyanate-linked goat anti-rabbit or tetramethyl rhodamine B isothiocyanate-linked goat anti-mouse antibody (Sigma) for 30 to 60 min at 37°C. Cells were then washed three times with PBS and once with water. Stained cells were photographed by fluorescence microscopy with ASA 200 Ektachrome slide film. Exposures ranged from 1 to 16 s for the green (fluorescein isothiocyanate) channel and 24 to 79 s for the red (tetramethyl rhodamine B isothiocyanate) channel.

In vitro translation. The construction of plasmids pSP6-VP1, pSP65-VP2, and pSP65-VP3 has previously been described (8, 29). Plasmids were linearized with *Bgl*II (pSP6-VP1) or *Hin*dIII (pSP65-VP2 and pSP65-VP3), extracted with phenol-chloroform, and precipitated with ethanol. RNA was synthesized with SP6 polymerase by using the nuclease-treated rabbit reticulocyte lysate system (Promega, Madison, Wis.), extracted with phenol-chloroform, and precipitated by the addition of ammonium acetate to a final concentration of 3 M. Each 50-μl translation reaction mixture contained 1 μg of RNA, 1 μl of amino acid mix, 5 μl of [^{35}S]methionine, and 35 μl of hemin- and nuclease-treated rabbit reticulocyte lysate (Promega) and was incubated at 30°C for 1 to 6 h. Apyrase (EC 3.6.1.5) was added as indicated at 20 U/ml for 10 min.

RESULTS

Coimmunoprecipitation of polyomavirus coat proteins with hsc70 during viral infection. A possible association of the viral capsid proteins with cellular chaperone proteins was first tested by immunoprecipitation. Lysates from mock-infected, heat-shocked, and polyomavirus-infected A31 cells were immunoprecipitated with an anti-VP1 antibody (I58) and a polyclonal rabbit anti-hsp70 antibody which recognizes both hsp70 and hsc70 (Fig. 1A). As expected, hsp70 protein accumulation in cells exposed to heat shock (Fig. 1A, HS, lane 3) was higher than that in mock-infected cells (M, lane 3). Polyomavirus infection had no apparent effect on hsp70 protein accumulation at 25 or 50 h after infection compared with that in mock-

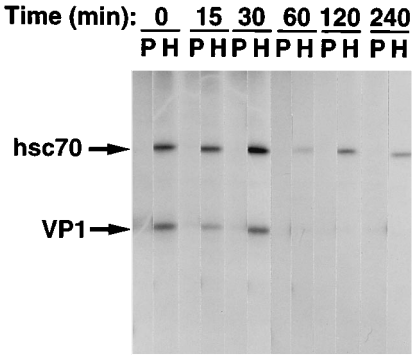

FIG. 2. Pulse-chase analysis of the VP1-hsc70 association. Virus-infected cells were labeled for 5 min and chased for the times indicated. Lysates were immunoprecipitated with either preimmune rabbit serum (lanes P) or anti-hsc70 antibody SPA815 (lanes H).

infected cells (Fig. 1A; compare lanes 3). In lysates from infected cells, VP1 coimmunoprecipitated with the anti-hsp70 antibody (Fig. 1A, lanes 3). In this experiment, a 70-kDa species was not evident in immunoprecipitations with the anti-VP1 antibody (Fig. 1A, lanes 2).

Immunoprecipitations with anti-VP2/VP3 antibodies (R3 and R4) of lysates from virus-infected cells demonstrated a 70-kDa protein in addition to VP2 and VP3 (Fig. 1B). In this experiment, a similar 70-kDa band was also faintly visible in immunoprecipitates with anti-VP1 antibodies, particularly with the monoclonal antibody Fd9 (Fig. 1B, lane 3). To confirm that this protein is a member of the hsp70 family, these immunoprecipitates were subjected to SDS-PAGE and immunoblotted with a monoclonal antibody recognizing hsc70 and hsp70 (Fig. 1C). The 70-kDa protein was identified with a specific monoclonal antibody which recognizes hsc70 (SPA815), the constitutively expressed cognate of hsp70, but not with an antibody which recognizes only hsp70 (SPA810) (data not shown). No viral proteins immunoprecipitated with antibodies to hsp90, hsp60, and hsp27 chaperone proteins (data not shown).

A pulse-chase analysis was performed to determine the time course of the hsc70-VP1 association relative to capsid protein synthesis. Virus-infected cells were pulse-labeled for 5 min and harvested at various times after labeling. The coimmunoprecipitation of VP1 with anti-hsc70 antibody was detected up to 30 min after labeling (Fig. 2).

Virions purified by CsCl gradient centrifugation from infected cells were also tested for hsc70 by immunoblot (data not shown). At the limits of detection (estimated to be less than one molecule per virion), no hsc70 was detected. Therefore, the association occurs immediately after capsid protein synthesis but does not persist once the capsid proteins are completely assembled.

Nuclear localization of hsc70 and capsid proteins during virus infection. Double-staining immunofluorescence was used to assess the subcellular localization of hsc70 during viral infection. In the majority of cells demonstrating nuclear T antigen (Fig. 3A), hsc70 remained predominantly cytoplasmic (Fig. 3B), with the exception of nuclear staining in some cells which showed intense nuclear staining for T antigen (not shown). When cells were double stained for hsc70 and VP2/VP3 (R4 antibody), however, those with a strong nuclear hsc70 signal (Fig. 3D) were limited to those that exhibited a nuclear VP2/VP3 signal (Fig. 3C). Note that the uninfected cells in Fig. 3C maintained the cytosolic hsc70 distribution seen in Fig. 3D. The staining observed with the anti-VP1 antibody (I58) was indistinguishable from that obtained with anti-VP2/VP3 antibody (data not shown). Identical results were obtained with antibodies specific for hsc70 and antibodies recognizing both hsp70 and hsc70, whereas no nuclear localization was seen with antibody specific for hsp70 (data not shown). Controls with preimmune sera demonstrated the specificity of staining for the anti-VP2 and anti-hsc70 antibodies with cells late in infection which exhibited nuclear localization of capsid proteins and hsc70 (Fig. 3E to H).

Binding to hsc70-like proteins occurs during recombinant capsid protein expression. To determine if the binding of hsc70 to viral capsid proteins was independent of other viral factors, polyomavirus capsid proteins VP1 and VP2, expressed in Sf9 insect cells by recombinant baculovirus vectors, were tested for coimmunoprecipitation with anti-hsp70 antibodies. As shown in Fig. 4, VP1 and VP2 expressed in Sf9 cells were immunoprecipitated with a rabbit polyclonal antibody recognizing DnaK, the *E. coli* homolog of hsp70. An anti-hsp60 antibody was used as a negative control (Fig. 4, lanes 3).

VP1 has previously been expressed in *E. coli* and purified to near homogeneity (19). After phosphocellulose chromatography elution in the purification scheme, VP1 is approximately 90% pure. One of the remaining contaminating proteins in this fraction migrated to 70 kDa and was recognized by anti-DnaK antibody by immunoblot analysis (data not shown). To determine if VP1 and DnaK were present in a complex, these fractions of recombinant VP1 were immunoprecipitated with anti-DnaK antibody. As estimated by densitometric scanning of gels stained with Coomassie blue, the anti-DnaK antibody immunoprecipitated approximately 25 to 30% of the total VP1 present (determined by immunoprecipitation with the anti-VP1 antibody) (data not shown). Furthermore, as expected for specific association with DnaK, the amount of VP1 coimmunoprecipitated was markedly reduced by the addition of 10 mM $MgCl_2$–10 mM ATP to the wash buffer for anti-DnaK immunoprecipitation. DnaK also coimmunoprecipitated with the anti-VP1 antibody (Fig. 5). Mg-ATP added during the wash steps decreased this coimmunoprecipitation, whereas Mg-ATP had no effect on the amount of DnaK immunoprecipitated with anti-DnaK antibody. These results suggest that DnaK is complexed with VP1 expressed in *E. coli* and copurifies with VP1 through two chromatography steps.

In vitro-translated coat proteins bind hsc70. The interactions of viral capsid proteins with hsc70 were also characterized after in vitro translation of VP1, VP2, and VP3. As determined by immunoblot analysis, the rabbit reticulocyte lysate contained abundant hsc70 (data not shown). Because of the ATP-regenerating system added to the lysate for translation, interactions between hsc70 and other proteins might be transient and difficult to detect. To detect such associations, an enzyme capable of hydrolyzing ATP, apyrase, was added in some experiments to prevent the dissociation of hsc70-protein complexes. By this method, all three polyomavirus capsid proteins were found to bind hsc70 (Fig. 6, lanes 5), although the strongest interaction was with VP2. In each case, binding was markedly decreased by the addition of 10 mM ATP–10 mM $MgCl_2$ during the immunoprecipitation washing steps (Fig. 6, lanes 6), as expected for proteins which specifically bind members of the hsp70 family. Binding to hsp70 was not detected (Fig. 6, lanes 3), perhaps because of its low abundance in reticulocyte lysates.

DISCUSSION

The polyomavirus VP1 capsid protein assembles only in the nucleus, not the cytosol, despite its ability to self-assemble in

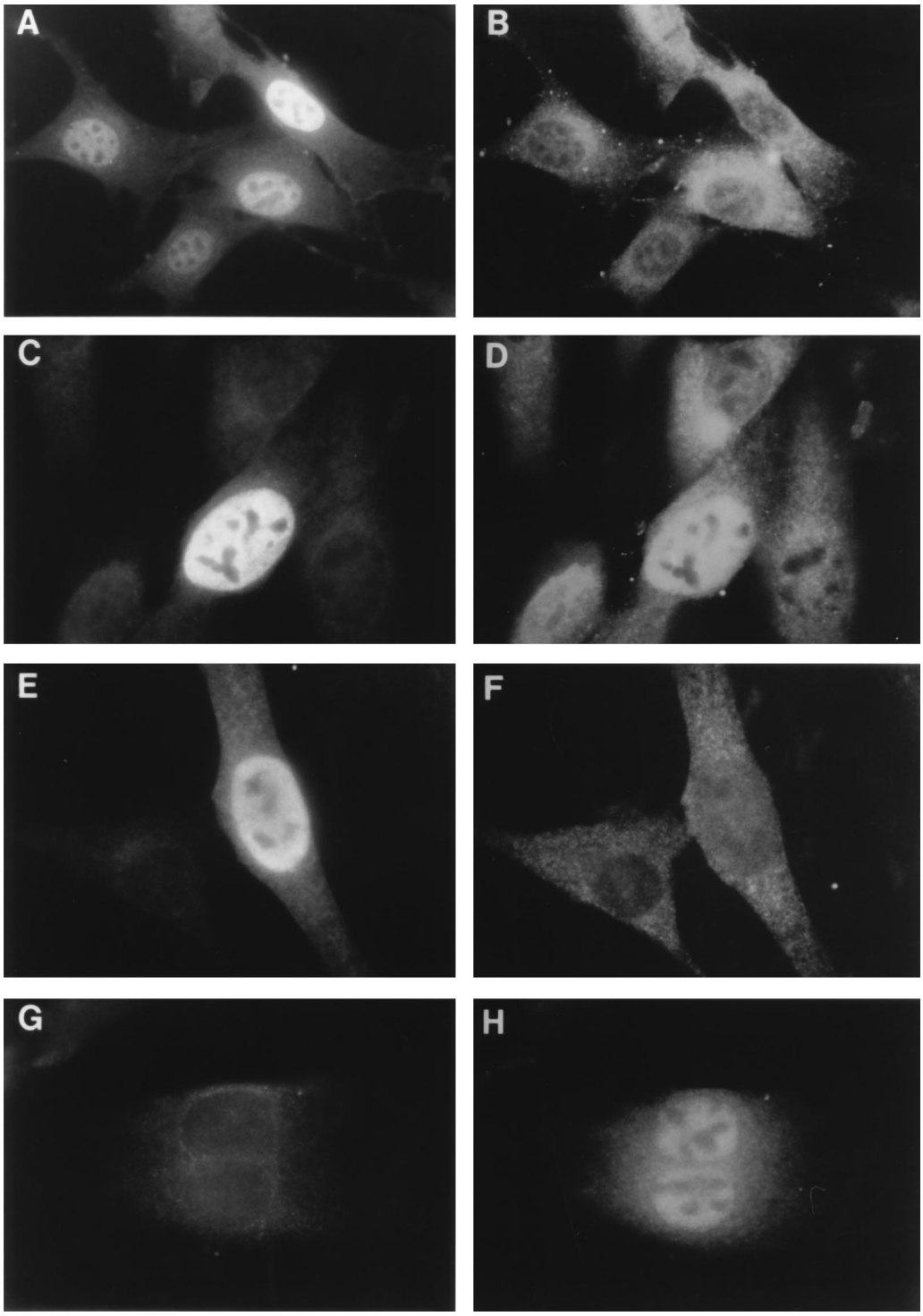

FIG. 3. Polyomavirus infection induces diffuse nuclear localization of hsc70. Infected A31 cells were double stained with mouse anti-hsp70/hsc70 antibody (A to D, G, and H) and either rabbit anti-T-antigen antibody (A and B), rabbit anti-VP2 antibody (C to F), or preimmune rabbit serum (G and H). Preimmune mouse serum was used as a control (E and F). Anti-rabbit secondary antibody (A, C, E, and G) and anti-mouse secondary antibody (B, D, F, and H) were used. Magnifications, ×306 (A and B) and ×484 (C to H).

vitro into capsids (27, 37). To investigate the regulation of in vivo capsid assembly, we examined the association of cellular chaperone proteins with capsid proteins. We found that during polyomavirus infection of mouse cells, the cellular hsc70 chaperone protein coimmunoprecipitated with all three capsid proteins. hsc70 also appeared by immunofluorescence to colocalize temporally in the nucleus with capsid proteins. The association with chaperone proteins was also seen during capsid protein expression in Sf9 insect cells, *E. coli*, and reticulocyte lysates. Thus, the polyomavirus capsid proteins

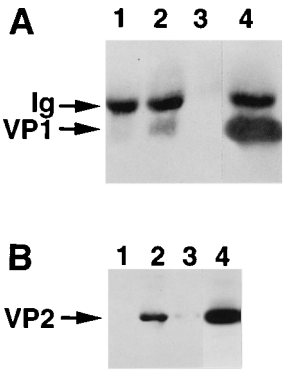

FIG. 4. DnaK-like protein associates with VP1 and VP2 expressed in Sf9 insect cells. Lysates from cells infected with recombinant baculoviruses expressing VP1 (A) and VP2 (B) were immunoprecipitated with preimmune rabbit serum (lanes 1), anti-DnaK antibody AXL623 (lanes 2), mouse anti-hsp60 antibody SPA804 (lanes 3), anti-VP1 antibody I58 (lane 4 in panel A), or anti-VP2/VP3 antibody R4 (lane 4 in panel B). Ig, immunoglobulin.

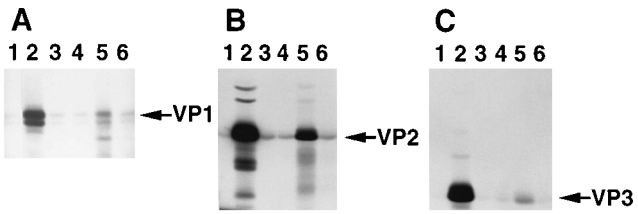

FIG. 6. Binding of hsc70 to in vitro-translated capsid proteins. Reticulocyte lysate translation reaction mixtures were incubated with apyrase to hydrolyze ATP and immunoprecipitated with preimmune rabbit serum (lanes 1), anti-VP1 antibody I58 (lane 2 in panel A), anti-VP2/VP3 antibody R4 (lanes 2 in panels B and C), polyclonal anti-hsp70 antibody (lanes 3 and 4), or anti-hsc70 antibody SPA815 (lanes 5 and 6). Lanes 4 and 6, $MgCl_2$ and ATP were added during immunoprecipitation washes.

appear to complex with chaperone proteins in several contexts, suggesting that this association is an intrinsic property of these proteins. Consistent with the association of other viral proteins with chaperone proteins, interaction with hsc70 could be an integral part of the polyomavirus assembly pathway, facilitating efficient virus production by several mechanisms.

Complex formation between polyomavirus capsid proteins and hsc70 appears to be specific and short-lived. Maximal association was detected after a 5-min pulse-label (Fig. 2), suggesting that hsc70 binds newly synthesized capsid proteins. As demonstrated by pulse-chase, the complex dissociated between 30 and 60 min after capsid protein synthesis, which may explain the faint coimmunoprecipitating bands seen when cells were radiolabeled for several hours (Fig. 1). The binding was specific, as evidenced by its disruption upon the addition of Mg-ATP, a hallmark of protein-protein interactions by the hsp70 family. In vivo, the binding was also specific for hsc70 rather than for other chaperone family members. The time frame of association corresponds to that determined for the transport of newly synthesized SV40 capsid proteins to the nucleus (10 to 30 min) (22) and to the minimal transit time (2 h) for polyomavirus replication complexes to become encapsidated (10). Given our observation that hsc70 was not detected in virions, these kinetic determinations support the hypothesis that hsc70 dissociates from the capsid proteins before the minichromosome is encapsidated.

hsp70 proteins are known to play a role in the nuclear translocation of SV40 large T antigen and other karyophilic proteins containing nuclear localization signals (16, 41, 46). Although microinjection experiments have shown that hsc70 can shuttle between the cytoplasm and nucleus (25), whether hsp70-like proteins involved in nuclear transport traverse the nuclear membrane or transfer their substrate to the nuclear pore complex is unknown. The nuclear accumulation of hsc70 seen by immunofluorescence was not observed until late in infection, coincident with the accumulation of nuclear capsid proteins, suggesting that hsc70 is important for the nuclear localization of polyomavirus capsid proteins. It is possible that hsc70 is not predominantly nuclear early in infection during the expression of large T antigen because it is released from large T antigen at the nuclear pore complex or it exits the nucleus too rapidly to accumulate. The observed late nuclear accumulation of hsc70 may be due to a relatively prolonged association with the capsid proteins compared with that of large T antigen or to a kinetic consequence of the large amounts of viral capsid proteins being shuttled to the nucleus. In contrast to polyomavirus, SV40 encodes a protein (agnoprotein) which is expressed late in infection and is required for efficient nuclear localization of SV40 VP1 in some cell types (30, 34). Agnoprotein may also be involved in viral assembly, since strains containing mutations of agnoprotein exhibit small plaque sizes. During SV40 infection, the role of agnoprotein may thus overlap with that of hsc70, with the agnoprotein function becoming essential in cells with insufficient endogenous hsc70-like chaperone proteins.

In cells exposed to heat shock, hsp70 and hsc70 undergo characteristic redistribution in their subcellular localization from diffusely cytoplasmic to predominantly nucleolar (2). After cells have been returned to a normal temperature, these proteins again localize to the cytoplasm. Virus-induced nuclear localization of hsp70-like proteins during infection with adenovirus has previously been demonstrated and was interpreted to be a protective host response. Specifically, E1A protein expression was sufficient to induce the normally diffuse hsp70 into a nucleolar pattern (44) similar to that seen after heat shock. Such a pattern is distinctly different from the diffuse nuclear pattern observed late in polyomavirus infection, which spared the nucleoli and paralleled the distribution of VP1 and VP2. Thus, the nonnucleolar distribution of hsc70 during polyomavirus infection argues against a simple protective stress response function for this chaperone like that thought to occur during heat shock.

The association of hsc70 with the VP1 and VP2 capsid proteins expressed in Sf9 cells, of DnaK with VP1 expressed in *E. coli*, and of hsc70 with all three capsid proteins expressed by in vitro translation may be related to improper folding of ectopically expressed proteins or to the intrinsic affinities of these proteins for chaperones. These results also demonstrate that this interaction is independent of those with other viral proteins, does not require posttranslational modification of VP1 (VP1 expressed in *E. coli* is unmodified) (19), and has been preserved across species. Some fraction of these proteins, particularly VP2, which becomes associated with cytoplasmic

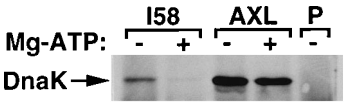

FIG. 5. DnaK copurifies with VP1 expressed in *E. coli*. Immunoblot with anti-DnaK antibody of purified VP1 immunoprecipitated with rabbit anti-VP1 antibody I58 or rabbit anti-DnaK antibody AXL623. Immunoprecipitates were washed in the absence (−) or presence (+) of $MgCl_2$ and ATP. Lane P, preimmune rabbit antiserum.

membranes (7), may be improperly folded, but recombinant VP1 appears to be structurally and functionally intact (37, 38). Although 25 to 30% of the VP1 pentamers isolated after expression in *E. coli* copurified with DnaK, it is possible that more pentamers were once bound to DnaK within bacteria. Overexpression of VP1 occurs both in *E. coli* and in virus-infected cells, so the stoichiometry of association with chaperone proteins in recombinant systems may be similar to that occurring during viral infection. However, there is presently no evidence that purified VP1 associated with DnaK is blocked in its ability to self-assemble in vitro, although capsid-like aggregates have never been detected (as determined by electron microscopy) (9a) in the cytoplasm of *E. coli* expressing VP1.

Recombinant VP1 expressed in *E. coli* is purified as pentamers, equivalent to those found in virions whose structures are known to atomic resolution (21, 33, 42). The carboxy terminus of each VP1 monomer protrudes from the pentamer and interacts with adjacent pentamers, while the amino-terminal 28 amino acids, in which the DNA binding and nuclear localization signal domains are located (4, 5, 28, 29), are disordered in the high-resolution structure within the interior of the virion. The large loop domains of each monomer which are located on the outer surface of the capsid may function in binding the cell surface receptor for the virus (42). Therefore, both termini and perhaps the external loop domains of each unassembled pentamer are essentially exposed or unfolded relative to their final conformations in the virion. DnaK or hsc70 may bind one or more of these domains in an unassembled pentamer, possibly protecting them from interactions with other cellular proteins that would interfere with virion assembly. Indeed, genetic evidence suggests that agnoprotein binds one of the external loop domains of SV40 (1, 3, 26). Further studies are needed to determine whether in vitro capsid self-assembly with the recombinant VP1 protein releases DnaK from VP1 or whether variable fractions of VP1 molecules associated with DnaK do not participate in the in vitro assembly reaction.

This study provides evidence of a role for hsc70 late during polyomavirus infection. Direct genetic evidence for the role of hsc70 in virus infections is not possible because cells with a functional hsc70 deleted are nonviable. However, given the association of chaperone proteins with other viral proteins, we propose that hsc70 contributes actively to facilitate efficient polyomavirus production by (i) transiently binding to capsid proteins, preventing cytoplasmic assembly of capsids; (ii) facilitating nuclear transport of the capsid proteins; (iii) controlling intranuclear capsid assembly; and (iv) sequestering improperly folded capsid protein subassemblies. These activities may be accomplished by the known ability of hsc70 to shield unfolded domains of proteins from interfering contacts. In addition, the large T antigens of both SV40 and polyomavirus have been shown to stimulate hsp70 protein expression. This increased expression may represent a strategy to induce chaperone proteins for use at times late in the virus life cycle. Virus-induced stimulation of hsp70 or hsc70 protein accumulation was not seen in this study, though such an effect may be a function of the particular cells or conditions used. The natures of hsc70- and DnaK-capsid protein complexes and their role in virus capsid assembly may now be further explored with recombinant and in vitro-translated capsid proteins.

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