Auxilin is a brain-specific DnaJ homolog that is required for Hsc70 to dissociate clathrin from bovine brain clathrin-coated vesicles. However, Hsc70 is also involved in uncoating clathrin-coated vesicles formed at the plasma membrane of non-neuronal cells suggesting that an auxilin homolog may be required for uncoating in these cells. One candidate is cyclin G-associated kinase (GAK), a 150-kDa protein expressed ubiquitously in various tissues. GAK has a C-terminal domain with high sequence similarity to auxilin; like auxilin, this C-terminal domain consists of three subdomains, an N-terminal tensin-like domain, a clathrin-binding domain, and a C-terminal J-domain. Western blot analysis shows that GAK is present in rat liver, bovine testes, and bovine brain clathrin-coated vesicles. More importantly, liver clathrin-coated vesicles, which contain GAK but not auxilin, are uncoated by Hsc70, suggesting that GAK acts as an auxilin homolog in non-neuronal cells.

In support of this view, the clathrin-binding domain of GAK alone induces clathrin polymerization into baskets and the combined clathrin-binding domain and J-domain of GAK supports uncoating of AP180-clathrin baskets by Hsc70 at pH 7 and induces Hsc70 binding to clathrin baskets at pH 6. Immunolocalization studies suggest that GAK is a cytosolic protein that is concentrated in the perinuclear region; it appears to be highly associated with the trans-Golgi where the budding of clathrin-coated vesicles occurs. We propose that GAK is a required cofactor for the uncoating of clathrin-coated vesicles by Hsc70 in non-neuronal cells.

The molecular chaperone Hsc70 appears to be responsible for the uncoating of clathrin-coated vesicles during endocytosis (1–3). This process requires both ATP and auxilin, originally identified as a minor clathrin assembly protein (4) but more recently identified as a member of the DnaJ family of proteins that support Hsc70 function (5, 6). Since auxilin is a brain-specific protein, it may be supporting Hsc70 in the uncoating of clathrin-coated vesicles formed during the recycling of synaptic vesicles. However, Hsc70 may also be involved in uncoating clathrin-coated vesicles formed at the plasma membrane of non-neuronal cells (7). Furthermore, while recruitment of clathrin and AP-1 to clathrin-coated vesicles formed at the trans-Golgi network is mediated by the small GTP-binding protein ARF1 (8, 9), the resulting clathrin-coated vesicles contain little ARF1 (10). Thus uncoating of these vesicles is probably not mediated by ARF1, but rather by Hsc70. The question therefore arises as to whether different homologs of auxilin are involved in uncoating different types of clathrin-coated vesicles.

Auxilin has three domains, an N-terminal tensin domain, a clathrin-binding domain that can assemble clathrin into clathrin baskets, and a C-terminal J-domain that interacts with Hsc70 (6, 11, 12). Auxilin shares many properties with other DnaJ homologs such as increasing the rate of ATP hydrolysis by Hsc70 and inducing Hsc70 to polymerize reversibly in ATP but not ADP (13–15), but it differs from other DnaJ homologs, like yeast YDJ1 and human HDJ1, in that it binds strongly to Hsc70 in ATP (15–18). DnaJ homologs appear to function by presenting substrates to Hsc70 (19–21) and, in this regard, auxilin acts as a classical DnaJ homolog by inducing Hsc70 to bind to clathrin baskets (22). Since other DnaJ homologs cannot substitute for auxilin in this reaction (23, 24), the clathrin-binding domain of auxilin is apparently crucial to its ability to support uncoating by Hsc70-ATP.

Recently, GAK, a 150-kDa Ser/Thr protein kinase, was cloned from both rats and humans (25, 26). While the N terminus of GAK is a functional Ser/Thr protein kinase, the rest of the molecule is highly homologous to the three domains of auxilin. Excluding its kinase domain, GAK is 43% identical to auxilin in its amino acid composition, and, including conserved residues, the homology between these proteins increases to 57% with the tensin and J-domains showing the highest homology.

GAK was originally isolated as a protein that can be immunoprecipitated with cyclin G (25). Since there is little evidence that GAK is involved in regulating the cell cycle and, since, in contrast to auxilin (4), GAK is expressed ubiquitously in various tissues (25, 26), it seemed possible that one of the functions of GAK is to support uncoating by Hsc70 in non-neuronal cells. To test this possibility, we investigated whether GAK is present in clathrin-coated vesicles isolated from non-neuronal cells, and whether GAK acts like auxilin in assembling clathrin tinselions into clathrin baskets and in supporting uncoating by Hsc70.

We found that while clathrin-coated vesicles isolated from testes and liver do not contain auxilin they do contain GAK, and furthermore, the liver clathrin-coated vesicles showed normal uncoating when treated with Hsc70. We also found that GAK acts like auxilin in regard to its ability to assemble clathrin into baskets, to support the uncoating activity of Hsc70, and...
to induce Hsc70 to bind to clathrin baskets at pH 6 where uncoating does not occur. Furthermore, like auxilin, GAK requires a functional J-domain to support uncoating and induce Hsc70 binding, and in both cases it carries out these activities catalytically. Therefore, our results suggest that GAK supports clathrin uncoating by Hsc70 in non-neuronal cells.

EXPERIMENTAL PROCEDURES

Preparation of Proteins—Hsc70, clathrin-coated vesicles, clathrin, and AP180-clathrin baskets were prepared as described previously (3, 5). Coated vesicles from calf testes and rat liver were isolated by the same procedures used for calf brains (3). AP180 was prepared as a recombinant GST fusion protein (27).

Construction and Expression of Recombinant GAK and Auxilin Fragments (Fig. 1) The recombinant proteins GAK62, GAK62ΔJ, AUX54, and AUX54ΔJ were prepared as 6His fusion proteins. GAK cDNA was subcloned in pQE30 to give GAK62, a 6His fusion protein including GAK 560 C-terminal amino acids. Deletion from GAK62 of the 93 C-terminal amino acids, containing the J-domain produced GAK62ΔJ. Recombinant GAK kinase domain was prepared as a GST fusion protein by subcloning the first 420 N-terminal amino acids in pGEX4T1. Recombinant proteins were expressed in Escherichia coli TG100 strain (E. coli B, lon−, ompT−, recA−, lacI−). Starter cultures (300 ml) were grown overnight at 30 °C, and then were diluted 1:10 in 3 liter of fresh LB medium, and recovered for 1 h at 30 °C. AP180 was prepared as a 6His GST fusion protein (26). Reactions mixtures (50 μl, final volume) contained 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, and 5 μM of GST-GAK kinase and 8 μg of histone H1, and the control reaction contains 3 μg of GST and 8 μg of histone H1. The reactions were incubated for 4 h at 37 °C, and then analyzed by electrophoresis (SDS-PAGE) and subsequent exposure to x-ray film.

Antibody Production and Immunodecoration—The recombinant polyclonal antibody against GAK used in this work was obtained from rabbits that were immunized with GAK62 (Research Genetics). Monoclonal auxilin antibody (100/4) was obtained from E. Ungewickell. Western blot analysis was performed as described in the ECL manual by Amersham Pharmacia Biotech. Briefly, blots were blocked with 5% bovine serum albumin in PBS buffer with 1:1000 Tween 20 for 1 h at 25 °C. Blocked blots were incubated for 1 h at 37 °C with 1:50 diluted pGAK62 in PBS buffer with 0.5% bovine serum albumin and 1:1000 Tween 20.

Cell Culture and Antibodies—HeLa cells were purchased from ATCC. The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 μg/ml), and streptomycin (100 μg/ml) in a humidified incubator with 5% CO2 at 37 °C. Monoclonal antibodies against clathrin heavy chain (X22) was purchased from Affinity Bioreagents, Inc. Monoclonal antibodies against vinculin and γ-adaptin (100/3) were from Sigma. Fluorescein isothiocyanate- or rhodamine- conjugated donkey anti-mouse or donkey anti-rabbit antiseraums were purchased from Jackson ImmunoResearch Laboratories, Inc.

Indirect Immunofluorescence Microscopy—Cells growing on glass coverslips were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature and washed with PBS containing 10% fetal bovine serum. Cells were then incubated with primary antibody in PBS containing 10% fetal bovine serum and 0.2% saponin for 1 h at room temperature. Cells were washed and incubated with appropriate secondary antibodies conjugated with fluorescein isothiocyanate or rhodamine. Cells were washed again and mounted using Gel/Mount from Biomeda Corp. Samples were viewed under Zeiss conventional microscope.

Materials—Superose-6 was obtained from Amersham Pharmacia Biotech; ATP, MES, creatine kinase, hexokinase, imidazole, GST affinity resin, and reduced glutathione were from Sigma. SDS-polyacrylamide gels (4–20%) from Oval Inc. Primers were made and sequences determined by BioSynthesis Inc. 6His affinity resin and pQE vector were obtained from QIAGEN. pGEX4T1 was obtained from Amersham Pharmacia Biotech, polymerase chain reaction kits from Roche Molec-
ular Biochemicals, and restriction enzymes were obtained from Roche Molecular Biochemicals and New England BioLabs.

RESULTS

GAK Is Present in Non-neuronal Clathrin-coated Vesicles—

Fig. 2 shows the uncoating of bovine brain and rat liver clathrin-coated vesicles by Hsc70. As previously reported (7), Hsc70 uncoats rat liver clathrin-coated vesicles comparably to bovine brain clathrin-coated vesicles. The question therefore arises as to whether auxilin is indeed absent from liver as well as other non-neuronal clathrin-coated vesicles, and if so whether liver and other non-neuronal clathrin-coated vesicles instead contain the auxilin homolog, GAK. To answer this question, we prepared a polyclonal anti-GAK antibody against recombinant GAK62, which is the C-terminal portion of GAK (Fig. 1). In addition, we used a monoclonal anti-auxilin antibody (100/4) to test for the presence of auxilin. The Western blot in Fig. 3 shows the reaction of these antibodies against both GAK62 and AUX54, a comparable truncated auxilin recombinant. When the same concentrations of GAK62 and AUX54 were immunoblotted with the polyclonal anti-GAK antibody, the antibody reacted much more strongly with GAK62 than AUX54. In contrast, mAb 100/4 only reacted with AUX54 and not with GAK62 (data not shown).

Fig. 3B shows a Western blot of clathrin-coated vesicles obtained from bovine brain (lane 1), calf testes (lane 2), and rat liver (lane 3) immunoblotted with the anti-GAK polyclonal antibody. Clathrin-coated vesicles from liver and testes showed only one significant band that corresponds to GAK in molecular weight. On the other hand, clathrin-coated vesicles from bovine brain showed two bands that correspond to GAK and auxilin in molecular weight. There is about the same amount of GAK in brain, liver, and testes clathrin-coated vesicles. By using GAK and auxilin standards to quantify the Western blots with anti-GAK polyclonal and anti-auxilin monoclonal antibodies, we found that there is about 10 times more auxilin than GAK in the brain-coated residues.

Functional Properties of GAK—

The activities of GAK62 and GAK62ΔJ (GAK62 with its J-domain deleted) were compared with the activities of the comparable constructs prepared from auxilin, AUX54, and AUX54ΔJ. We used truncated GAK and auxilin constructs because it was previously shown that the

FIG. 3. Western blot of GAK and auxilin from clathrin-coated vesicles isolated from different tissues. A: 1 μM GAK62 (lane 1); 1 μM AUX54 (lane 2) blotted with anti-GAK polyclonal antibody. B: 0.1 μM bovine brain-coated vesicles (lane 1), 0.1 μM calf testes-coated vesicles (lane 2), and 0.1 μM rat liver-coated vesicles (lane 3) blotted with anti-GAK polyclonal antibody. C, the same as B but blotted using mAb100/4, auxilin monoclonal antibody. To quantify the amounts of GAK and auxilin in the brain, Western blots of brain clathrin-coated vesicles were run with known concentrations of recombinant GAK and auxilin and then immunoblotted with anti-GAK polyclonal and anti-auxilin monoclonal antibodies. The ECLs of these Western blots were scanned to quantify the amount of auxilin and GAK in the brain clathrin-coated vesicles and the results showed that there is about 10 times more auxilin than GAK in the brain-coated residues.

FIG. 4. GAK polymerizes clathrin into baskets. A, the amounts of clathrin sedimenting as a result of polymerization with increasing concentrations of GAK62 (squares), GAK62ΔJ (circles), and AUX54 (triangles) were obtained after ultracentrifugation from densitometry of SDS-PAGE gels. B, electron microscopy of GAK62-clathrin baskets after negative staining with uranyl acetate.
full-length auxilin containing the tensin domain is expressed very poorly in E. coli, whereas the truncated auxilin is expressed very well and acts exactly like intact auxilin in vitro (12). Fig. 4A shows that GAK62 and GAK62ΔJ induced clathrin assembly as effectively as auxilin with almost all of the clathrin assembling at a ratio of one GAK62 per clathrin triskelion. Furthermore, the structure of the clathrin baskets polymerized by GAK62 appeared normal by electron microscopy (Fig. 4B). More importantly, like auxilin, GAK supported the uncoating of AP180-clathrin baskets by Hsc70. The SDS gel in Fig. 5 shows that addition of catalytic amounts of GAK62 caused uncoating of clathrin from the baskets (lane 3), similar to that obtained with AUX54 (lane 5). This uncoating requires an intact J-domain since neither GAK62ΔJ (lane 4) nor AUX54ΔJ (lane 6) supported uncoating.

The time course of clathrin release from the AP180-clathrin baskets at varying concentrations of GAK showed a rapid initial burst of uncoating followed by very slow steady-state uncoating (Fig. 6A). Both the rate and magnitude of the uncoating saturated at about a 1 to 10 molar ratio of GAK to both Hsc70 and clathrin baskets (Fig. 6B). Our previous results with auxilin showed the same time course of uncoating and a similar substoichiometric dependence of uncoating on auxilin concentration (5). Even very high concentrations of GAK62ΔJ were unable to support uncoating (Fig. 6B, circles), further confirming the importance of the J-domain for this process. These quantitative data offer additional support for the view that GAK62 functions like auxilin.

We also examined whether GAK62 induces the binding of Hsc70 to pure clathrin baskets in ATP at pH 6, as was observed previously with auxilin (22). Fig. 7 shows that catalytic amounts of GAK62 induced Hsc70 to bind to clathrin baskets. Furthermore, as found for uncoating, the J-domain is required for this effect; GAK62ΔJ (circles) did not induce the binding of clathrin baskets to Hsc70. Finally, we found that this binding requires ATP. After the complex was formed in ATP, addition of hexokinase/glucose to remove the ATP caused the complex to decay with a half-life of about 5 min (Fig. 8). These results are similar to those obtained with auxilin (22) and indicate that GAK promotes the formation of a metastable complex between Hsc70-ADP and clathrin baskets at pH 6.

Finally we found that not only does the isolated auxilin domain of GAK have almost exactly the same physiological activity as auxilin, but, in addition, the isolated kinase domain of GAK retains the ability of the intact GAK molecule (26) to phosphorylate histones (Fig. 9). Therefore, in regard to their basic functions, both the isolated auxilin domain and the isolated kinase domain appear to act independently of the remainder of the molecule.

Localization of GAK—Using the anti-GAK polyclonal anti-
body to immunodecorate HeLa cells, we found that GAK is mainly localized in the cytosol (Fig. 10A), particularly in the perinuclear area where it extensively colocalized with AP-1, a marker for the trans-Golgi network (Fig. 10B). Similarly, there is extensive colocalization of clathrin and GAK on the trans-Golgi network (Fig. 10C and D). Our results also show that GAK is localized on the plasma membrane, but appears to be localized at focal adhesions rather than at coated pits. This can best be seen by comparing the immunodecoration of HeLa cells with anti-GAK (Fig. 10E) and anti-vinculin antibodies (Fig. 10F), which localize at focal adhesions. Possibly, the localization of GAK at the focal adhesions is related to the tensin domain in GAK.

**DISCUSSION**

In the present study we investigated whether, like auxilin, GAK functions as a cofactor in the uncoating of clathrin-coated vesicles by Hsc70. We previously showed that auxilin is the only DnaJ homolog that is able to support uncoating of clathrin-coated baskets by Hsc70 (23); other DnaJ homologs not only do not support uncoating, but actually inhibit the effect of auxilin (23, 24). In the present study we have obtained considerable evidence that GAK plays the same role in non-neuronal cells where it is widely distributed (25, 26) as auxilin plays in neuronal cells. First, we isolated clathrin-coated vesicles from testes and liver and showed that GAK is associated with these vesicles while auxilin is not. Second, we showed that the clathrin-coated vesicles from liver are uncoated by Hsc70 in vitro. Therefore, it seems very likely that GAK is the component of the clathrin-coated vesicles that is supporting uncoating by Hsc70 in these tissues.
Supporting this view is our observation that GAK carries out the same functions as auxilin in vitro. To carry out these in vitro experiments, we prepared a truncated recombinant GAK that contains both the clathrin-binding and J-domain regions of GAK, but lacks the tensin and kinase domains. Our results show that GAK62 is remarkably similar to auxilin in its interaction with clathrin and Hsc70. GAK62 stoichiometrically induces assembly of clathrin baskets from clathrin triskelions. More importantly, it supports uncoating of clathrin baskets by Hsc70 at pH 7 and induces the binding of clathrin baskets to Hsc70 at pH 6, and in both cases does so only when it contains the J-domain that interacts with Hsc70. Finally, it carries out these functions catalytically rather than stoichiometrically demonstrating that the auxilin domain of GAK is not only qualitatively similar to auxilin but is also quantitatively as active as auxilin (5). Our observation that GAK as well as auxilin functions catalytically in supporting uncoating may explain why much less GAK is present in non-neuronal cells than auxilin in neuronal cells; only small amounts of GAK may be required in these cells because it acts catalytically rather than stoichiometrically. On the other hand, the large amount of auxilin present in neuronal cells may reflect the necessity for very rapid endocytosis to occur during synaptic transmission.

GAK was originally discovered as a protein that immunoprecipitated with both cyclin G and CDK5, a kinase that binds to cyclin G. However, neither CDK5 nor GAK kinase activity is activated by cyclin G nor does GAK affect CDK5 kinase activity (25, 26). In the present study, we found that the N-terminal kinase domain is able to phosphorylate histone H1, indicating that this domain retains the activity of the parent molecule (26). Adjacent to the kinase domain, GAK, as well as auxilin, has a tensin domain, which has yet to be studied. Interestingly, the tensin domains of both GAK and auxilin share high homology with the tumor suppressor, PTEN (28, 29). The latter has a tensin domain, which has yet to be studied. Interestingly, the kinase domain is able to phosphorylate histone H1, indicating that the auxilin domain of GAK is not only qualitatively similar to auxilin but is also quantitatively as active as auxilin (5). Our observation that GAK as well as auxilin functions catalytically in supporting uncoating may explain why much less GAK is present in non-neuronal cells than auxilin in neuronal cells; only small amounts of GAK may be required in these cells because it acts catalytically rather than stoichiometrically. On the other hand, the large amount of auxilin present in neuronal cells may reflect the necessity for very rapid endocytosis to occur during synaptic transmission.

GAK Functions as Non-neuronal Cofactor in Uncoating

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