Depletion of GAK/auxilin 2 inhibits receptor-mediated endocytosis and recruitment of both clathrin and clathrin adaptors

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Accepted 17 June 2005
Journal of Cell Science 118, 4311-4321 Published by The Company of Biologists 2005
doi:10.1242/jcs.02548

Summary

Cyclin G-associated kinase (GAK/auxilin 2), the ubiquitous form of the neuronal-specific protein auxilin 1, is an essential cofactor for the Hsc70-dependent uncoating of clathrin-coated vesicles. We have now investigated the effect of knocking down GAK in HeLa cells by vector-based small hairpin RNA. Functionally, depletion of GAK caused a marked decrease in internalization of both transferrin and epidermal growth factor and altered mannose 6-phosphate receptor trafficking, but had little effect on the recycling of transferrin receptor back to the plasma membrane. Structurally, depletion of GAK caused a marked reduction in perinuclear clathrin associated with the trans-Golgi network and in the number of clathrin-coated pits on the plasma membrane, and reduced clathrin exchange on the few clathrin-coated pits that remained. Surprisingly, while clathrin depletion does not prevent adaptors from assembling on the membrane, depletion of GAK caused a dramatic reduction in AP2 and epsin on the plasma membrane and AP1 and GGA at the trans-Golgi network. A similar effect was caused by expression of a dominant negative Hsp70 mutant. These results suggest that GAK, in conjunction with Hsc70, not only uncoats clathrin-coated vesicles and induces clathrin exchange on clathrin-coated pits, but also mediates binding of clathrin and adaptors to the plasma membrane and the trans-Golgi network.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/118/18/4311/DC1

Key words: GAK depletion, Clathrin, Clathrin adaptors, Endocytosis

Introduction

The J-domain proteins, auxilin and cyclin G-associated kinase (GAK/auxilin 2), are a family of chaperone cofactors important in the endocytic pathway (Lemmon, 2001). Auxilin is neuronal specific whereas GAK is expressed ubiquitously. These proteins cooperate with the Hsc70 family of heat-shock proteins to dissociate clathrin from clathrin-coated vesicles (CCVs) and pits in vitro (Ungewickell et al., 1995; Greener et al., 2000; Umeda et al., 2000). Unlike Hsc70, which dissociates clathrin stoichiometrically, auxilin works catalytically (Greene and Eisenberg, 1990; Prasad et al., 1993). As a member of the J-domain family, auxilin has the characteristic HPD motif that interacts with Hsc70, but auxilin is unusual in that its J-domain is located at its C-terminal end. Recently, X-ray diffraction and NMR studies of the J-domain of auxilin have shown that auxilin is unique in that it has an unusually long loop extending between helices one and two (Jiang et al., 2003; Gruschus et al., 2004), which binds to Hsc70 (Jiang et al., 2003). In addition to its J-domain, auxilin has an N-terminal Pten-like domain that may bind to PtdIns(4,5)P_2 (Walker et al., 2001; Marsh et al., 1998), and a central region that constitutes the clathrin binding domain (Schroder et al., 1995). The presence of this clathrin binding domain enables auxilin to induce polymerization of clathrin, which led to it initially being identified as an assembly protein (Ahle and Ungewickell, 1990). However, further studies have shown that, in addition to binding to clathrin, auxilin also binds both AP2 and dynamin (Scheele et al., 2001; Newmyer et al., 2003). There is a very high homology between auxilin and GAK but they differ in that GAK, but not auxilin, has an N-terminal kinase domain that has been shown to phosphorylate the μ subunits of the clathrin adaptor proteins AP1 and AP2 (Kanaoka et al., 1997; Umeda et al., 2000; Korolchuk and Banting, 2002).

Auxilin depletion has produced endocytic phenotypes in both yeast and *C. elegans*. In yeast, depletion of auxilin causes accumulation of CCVs, impaired cargo delivery to the vacuole, an increased in the clathrin associated with vesicles relative to cytoplasmic clathrin, and slow cell growth (Pishvaee et al., 2000; Gall et al., 2000). Similarly, in *C. elegans*, when RNA interference (RNAi) is used to inhibit auxilin expression, oocytes show markedly reduced receptor-mediated endocytosis of yolk protein tagged with GFP (Greener et al., 2001). In addition, most of these worms arrest during larval development, exhibit defective distribution of GFP-clathrin in many cell types, and show a marked change in clathrin dynamics.

Recently, in mammals, the level of the ubiquitously expressed auxilin homolog, GAK, has been reduced using

RNAi (Zhang et al., 2004). In this study, a cell line that was stably depleted of GAK showed a 50-fold increase in levels of expression of both epidermal growth factor receptor (EGFR) and tyrosine kinase, as well as significant changes in downstream EGFR signaling. EGF uptake was inhibited in the cell line used, but surprisingly, there was only minor inhibition of transferrin uptake. As the authors noted that the phenotype was a result of selecting for a stably depleted GAK cell line since there were significant differences in phenotype between cells that were transiently and stably depleted of GAK.

Since Hsc70 and auxilin together are essential to dissociate clathrin from CCVs and pits (Ungewickell et al., 1995), the phenotype of transient GAK-depleted cells might be similar to that observed using a dominant-negative mutant of Hsc70. Newmyer and Schmid (Newmyer and Schmid, 2001) found that expression of the dominant negative Hsc70 mutant, Hsc70(K71M), caused the unassembled pool of clathrin to shift to an assembled pool that cofractionated with AP1 and AP2 but was devoid of receptors, suggesting that it might be clathrin baskets. In addition, transferrin internalization was reduced and there was a block in the recycling of transferrin receptor back to the plasma membrane. Because the dominant negative Hsc70 mutant might cause phenotypic changes unrelated to its interaction with GAK, we were interested in determining whether transient GAK depletion gave the same phenotype as expression of the Hsc70 mutant. Therefore, we investigated the effect of down regulation of GAK in HeLa cells.

As was observed for expression of the dominant negative Hsc70 (Newmyer and Schmid, 2001) depletion of GAK by RNAi caused a marked decrease in the internalization of the transferrin receptor, but it differed from expression of the dominant negative Hsc70 in that it had almost no effect on the recycling of the transferrin receptor back to the plasma membrane. In addition, there was a marked decrease in the number of clathrin-coated pits (CCPs) on the plasma membrane and a reduction in perinuclear clathrin associated with the trans-Golgi network (TGN). Surprisingly, depletion of GAK actually prevented binding of AP2 and epsin, as well as clathrin, to pits on the plasma membrane. In addition, there was a loss of the clathrin adaptors, AP1 and GGA, at the TGN. These results suggest that Hsc70 and GAK not only uncoat CCVs and mediate rebinding of clathrin to the plasma membrane and the TGN, but may also function in the recruitment of adaptors to the plasma membrane and TGN.

Materials and Methods

RNAi

GAK was depleted in HeLa cells using the two oligonucleotides sequences, AAGCUCAAGAUGUGGGGAGUG (sequence #1) and AAAGCAUUCCAAAGCCUCUGA (sequence #2). We used vector based RNAi (p*Silencer*3.1-H1 hygro, Ambion Inc. Cat. no. 5766) to obtain the GAK knockdown cells expressing these oligonucleotides after selecting for 5-7 days with 400 μg/ml of hygromycin B (RPI, Cat. no. H75000-1). Mock-depleted cells were transfected with the vector that has scrambled DNA sequences. To determine the level of GAK in the cell lysates, the lysates were run on SDS-PAGE gels (Invitrogen), and then immunoblotted using anti-GAK antibodies. The GAK band was detected using chemiluminescent substrate (Pierce, Cat. no. 34080). The GAK band was analyzed using the densitometer (ChemiImager, Alpha Innotech Corp).

Plasmids

The following constructs were used to transfect HeLa cells: GFP-clathrin light chain a, DsRed-clathrin light chain a, GFP-AP2 (α-chain labeled), GFP-GGA3 (a gift from R. Puertollano, NIH, Bethesda, MD, USA), GFP-epsin (a gift from P. De Camilli, Yale University, New Haven, CT, USA), flag-tagged Hsp70, and flag-tagged Hsp70(K71E) (Wu et al., 2001; Wu et al., 2003; Zeng et al., 2004). To make mRFP-GAK, we used mRFP expression vector (gift from R. Tsien, Stanford University, Stanford, CA, USA) and then subcloned GAK into *Eco*RI and *Kpn*I restriction sites.

Microscopy

Cells grown on 2-chamber 25-mm^2 coverslips (Labtek, NY, USA) were imaged using either the Zeiss LSM 510 confocal microscope or Olympus IX70. GFP-expressing cells or Alexa Fluor 488 was imaged on a Zeiss LSM 510 confocal microscope using an argon laser for excitation at 488 nm with a 40×, 1.4 NA objective. Rhodamine, Alexa Fluor 546, Alexa Fluor 647 and Cy5 were imaged using the helium/neon laser by exciting at 543 nm and 633 nm, respectively. Image analysis was done using the LSM510 confocal program, which measures the relative intensity of fluorescence in a given area. Images were checked to ensure that the photomultiplier tube was not saturated. For total internal reflectance fluorescence microscopy (TIR-FM), the Olympus microscope was used with a 63×, 1.78 NA oil immersion objective. GFP and DsRed were excited with the 488 nm line of an argon laser (Melles Griot, Carlsbad, CA, USA) and the 567 nm line of a krypton laser (Melles Griot, Carlsbad, CA, USA), respectively. To image both green and red fluorophores simultaneously, we used an image splitter (Optical Insights, Santa Fe, NM, USA). Images were collected using a Photometrics Cool Snap HQ CCD camera (Photometrics, Tucson, AZ, USA). Data sets were acquired using Metamorph software (Universal Imaging, PA, USA).

Tissue culture, immunostaining and immunoprecipitation

HeLa cells were maintained in DMEM (Biofluids, MD, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 unit/ml) and streptomycin (100 unit/ml) in a humidified incubator with 5% CO_2 at 37°C. Five days after selecting cells for the vector based RNAi, cells were transfected with the assorted plasmids using Fugene6 (Roche Diagnostics, IN, USA). Cotransfections using a GFP-vector and flag-tagged Hsp70 or Hsp70(K71E) were always done using half as much of the GFP-vector as the non-fluorescent vector. Cells were treated with 5 μg/ml Brefeldin A (BFA; Sigma) for 10 minutes at 37°C.

The expressed Hsp70 constructs were visualized by immunostaining with anti-flag antibody (Sigma). Cells were first fixed with 4% paraformaldehyde or absolute methanol at –20°C. The following antibodies were used: anti-clathrin antibodies, mAb X22 (Affinity BioReagents, Golden, CO, USA) or CHC5.9 (Biodesign International, Saco, Maine, USA); anti-AP2 antibody, AP.6 (Affinity BioReagents, Golden, CO, USA); anti-AP1, 100/3 (Sigma); anti-GGA3 (BD Biosciences); anti-EEA1 (BD Biosciences), anti-p230 and anti-GM130 (BD Biosciences), anti-TGN46 (Serotec), anti-mannose 6-phosphate cation-independent receptor (MPR) (Abcam, Cambridge, UK), and rabbit polyclonal GAK (Greener et al., 2000). Secondary antibodies used were: rhodamine-conjugated donkey anti-mouse IgG antibody and Cy5-conjugated goat anti-mouse IgM antibody (Jackson Immunoresearch Laboratory Inc.).

Immunoprecipitation of EGFR was performed by collecting mock-depleted or GAK-depleted HeLa cells grown in 100 mm culture dishes using lysis buffer (5 mM EDTA, 0.5% Triton X-100, 1× PBS). The cell lysate (500 μg) was then mixed with 4 μg of anti-EGFR antibody (Upstate, Charlottesville, VA, USA) followed by the addition of 50 μl protein A Sepharose 4 Fast Flow (50% slurry; Amersham Bioscience). After incubating at 4°C, the Sepharose beads were collected by

centrifugation at 14,000 ***g***, washed with PBS and resuspended in 50 μl of 2× Laemmli sample buffer.

Internalization and efflux of ligand

Mock-depleted or GAK-depleted HeLa cells grown in 6-well 35 mm^2 culture dishes were incubated for 30 minutes in minimal medium. They were placed at 4°C, and 60 nCi ^{125}I-transferrin (PerkinElmer) was added for 30 minutes. These conditions inhibited transferrin internalization, thereby increasing the surface number of transferrin receptors. The cells were warmed to 37°C and the influx of transferrin was measured at varied times as described previously (Wu et al., 2001). The internalization of Alexa-conjugated transferrin, EGF, and BSA (Molecular Probes, Oregon, USA) was performed using the same protocol. The efflux of transferrin was determined by incubating cells with 60 nCi ^{125}I-transferrin at 25°C for 30 minutes. At zero time, the radioactivity was washed from the cells; the cells were then incubated with 100 μg/ml of non-radioactive transferrin (Sigma) at 37°C. At varying times, the ^{125}I-transferrin in the cells and medium was measured.

Fluorescence recovery after photobleaching (FRAP)

GFP-clathrin was imaged and photobleached using a 488-nm laser light with a 40×, 1.4 NA objective. A defined region was photobleached at high laser power resulting in 50-80% reduction in the fluorescence intensity. Scanning at low laser power monitored the fluorescence recovery after photobleaching. For each experimental condition, a minimum of eight data sets was averaged to obtain the mean and standard deviation for each time point. Setting the maximum fluorescence to 100% and the minimum fluorescence to 0% normalized the fluorescence intensity data in each experiment. The very low laser intensity used in scanning the cell after the initial photobleach did not cause significant bleaching during our experiments and therefore no correction was necessary for this effect.

Results

As shown in Fig. 1A, two different oligonucleotide sequences were used to knockdown GAK in HeLa cells. Both sequences gave about 95% reduction in GAK expression after 5 days of oligonucleotide treatment as shown by western blot analysis (Fig. 1A). Sequence #1, in the coding region, was a little more effective than sequence #2, in the 3′-UTR in depleting GAK. The mock-depleted cells always contained vector-based RNAi with a scrambled GAK RNA sequence. Both mock- and GAK-depleted cells were immunostained using anti-GAK antibody. Fig. 1B shows that GAK was no longer localized at the Golgi in the GAK-depleted cells. In determining different effects of GAK depletion, both oligonucleotides showed the same phenotypes throughout the study.

Functional changes in GAK-depleted cells

Because depletion of auxilin by RNAi in *C. elegans* inhibited receptor-mediated endocytosis (Greener et al., 2001), the internalization of transferrin was examined to determine whether GAK-depleted cells showed a reduction in endocytosis. As shown in Fig. 2A, there was indeed a marked reduction in the uptake of transferrin. The kinetics of transferrin recycling was examined in greater detail using radioactivity to determine whether GAK-depletion affected both the in-rate and out-rate. GAK-depleted cells showed a marked reduction in the rate of transferrin uptake compared with the mock-depleted cells when transferrin internalization was measured in a single round of trafficking (Fig. 2B). Interestingly, however, depletion of GAK had little effect on the rate of transferrin recycling back to the plasma membrane (Fig. 2C).

In addition to inhibition of transferrin uptake, there was also marked inhibition of EGF uptake (Fig. 2D). Furthermore, depletion of GAK had a marked effect on trafficking of MPR from the TGN to the lysosome, which normally occurs via vesicles containing clathrin, GGA and AP1 (Doray et al., 2002). Most of the MPR is distributed throughout the cytosol in the knockdown cells, whereas it is present at the TGN in mock-depleted cells (Fig. 2E). Therefore, depletion of GAK affected trafficking from the TGN as well as from the plasma membrane. These were specific effects since GAK depletion had no significant effect on fluid phase uptake measured using fluorescent-conjugated BSA (supplementary material Fig. S1A).

Structural changes in GAK-depleted cells

Since GAK-depleted cells showed a marked decrease in receptor-mediated endocytosis, we imaged the cells to

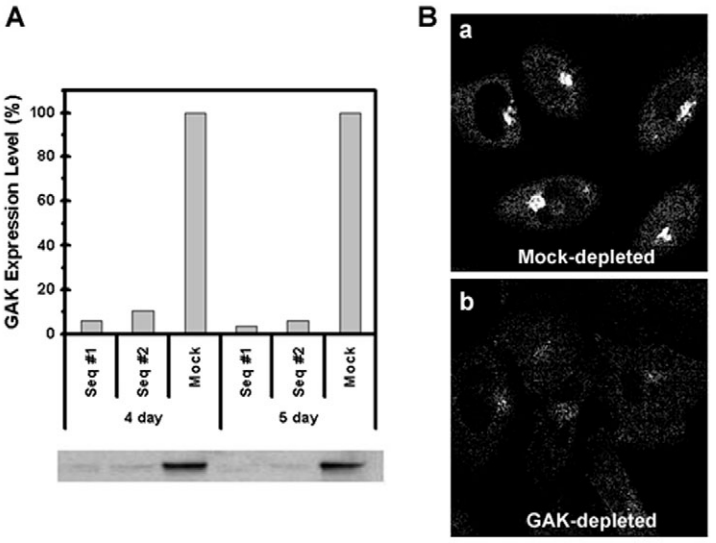

Fig. 1. Depletion of GAK by vector based oligonucleotides. (A) Western blot and quantification of GAK in mock-depleted and GAK-depleted cells using the two different sequences as described in the Materials and Methods. Days are the number of days after transfection with vector-based RNAi. 10 μg of total protein was loaded per lane. The mock-depleted value of the GAK intensity band was normalized to 100% for comparison with the amount of GAK in the GAK-depleted cells. (B) Absence of GAK at Golgi in GAK-depleted cells. GAK was immunostained in mock-depleted (a) and GAK-depleted (b) cells.

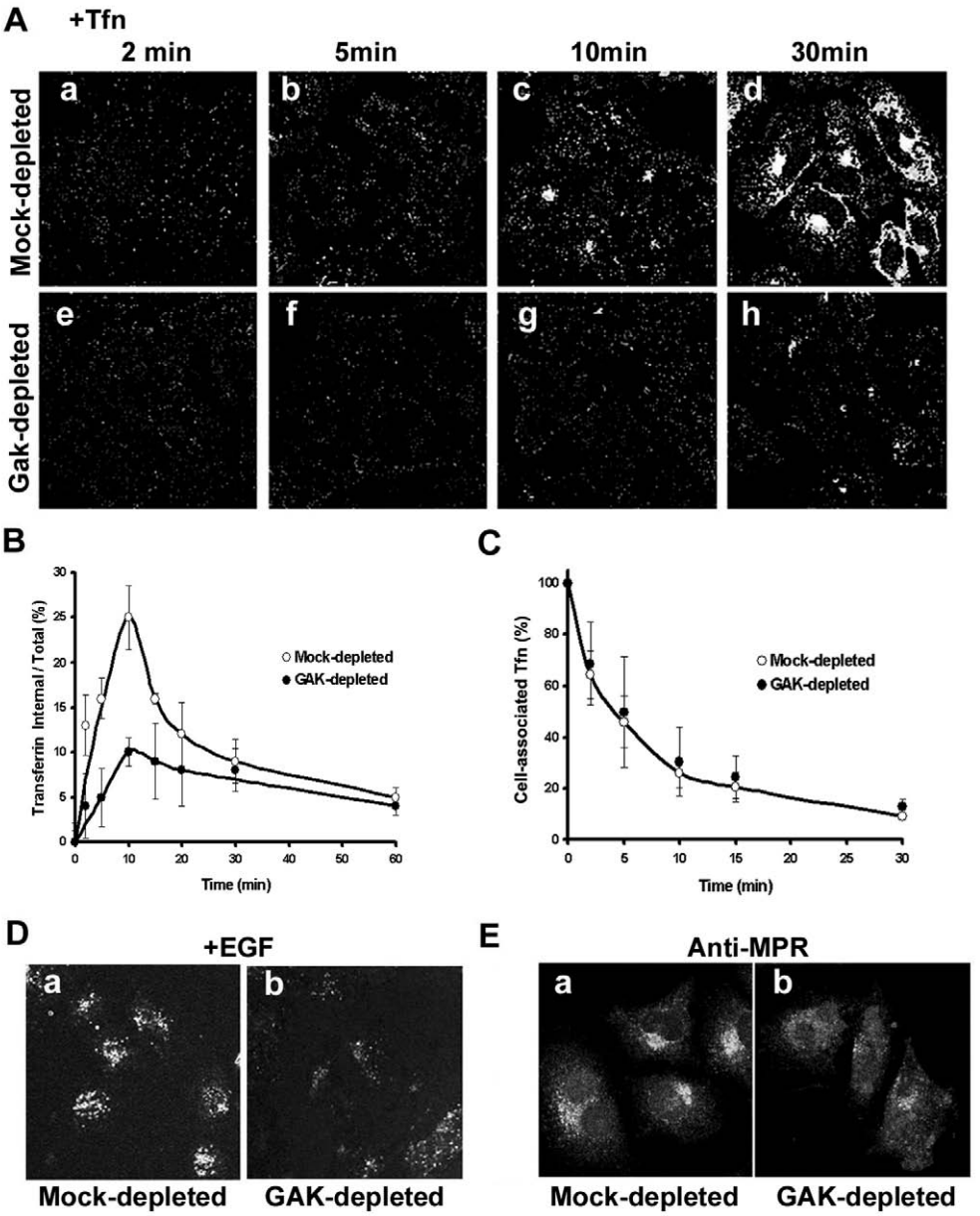

Fig. 2. Effect of GAK depletion on receptor-mediated endocytosis and MPR trafficking. (A) Uptake of transferrin is inhibited in GAK-depleted cells. Alexa Fluor 546-transferrin (10 μg/ml) was incubated with the cells for the times indicated. (a-d) Mock-depleted and (e-h) GAK-depleted cells. (B) Internalization of ^{125}I-transferrin was measured as a function of time in mock-depleted (open circles) and GAK-depleted cells (solid circles). The total transferrin is the amount bound plus the amount endocytosed and later exocytosed. The initial surface values for both the control and GAK-depleted cells were about 3500 c.p.m. (C) Efflux of ^{125}I-transferrin was measured as a function of time in mock-depleted (open circles) and GAK-depleted cells (solid circles). Uptake and efflux of transferrin was performed as described in the Materials and Methods. (D) Uptake of EGF is inhibited in GAK-depleted cells. Cells were incubated with Alexa Fluor 647 EGF (4 μM) for 30 minutes in mock-depleted (a) and GAK-depleted (b) cells. (E) Localization of MPR is altered after GAK-depletion. MPR was immunostained in mock-depleted (a) and GAK-depleted (b) cells.

determine whether there was a change in the number or distribution of clathrin coated pits (CCPs). Immunostaining of clathrin in the GAK-depleted cells showed that there was a marked reduction in the number of CCPs, as well as a tendency for the few remaining pits to cluster (Fig. 3b). Confocal microscopy of live cells transfected with GFP-clathrin also showed a marked decrease in the number of CCPs on the plasma membrane (Fig. 3d). In addition, GAK-depleted cells showed a considerable increase in background clathrin compared with mock-depleted cells. Therefore, to image CCPs on the plasma membrane more clearly in live cells transfected with GFP-clathrin we used total internal reflectance fluorescence microscopy (TIR-FM), which has the advantage of imaging only 100-200 nm at the bottom surface of the cell. As shown in Fig. 3f, TIR-FM imaging confirmed that there was a marked reduction in the number of CCPs on the plasma membrane of GAK-depleted cells compared with mock-depleted cells.

Since clathrin is also localized at the TGN, we examined whether its localization is likewise altered in GAK-depleted cells. The GAK-depleted cells did not show pronounced perinuclear staining of clathrin at the TGN unlike the mock-depleted cells (Fig. 4a,b). We next examined whether other resident Golgi proteins are altered in GAK-depleted cells by staining for the cis-Golgi protein, GM130, and the TGN proteins, p230 and TGN46 (Fig. 4c-h). Unlike the compact Golgi protein staining found in mock-depleted cells, in the majority of GAK-depleted cells all three proteins showed a similar distribution, giving the appearance of swollen and fragmented Golgi. Occasionally, in cells showing complete absence of GAK-immunostaining, both the cis Golgi protein, GM130, and the TGN proteins dispersed into small vesicles so that there was no longer any apparent Golgi (see cells with asterisks, Fig. 4). Therefore, depletion of GAK not only caused the redistribution of clathrin at the TGN, it seemed to affect the morphology of the entire Golgi.

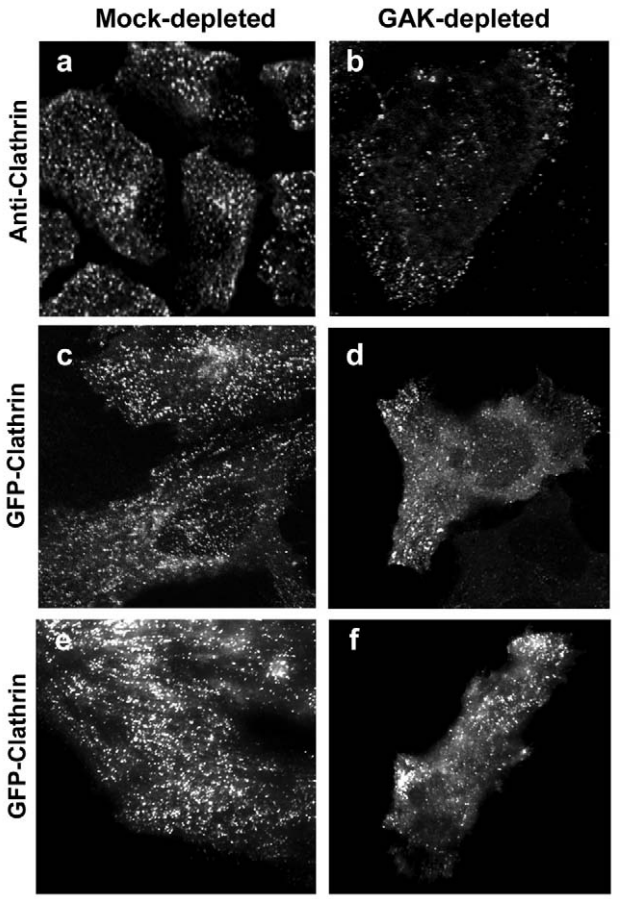

Fig. 3. GAK depletion alters distribution of CCPs on plasma membrane. Immunostained clathrin on the plasma membrane was imaged using confocal microscopy in mock-depleted (a) and GAK-depleted (b) cells. Transfected GFP-clathrin on the plasma membrane was imaged using confocal microscopy in mock-depleted (c) and GAK-depleted (d) cells. Transfected GFP-clathrin on the plasma membrane was imaged using TIR-FM in mock-depleted (e) and GAK-depleted (f) cells.

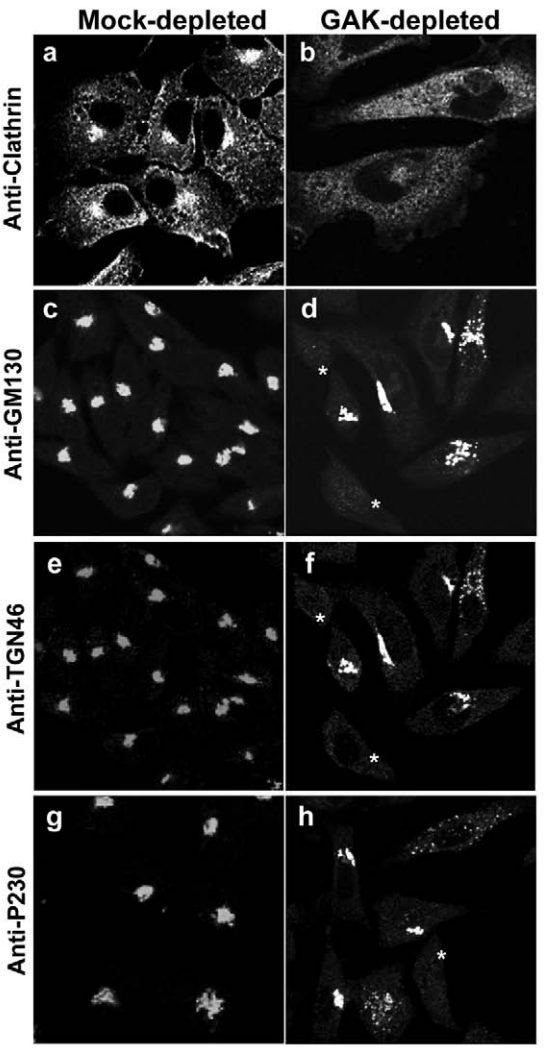

Fig. 4. Localization of proteins on TGN is altered in GAK-depleted cells. Both mock-depleted (a,c,e,g) and GAK-depleted (b,d,f,h) cells were imaged using confocal microscopy, after being immunostained for clathrin (a,b), GM130 (c,d), TGN46 (e,f) and p230 (g,h). The identical cells were immunostained for GM130 and TGN46 proteins. The cells with no apparent cis or TGN Golgi proteins are indicated with asterisks.

To verify that the GAK-depletion phenotype was due only to knockdown of the GAK gene, we reversed the loss of TGN-associated clathrin by expressing mRFP-GAK in GAK-depleted cells. Cells were depleted of GAK using sequence #2, which targets to the untranslated region of the GAK gene. Since over expression of GAK causes clathrin to be sequestered (Zhao et al., 2001) we only studied cells expressing low levels of mRFP-GAK in these experiments. Expression of low to medium levels of mRFP-GAK in mock-depleted cells caused little change in TGN-associated clathrin, whereas cells expressing mRFP-GAK showed a significant increase in TGN-associated clathrin in GAK-depleted cells (Fig. 5A). Transfecting mRFP-GAK into the GAK-depleted cell caused partial rescue of the cells (Fig. 5B) although, as noted above, this rescue is complicated by the fact that over expression of GAK causes clathrin to be sequestered (Zhao et al., 2001).

Dynamic properties of free and bound clathrin in GAK-depleted cells

To test our previous suggestion that GAK and Hsc70 mediate clathrin exchange on CCPs (Wu et al., 2001), the fluorescence recovery after photobleaching of GFP-clathrin on CCPs was measured in GAK-depleted cells. We photobleached clustered CCPs in these cells since the rest of the plasma membrane had very low density CCPs. As shown in Fig. 6A, in the GAK-depleted cells GFP-clathrin fluorescence recovery was about 50% of that in the mock-depleted or control cells. This indicates an immobilized population of clathrin on the pits in GAK-depleted cells. In addition, the rate of recovery of the GFP-clathrin was two- to threefold slower in the GAK-depleted cells compared to mock-depleted or control cells. Since the rate of fluorescence recovery is a measure of the rate of dissociation of clathrin from the pits, these data are consistent with our in vitro data showing that GAK, along with Hsc70, is required for the dissociation of clathrin that occurs

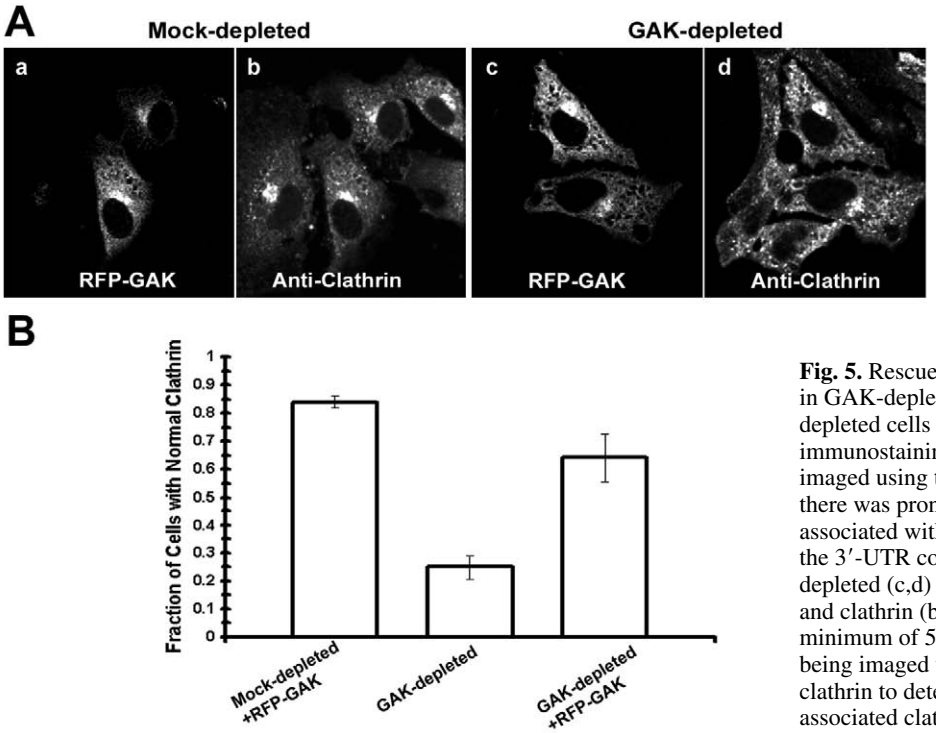

Fig. 5. Rescue of TGN-associated clathrin by mRFP-GAK in GAK-depleted cells. Both mock-depleted and GAK-depleted cells were transfected with mRFP-GAK. After immunostaining with clathrin antibodies, the cells were imaged using the confocal microscope to determine whether there was pronounced perinuclear clathrin staining associated with the TGN. Cells were GAK depleted using the 3′-UTR construct. (A) Mock-depleted (a,b) and GAK-depleted (c,d) cells were imaged for both mRFP-GAK (a,c) and clathrin (b,d). (B) Cells from two experiments (a minimum of 50 cells per experiment), were counted after being imaged using either clathrin immunostaining or GFP-clathrin to determine whether there was pronounced TGN-associated clathrin.

both during clathrin uncoating and clathrin exchange (Greener et al., 2000; Umeda et al., 2000; Yim et al., 2005).

FRAP experiments were also performed on the GFP-clathrin in the cytosol of the GAK-depleted cells. As shown in Fig. 6B, GFP-clathrin fluorescence in the cytosol recovered in 20 seconds. This is an order of magnitude slower than that obtained from photobleaching freely diffusible cytoplasmic GFP-clathrin (inset in Fig. 6B), measured after treatment of mock-depleted cells with BFA to dissociate clathrin bound at the TGN and endosomes. These experiments confirmed that the majority of the clathrin was not freely diffusible. Moreover, the rate of fluorescence recovery of the GFP-clathrin in the clathrin clusters was insensitive to BFA (Fig. 6B, open triangles). Consistent with these results, the GFP-clathrin in the GAK-depleted cells appeared clustered and BFA-treatment had no significant effect on its distribution (supplementary material Fig. S1B). This BFA insensitivity suggests that the clathrin clusters are not fragmented TGN, which is consistent with the absence of the TGN clathrin adaptors, AP1 and GGA3, in the clathrin clusters (see below).

To determine whether the clathrin was bound to CCVs or early endosomes in GAK-depleted cells, we investigated whether clathrin colocalized with internalized transferrin or the early endosome marker, EEA1. The clathrin clusters had a much more dispersed distribution than either transferrin or EEA1 (Fig. 7). These results suggest that a significant proportion of the clathrin clusters is not in CCVs that failed to uncoat or in early endosomes.

Dominant-negative mutant of Hsp70

Interestingly, the functional effects that we observed in cells depleted of GAK resemble those observed in cells expressing dominant-negative mutants of Hsc70 such as Hsc70(K71M) (Newmyer and Schmid, 2001). For example, in both cases internalization of transferrin and trafficking of MPR were affected, although recycling of transferrin was markedly inhibited by expression of Hsc70(K71M) but not by depletion of GAK. Our laboratory has previously shown that the dominant-negative mutant Hsp70, Hsp70(K71E) neither supports clathrin uncoating nor interacts with clathrin triskelia, as is the case for wild-type Hsp70 and Hsc70 (Rajapandi et al., 1998). We were therefore very interested in comparing the effects caused by over expressing this dominant negative Hsp70 with that produced by depletion of GAK. We first examined the morphological effects of over expression of Hsp70(K71E). In contrast to our observations with GAK-depleted cells, we were unable to clearly image CCPs by immunostaining because of the high clathrin background in the cells over expressing Hsp70(K71E). To image the CCPs, we had to cotransfect GFP-clathrin and Hsp70(K71E) and image the CCPs using TIR-FM. Cells expressing Hsp70(K71E) showed a marked reduction in CCPs on the plasma membrane compared with cells cotransfected with GFP-clathrin and wild-type Hsp70 (Fig. 8A). There was also a reduction in clathrin associated with the TGN in cells transfected with Hsp70(K71E) as observed by clathrin immunostaining (Fig. 8B).

Because the clathrin background in cells over expressing Hsp70(K71E) was even higher than in the GAK-depleted cells, we could not obtain meaningful fluorescence recovery data when the CCPs in these cells were photobleached. However, we were able to photobleach the cytosolic clathrin in cells cotransfected with GFP-clathrin and Hsp70(K71E), which, as in the case of GAK depletion, appeared to occur in clathrin clusters distributed throughout the cytosol. The rates of the

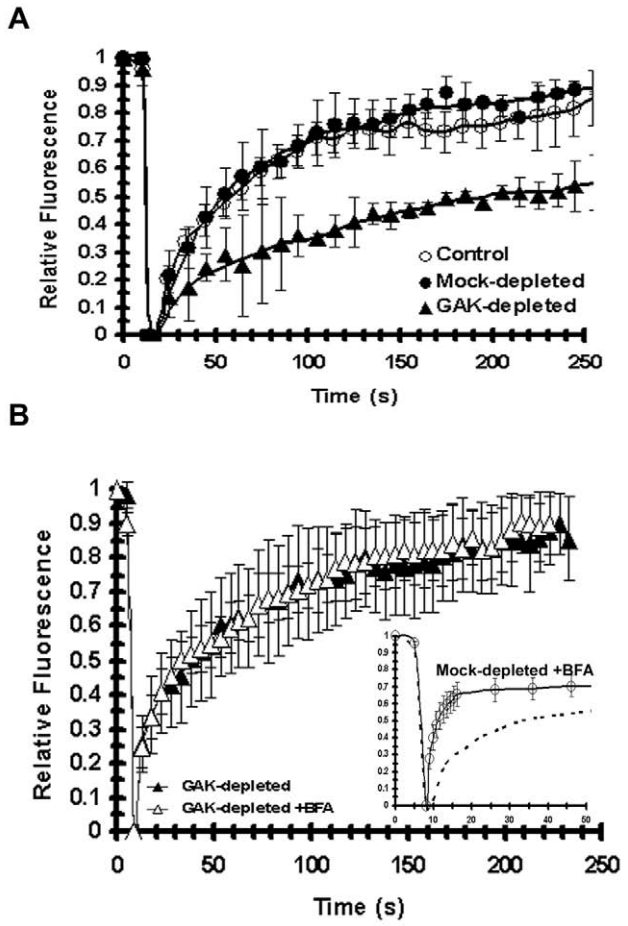

Fig. 6. Clathrin exchange in GAK-depleted cells. (A) The exchange of GFP-clathrin on CCPs was determined in control (open circles), mock-depleted (solid circles) and GAK-depleted (solid triangles) HeLa cells by measuring the fluorescence recovery after photobleaching. (B) The exchange of cytosolic clathrin in GAK-depleted cells was measured in the presence (open triangles) and absence (solid triangles) of BFA. The inset shows the time course of recovery of cytosolic clathrin measured after treating mock-depleted cells with BFA (open circles). For comparison, the dashed line shows the rate of recovery of the cytosolic clathrin in GAK-depleted cells.

fluorescence recovery of the cytosolic clathrin clusters showed that the cells had varied mobile fractions of clathrin (Fig. 8C). In about one-third of the cells expressing Hsp70(K71E), the fluorescence recovery of the photobleached clathrin had a half-life of about 20 seconds (solid triangles), a rate similar to that observed in the GAK-depleted cells (dashed line). However, in one-third of the cells the GFP-clathrin in the cytosolic clusters showed essentially no fluorescence recovery (open triangles). In the remainder of the cells, the rate and extent of the fluorescence recovery of the cytosolic clathrin clusters ranged between these two extremes. As in GAK-depleted cells, the cytosolic clathrin clusters were insensitive to BFA treatment, suggesting that they are neither early endosomes nor fragmented TGN. Since the clathrin clusters had such unique properties in cells depleted of GAK or over expressing the dominant-negative mutant of Hsp70, one possibility is that when clathrin is no longer chaperoned by Hsc70 it forms clathrin baskets in the cytosol and these form clusters that are constantly dissociating and reforming. The heterogeneity in the mobility of the clathrin associated with the cytosolic clathrin clusters is probably due to differences in expression levels of Hsp70(K71E). Nevertheless, these results suggest that over expression of Hsp70(K71E) is more effective at preventing clathrin exchange in the cytosolic clathrin clusters than knocking down GAK.

The effect of GAK depletion and Hsp70(K71E) expression on adaptor binding to the plasma membrane and TGN

All of the functional changes in trafficking that we observed after depletion of GAK involved organelles known to normally contain clathrin. Therefore, one explanation for our observations is that GAK is required for the normal functioning of clathrin coats in the cell, perhaps through mediating clathrin exchange and chaperoning cytosolic clathrin. This, in turn, suggests that depletion of GAK might produce a phenotype similar to the one obtained after depletion of clathrin. Interestingly however, depletion of clathrin did not significantly prevent the clathrin adaptor, AP2, and the accessory protein, epsin, from forming a lawn of puncta on the

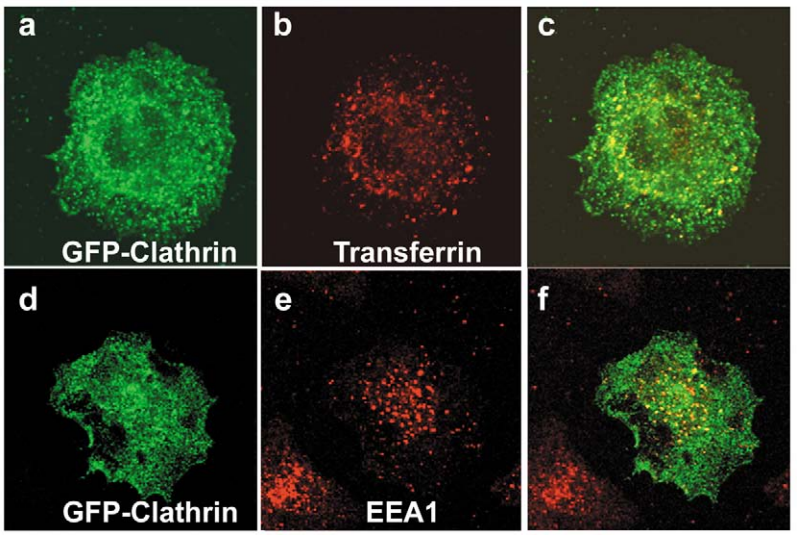

Fig. 7. Colocalization of GFP-clathrin clusters in GAK-depleted cells with transferrin and EEA1. Confocal images of GFP-clathrin (a,d) and either transferrin (b) or immunostained EEA1 (e) in GAK-depleted cells. The cells internalized Alexa Fluor 546-transferrin for 30 minutes. (c,f) The merged images showing the overlaps.

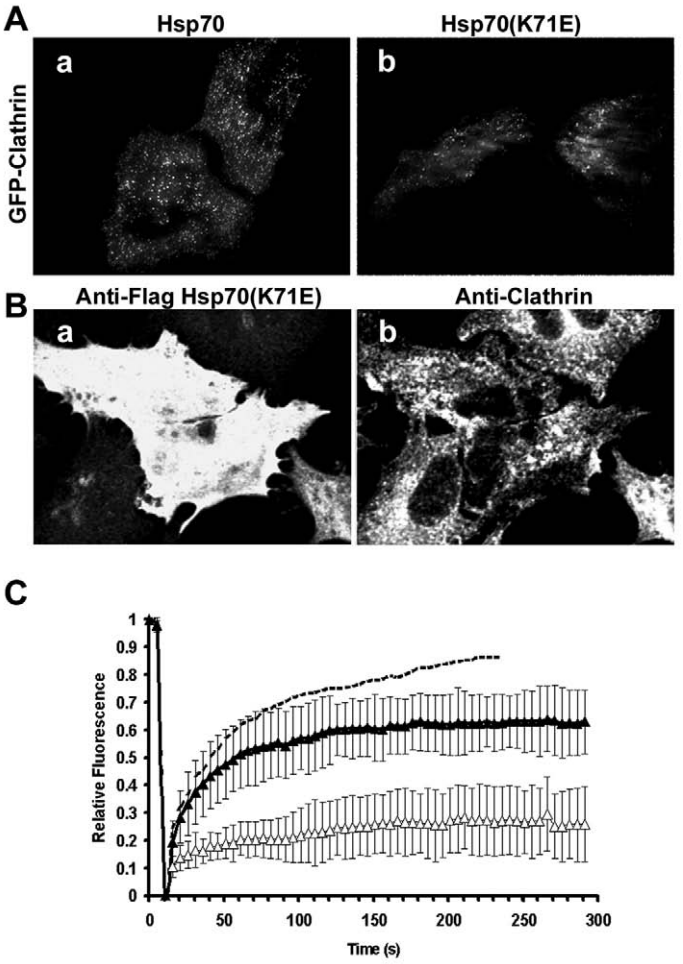

Fig. 8. Clathrin localization on the plasma membrane and TGN is altered in cells transfected with Hsp70(K71E). (A) TIR-FM images of GFP-clathrin in cells that were cotransfected with GFP-clathrin and either Hsp70 (a) or Hsp70(K71E) (b). (B) Confocal images of cells transfected with Hsp70(K71E) and immunostained with anti-flag (a) and anti-clathrin (b) antibodies. (C) The exchange of cytosolic clathrin clusters measured in cells cotransfected with GFP-clathrin and Hsp70(K71E). Data were collected from photobleaching 30 cells. One-third of the cells recovered with a half-life of 20 seconds (solid triangles), one-third showed no significant recovery (open triangles) and the remainder of the cells recovered with a time course between these extremes. The dashed line is the time course of recovery of the cytosolic clathrin measured in the GAK-depleted cells in Fig. 6B.

plasma membrane (Motley et al., 2003; Hinrichsen et al., 2003) nor did it significantly affect the association of the TGN clathrin adaptors AP1 (Hinrichsen et al., 2003) and GGA3 (our unpublished data). We were, therefore, interested in determining whether depletion of GAK likewise had no effect on the localization of these proteins. Surprisingly, in contrast to the depletion of clathrin, Fig. 9A,B shows that depletion of GAK had a profound effect on the formation of AP2 and epsin puncta at the plasma membrane. In addition to causing a marked reduction in these puncta, the few remaining puncta were clustered and contained bound clathrin. The remainder of the AP2 and epsin appeared diffuse in the cytosol and freely mobile, as determined by measuring their rate of recovery after photobleaching (data not shown). GAK depletion also caused a dramatic reduction in the association of the TGN adaptors, AP1 and GGA3, with the fragmented TGN (Fig. 9C). Most of these adaptors were dispersed throughout the cytosol. FRAP experiments confirmed that these adaptors were free in the cytosol, just like AP2 (data not shown). Since GAK depletion caused a dramatic reduction in deposition of clathrin adaptors, GAK apparently not only uncoats and chaperones clathrin, but also functions in the recruitment of clathrin adaptors at the plasma membrane and TGN.

This would predict that, like depletion of GAK, transient expression of Hsp70(K71E) should prevent recruitment of AP2 and epsin to the plasma membrane and TGN. As shown in Fig. 10A, there was indeed a dramatic reduction in pits containing AP2 and epsin in cells over expressing Hsp70(K71E), an effect that was not observed in cells overexpressing wild-type Hsp70. Fig. 10B shows that there was also a dramatic reduction in the association of AP1, GGA3 and GAK with the TGN in cells overexpressing Hsp70(K71E).

Discussion

Previously, Zhang et al. (Zhang et al., 2004) examined the effect of GAK depletion using a stable HeLa cell line in which the GAK level was reduced by more than 90%. This stable cell line has several phenotypic differences to our transiently transfected cells. First, rather than observing a significant increase in expression of EGFR in GAK-depleted cells when the level of receptor was measured by the same immunoprecipitation method used in their study, we found that the GAK-depleted cells showed a reduction in EGFR (supplementary material Fig. S1C). In addition, in contrast to our results, their stably depleted GAK cells did not show a decrease in transferrin uptake or a change in the distribution of clathrin or AP2. However, we observed a marked decrease in the rate of receptor uptake after addition of EGF and a mislocalization of early endosomes, phenotypes also observed in the GAK-depleted stable cell line. The phenotypic differences we did observe are most likely due to transient, as opposed to, stable depletion of GAK. In the latter case, the cells have been selected to survive in the absence of GAK. In fact, Zhang et al. (Zhang et al., 2004) found that, although transferrin uptake was inhibited upon transient depletion of GAK, it was not inhibited in their stably depleted cell line. It is important to note that, since GAK acts catalytically to induce Hsc70 binding to clathrin (Greener et al., 2000), if there was slightly more GAK present in the stable cell line than in the transiently transfected cells, it could markedly affect their phenotype. Upregulation of the auxilin gene in the stable cell line is also a possibility.

Since depletion of GAK and expression of Hsp70(K71E) caused similar phenotypes, it is likely that most of the effects of GAK depletion are due to an interference with Hsc70 function. Interestingly, in addition to affecting clathrin localization, depletion of GAK and expression of Hsp70(K71E) affected recruitment of clathrin adaptors. This loss of the TGN adaptors might have been responsible for the disruption of the entire Golgi. Alternatively, since the kinase domain of GAK phosphorylates the mu1 and mu2 chain of AP1 and AP2, respectively, it is possible that some of our

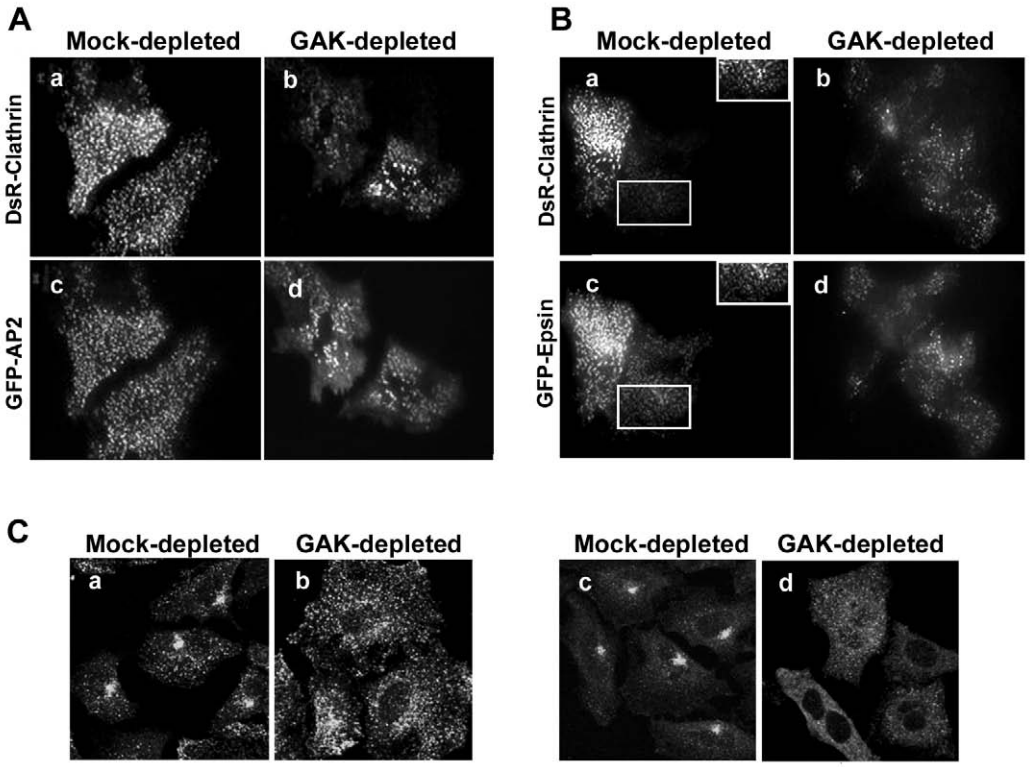

Fig. 9. Localization of clathrin adaptors on the plasma membrane and TGN is altered in GAK-depleted cells. (A) TIR-FM images of mock-depleted (a,c) and GAK-depleted (b,d) cells cotransfected with DsRed-clathrin (a,b) and GFP-AP2 (b,d). (B) TIR-FM images of mock-depleted (a,c) and GAK-depleted (b,d) cells cotransfected with DsRed-clathrin (a,c) and GFP-epsin (b,d). In the inset, the brightness was increased to better visualize the pits of the dimmer cell (boxed region) that was expressing clathrin and epsin at lower levels than the neighboring cell. (C) Confocal images of immunostained TGN-associated clathrin adaptors, AP1 (a,b) and GGA3 (c,d) in mock-depleted (a,c) and GAK-depleted (b,d) cells.

observed phenotypes are caused by loss of GAK kinase function (Umeda et al., 2000; Korolchuk and Banting, 2002). In yeast, the Ark1p and Prk1 kinases, which are related to GAK kinase, have been shown to affect endocytic trafficking (Toshima et al., 2005). Although at this time we can not determine the effect of specifically deleting the GAK kinase domain, mammalian cells also have the AAK1 kinase that, like GAK, phosphorylates the mu2 chain (Conner and Schmid, 2002). Therefore, the effects of deletion of the GAK kinase may not be observable until both kinases are deleted.

Our structural studies on the effect of GAK depletion showed a disruption of the clathrin lattice at the various membranes where it normally occurs. The disruption could occur for several reasons. If all of the clathrin were trapped in vesicles that could not be uncoated, there might be a lack of free clathrin to form normal clathrin lattices. However, the clathrin that is present in the cytosol does not appear to be associated with CCVs. Therefore, the decrease in CCPs on the plasma membrane and at the TGN suggests that Hsc70 may be required to chaperone free clathrin, thus facilitating its binding to CCPs. It has previously been noted that free clathrin does not appear to bind to CCVs or uncoated vesicles in vitro (Jiang et al., 2000; Wu et al., 2001). This observation might be explained by a requirement for Hsc70 to form a complex with clathrin in order for clathrin to efficiently bind to the plasma and TGN membranes.

We previously suggested that Hsc70 is required for the clathrin exchange that we observed on CCPs in vivo (Wu et al., 2001). In confirmation of this view, we have now found that the rate of clathrin exchange on the few remaining CCPs in the GAK-depleted cells was markedly decreased. Surprisingly, however, the clathrin in the cytosolic clathrin clusters exchanged relatively rapidly despite the depletion of GAK. By contrast, in about one-third of the cells over expressing Hsp70(K71E), the cytosolic clathrin clusters showed very slow clathrin exchange. One possibility is that these clusters are actually clathrin baskets, which previous fractionation studies have suggested occur in cells expressing dominant negative Hsc70 (Newmyer and Schmid, 2001). If the clusters are indeed clathrin baskets, the clathrin exchange we observe in the GAK-depleted cell could be the result of the small amount of GAK still remaining in the cell. This result would be consistent with our previous observation that much lower levels of GAK are required to uncoat clathrin baskets in vitro than to uncoat CCVs (Jiang et al., 2000).

Perhaps the most surprising result of our present study is that, in contrast to the abortive pits composed of AP2 and epsin observed in cells depleted of clathrin, such pits did not form in either the GAK-depleted cells or in the cells expressing Hsp70(K71E). If Hsc70 is involved only in uncoating clathrin from CCVs, it is difficult to see why depletion of GAK should have a greater effect on formation of these abortive pits than depletion of clathrin itself. Therefore, these data suggest that GAK and Hsc70 may play a role in the recruitment of clathrin adaptors and accessory proteins to the pits, independent of clathrin binding. This suggestion is consistent with the observation that GAK binds to AP1, AP2 and dynamin, as well as clathrin (Umeda et al., 2000; Scheele et al., 2001; Newmyer et al., 2003; Ahle and Ungewickell, 1990). Therefore, GAK may be incorporated in the abortive pits even though clathrin is not present. Of course, this effect could occur for the trivial reason that, like clathrin, clathrin adaptors are aggregated in the cytosol of GAK-depleted cells. However, FRAP studies suggest that, unlike clathrin, clathrin adaptors are completely free in the cytosol.

In the recent study of Ehrlich et al. (Ehrlich et al., 2004),

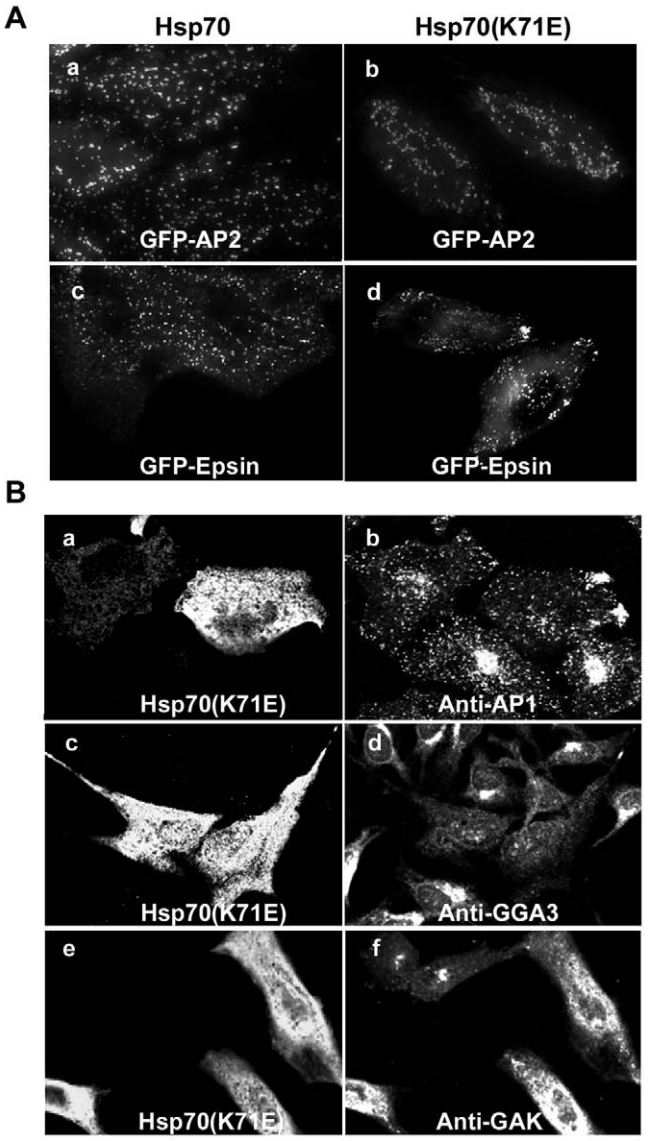

Fig. 10. Clathrin adaptors on the plasma membrane and TGN are altered in cells transfected with Hsp70(K71E). (A) TIR-FM images of GFP-AP2 (a,b) and GFP-epsin (c,d) in cells cotransfected with either Hsp70 (a,c) or Hsp70(K71E) (b,d). (B) Confocal images of cells transfected with Hsp70(K71E) stained with flag antibodies (a,c,e) and TGN-associated adaptors: AP1 (b), GGA3 (d) and GAK (f).

clathrin and AP2 were shown to form nascent CCPs, which are likely to dissolve unless the binding of cargo stabilizes them. If GAK and Hsc70 bind to pits very early in their formation, the chaperone activity of Hsc70 may be involved in the further binding of various components to the nascent pits as they develop and mature after binding cargo. Alternatively, if cargo does not bind, Hsc70 may be involved in the dissolution of the nascent pits just as it is responsible for the uncoating of CCVs. Therefore, GAK and Hsc70 may play a critical role in the dynamics of CCPs very early in their development as well as in the exchange of clathrin that occurs as the pits invaginate. Finally, it has been proposed that GAK and Hsc70 are involved in the dynamin-induced pinching-off of the CCVs (Newmyer et al., 2003). It remains to be determined what triggers GAK and Hsc70 to completely uncoat the vesicles after they have invaginated in contrast to the much more subtle role they play earlier during the maturation and invagination of CCPs.

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