The Tyrosine Kinase ACK1 Associates with Clathrin-coated Vesicles through a Binding Motif Shared by Arrestin and Other Adaptors*

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The Rho family of small GTPases, of which the best characterized members include Rac, Rho, and Cdc42, are implicated in various cellular processes including morphological reorganization and transscriptional activation (1). Initial microinjection studies revealed that Rho drives the assembly of filamentous actin into stress fibers, whereas Rac and Cdc42 induce lamellipodia and filopodia, respectively (2–5). Microinjected Cdc42G12V causes filopodia production and neurite formation in neuroblastoma cells (6). In this context Cdc42 antagonizes the effects of RhoA, which needs to be down-regulated for neurite formation (6). Recently Cdc42 has been found to associate with the γ-subunit of the coatamer complex; it is hypothesized that Cdc42 may compete with cargo receptors for binding to the coatomer protein γ-COP subunit and facilitate the release of coatomer subunits from transport vesicles (7).

Many targets for the Rho GTPases have now been identified (1). The p21-activated serine/threonine kinase (PAKs)1 have an N-terminal high affinity Rac/Cdc42 binding domain that regulates the C-terminal kinase domain (8). Besides PAK, other Cdc42-interacting proteins include ACK (9), Wiskott-Aldrich syndrome protein (WASP) (10), IQGAP (11) and mixed lineage kinase 3 (12). WASP and IQGAPs regulate actin cytoskeletal reorganization (10, 11) and WASP directs actin-based motility in cell extracts by stimulating actin nucleation with the Arp2/3 complex (13).

ACK is the only tyrosine kinase known to interact with Cdc42 (9). ACK1 is a 1,036-residue protein with an N-terminal tyrosine kinase domain, flanked by SH3 and Cdc42 binding domain, and an extensive proline-rich C-terminal region. ACK1 interacts specifically with Cdc42 and not Rac1 or Rho (9). The structure of Cdc42 complexed to the p21 binding domain of ACK indicates that hydrophobic residues in the “effector loop” and also the C-terminal region of Cdc42 are important in determining the specificity of effector binding (14). The ACK-2 tyrosine kinase (a smaller spliced variant of ACK1) becomes tyrosine phosphorylated upon stimulation by epidermal growth factor (EGF) and bradykinin (15). Recently a Caenorhabditis elegans homolog termed ARK-1, which also contains a CRIB domain, has been reported to act as a negative regulator of EGF signaling downstream of let-23 (16).

Here we report that a region of ACK1 specifically associates with the N-terminal globular region of clathrin heavy chain in vitro. This region has homology to sequences in β-arrestin, which provides a link between clathrin and various serpentine receptors (17). Clathrin is the major structural component of clathrin-coated vesicles, and clathrin-mediated endocytosis plays a particularly important role in receptor internalization and export of components out of the trans-Golgi network. ACK1 is found here to co-localize with a subset of brefeldin A-insensitive clathrin vesicles and a WASP-regulated mechanism (18).

MATERIALS AND METHODS

Mammalian Cell Expression Vectors—The pXJ-HA or pXJ-FLAG and pXJ-GFP vectors containing the cytomegalovirus enhancer/promoter and encoding the Kozak initiation and N-terminal HA or FLAG epitopes were as described previously (9). GST-Nck and FLAG-Nck vectors contained the full-length human cDNA. HA-ACK was constructed by introducing a BamHI site adjacent to the initiation codon and an HindIII site flanking the termination codon of ACK1 by polymerase chain reaction (using Vent polymerase, New England Biolabs). The entire coding sequence was cloned into the pXJ-HA vector via these unique sites. The kinase-inactive ACK1K158R mutant was generated using the QuickChange protocol (Stratagene). ACK1 deletion (see Fig. 4A) and point mutants (see Fig. 4C) were constructed with BamHI- and EcoRl-containing primers by polymerase chain reaction and were cloned into pGEX vector for protein expression. The ACK1 C-terminal constructs were constructed by polymerase chain reaction using BamHI and HindIII site linkers and were cloned into pXJ-FLAG vectors for cell transfection. In Fig. 4B human ACK1, rat arrestin-3, and human sele-
nium-binding protein (SBP) 56 sequences were cloned by annealing primers with a BamHI site and EcoRI site overhang and cloned into pGEX 2T. The sequences correspond to: ACK 19-mer (564–583), ACK 22-mer (561–582), SBP56 19-mer (437–455), SBP56 24-mer (432–455), arrestin-3 19-mer (367–386), arrestin-3 26-mer (360–385).

Cell Culture—COS-7, NIH 3T3, and A431 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For transfection studies cells were plated at low density on glass chamber slides (Nunc) for 1–2 days. The cells were starved for 1 h and then transfected by using LipofectAMINE (Life Technologies, Inc.) using 0.5 μg of each plasmid and 3 μl of LipofectAMINE/ml of medium. After a 16-h overnight period in 1% fetal bovine serum cells were incubated with Texas Red-conjugated transferrin (Molecular Probes) for 15 min at 37 °C, washed, and fixed for indirect immunofluorescence.

Immunofluorescence and Imaging—Cell staining was performed as described previously (8). Primary antibodies were incubated in 0.5% Triton X-100 for 2 h at 37 °C at the following dilutions: anti-HA (Santa Cruz) or anti-FLAG monoclonal antibody (Sigma) was used at 2.5 μg/ml and anti-clathrin (ICN) or anti-adaptin (Transduction Laboratories) monoclonal antibody at a 1:200 dilution. The cells were then washed twice with 0.1% Triton X-100 and incubated with secondary antibody in 0.5% Triton X-100 for 1 h. Fluorescein isothiocyanate-conjugated second antibodies at 1:100 dilutions and rhodamine-conjugated second antibodies (Boehringer Mannheim) at 1:100 were used at room temperature. The photomicrographs were collected on a Zeiss Axioscan microscope using a ×63 oil immersion objective. Digitized data (8-bit) were subject to analysis performed using the Macintosh version of the public domain NIH Image program (developed at the U. S. National Institutes of Health and available on the Internet).

In Vitro Binding Assays—GST fusion proteins were expressed in Escherichia coli BL21 and purified as described (9). Rat brain lysates were prepared in buffer A (40 mM HEPES pH 7.5, 0.1 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM sodium vanadate, 25 mM sodium fluoride, 5% glycerol, 5 mM (dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and leupeptin). GST-ACK fusion proteins were loaded onto a glutathione-Sepharose column (50 ml, final 2 mg/ml) and incubated with 0.5% Triton X-100 for 2 h at 4 °C. Columns were washed extensively and proteins were eluted with 10 mM glutathione. Bound proteins were then eluted with 100 μl glutathione-Sepharose 4B columns, washed with GST buffer, and incubated with 0.25 ml of cytosol for 2 h. The column was washed with GST buffer and then with 1 column volume of solution D (25 mM MES, pH 6.5, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM CaCl2). 1 volume of subtilisin (2 g/ml aprotinin and leupeptin). GST-ACK fusion proteins were loaded onto a glutathione-Sepharose column (50 ml, final 2 mg/ml) and incubated with 0.5% Triton X-100 for 2 h at 4 °C. Columns were washed extensively and bound proteins released by 10 mM glutathione. Crude coated vesicles were partially purified from rat brain according to method by Kirchhausen and Harrison (19).

Competition Assay—In vitro translated clathrin was prepared according to the manufacturer’s protocol. Reticulolysates containing in vitro translated [35S]Met N-terminal clathrin was incubated with β-adrestin bound to glutathione-Sepharose in the presence of either ACK peptides (19-mer), pep-1 (normal), or pep-2 (reverse sequence). Bound proteins were eluted with 10 μl glutathione.

Proteolytic Digestion—2.5 ml of GST-ACK lysate was loaded onto 100-μl glutathione-Sepharose 4B columns, washed with GST buffer, and incubated with 0.25 ml of cytosol for 2 h. The column was washed with GST buffer and then with 1 column volume of solution D (25 mM MES, pH 6.5, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM CaCl2). 1 volume of subtilisin (2 μg/ml) in solution D was added for 30 min at room temperature. 1 column volume of solution D was defined as flow-through. Bound proteins were then eluted with glutathione (referred to as bound fraction).

Western Analysis—Proteins were transferred to nitrocellulose filters, blocked in 5% skim milk for 1 h at room temperature, and incubated with primary antibodies for 2 h at room temperature. Mouse anti-clathrin (Transduction Laboratories) was used at a 1:500 dilution in phosphate-buffered saline containing 1% milk. After washing, incubation with horseradish peroxidase-conjugated secondary antibody (Dako) was performed for 1 h. Signals were visualized in the presence of luminol (Amersham Pharmacia Biotech).

RESULTS

A Clathrin Binding Region Is Present in ACK1—The ACK1 cDNA was isolated from a human hippocampal expression library by screening with [32P]GTP-Cdc42 (9). A related human kinase is TNK1 (20), which exhibits homology in the tyrosine kinase domain, the flanking SH3 domain, and in a region at the extreme C terminus of the two proteins (Fig. 1A). A bovine brain cDNA encoding a related ACK isoform referred to as ACK2 which lacks C-terminal regions has been isolated (15). Interestingly the “gene 33” protein binds Cdc42 through an ACK-related CRIB domain and contains extensive homology to C-terminal regions also (21). To search for interacting partners of ACK, a stable GST-ACK413-446 fusion protein (Fig. 1A, construct 1) was expressed and used as an affinity matrix with rat brain lysate. An ~160-kDa protein that bound to GST-ACK413-446, but not GST, was determined to be clathrin heavy chain by peptide microsequencing of the excised band. Deletion analysis allowed us to map a minimal region required for clathrin association (Fig. 1A). The p21 binding domain (CRIB) was not required for clathrin association. A 32-residue construct (ACK461-503) was sufficient for clathrin binding, albeit with reduced efficiency.

Clathrin-mediated endocytosis involves multiprotein components that comprise the clathrin coat; accessory components include dynamin, amphiphysin, adaptors, and AP-180. We did not detect any purified protein by Coomassie staining, nor were the coated vesicle proteins AP-180 and α-adaptin enriched in the ACK-bound fraction (Fig. 1B).

ACK1 Co-localizes with Clathrin in Vivo—COS-7 cells were transiently transfected with FLAG-tagged ACK and stained for endogenous clathrin. Clathrin typically shows punctate vesicular and perinuclear staining in all cell types, reflecting its
involvement in a variety of membrane trafficking pathways (Fig. 2A, top left frame). Transient (higher level) ACK1 expression caused redistribution of clathrin that co-localized with ACK1 (Fig. 2A, top right panel). By contrast the distribution of actin was unaffected by ACK1 expression (data not shown). Various regions of ACK1 were then tested for their ability to induce this alteration in clathrin distribution (as summarized in Fig. 2C). The N-terminal kinase/SH3 domains (1–513) did not exhibit such an effect (not shown), whereas the C-terminal half of ACK1 (middle frames) induced clathrin redistribution to the same extent as the full-length protein. A minimal clathrin binding domain ACK1(561–598) did not induce aggregation but did disrupt the normal perinuclear distribution of clathrin (not shown). Thus, clathrin redistribution (clustering) may require ACK1 C-terminal sequences to form multivalent interactions with other proteins. This redistribution of clathrin was reflected in the loss of receptor-mediated endocytotic function as assayed by transferrin uptake in A431 cells (Fig. 2B). Similarly, ACK1(514–1037) containing sequences C-terminal to the p21 binding domain inhibited transferrin uptake into transfected cells. However, smaller ACK constructs (compare ACK1514–927) apparently induced clathrin aggregation while having little effect on transferrin uptake as summarized in Fig. 2C.

ACK Provides a Link between Clathrins and Signaling Adaptor Proteins—The tyrosine phosphorylation of ACK2 in response to integrin (15) and of ACK1 in response to melanoma chondroitin sulfate proteoglycan has been noted (22), where the kinase is linked to these pathways via p130Cas (23). A striking feature of ACK is the presence of multiple proline-rich motifs in the C-terminal half with substantial similarity to the Src SH3 binding domains present in the cell (Fig. 3A). The striking segregation of ACK1 and clathrin in cultured cells (Fig. 2) allows in vivo association to be tested by co-localization. As shown in Fig. 3A, the distribution of ACK was shifted dramatically by co-expression of ACK1, indicating an association of the proteins through SH3 rather than SH2 domains. This was confirmed by blot overlay (18) using the Nck SH3 domains as a probe (data not shown). Consistent with this, α-PAK528A (the catalytic inactive form that binds most tightly to the second SH3 domain of Nck) did not segregate with ACK1 complexes present in the cell (Fig. 3A). Most likely a trimeric

![Image](333x453 to 529x730)

**FIG. 2.** ACK can cause reorganization of clathrin in vivo. Panel A, fluorescence micrograph of COS-7 cells showing the distribution of ACK1 and of clathrin in untreated cells. COS-7 cells were transfected with full-length ACK1 and immunostained with affinity-purified anti-ACK1 antibody and anti-clathrin antibody (left frame) and anti-FLAG antibody (right frame) and labeled transferrin (15 min). Localization of the FLAG epitope (ACK1, right frame) and labeled transferrin (left frame) is shown. Panel B, fluorescence micrograph of A431 cells showing that overexpression of ACK1 blocks transferrin uptake. A431 cells were transfected with FLAG-tagged ACK1 and incubated with Texas Red-conjugated transferrin for 15 min. Localization of the FLAG epitope (ACK1, right frame) and labeled transferrin (left frame) is shown. Panel C, summary of the effects of various ACK constructs on clathrin redistribution in COS-7 cells or transferrin uptake in A431 cells. The data represent observations made on at least 30 cells in two separate sets of transfection experiments.
Fig. 4. Residues required for efficient clathrin binding. Panel A, sequence comparison of the core clathrin binding sequences derived from ACK, rat arrestin-3, human selenium-binding protein (SBP56), amphiphysin-2, the AP-3 subunit β3A, yeast Ent1p, and rat epsin 1. The two putative clathrin binding sequences in Drosophila ACK and C. elegans ARK-1 are shown below. Panel B, various GST-ACK, GST-SBP56, or GST-arrestin-3-derived peptides were expressed, immobilized on a glutathione-Sepharose 4B column, and tested for clathrin binding as for Fig. 1. Proteins were separated on 9% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, Coo- massie stained (upper frame), and probed with anti-clathrin monoclonal antibody (lower frame). Exposures shown were for 1 min. The contruc- ts correspond to residues of human sequences (in parentheses): ACK1 19-mer (564–582), ACK1 22-mer (561–582), SBP56 19-mer (437–455), SBP56 24-mer (432–455), arrestin-3 19-mer (367–385), and arrestin-3 26-mer (360–385). Panel C, GST-ACK561–593 containing single point substitutions as indicated above each lane were analyzed for clathrin binding as for Fig. 1 using anti-clathrin antibodies.

ACK-Nck-PAK complex is not seen because the second SH3 domain of Nck is involved in binding both partners. An interaction of Src SH3 with arrestin is implicated in the coupling of signals between G protein-coupled receptors and downstream pathways (25), indicating that Src kinases may play a general role in down-regulation of receptors. We also detected a co-localization of ACK1 and Src (data not shown) as for Nck. From analysis of various ACK constructs, at least two proline-rich Src-SH3 interacting regions (490–565 and 705–737) were identified by overlay (Fig. 3B). Because a significant fraction of cellular Src in fibroblasts is associated with endosomes and can enhance EGF receptor internalization (26), a fraction of cellular Src in fibroblasts is associated with endo-

Although only leucine is completely conserved among these binders, other candidate clathrin binders including SBP56 (29), SH3 and ankyrin repeat containing SHANK isoforms (30) were identified. Interestingly, SBP56 has recently been purified as a factor that can stimulate intra-Golgi transport (31).

GST fusion proteins displaying peptides corresponding to sequences from ACK1, β-arrestin, and SBP56 were generated and assessed for their clathrin binding potential (Fig. 4B). An ACK 19-mer bound efficiently to clathrin, but the β-arrestin 19-mer or an SBP56 19-mer interacted only weakly. Although a larger β-arrestin 26-mer bound clathrin, the SBP56 24-mer contained core sequences essentially identical to those of ACK1, it interacted only weakly under these conditions. The clathrin binding of SBP56 may require flanking sequences to adopt a higher affinity binding state. The suggestion that SBP56 stimulates intra-Golgi transport (31) via a clathrin-binding protein is intriguing.

Site-directed mutagenesis was used to introduce amino acid substitutions within GST-ACK1561–593 in the core clathrin box (Fig. 4A). Substitutions of four of the core residues prevented clathrin binding (Fig. 4C), whereas the fifth position was un-affected by the glycine → alanine change. The hydrophobic residues within the core motif (i.e. ACK1 Leu-570, Ile-571, and Phe-573) play similarly important roles in other clathrin bind-ers (32). We conclude that a central motif (LIDF) corresponding to residues 575–580 is indeed essential for clathrin binding, as has been found with other adaptors (28).

We were able to identify similar motifs (Fig. 4A) in Drosophila ACK and the recently described C. elegans homolog ARK-1 (16), although the C-terminal halves of these proteins are otherwise dissimilar.

ACK and Arrestin Compete for a Common Binding Site on the N-terminal Head Region of Clathrin—Clathrin forms a trimeric complex of three heavy and light chains in solution. These assemble into cage-like structures under appropriate condition with the globular head domain facing inward (19). To confirm that ACK interacted with this head domain, clathrin immobilized on GST-ACK443–646 was subjected to proteolytic digestion with subtilisin in situ, then flow-through and bound fractions were analyzed (Fig. 5A). After the ~160-kDa clathrin heavy chain (Fig. 5A, second lane) was fragmented by subtili- sin, and the ~50-kDa N-terminal globular “head” domain (19) remaining in the bound fraction (fourth lane) was detected by an anti-clathrin (N terminal) antibody.

Residues within the globular clathrin N-terminal domain are involved in associating with β-arrestin (17). Because ACK and β-arrestin probably bind to the same surface we next tested whether ACK and β-arrestin compete for clathrin binding. Synthetic peptides (19-mers) corresponding to the ACK clath- rin binding sequence pep-1 and a control reverse peptide (pep-2) were synthesized. In vitro translated clathrin1–579 was passed through columns containing immobilized GST/β-arrestin360–385 in the presence of these peptides (Fig. 5B). ACK pep-1 but not pep-2 (reverse sequence) could compete with β-arrestin for binding to both clathrin1–579 (head) and full-length clathrin (from the reticulocyte). The latter binds more efficiently as a consequence of its multivalent character. The high peptide concentration required for competition prob-ably reflects the dimeric nature of the immobilized GST/β-arrestin360–385 and the smaller length of competitor (i.e. GST-26-mer versus 19-mer competitor). Thus both ACK and β-arrestin bind to the same site on the clathrin head domain.

Localization of GFP-ACK1 in NIH 3T3 Cells—Because transient overexpression of ACK leads to deleterious effects on the general distribution and function of clathrin, we sought to localize directly a GFP-tagged version of ACK1 protein expressed at low level in cultured cells. Antibodies raised against the conserved N-terminal region of ACK1 recognized an endog-

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enous ACK species in COS-7 and NIH 3T3 cells that co-migrated with transfected HA-ACK1 (Fig. 6A), suggesting that this is the endogenous species not the smaller ACK2 protein. However, our antibody did not detect ACK by immunofluorescence efficiently. Therefore, a pool of NIH 3T3 cells stably expressing GFP-ACK was selected and passaged six times (−12 divisions) to confirm viability; among this population ~20% of the cells stained positive for anti-GFP, although the GFP fluorescence itself was barely detectable. The distribution of GFP-ACK1 in these cells (using anti-GFP antibodies) was punctate with no overall disruption to the perinuclear and vesicular clathrin localization, compared with the nonexpressing cells (Fig. 6B). At high magnification ACK1 indeed co-localized to a subpopulation of clathrin vesicles (Fig. 6C), although we noted that most of the vesicles showed only weak clathrin staining (marked by arrows). Thus in the merged image (right) it appears that many (green) ACK vesicles lack clathrin.

Two major populations of clathrin-containing vesicles termed AP-1 and AP-2 complexes are well characterized. AP-1-containing vesicles, which are marked by γ-adaptin, are thought to be primarily trans-Golgi originating vesicles that are sensitive to brefeldin A. As seen in Fig. 7A, brefeldin A causes loss of punctate γ-adaptin distribution but had no effect on ACK. At higher magnification it was clear that the predominantly perinuclear γ-adaptin never co-localized with GFP-ACK. By contrast there was significant co-localization with the α-adaptin in the perinuclear region, although it was apparent that many of the peripheral located AP-2 vesicles did not contain ACK.

Because AP-2 is involved in receptor-mediated endocytosis and the related β-arrestin acts as a clathrin adaptor (17) it seems likely that ACK1 affects clathrin-mediated endocytosis. In the stable GFP-ACK cell lines we noted enhanced transferrin-receptor endocytosis relative to control cells (data not shown). To confirm this effect we analyzed cells expressing moderate levels of transiently expressed ACK1 or the kinase-inactive ACK1-K168R. Texas Red uptake over a 15-min labeling period was monitored in transiently transfected COS-7 cells and quantified from the fixed cell images (using NIH Image software). This revealed that both ACK1 constructs could stimulate transferrin uptake as illustrated in Fig. 8, A and B. Thus the stimulatory effect was not dependent on kinase activity.

**DISCUSSION**

Several Cdc42 targets have been identified, and possible roles of these effectors have been reported (12). The Cdc42 binding region (present also in Drosophila and C. elegans ACK-related kinases) is thought to differ somewhat from those of WASP and PAK where residues C-terminal to the GTPase binding domain regulate intramolecular interactions (33). Thus it is possible that Cdc42-GTP targets ACK but plays no role in modulating kinase function.

Taking our data with the recent report that an ACK1 homolog ARK-1 inhibits EGFR receptor signaling in C. elegans (16), it is tempting to speculate that this occurs by a stimulation of clathrin-mediated receptor endocytosis. This idea is strengthened by the observation that ARK-1 kinase activity is dispensable for this function which instead resides in the C-terminal...
half of ARK-1 (16). Similarly expressing ACK1 or a kinase-inactive mutant alters endocytosis similarly. We have suggested a region of ARK-1 to be involved with binding clathrin (Fig. 4A), but this remains to be validated. ARK-1 is also implicated in an interaction with the Grb2 homolog SEM-5 thereby linking to the let-23 EGF-like receptor. Multiple proline-rich domains in the C-terminal half of ACK1 appears to provide SH3 docking sites for other adaptors, including Nck and Src as we show here (Fig. 3) potentially linking to tyrosine kinase receptors via SH2 interactions. Another negative regulator of EGF receptor signaling is the unc-101 gene encoding a homolog of the clathrin-associated AP-47, a medium sized component of the AP-1 complex (34). One can envisage that either a defective trans-Golgi transport system (in unc-101 mutants) or endocytic system (in ark-1 mutants) might lead to the potentiation of EGF signaling because of a sustained presence of the active receptor on the cell membrane.

The ability of overexpressed ACK to induce clathrin aggregation relates to activities associated with its C-terminal region (i.e. kinase-independent). As a consequence transient overexpressed ACK blocks transferrin uptake (Fig. 2). Such general disruption to clathrin-mediated endocytic events as has also been seen with epsin over expression (28). Gene 33 also known as receptor-associated late transducer (RALT), which is homologous to ACK1, has recently been found to bind to ErbB2 receptor complexes (21), consistent with it providing a negative feedback loop via receptor down-regulation.

The clathrin binding region in ACK1 is almost identical to a sequence in SBP56 (Fig. 4A), and yet it binds poorly to clathrin, perhaps because of the presence of lysine C-terminal to this box (acidic residues are common here). Porat et al. (31) conclude from their studies that SBP56 is required at a late stage of intra-Golgi transport (i.e. docking or fusion), although the protein is primarily cytosolic (31). Binding of ACK (or SBP56) to clathrin is perhaps controversial because the recent structure of the clathrin head with peptides derived from β-arrestin-2 or the β-subunit of AP-3 reveals significant interactions of their fifth acidic residue in the clathrin box with basic residues in clathrin (35). This position in ACK contains a glycine, however, but one should note that an equivalent residue in epsin is alanine (Fig. 4A and Ref. 28). Further, our mutagenesis data confirm that this glycine can be replaced by alanine with no effect on binding, whereas substitution of the other four residues essentially leads to complete loss of interaction. This suggests that binding of the ACK peptide to the major groove in the clathrin head occurs through a distinct set of interactions, perhaps involving a different peptide conformation. Certainly the ACK 24-mer binds to clathrin as efficiently as the arrestin 26-mer peptide (Fig. 4B).

Clathrin adaptors are not necessarily concentrated in clathrin-coated vesicles, for example neither amphiphysin nor epsin is enriched in purified clathrin-coated vesicles (36). Although the specific role of ACK in clathrin-coated vesicle-mediated...
endocytosis remains to be established, the observation that ACK is associated with vesicles relatively depleted in clathrin (Fig. 6) suggests an association with partially uncoated vesicles, an event occurring prior to docking or fusion with endosomes. ACK-2 is responsive to signaling by receptor tyrosine kinases or heterotrimeric G protein-coupled receptors (15). The nonvisual arrestins, β-arrestin and arrestin-3, promote G protein-coupled receptor internalization by binding to clathrin and promoting assembly of endocytic vesicles (17). We show here that elevated ACK1 can both stimulate and inhibit the rate of transferrin uptake (depending on the level of the protein). Such stimulated transferrin uptake might result from a general stimulation of clathrin assembly on the plasma membrane, similar to overexpression of transferrin receptor itself (36) or HIV Nef protein (37). Although tyrosine phosphorylation of integral membrane proteins can mediate endocytosis (38) we do not observe any requirement for ACK1 catalytic activity.

Although only Rho and Rac were previously implicated in receptor-mediated endocytosis (39), the involvement of Cdc42 is not unexpected. It is already known that disruption of the receptor-mediated endocytosis (39), the involvement of Cdc42 stimulation of clathrin assembly on the plasma membrane, transferrin uptake (depending on the level of the protein). Such elevated ACK1 can both stimulate and inhibit the rate of endocytosis remains to be established, the observation that ACK is associated with vesicles relatively depleted in clathrin (Fig. 6) suggests an association with partially uncoated vesicles, an event occurring prior to docking or fusion with endosomes. ACK-2 is responsive to signaling by receptor tyrosine kinases or heterotrimeric G protein-coupled receptors (15). The nonvisual arrestins, β-arrestin and arrestin-3, promote G protein-coupled receptor internalization by binding to clathrin and promoting assembly of endocytic vesicles (17). We show here that elevated ACK1 can both stimulate and inhibit the rate of transferrin uptake (depending on the level of the protein). Such stimulated transferrin uptake might result from a general stimulation of clathrin assembly on the plasma membrane, similar to overexpression of transferrin receptor itself (36) or HIV Nef protein (37). Although tyrosine phosphorylation of integral membrane proteins can mediate endocytosis (38) we do not observe any requirement for ACK1 catalytic activity.

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