

Dominant-interfering Hsc70 Mutants Disrupt Multiple Stages of the Clathrin-coated Vesicle Cycle In Vivo[Ⓞ]

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Abstract. Within the clathrin-coated vesicle (CCV) cycle, coat assembly drives the internalization of receptors from the cell surface and disassembly allows for the processing of internalized ligands. The heat shock cognate protein, hsc70, has been implicated in regulating coat disassembly. We find that in cells overexpressing ATPase-deficient hsc70 mutants, uncoating of CCVs is inhibited in vivo, and the majority of unassembled cytosolic clathrin shifts to an assembled pool that cofractionates with AP1 and AP2. Surprisingly, this assembled pool of coat proteins accumulates in the absence of cargo receptors, suggesting that disruption of hsc70 activity may cause misassembly of empty clathrin cages. The strongest effect of overexpression of hsc70 mutants is a block in transferrin receptor (TfnR) recycling,

which cannot be accounted for by the degree of inhibition of uncoating of endocytic CCVs. These results suggest that hsc70 participates in multiple transport and/or sorting events between endosomal compartments. Additionally, the mutant-expressing cells are defective at internalizing transferrin. In the most potent case, the initial rate of uptake is inhibited 10-fold, and TfnR levels double at the cell surface. Our findings demonstrate that hsc70 indeed regulates coat disassembly and also suggest that this chaperone broadly modulates clathrin dynamics throughout the CCV cycle.

Key words: Hsc70 • endocytosis • clathrin-coated vesicles • transferrin receptor • recycling

Introduction

Receptor-mediated endocytosis (RME)¹ is driven by the clathrin-coated vesicle (CCV) cycle (Schmid, 1997). This cycle begins with the assembly of the coat proteins, AP2, and clathrin into coated pits. AP2 complexes concentrate receptors and also mediate clathrin assembly at the plasma membrane (PM) (Kirchhausen, 1999). Clathrin assembles into a lattice-like structure and induces inward curvature of the pit as it grows with the successive addition of coat proteins. The coated pit invaginates and eventually detaches from the PM, forming a coated vesicle (Kirchhausen, 2000). The CCV cycle is completed when the coat components are released and recycled.

One factor implicated in releasing coat proteins from CCVs is hsc70. This chaperone was purified as a factor that released clathrin from isolated coated vesicles in an

ATP-dependent manner (Schlossman et al., 1984). More recently, hsc70 was also identified as a requirement for AP2 release from isolated CCVs (Hannan et al., 1998). Hsc70 is a constitutive member of the heat shock protein family and is thought to function in multiple cellular processes, including the folding of nascent chain polypeptides, the translocation of proteins across membranes, and the prevention of protein aggregation under stress conditions (Mayer and Bukau, 1998). These diverse hsc70 activities are, in part, facilitated through interactions with a series of DnaJ-like proteins (Kelley, 1999). The supporting role played by DnaJ-like proteins is demonstrated by auxilin, which mediates the CCV uncoating reaction (Ungewickell et al., 1995). Auxilin serves to recruit hsc70 to CCVs and concomitantly stimulates hsc70 ATPase activity (Holstein et al., 1996; Barouch et al., 1997). In its ADP-bound state, hsc70 exhibits high affinity for protein substrates, including clathrin (Prasad et al., 1994).

Hsc70 has been implicated in many cellular processes, but it has been difficult to establish these activities in vivo. To this end, an anti-hsc70 mAb was used in microinjection studies to characterize the involvement of hsc70 in the clathrin uncoating reaction in vivo (Honing et al., 1994). Microinjection of anti-hsc70 caused a prolonged colocalization between internalized ligand and clathrin and an ac-

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¹Abbreviations used in this paper: AP, adaptor protein; BXX-Tfn and BSS-Tfn, biotinylated Tfn; CCV, clathrin-coated vesicle; Endo H, endoglycosidase H; HA, hemagglutinin; MPR, 300-kD mannose 6-phosphate receptor; PM, plasma membrane; RME, receptor-mediated endocytosis; Tfn, transferrin; TfnR, Tfn receptor.

cumulation of coated pits at the cell surface. These *in vivo* studies supported a role for hsc70 in CCV disassembly and suggested that it might have additional functions within the CCV cycle. However, due to the single cell nature of this assay, neither the accumulating coated structures nor the nature of the endocytic block could be further analyzed.

An alternate approach was used to probe the cellular functions of the hsc70-related protein BIP. Overexpression of dominant interfering mutants of BIP was used to reveal its involvement in the maintenance of ER morphology in mammalian cells (Hendershot et al., 1995). To initially determine whether this approach could be used to explore hsc70 function *in vivo*, we generated and characterized several hsc70 ATPase domain mutants defective in CCV uncoating and found that they inhibited the wild-type hsc70-mediated release of clathrin from isolated CCVs. We next examined the effect of overexpression of the dominant-interfering hsc70 mutants on the CCV cycle using assays that allow dissection of discrete events in endocytosis. Our findings support a role for hsc70 in the clathrin uncoating reaction *in vivo* but, more strikingly, suggest novel roles for hsc70 in chaperoning cytosolic clathrin and in endosomal trafficking.

Materials and Methods

Reagents

The following mAbs were gifts from the indicated individuals: anti- α adaptin AP.6 and anticlathrin TD1 from F.M. Brodsky (University of California at San Francisco, San Francisco, CA), anti-transferrin receptor (TfnR) D65 from I. Trowbridge (Salk Institute, La Jolla, CA), anti-300-kD mannose-6 phosphate receptor (MPR) from S. Pfeffer (Stanford University, Stanford, CA), anti- β 1/ β 2 adaptin 100/1 from E. Ungewickell (Hanover Medical School, Hanover, NH), anti-hsc70/hsp70 3C5 from B.M. Jockusch (University of Cologne, Cologne, Germany), and anti-hemagglutinin (HA) 12CA5 from I. Wilson (The Scripps Research Institute, La Jolla, CA). Polyclonal antibodies were kindly provided as follows: anticlathrin light chain from F. M. Brodsky, anti-MPR from S. Pfeffer, and anti- δ adaptin from M.S. Robinson (Cambridge University, Cambridge, MA). Anti-DnaK SPA-880 and anti-hsp70 SPA-810 were obtained from StressGen Biotechnologies. Additional chemicals were of reagent grade. Biotinylated transferrin (BXX-Tfn) was generated as described previously (Smythe et al., 1992).

Preparation of cDNA Constructs

Constructs BovHsc70.pRSET.wt, encoding full-length hsc70_{WT}, and BovHsc70.pT7-7.D199S and BovHsc70.pT7-7.K71M, encoding 60-kD NH₂-terminal hsc70 mutant fragments, were generously supplied by D.B. McKay (Stanford University, Stanford, CA). For protein expression, pT7-7.Hsc70WT was generated using NdeI/HindIII sites to exchange the sequence encoding the full-length wild-type protein from pRSET.wt into the pT7-7 vector. DNA encoding the COOH-terminal domains of hsc70 was restored to the above truncated mutant constructs using NdeI/BlnI restriction sites. Full-length pT7-7.Hsc70.T204V was generated using QuikChange Mutagenesis kit (Stratagene) (forward primer, GATTTAGGGGTGGCGTTTTTGATGTG; reverse primer, CACATGAAAAACGCCACCCCCTAAATC). An HA tag was introduced at the NH₂ terminus of hsc70 by ligating a cassette of synthetic oligomers (forward primer, CATGGA-GTATGATGTTCCCTGATTATGCTCA; reverse primer, TATGAGC-ATAATCAGGAACATCATACTCCA) within the NdeI site of pT7-7.Hsc70WT.

For adenoviral expression, the XbaI-excised HA-hsc70-encoding fragment of pT7-7.HA-Hsc70 was subcloned into pADtet7, which carries the tetracycline-regulated promoter. Mutations were introduced into this construct by swapping in the PstI-excised fragment from the corresponding mutant pT7-7.Hsc70. The generated constructs were sequenced to verify correct engineering by the The Scripps Research Institute Protein and Nucleic Acids Core Facility.

Proteins

CCVs were purified from either fresh or frozen bovine brain (Hannan et al., 1998). Protein concentration was determined by Coomassie Protein Assay (Pierce Chemical Co.). All recombinant proteins were expressed in BL21(DE3) and harvested by lysis in buffer A (20 mM Hepes, pH 7, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) supplemented with 25 mM KCl. Native hsc70 was purified from the first high speed supernatant obtained from the bovine brain CCV preparation. Native and recombinant hsc70 proteins, including HA-hsc70, were purified by sequential chromatography first on Q-Sepharose (2.5 \times 16 cm; Amersham Pharmacia Biotech) and then on ATP agarose (catalogue no. 2767; 1.5 \times 15 cm; Sigma-Aldrich) as described (Schlossman et al., 1984; Greene and Eisenberg, 1990). The resulting protein was precipitated with 75% (NH₄)₂SO₄ in the presence of 10 mM EDTA to promote the dissociation of ATP (Buxbaum and Woodman, 1996). Recombinant hsc70 was further purified by size exclusion chromatography on Superose S-300 (1.5 \times 95 cm; Amersham Pharmacia Biotech) in buffer A containing 100 mM KCl, followed by chromatography on Q-Sepharose (1.5 \times 10 cm). Hsc70 was eluted with a 25–250-mM KCl linear gradient in buffer A (200 ml, 1 ml/min). The latter column completely removed contaminating DnaK as verified by Western blotting. The molar extinction coefficient, $\epsilon_{1\%} = 6.2$, was used to quantify native and recombinant hsc70.

Adenoviral Expression of Hsc70 WT and Mutant Proteins in tTA HeLa Cells

Adenoviruses carrying the hsc70 genes were prepared from the pADtet7 hsc70 constructs as described earlier (Hardy et al., 1997; Altschuler et al., 1998). Within this expression system, the gene is introduced under the control of a tetracycline-repressible promoter. For all of the assays described below, subconfluent dishes of cells were infected for 2 h with the respective viruses at 400 pfu/cell and were subsequently incubated in fresh DME/10% FBS for 16 h.

Fractionation Procedures

Oligomerization of Endogenous Hsc70. For analysis of hsc70 oligomerization, 2 \times 10⁶ cells were infected on 10-cm dishes. After infection, the cells were released with PBS plus 5 mM EDTA, washed once with ice-cold KH250KCl buffer (100 mM KOAc, 20 mM Hepes, 250 mM KCl, complete protease inhibitors [Roche], pH 7.4) containing either 1 mM MgOAc or 1 mM NaN₃, 5 mM EDTA and 2 mM deoxyglucose (ATP-depleting), and finally resuspended in 100 μ l of the respective buffer. After incubating the cells at 4°C for 10 min, cytosol was prepared through rapid freeze/thaw and centrifugation at 20,000 g for 5 min. The supernatant was fractionated on Superdex 200 HR 10/30 (Amersham Pharmacia Biotech) at 0.25 ml/min run with 20 mM Hepes, 100 mM KCl, 2 mM MgCl₂, pH 7. Samples prepared with KH250KCl and 1 mM MgOAc were incubated with 1 mM Mg²⁺ATP and a regenerating system for 10 min at 37°C before fractionation. Fractions were analyzed by SDS-PAGE and Western blotting.

Subcellular Fractionation of Adenovirally Infected Cells. Infected cells were released from 15-cm dishes with PBS plus 5 mM EDTA. The cells (10⁷) were washed with 100 mM Mes, pH 6.2, 1 mM EGTA, 0.5 mM MgCl₂, 250 mM KCl, 250 mM sucrose and complete protease inhibitors, resuspended in 200 μ l of the same buffer, and disrupted by freeze-thaw. Rigorous mixing via a Vortex and drawing the suspension through a 22-gauge needle released coated vesicles from larger membranes. Subsequent centrifugation at 14,000 g pelleted the heavy membranes including the PM, Golgi, and endosomal compartments. The low speed supernatant was centrifuged at 100,000 g, generating a high speed pellet containing the vesicular pool and a high speed supernatant corresponding to the cytosolic pool. Alternatively, the low speed supernatant was loaded onto an S-1000 column (1.0 \times 90 cm; Amersham Pharmacia Biotech) run at 0.07 ml/min (Phan et al., 1994). 2-ml fractions were collected, TCA precipitated, and analyzed by SDS-PAGE and Western blotting.

Biochemical Assays

Clathrin Release Assay. Hsc70-mediated clathrin release was performed for 8 min at 25°C in 20 mM Hepes, pH 7.0, 25 mM KCl, 2 mM Mg²⁺/ATP as previously described (Hannan et al., 1998).

Single Round Endocytosis and Recycling from the Endosomal Compartment. The internalization of prebound BXX-Tfn was performed as described previously (Sever et al., 2000).

Determination of Surface-bound TfnR. The steady state distribution of surface-bound TfnR was determined by monitoring the relative amounts of BXX-Tfn that bound to virally infected cells in the absence and presence of saponin. After the infection of 10^5 cells grown in 12-well plates, the cells were washed twice with ice-cold PBS, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ (PBS^{2+}). For the subsequent $4^\circ C$ washes that follow, the cells in half the wells were permeabilized with $PBS^{2+}/0.05\%$ saponin, and the cells in the remaining wells were left intact and washed with PBS^{4+} . The cells were initially incubated with $PBS^{4+} \pm$ saponin for 30 min and subsequently were incubated with $4 \mu g/ml$ BXX-Tfn in $PBS^{4+} \pm$ saponin for 2 h. The cells were then washed four times with $PBS^{4+} \pm$ saponin and twice with PBS. To determine total cell-associated BXX-Tfn, the cells were lysed in blocking buffer (10 mM Tris HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.2% BSA), and BXX-Tfn was quantified by ELISA, as described previously (Damke et al., 1994).

Tfn Receptor Biosynthesis. Trafficking of a pulse of ^{35}S -labeled TfnR through the Golgi region was monitored by the acquired resistance to endoglycosidase H (Endo H) cleavage and by the addition of complex oligosaccharides as described previously (Omary and Trowbridge, 1981; Damke et al., 1994).

Fluid Phase Uptake. HRP was used as a bulk flow marker as described earlier (Damke et al., 1994).

Internalization of Alexa₄₈₈-Tfn. For immunofluorescence experiments, cells were grown and infected on coverslips. To follow RME, a final concentration of $50 \mu g/ml$ Alexa₄₈₈-Tfn (Molecular Probes) was incubated with PBS-washed cells for 5 min at $37^\circ C$. The coverslips were rinsed with PBS^{2+} and incubated with $100 \mu g/ml$ unlabeled Tfn. At designated times, the coverslips were placed at $4^\circ C$, and at the completion of the time course the coverslips were washed with PBS^{2+} , subjected to a 2 min $37^\circ C$ acidic wash (Dautry-Varsat et al., 1983) that removed the majority of bound Tfn as indicated by the respective $4^\circ C$ control, and washed again with PBS^{2+} . Cells labeled with Alexa₄₈₈-Tfn, as well as unlabeled cells used for additional labeling studies, were fixed with 3% formaldehyde and solubilized with $PBS^{2+}/0.1\%$ saponin/5% goat serum. Subsequent to fixation and permeabilization, cells were incubated with primary antibodies as indicated in the figure legends and these were detected with the following secondary antibodies: Texas red-labeled goat anti-rabbit and goat anti-mouse (Molecular Probes) and FITC-labeled goat anti-mouse (Sigma-Aldrich). A Zeiss Axiovert 100TV and Spot 32 camera were used to collect digital epifluorescent images. Additional immunofluorescence data were collected with a confocal microscope (MRC 1024; Bio-Rad Laboratories).

Online Supplemental Material

Figure S1 shows EM images taken of thin sections prepared from epon-embedded, adenovirally infected tTA HeLa cells. Similar cell morphology is observed between $hsc70_{WT}$ - and $hsc70_{K71M}$ -expressing cells. The supplemental figure and legend are available at <http://www.jcb.org/cgi/content/full/152/3/607/DC1>.

Results

Hsc70 ATPase Domain Mutants Are Dominant-interfering In Vitro

To identify hsc70 ATPase-deficient mutants that could dominantly interfere with hsc70 function in vivo, we assayed three hsc70 mutants in the in vitro clathrin uncoating reaction. The mutated residues (K71, D199, and T204) are all found within the nucleotide binding site (Flaherty et al., 1991) and were originally introduced to understand their contribution to hsc70 ATPase cycling. Hsc70_{T204V} was formerly characterized within the full-length protein and exhibited normal rates of ATP hydrolysis but bound ATP ~ 100 -fold more weakly than hsc70_{WT} (O'Brien and McKay, 1993). Hsc70_{D199S} and hsc70_{K71M} were previously characterized within the 44-kD ATPase fragment and exhibited ATPase rates that were either 50-fold reduced or undetectable compared with hsc70_{WT}, respectively (Wilbanks et al., 1994; O'Brien et al., 1996). Analysis of a 60-kD fragment containing the ATPase and

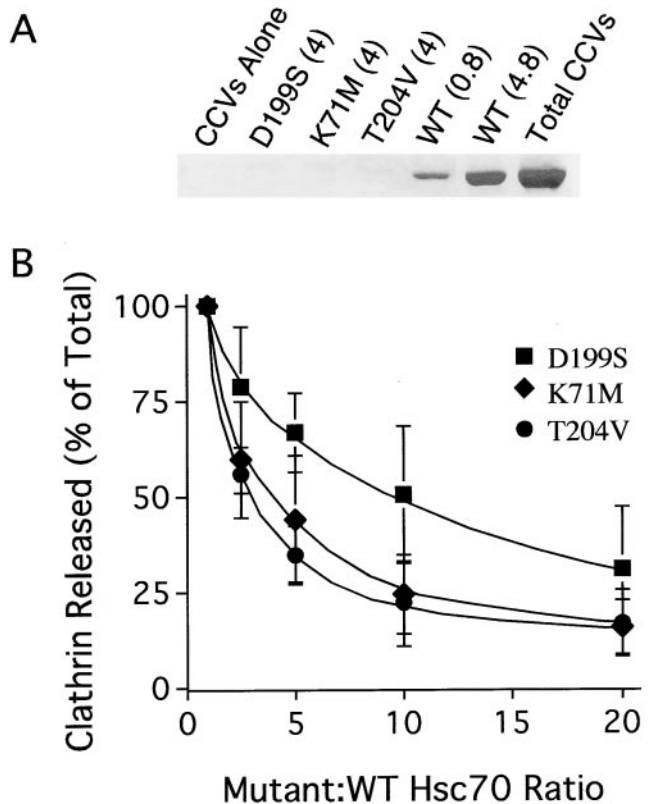


Figure 1. Hsc70 ATPase domain mutants inhibit hsc70-mediated clathrin uncoating. CCVs were incubated in the presence of wild-type and mutant hsc70 proteins. (A) Shown is a Coomassie blue-stained gel of clathrin released into the 100,000-g supernatant. The final μM concentration of recombinant protein used is indicated in parenthesis. (B) The uncoating reaction mediated by hsc70_{Native} (0.4 μM) was assayed in the presence of increasing hsc70 mutant concentration. The error bars reflect the SD from three independent experiments. WT, wild type.

substrate binding domain suggested that these latter two mutants were also impaired in their ability to yield an ATP-induced conformational change (Johnson and McKay, 1999). The ATPase properties of these mutants in the context of full-length hsc70 will be described in detail elsewhere (Newmyer, S.L., and S.L. Schmid, manuscript in preparation).

None of the hsc70 ATPase domain mutants could efficiently release clathrin from CCVs, even when present at fivefold higher concentrations compared with the recombinant wild-type protein (Fig. 1 A). Thus, as has been observed in vitro with hsp70 ATPase domain mutants (Rajapandi et al., 1998), proper ATPase cycling was required for hsc70-mediated CCV uncoating activity. To learn whether the hsc70 mutants could be used in vivo to disrupt hsc70 function, the mutant proteins were titrated into the CCV uncoating reaction mediated by native hsc70, purified from brain cytosol. All three of the mutants interfered with the ability of hsc70_{Native} to mediate clathrin release (Fig. 1 B). Inhibition of the uncoating reaction occurred in a saturable manner with half maximal inhibition occurring at approximately fourfold excess of either hsc70_{K71M} or hsc70_{T204V} to hsc70_{Native}. Hsc70_{D199S} also in-

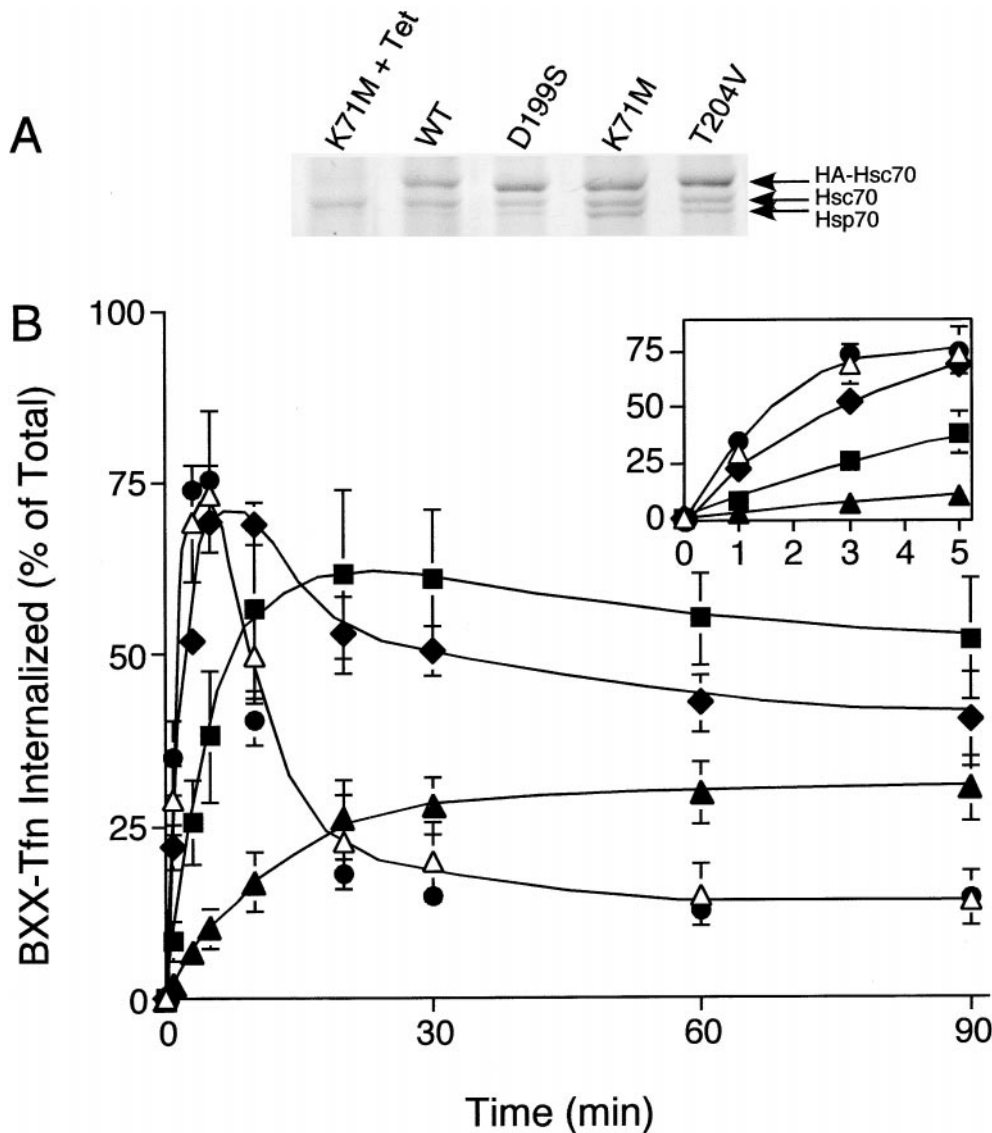


Figure 2. Overexpression of hsc70 mutants dominantly interferes with TfnR endocytosis and recycling. (A) Adenovirally infected tTA HeLa cells overexpressing hsc70 wild-type and mutant proteins were harvested 18 h after infection and analyzed by SDS-PAGE and Coomassie blue staining. The 70-kD hsc/hsp70 bands are shown. Western blotting with antibodies 3C5, SPA-810, and 12CA5 identified the hsc/hsp70 species as indicated. (B) Adenovirally infected tTA HeLa cells expressing wild type (●), D199S (◆), K71M (▲), and T204V (■) hsc70 were assayed for a single round of BXX-Tfn uptake and recycling. Control cells (Δ) were infected with hsc70_{K71M}-encoding adenoviruses but were cultured in the presence of tetracycline to suppress expression. The inset expands the data collected within the initial 5 min. The error bars reflect the SD from three independent experiments. WT, wild type.

hibited hsc70_{Native}-mediated clathrin release, though at higher concentrations. Thus, these mutants have dominant-interfering effects and can be used to probe hsc70 function in the cell.

Overexpression of Hsc70 ATPase Mutants in Adenovirally Infected tTA HeLa Cells

Hsc70 is abundant, constituting ~1–2% of total cellular protein, and can be detected by Coomassie blue staining of cell lysates (Fig. 2 A, the identity of each species was confirmed by Western blotting). To generate robust hsc70 ATPase mutant expression, we used a tetracycline-regulated adenoviral expression system. The cDNA encoding the wild-type and mutant hsc70 proteins included a sequence encoding an NH₂-terminal HA tag in order to distinguish the overexpressed protein from the endogenous protein. Control experiments established that the HA tag did not significantly affect the properties of recombinantly generated hsc70 (data not shown). Reasonable overexpression (approximately threefold) of the mutant protein relative to endogenous hsc70 was attained at high viral levels, 400 pfu/cell (Fig. 2 A). Interestingly, some induction of

hsc70, the inducible form of hsc70, was seen in cells expressing hsc70_{K71M} and hsc70_{T204V}. Within these cells, the level of hsp70 equaled that of endogenous hsc70, suggesting that overexpression of inhibitory hsc70 mutants might stress the cell. Under these conditions, however, infected cells exhibited normal morphology as observed by light microscopy and EM analysis (online supplemental Figure S1, available at <http://www.jcb.org/cgi/content/full/152/3/607/DC1>). If higher viral concentrations were used with expression of the mutant proteins, cells would round up and detach. Potential aberrant effects arising from the use of high virus levels were controlled by assaying a sample infected with hsc70_{K71M} virus in the presence of tetracycline to repress expression of the adenovirally introduced gene (Fig. 2 A).

Hsc70 ATPase Domain Mutants Dominantly Interfere with Tfn Endocytosis and Recycling

Based on in vitro findings, we predicted that the hsc70 mutant-expressing cells would exhibit a CCV uncoating defect provided that hsc70 indeed functions in clathrin uncoating in vivo and that our expression system sup-

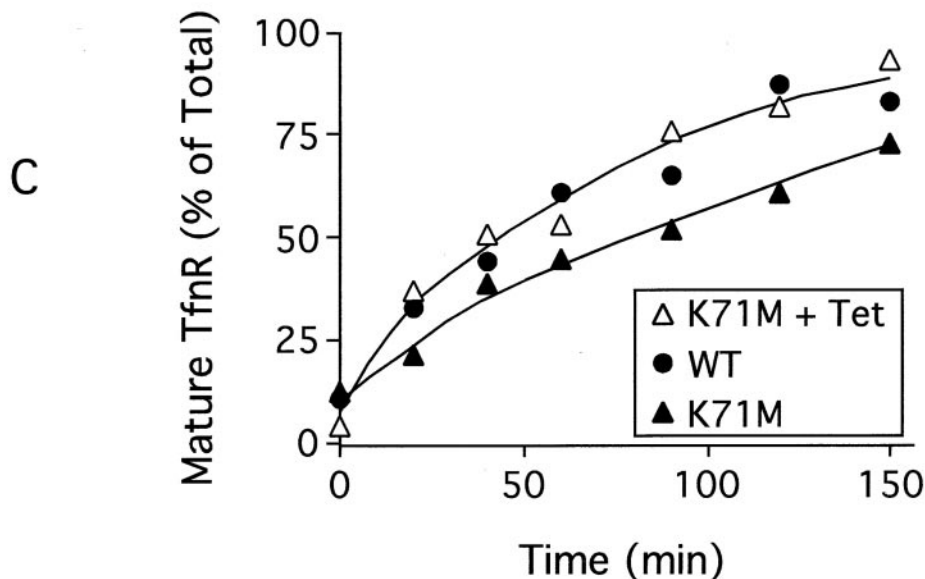
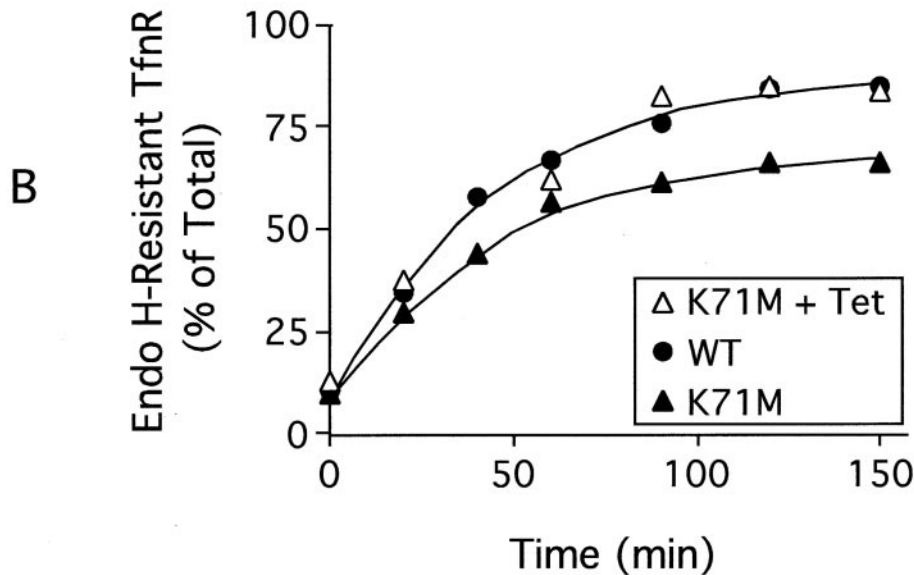
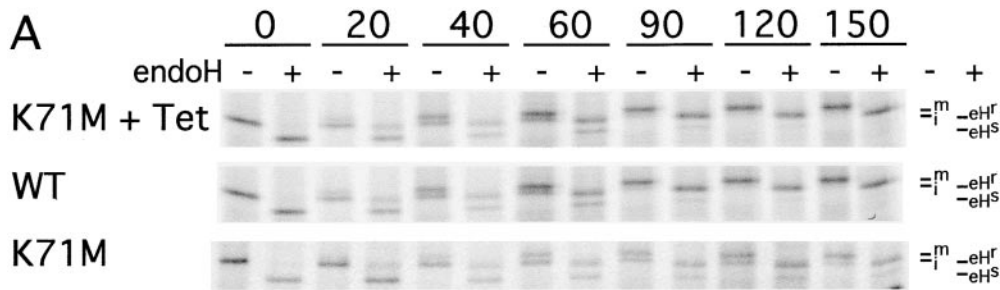


Figure 3. The trafficking of newly synthesized Tfnr is slightly inhibited by dominant-interfering hsc70 mutants. Autoradiogram (A) and corresponding quantitation (B and C) of immunoprecipitated Tfnr from a representative experiment. (B) Trafficking of Tfnr from the ER (eH^f) to the cis-Golgi region (eH^s) was monitored through the resistance to Endo H. (C) Trafficking to the trans-Golgi region was assayed through the shift of glycosylation from the immature (i) to mature (m) receptor state. WT, wild type.

plied enough mutant protein. An uncoating defect would limit the cellular sorting of Tfn and prevent its recycling. Accordingly, we assayed a single round of trafficking of surface-bound BXX-Tfn in the virally infected cells. In both control and hsc70_{WT}-expressing cells, Tfn was endocytosed rapidly, within 5 min, and the internalized ligand was efficiently recycled ($t_{1/2} \approx 15$ min) out to the cell surface (Fig. 2 B, Δ and \bullet). The mutant-expressing cells demonstrated dramatically different Tfn trafficking

kinetics. Consistent with *in vitro* studies, and supporting a role for hsc70 in CCV uncoating, these cells exhibited significant recycling defects, whereby Tfn remained in the hsc70 mutant expressing cells >60 min after internalization. The recycling defect was most striking with overexpression of hsc70_{T204V} (\blacksquare) and hsc70_{K71M} (\blacktriangle) with essentially all of the internalized Tfn remaining within the cell. Interestingly, the mutant-expressing cells also demonstrated endocytosis defects, which in strength were

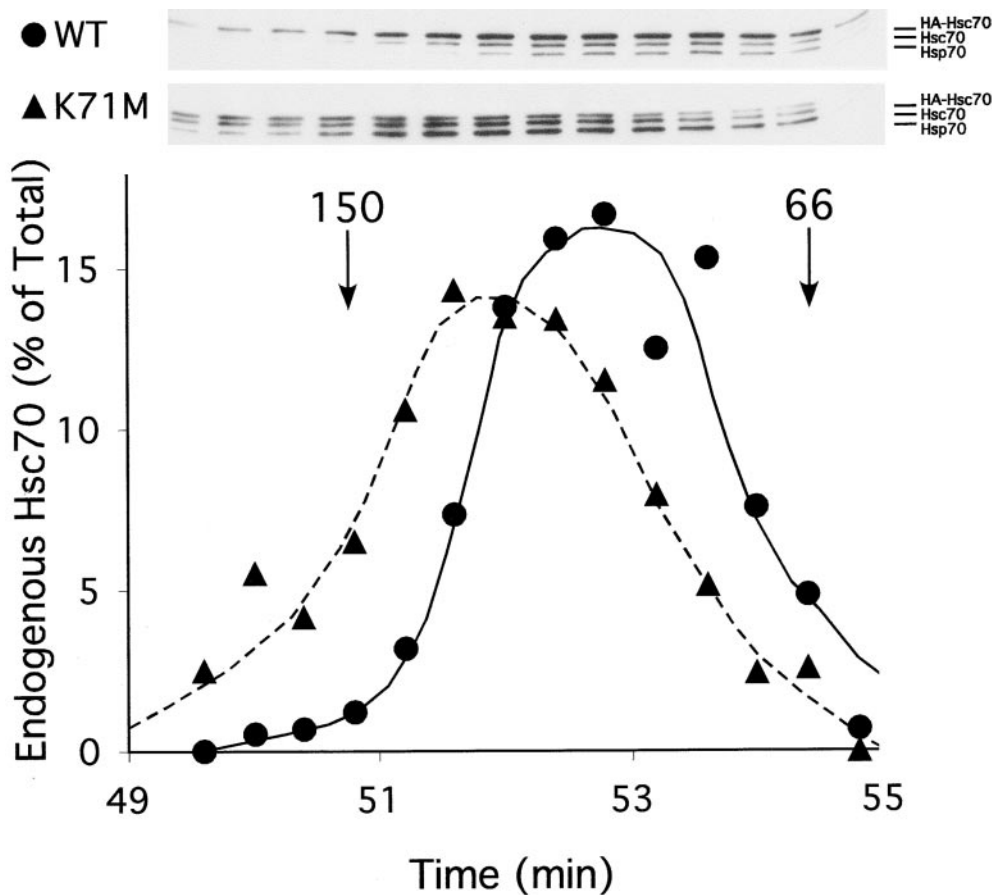


Figure 4. Overexpression of $hsc70_{K71M}$ causes endogenous $hsc70$ to migrate as a complex. Size exclusion chromatography was employed to monitor the potential oligomerization of endogenous $hsc70$. The independent migration of alcohol dehydrogenase (150 kD) and BSA (66 kD) are shown as markers. Western blotting with 3C5 revealed the migration of endogenous $hsc70$, $hsp70$, and the HA-tagged adenovirally expressed mutant and wild-type $hsc70$ and was quantified through densitometric scanning. WT, wild type.

proportional to their corresponding recycling defects (Fig. 2 B, inset). Expression of $hsc70_{D199S}$, $hsc70_{T204V}$, and $hsc70_{K71M}$ decreased the initial rate of Tfn uptake 1.4-, 2.7- and 10-fold, respectively, relative to control and wild type-expressing conditions.

The data described above were generated by quenching external BXX-Tfn with avidin, a relatively large probe that masks the biotin molecule at early stages of endocytosis but cannot access BXX-Tfn when it becomes sequestered into constricted coated pits (Schmid, 1997). We also monitored late stages of endocytosis with BSS-Tfn where the disulfide linkage between the biotin and ligand can be cleaved by smaller probes that reduce this bond. Compared with BXX-Tfn uptake, similar results were obtained when endocytosis was measured using BSS-Tfn as ligand, and the uptake into sealed CCVs was assessed by resistance to the small membrane impermeant reducing agent, MesNa (data not shown). This suggested that endocytic CCV formation was inhibited in the mutant-expressing cells before the formation of constricted coated pits. Thus, these dominant-interfering $hsc70$ mutants appeared to inhibit at least two stages of the CCV cycle, initial coated pit formation and a postbudding step.

Specificity of Inhibitory Effects of Dominant-interfering Hsc70 Mutants

Given the many possible functions of $hsc70$ within the cell, overexpression of dominant-interfering $hsc70$ mutants might have a global effect on cell viability. Therefore, to

learn whether the inhibitory effects of $hsc70$ ATPase domain mutants on endocytosis and recycling were specific, we determined the effects of overexpression of $hsc70_{WT}$ or $hsc70_{K71M}$, the most potent mutant, on vesicular trafficking along the secretory pathway. Cells were pulse labeled with [35 S]methionine, and the delivery of newly synthesized TfnR from the ER to cis- and trans-Golgi compartments was monitored by acquisition of Endo H resistance and the addition of complex oligosaccharides, respectively. Quantitation of the results shown in Fig. 3 A reveal that Endo H-resistant (Fig. 3 B) and mature (Fig. 3 C) forms of TfnR appeared at similar rates in control and $hsc70_{WT}$ -expressing cells. The corresponding rates observed in $hsc70_{K71M}$ -expressing cells were slightly inhibited (approximately twofold). The rates of biosynthesis and translocation of TfnR into the ER, as assessed by radiolabel incorporation and addition of core oligosaccharides, were also not significantly inhibited with overexpression of $hsc70_{K71M}$ (data not shown). Thus, TfnR biosynthesis was only slightly inhibited by the most potent inhibitor of BXX-Tfn uptake and recycling.

To further gauge the specificity of inhibition of RME and recycling by $hsc70$ mutants, we measured the internalization kinetics of the fluid phase marker, HRP. Normalizing to the control condition, cells expressing $hsc70_{WT}$, $hsc70_{D199S}$, $hsc70_{T204V}$, and $hsc70_{K71M}$ exhibited the following rates of fluid phase uptake: 0.91 ± 0.14 , 1.01 ± 0.18 , 0.72 ± 0.09 , and 0.70 ± 0.20 arbitrary peroxidase U/mg cellular protein/min. A slight inhibitory trend was observed with expression of the two most potent mutants, $hsc70_{T204V}$

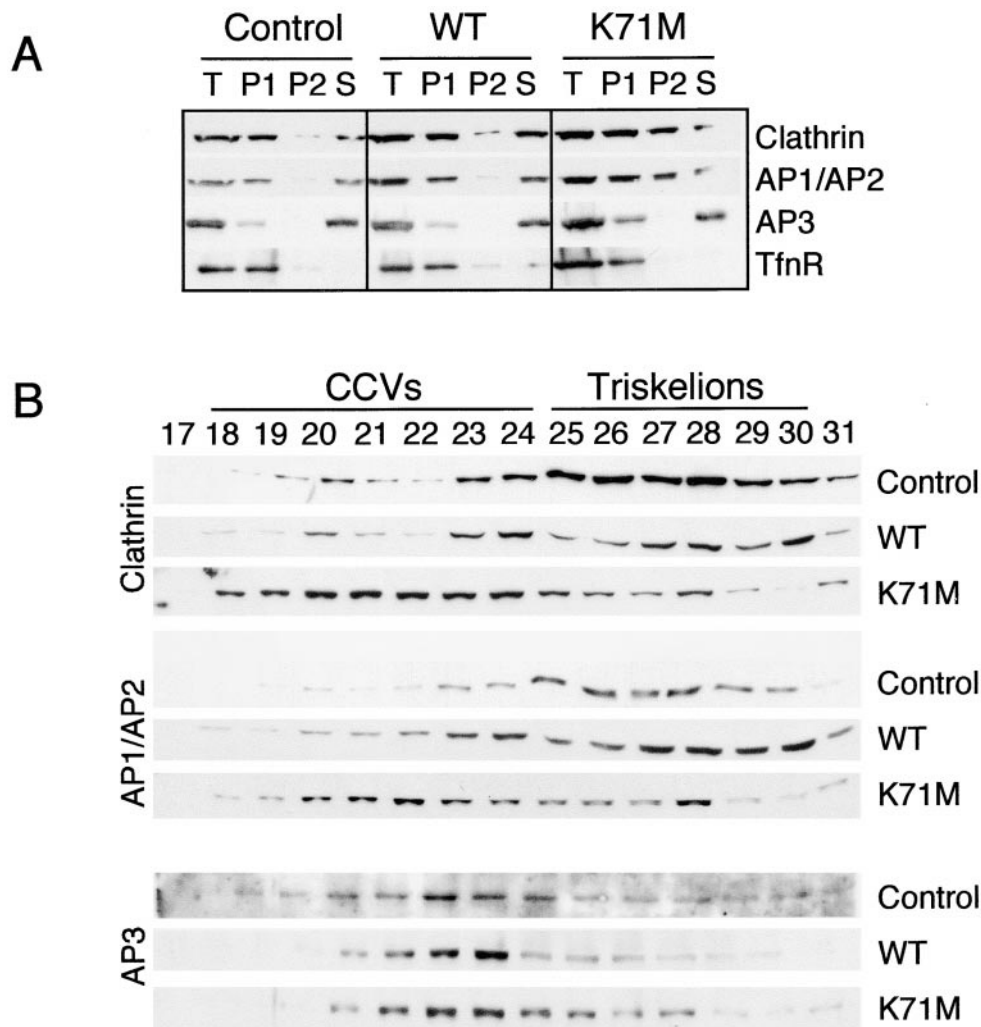


Figure 5. Subcellular fractionation of $hsc70_{WT}$ - and $hsc70_{K71M}$ -expressing and control cells. (A) Western blot analysis of the crude subcellular distribution of coat proteins and receptors includes the total cellular (T), the 14,000-g pelleted heavy membrane (P1), the 100,000-g pelleted vesicular membrane (P2), and the cytosolic (S) pools. (B) Western blot analysis of fractions 17–31 collected from an S-1000 column show the migration of vesicular and cytosolic proteins within the low speed supernatant. The migration of purified CCVs and clathrin is indicated. Anti-clathrin TD1, anti- β 1/ β 2 adaptin 100/1, anti- δ adaptin, and anti-TfnR D65 were used for immunoblotting. WT, wild type.

and $hsc70_{K71M}$. Bulk fluid uptake and trafficking through the Golgi compartments, thus, were both slightly affected in the mutant-expressing cells, suggesting that $hsc70$ might play a limited role in these processes or that cell viability is slightly compromised by the $hsc70$ mutants. In contrast to these small effects, overexpression of all $hsc70$ ATPase mutants significantly inhibited endocytosis and recycling (Fig. 2 B). In the most potent case, endocytosis was inhibited 10-fold, and recycling was no longer detected. Thus, in comparison with other trafficking events, the potent effects of the dominant-interfering $hsc70$ ATPase mutants appear to be specific for TfnR endocytosis and recycling.

Ultrastructural analysis of adenovirally infected cells overexpressing $hsc70_{WT}$ and $hsc70_{K71M}$ (online supplemental Figure S1, available at <http://www.jcb.org/cgi/content/full/152/3/607/DC1>) also did not reveal differences in the ER, mitochondria, Golgi regions, or PM when compared with uninfected cells, providing further evidence against a general effect on cell viability.

Overexpression of Mutant Hsc70s Sequesters Endogenous Hsc70 in a Complex

Given that the $hsc70$ mutants specifically disrupt Tfn uptake and recycling, we were interested in exploring the

mechanism by which the endogenous protein was inhibited. We hypothesized that these mutants could inhibit through (a) sequestration of clathrin, (b) sequestration of auxilin, the DnaJ protein that is thought to target $hsc70$ to clathrin, and/or (c) sequestration of endogenous $hsc70$. In vitro, $hsc70$ forms a stable complex with clathrin and auxilin (Schmid and Rothman, 1985; Holstein et al., 1996); however, we were unable to detect similar complexes with the $hsc70$ mutants (Newmyer, S.L., and S.L. Schmid, manuscript in preparation), suggesting that inhibition is not due to sequestration of either clathrin or auxilin. In contrast, the mutant $hsc70$ s could form dimers and higher order oligomers with $hsc70_{WT}$ as detected through crosslinking (data not shown). $Hsc70$ oligomerization is promoted in the presence of ADP, and it has been suggested that oligomerization inhibits $hsc70$ activity (Benaroudj et al., 1996; Gao et al., 1996). Given that our mutants have aberrant ATPase cycles, we looked for their ability to oligomerize with endogenous $hsc70$ by using size exclusion chromatography to monitor the presence of such complexes in the $hsc70$ expressing cells (Fig. 4). In the $hsc70_{WT}$ -expressing cells (solid line), the endogenous and HA-tagged proteins coeluted just before the BSA standard. Incubation of the lysate with ATP did not alter this elution profile (data not shown), suggesting that under

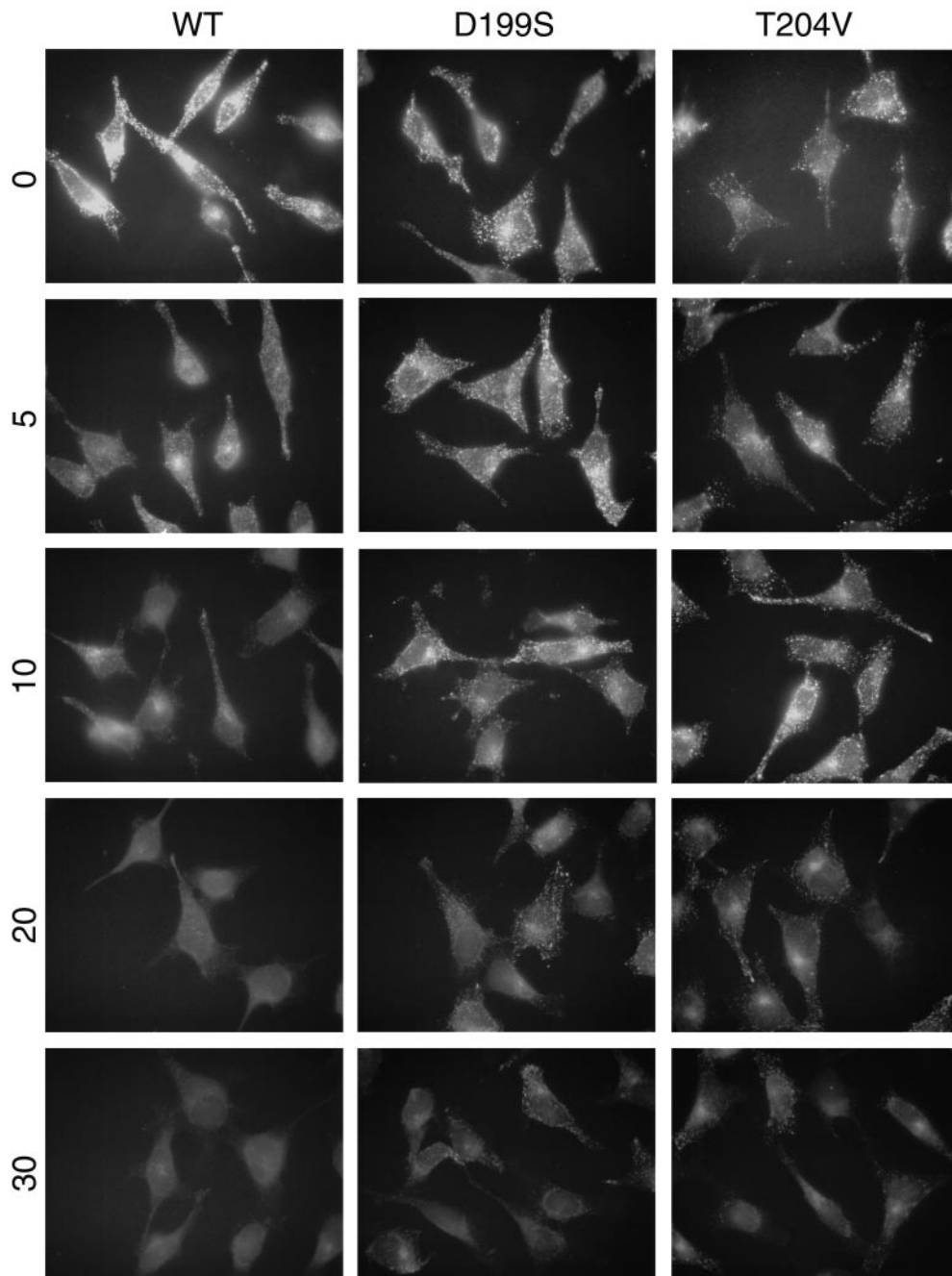


Figure 6. Internalized Tfñ exhibits prolonged trafficking through punctate structures in *hsc70_{D199S}*- and *hsc70_{T204V}*-expressing cells as visualized by epifluorescence microscopy. A 5-min pulse of Alexa₄₈₈-Tfñ (0 min) was chased with unlabeled Tfñ and followed for 5, 10, 20, and 30 min. Cells were fixed and processed for fluorescence microscopy as described in Materials and Methods. All images were collected with 2-s exposures. WT, wild type.

these conditions *hsc70* existed as a monomer (Angelidis et al., 1999). Strikingly, in cells overexpressing the K71M mutant, endogenous *hsc70* coeluted with *hsc70_{K71M}* as a higher molecular weight species (dashed line), suggesting that these proteins exist in a complex. We could not determine whether other cytosolic proteins were also present in this complex. As with *hsc70_{WT}* cells, the elution profiles observed were unaffected by incubation under ATP-depleting and -supplementing conditions, indicating that the faster migrating *hsc70* species is stable to the addition of ATP (data not shown). These results suggested that the overexpression of *hsc70* ATPase-deficient mutants created a new equilibrium between active monomeric and potentially inactive oligomeric *hsc70* species and that by overexpressing these mutants we have, in effect, sequestered the endogenous protein.

The Unassembled Cytosolic Pool of Clathrin Is Depleted in Cells Overexpressing Dominant-interfering Hsc70 Mutants

We next tested whether CCVs indeed accumulate in *hsc70* mutant expressing cells as suggested by our observed recycling defect. To examine this, we performed subcellular fractionation of control and *hsc70*-expressing cells. Lysed cells were fractionated by low and high speed centrifugation to obtain pellets containing heavy membrane (P1)- and vesicular membrane (P2)-associated proteins, respectively, and the remaining supernatant was used to identify cytosolic protein (S). In control and *hsc70_{WT}*-expressing cells, clathrin was distributed evenly between the heavy membrane and cytosolic pools (Fig. 5 A). Strikingly, in *hsc70_{K71M}*-expressing cells, the majority of the cytosolic

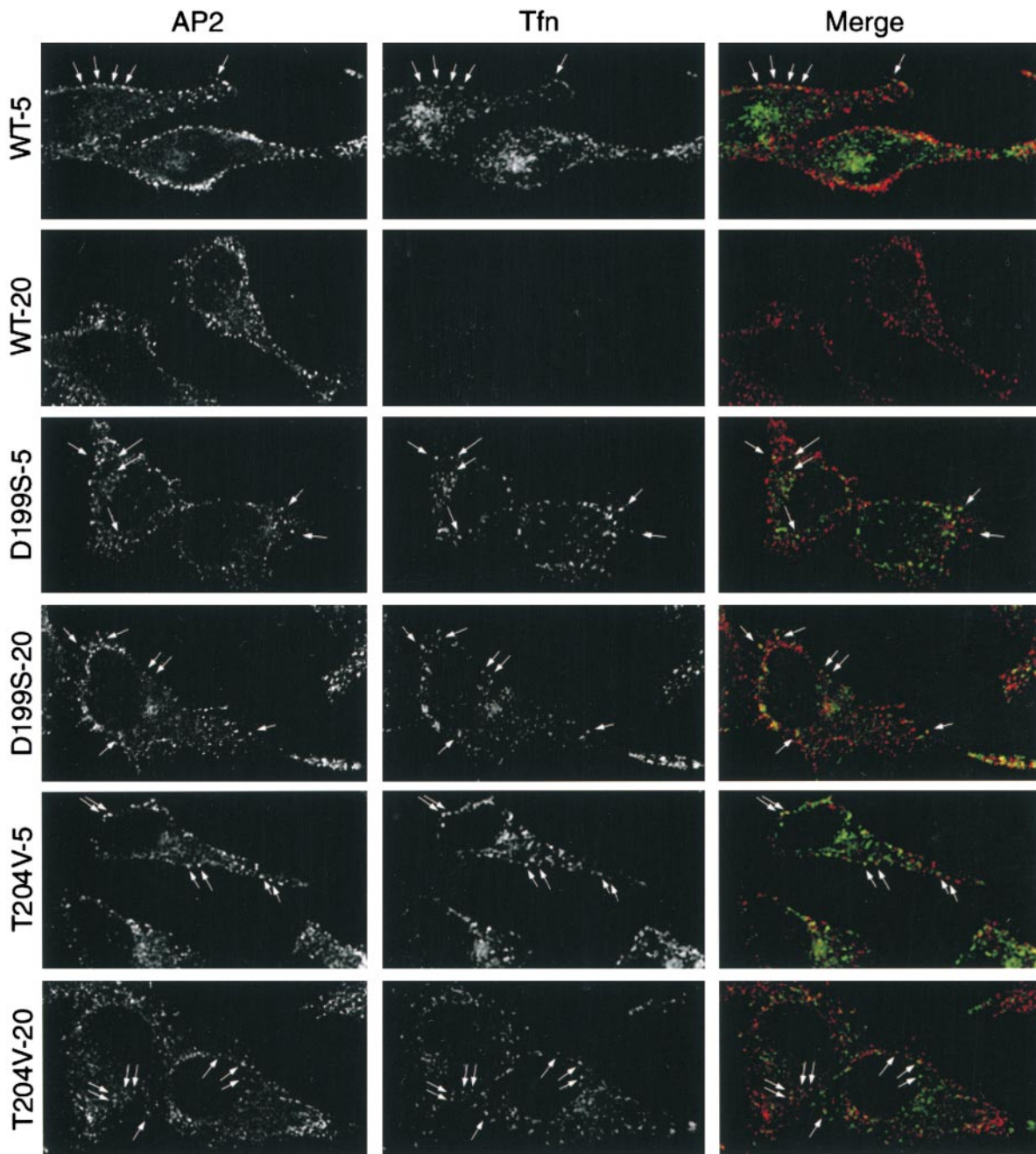


Figure 7. Internalized Tfn is largely associated with punctate AP2-free structures in mutant *hsc70*-expressing cells. Confocal microscopy was employed to monitor the colocalization of AP2 (red) with Alexa₄₈₈-Tfn (green). *Hsc70* mutant-expressing cells internalized Alexa₄₈₈-Tfn for 5 min and then were chased for 5 and 20 min. The cells were subsequently fixed and processed. The mAb AP.6 immunolabeled α -adaptnin. Arrows mark punctate colabeled structures. WT, wild type.

pool of coat proteins was lost, and the vesicular pool correspondingly increased. Similar results were observed in *hsc70*_{D199S}- and *hsc70*_{T204V}-expressing cells, with the degree of accumulation of clathrin in the P2 pool being proportional to the relative inhibitory strengths of the mutants (i.e., K71M > T204V > D199S; data not shown). To further characterize the vesicular versus cytosolic pools, the low speed supernatant was fractionated by size exclusion chromatography to resolve triskelions from assembled clathrin cages. The majority of clathrin migrated as triskelions in control and *hsc70*_{WT}-expressing cells but, consistent with our subcellular fractionation findings, migrated like

assembled clathrin in the *hsc70*_{K71M}-expressing cells (Fig. 5 B). A strong uncoating defect would predict such an increased CCV phenotype. Moreover, AP1 and AP2 cofractionated with clathrin in the *hsc70*_{K71M}-expressing cells as would be expected if uncoating were inhibited. Recall that *hsc70* is required for adaptor protein (AP) release from purified CCVs (Hannan et al., 1998). We also looked at AP3, which has been implicated in clathrin-dependent and -independent sorting at the TGN and endosomes (Simpson et al., 1996; Dell'Angelica et al., 1998). In contrast to AP1 and AP2, we find that AP3 fractionated independently of the assembled pool of clathrin in *hsc70*_{K71M}-

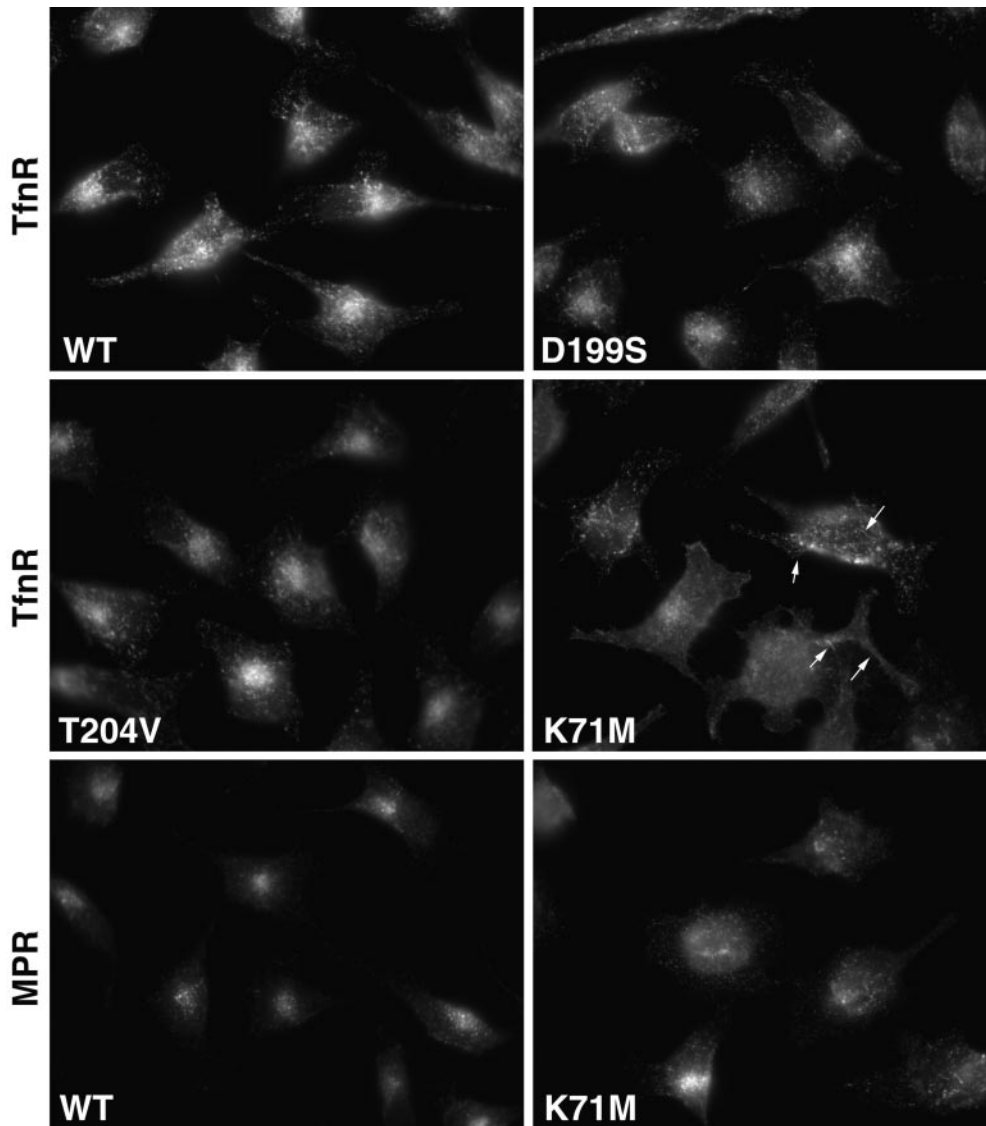


Figure 8. Steady state distribution of TfnR and MPR in wild-type and mutant hsc70-expressing cells. The cellular localization of PM- and TGN-cycled receptors were visualized by epifluorescent microscopy. Anti-TfnR D65 and anti-MPR antibodies were used to label adenovirally infected cells after their fixation. Arrows indicate the tubular TfnR-labeled structures observed in hsc70^{K71M}-expressing cells. WT, wild type.

expressing cells (Fig. 5, A and B). Relative to control and hsc70^{WT}-expressing cells, AP3 was not increased in the high speed pellet, and its migration on the S-1000 column was not altered, consistent with reports that AP3 can function independently of clathrin (Faundez and Kelly, 2000). Disruption of hsc70 activity, therefore, causes a striking redistribution of clathrin, AP1, and AP2 to an assembled pool that is consistent with there being a block in uncoating of the corresponding TGN-, endosome-, and PM-derived CCVs.

Hsc70 Mutants Inhibit the Transit of TfnR through the Endosomal Compartment

Given that the hsc70 ATPase-deficient mutants inhibited CCV uncoating mediated by the wild-type protein in vitro, we expected that overexpression of these mutants might also disrupt CCV disassembly in vivo. We have thus far provided strong evidence that disruption of hsc70 function in vivo alters the steady state distribution of clathrin from the disassembled to assembled states. The potent inhibition of BXX-Tfn recycling would suggest that internalized TfnR might accumulate in newly formed endocytic CCVs. However, when we examined the distribution of TfnR us-

ing subcellular fractionation, we were unable to detect an accumulation in the vesicular pool as determined either by differential centrifugation (Fig. 5 A) or by chromatography on S1000 (data not shown). Nor could we detect a TGN-derived cargo molecule, the MPR (data not shown). Therefore, to further elucidate the nature of the recycling defect, we followed the trafficking of a 5-min pulse of Alexa₄₈₈-Tfn. For these experiments, we studied cells expressing the weaker D199S and T204V hsc70 mutants because they incorporated a significant amount of labeled Tfn within the 5-min pulse time. In control (data not shown) and hsc70^{WT}-expressing cells, internalized Tfn was initially found in punctate structures resembling vesicles but rapidly trafficked to the perinuclear recycling endosome and back out of the cell (Fig. 6). In contrast, the mutant cells exhibited prolonged punctate staining that was still detectable 30 min after internalization. To determine whether these punctate structures were endocytic CCVs, the Alexa₄₈₈-Tfn-internalized cells were immunolabeled with anti-AP2. Normally, the release of coat proteins from CCVs occurs within 1 min of budding, and punctate structures labeling for both coat protein and internalized Tfn are rare. Accordingly, in control (data not shown) and

hsc70_{WT}-expressing cells, AP2 and Alexa₄₈₈-Tfn colocalize in only a few punctate structures at 5 min after internalization and are completely separated after 20 min (Fig. 7). In contrast, AP2 and Tfn colocalization within punctate structures could be detected even 20 min after internalization in the D199S- and T204V hsc70-expressing cells, suggesting that the uncoating reaction was significantly inhibited (Fig. 7). Similar results were obtained with clathrin labeling and after necessarily longer times of Alexa₄₈₈-Tfn uptake (30 min) into cells expressing hsc70_{K71M} (data not shown). Surprisingly, in the mutant-expressing cells, only a few contained punctate structures were found even at 5 min after internalization, demonstrating that CCV uncoating is not potently inhibited in these cells. Nonetheless, finding that the majority of Alexa₄₈₈-Tfn-labeled structures did not contain for AP2 was consistent with the fractionation experiment, demonstrating that assembled AP2-clathrin structures accumulated independently of TfnR. Together these data suggest that the accumulating coated structures might represent empty cages.

In hsc70 mutant-expressing cells, recycling of internalized BXX-Tfn was blocked (Fig. 2 B); yet, immunolabeling studies demonstrated that CCV uncoating was only partially inhibited. These data suggest that other postuncoating trafficking events were also inhibited by the hsc70 mutants. Analysis of trafficking of Alexa₄₈₈-Tfn through endosomal compartments confirmed this suggestion. In control and hsc70_{WT}-expressing cells, Tfn strongly labels the perinuclear recycling endosome within the initial 5-min uptake period (Fig. 6, 0 chase). In contrast, there was considerably less accumulation of Alexa₄₈₈-Tfn in the recycling endosome in mutant hsc70 cells, and accumulation appeared to be delayed 5–10 min relative to hsc70_{WT} cells (Fig. 6). The fluorescence data also demonstrated that punctate staining was substantially lost after 20 min of chase; yet, hsc70 mutant-expressing cells accumulate BXX-Tfn so that, after 60 min of incubation, the majority of internalized molecules remain in the cell (Fig. 2 B). Collectively, our findings indicate that, 20 min after internalization, Tfn is localized diffusely throughout the cell and suggest that vesicular trafficking to the cell surface is also inhibited. Thus, the recycling defect found in hsc70 mutant-expressing cells is apparently due to inhibition of multiple vesicular trafficking or sorting steps within the endosomal compartment.

Hsc70 ATPase Mutants Cause a Redistribution of TfnR within the Cell

The overexpression of hsc70 ATPase mutants significantly inhibited the trafficking of Tfn, suggesting that the trafficking of the receptor was also inhibited. Therefore, we monitored the steady state distribution of TfnR by immunofluorescence to learn how its trafficking was affected by the altered endocytic and recycling kinetics observed for the hsc70 mutant-expressing cells. Accumulation of TfnR within the recycling compartment was decreased, corresponding to the increased inhibitory potency of the hsc70 mutant (Fig. 8). This was especially evident in cells expressing hsc70_{K71M} where TfnR appeared to accumulate in vesicular and tubular structures (arrows).

We also characterized the localization of MPR, which is transported by CCVs between the TGN and late endosome, and found that this receptor was also more diffusely

localized in punctate structures in hsc70 mutant-expressing cells (Fig. 8). Consistent with results presented above, neither TfnR nor MPR showed significant colocalization with clathrin in the hsc70 mutant cells (data not shown). The finding that multiple receptors were mislocalized in hsc70 mutant-expressing cells further supports our hypothesis that these hsc70 inhibitory mutants interfere with sorting and trafficking within the endosomal compartment.

Given that TfnR trafficking was decreased with overexpression of hsc70 mutants, we asked whether the endocytosis defect (Fig. 2 B) was indirectly due to a decreased number of receptors at the cell surface. To determine the steady state extracellular distribution of TfnR, we measured and compared the binding of BXX-Tfn to virally infected cells in the presence and absence of saponin. The percentage of extracellular TfnR was found to be as follows: 16.6 ± 7.8 for control, 18.1 ± 6.5 for hsc70_{WT}, 17.0 ± 7.5 for hsc70_{D199S}, 18.8 ± 5.9 for hsc70_{T204V}, and 37.6 ± 15.3 for hsc70_{K71M} cells ($n = 3$ or 4), indicating that TfnR was actually increased at the cell surface of hsc70_{K71M}-expressing cells. A similar doubling of receptors at the cell surface is observed when RME is inhibited by dynamin mutants (Damke et al., 1994). The dramatic changes in the steady state distribution of PM and TGN receptors observed in hsc70_{K71M}-expressing cells suggests that hsc70 plays multiple roles in vesicle trafficking and RME.

Discussion

Hsc70 and the CCV Cycle

We have characterized several hsc70 ATPase-deficient mutants and found them to dominantly interfere with the CCV cycle *in vivo*. Within HeLa cells, clathrin is equally distributed between assembled and unassembled pools. Overexpression of hsc70 mutants dramatically shifts this equilibrium towards the assembled state, demonstrating that hsc70 plays a role in clathrin dynamics. We observed a significant shift of clathrin and AP1-AP2 complexes into fractions coeluting by size exclusion chromatography with CCVs. We also found prolonged colocalization of AP2 and internalized Tfn in punctate structures, suggesting that the increased assembled pool of clathrin and APs is in part due to a CCV uncoating defect. Hsc70 was previously implicated in releasing clathrin from CCVs based on *in vitro* studies (Rothman and Schmid, 1986). The physiological significance of hsc70-mediated uncoating was supported by microinjection studies (Honing et al., 1994) and by the discovery of a requirement for auxilin, a DnaJ-like protein associated with neuronal CCVs in recruiting hsc70 to assembled clathrin for uncoating (Ungewickell et al., 1995). Recently, nonneuronal (GAK) and yeast (swa2p/aux1p) homologues of auxilin have been identified (Gall et al., 2000; Greener et al., 2000; Pishvae et al., 2000; Umeda et al., 2000). Deletion of swa2p/aux1p in yeast results in trafficking phenotypes similar to that observed in clathrin-deleted strains, as well as accumulation of coated vesicles (Gall et al., 2000; Pishvae et al., 2000). The *in vivo* effects of interference with hsc70 function reported here are complementary to results from yeast and, together, they establish that CCV uncoating is a general cellular function of hsc70 (Fig. 9, step 1).

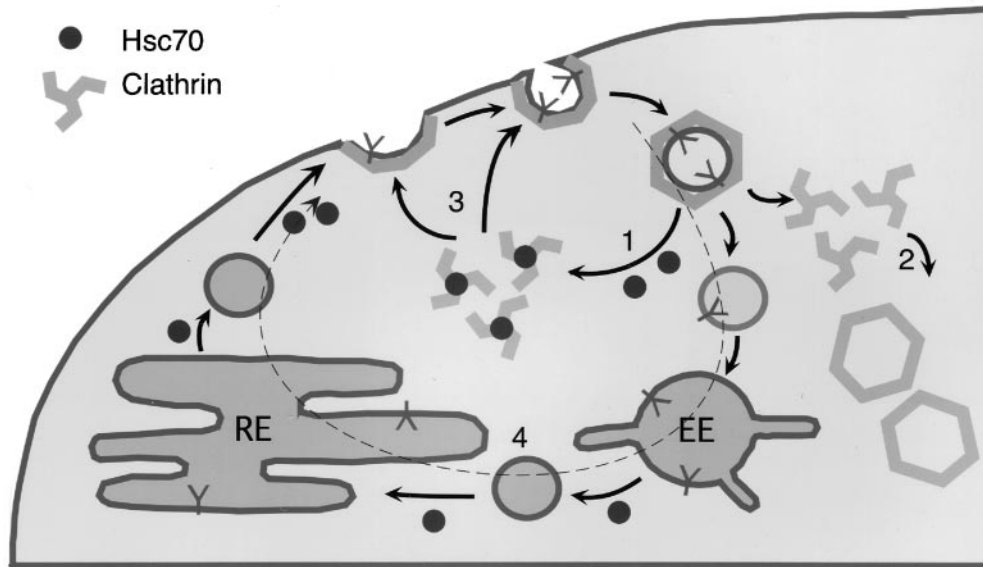


Figure 9. Hsc70 is involved in multiple steps in CCV cycling and receptor recycling. Hsc70 releases clathrin from newly budded CCVs (1). Our studies suggest that hsc70 may then be required to chaperone cytosolic clathrin triskelions (2) to allow their functional recruitment to coated pits (3). Within the endosomal compartment, hsc70 also promotes receptor recycling (dashed line, 4).

Under normal conditions, endocytic CCVs are rapidly uncoated ($t_{1/2} \approx 1-2$ min) and their contents are delivered, through fusion, to early endosomes ($t_{1/2} \approx 3$ min) (Mukherjee et al., 1997). Overexpression of hsc70 mutants appeared to delay the uncoating reaction and prolong the life-time of endocytic coated vesicles; however, even at early time points, only a relatively small population of internalized Tfn colocalized with AP2- or clathrin-labeled structures. We were also unable to detect cargo molecules in the AP2-AP1-clathrin-containing small vesicular fraction. These results suggest that the uncoating reaction was only partially inhibited, a result not unexpected given that the overexpression levels achieved in vivo (threefold over endogenous) were considerably less than those needed to potently inhibit hsc70-mediated uncoating in vitro (>10 -fold over wild type). Nonetheless, even at these levels of overexpression, we observed a quantitative redistribution of cellular clathrin from the cytosolic pool to the high speed pelleted fraction. One intriguing possibility that could explain this discrepancy is that the assembled coated structures that accumulate in the mutant cells and are pelletable at high speed are empty coats or misassembled lattices (Fig. 9, step 2). This scenario would suggest a role for hsc70 in maintaining clathrin (and perhaps APs) in an unassembled state and/or in regulating clathrin-AP assembly.

Clathrin and APs are known to spontaneously assemble in vitro under physiological salt conditions, but how assembly is regulated in the cell remains obscure. In vitro studies demonstrate that hsc70 forms a stable complex with clathrin during the uncoating reaction, which deters hsc70 from performing additional rounds of uncoating (Jiang et al., 2000). Hsc70 is known to stabilize many disassembled or unfolded proteins, including steroid hormone receptors and protein kinases (Morishima et al., 2000), and has been implicated in chaperoning newly synthesized clathrin during axonal transport (Black et al., 1991). Our studies suggest that hsc70 may also chaperone cytosolic clathrin within the CCV cycle, preventing assembly that is not directed towards the PM or TGN. The hsc70 ATPase mutants cannot bind clathrin stably in vitro and therefore could probably not serve such a chaperone function in vivo. This potential chaperone function would explain how

empty cages could accumulate in hsc70 mutant-expressing cells disproportionately to CCVs labeled with Tfn.

Inhibition of functional clathrin assembly in hsc70 mutant-expressing cells is also indicated by the observed RME defect. We find that early rather than late stages of RME are inhibited by overexpression of hsc70 mutants, implicating a role for the endogenous protein in modulating coated pit growth (Fig. 9, step 3). It is likely that the rate of coated pit assembly is decreased in mutant-expressing cells due to the partial depletion of cytosolic clathrin. Additionally, inhibition of RME may reflect a more direct role for hsc70 in chaperoning clathrin for functional assembly at the coated pit. Further work is necessary to establish a direct role for hsc70 in coated vesicle formation.

Hsc70 and Endosomal Trafficking

Characterization of our hsc70 ATPase domain mutants also reveals a role for this chaperone in TfnR recycling (Fig. 9, step 4). Surprisingly, the recycling defect in hsc70 mutant-expressing cells could not be ascribed to a potent block in CCV uncoating alone. Indeed, even the weakest hsc70 mutant, D199S, was potently inhibitory, suggesting that hsc70 is critically involved in other stages of TfnR trafficking through the endocytic pathway. In the most potently inhibited hsc70_{K71M}-expressing cells, the internalized BXX-Tfn does not appear to accumulate in the recycling endosome, and, at steady state, both MPR and TfnR become more diffusely distributed. Collectively, these data suggest that multiple endosomal trafficking events (early endosome \rightarrow recycling endosome \rightarrow PM and early endosome \rightarrow late endosome \rightarrow TGN) are inhibited when hsc70 function is disrupted in vivo. Clathrin coats are well-characterized in mediating vesicle formation at the PM and TGN but have also been implicated in modulating endosomal trafficking (Stoorvogel et al., 1996). APs have also been associated with endosomally derived CCV formation and may mediate specific endosomal sorting events (Futter et al., 1998; Sorkina et al., 1999; Meyer et al., 2000). Hsc70 is likely to function in the uncoating and/or formation of all CCVs regardless of their origin. Consequently, the potent recycling defect observed in hsc70 mutant-

expressing cells may, in part, reflect a cumulative CCV uncoating/coated pit assembly defect expected for successive vesicular trafficking events along the endocytic pathway.

Perturbation of endosomal trafficking may also arise, in part, from inhibited vesicle motility. Multiple studies suggest microtubule-based motility is required for endosomal sorting. Recently, an *in vitro* assay reconstituting the sorting of endosomal ligand from receptor was shown to require microtubules and the motor protein kinesin (Bananis et al., 2000). A viable DnaJ-like motif was characterized within kinesin and was suggested to recruit hsc70 for the dissociation of kinesin from vesicles (Tsai et al., 2000). Perhaps the potent recycling defect observed in hsc70-mutant cells is due to defective kinesin-dependent transport of vesicles along microtubules. Our identification of novel hsc70 roles will allow us to begin to elucidate the mechanisms that regulate these events. In regards to the CCV cycle, it will be especially interesting to learn how cytosolic clathrin is maintained and what directs clathrin assembly to the coated pit.

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