The Cdc42 Target ACK2 Directly Interacts with Clathrin and Influences Clathrin Assembly*

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The Ras-related GTP-binding protein Cdc42 has been implicated in a diversity of biological functions including the regulation of intracellular trafficking and endocytosis. While screening for Cdc42 targets that influence these activities, we identified the protein-tyrosine kinase ACK2 (for activated Cdc42-associated kinase 2) as a new binding partner for clathrin. ACK2 binds clathrin via a domain that is conserved among a number of other clathrin-binding proteins including the arrestins and AP-2. Overexpression of ACK2 in NIH3T3 cells results in an inhibition of transferrin receptor endocytosis because of a competition between ACK2 and AP-2 for clathrin. Activated Cdc42 weakens the interaction between ACK2 and clathrin and thus reverses the ACK2-mediated inhibition of endocytosis. Overexpression of ACK2 increases the amount of clathrin present in fractions enriched in clathrin-coated vesicles. Taken together, our data suggest that ACK2 may represent a novel clathrin-assembly protein and participate in the regulation of receptor-mediated endocytosis.

The interactions of Cdc42 with its various effector proteins give rise to actin cytoskeletal rearrangements that influence cell shape and motility, as well as stimulate cell-cycle progression and under some conditions, malignant transformation (1–3). Recently, it has become apparent that Cdc42 also plays important roles in both early and late stage exocytosis and in regulating endocytosis (4–7). The ACKs (ACK1 and ACK2) are nonreceptor-tyrosine kinases that share some similarity with the focal adhesion kinase (FAK) and protein-tyrosine kinase 2 (PYK2) and serve as highly specific target/effectors for the Ras-related GTP-binding protein Cdc42 (8, 9). They bind Cdc42 via a Cdc42/Rac interactive binding (CRIB) domain that is also present in other Cdc42/Rac effectors, including the PAKs (for p21-activated kinases), WASPs (Wiscott-Aldrich syndrome proteins), and MLKs (mixed lineage kinases) (10). ACKs have multiple known functional motifs such as SH3 and proline-rich domains. Aside from Cdc42, there are no known binding partners for ACKs. Although our previous studies indicate that ACK2 may play a role in cell adhesion signaling (11), the exact function is not known yet.

To better define the cellular role for ACK2, we have searched for novel binding partners. In this study, we show that ACK2 directly interacts with clathrin heavy chain via a conserved clathrin-binding motif shared in all endocytic adaptor proteins and that Cdc42 can regulate the ACK2-clathrin interaction. These findings now provide a potentially interesting link between Cdc42 and one of its specific target/effectors with clathrin-coated vesicle endocytosis.

EXPERIMENTAL PROCEDURES

Materials—Anti-clathrin heavy chain antibody was purchased from Transduction Laboratories. Anti-clathrin light chain antibody was a generous gift from Dr. Tom Kirchhausen of Harvard Medical School. Anti-AP2 αC subunit antibody was purchased from UBI. Cy3-conjugated transferrin was purchased from Molecular Probes. The GFP vector pGFP-C1 was obtained from CLONTECH. The ACK2 tetracycline-inducible (Tet-off) expression cell line was established as previously described (12).

Cell Culture, Transfection, Immunoprecipitation, and Immunoblotting—NIH3T3 cells were cultured in DMEM plus 10% calf serum. PC12 cells were cultured in DMEM plus 10% horse serum, 5% fetal bovine serum, whereas C2C12, COS-7, and SK-N-DZ cells were cultured in DMEM plus 10% fetal bovine serum. All cells were maintained in 5% CO2 at 37 °C. The experimental procedures for cDNA transfection, immunoprecipitation, immunoblotting, and GST fusion protein precipitation were the same as described previously (9). The immunofluorescent staining was performed as described in Ref. 7.

Construction of the Plasmids Encoding ACK2 and Its Mutants—The construction of HA- or Myc-tagged ACK2 was described previously (9). All of the ACK2 mutants were made by polymerase chain reaction-directed mutagenesis, and the mutations were confirmed by DNA sequencing. ACK2-2W2A, the SH3 domain mutant, was prepared by mutating Trp-424 and Trp-425 to Ala-424 and Ala-425; ACK2-2H2A, the Cdc42-binding defective mutant, was generated by mutating His-464 and His-467 to Ala-464 and Ala-467; ACK2-3N, the amino-terminal truncation mutant, was prepared by deleting the first 76 amino acid residues; ACK2-4C, a carboxyl-terminal truncation mutant, was prepared by deleting the last 241 amino acid residues.

Transferrin Receptor Endocytosis Assays—The cells were cultured to 80% confluency on coverslips in a 24-well plate. After the medium was removed, the cells were incubated with warm (37 °C) internalization medium (DMEM plus 1% bovine serum albumin) for 5–10 min. To initiate endocytosis, the cells were incubated with 200 μl of transferrin conjugated with Cy3 in internalization medium (1:100–200) at 37 °C for 15–30 min. Endocytosis was stopped by adding ice-cold phosphate-buffered saline, the cells were fixed with 3.7% formaldehyde at 25 °C for 10 min and then washed with phosphate-buffered saline (3×). The internalized Cy3-transferrin was monitored by fluorescence microscopy.

Membrane Fractionation—Membrane fractions were performed basically as adapted from Grimes et al. (13). The cells were harvested by scraping with a cell spade in hypotonic cell lysis buffer (40 mM Hepes, pH 7.4, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM MgCl2, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), and then homogenized manually with a 2-ml glass homogenizer. The homogenate was centrifuged at 1,000 × g for 10 min to remove cell bodies, nuclei, and cytoskeletal fractions. The supernatant was centrifuged at 8,000 × g for

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1 The abbreviations used are: ACK2, activated Cdc42-associated kinase 2; CRIB, Cdc42 interactive binding domain; DMEM, Dulbecco’s modified Eagle’s medium; PRD, proline-rich domain; GFP, green fluorescent protein; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin.
ACK2 binds clathrin heavy chain in vitro and in vivo. A, the ACK2 domains that were used for identifying ACK2-binding partners. KD, kinase domain; SH3, Src homology 3 domain; CRIB, Cdc42-binding domain; PRD, proline-rich domain. B, ACK2 binds to clathrin heavy chain in vitro. The GST-ACK2PRD1 and ACK2PRD2 fusion proteins were incubated with SK-N-DZ cell lysates, and the interactive proteins were precipitated, resolved by SDS-PAGE, transferred to Immobilon membranes and visualized by Coomassie Blue staining (left panel of B). A unique band at 180 kDa that bound to both of the fusion proteins was subjected to microsequence analysis. The sequences from two digested peptides are shown in C. A BLAST search of the GenbankTM/EBI database indicated that the 180 kDa protein was the clathrin heavy chain. Immunoblot analysis using the anti-clathrin heavy chain antibody confirmed that the band bound to both of the ACK2 fusion proteins was the clathrin heavy chain (right panel of B, lanes 5 and 6). D, ACK2 binds to the clathrin heavy chain in vivo. Myc-tagged ACK2 (4 μg/60-mm dish) or vector alone (4 μg/60-mm dish) was transfected into COS-7 cells and immunoprecipitated with an anti-Myc antibody. The total cell lysate proteins were resolved by SDS-PAGE together with samples that were precipitated with an anti-clathrin heavy chain antibody (>120 kDa) and an anti-Myc antibody (<120 kDa). Clathrin and Myc-ACK2 are indicated in the figure.

RESULTS AND DISCUSSION

To search for binding partners for ACK2, we prepared two GST constructs that each contained one of the two proline-rich domains (PRDs) from ACK2 (amino acids 490–589 and 580–711, Fig. 1A); these were designated GST-ACK2PRD1 and GST-ACK2PRD2, respectively. Following the incubation of glutathione-agarose resins containing GST-ACK2PRD1 and GST-ACK2PRD2 with lysates from the human neuroblastoma cell line SK-N-DZ, an 180 kDa protein was co-precipitated with the GST-ACK2 constructs (Fig. 1B, lanes 1 and 2). An apparently identical 180 kDa protein was precipitated following the incubation of these GST-ACK2 fusion proteins with lysates from either skeletal muscle C2C12 cells or from NIH3T3 cells transformed by oncogenic Ras (data not shown). Microsequence analysis indicated that the 180 kDa ACK2-binding protein was the clathrin heavy chain (Fig. 1C; Ref. 14). This was further confirmed by Western blot analysis of the ACK2PRD1- and ACK2PRD2-associated proteins with an anti-clathrin heavy chain antibody (Transduction Laboratory) (Fig. 1B, lanes 5 and 6). Myc-tagged ACK2 was also co-immunoprecipitated with endogenous clathrin heavy chain from COS-7 cells (Fig. 1D, lane 3).

There are 10 amino acid residues that are shared by ACK2PRD1 and ACK2PRD2 (Fig. 1A), and it therefore seemed likely that the clathrin-binding domain was contained within this overlapping region. To confirm this, two additional GST-ACK2 fusion proteins were prepared. The first, designated GST-ACK2PRD2-2, represented an amino-terminal deleted version of ACK2PRD2 that lacked 8 of the overlapping amino acids (i.e. residues that were also present in ACK2PRD1), whereas the second, designated ACK2CBD, essentially contained the region of overlap shared by ACK2PRD1 and ACK2PRD2 but lacked either proline-rich domain (Fig. 2A). As shown in Fig. 2B, clathrin binds to ACK2CBD as well as to ACK2PRD2, thus

FIG. 1. ACK2 binds to clathrin heavy chain in vitro and in vivo.

A, the ACK2 domains that were used for identifying ACK2-binding partners. KD, kinase domain; SH3, Src homology 3 domain; CRIB, Cdc42-binding domain; PRD, proline-rich domain. B, ACK2 binds to clathrin heavy chain in vitro. The GST-ACK2PRD1 and ACK2PRD2 fusion proteins were incubated with SK-N-DZ cell lysates, and the interactive proteins were precipitated, resolved by SDS-PAGE, transferred to Immobilon membranes and visualized by Coomassie Blue staining (left panel of B). A unique band at 180 kDa that bound to both of the fusion proteins was subjected to microsequence analysis. The sequences from two digested peptides are shown in C. A BLAST search of the GenbankTM/EBI database indicated that the 180 kDa protein was the clathrin heavy chain. Immunoblot analysis using the anti-clathrin heavy chain antibody confirmed that the band bound to both of the ACK2 fusion proteins was the clathrin heavy chain (right panel of B, lanes 5 and 6). D, ACK2 binds to the clathrin heavy chain in vivo. Myc-tagged ACK2 (4 μg/60-mm dish) or vector alone (4 μg/60-mm dish) was transfected into COS-7 cells and immunoprecipitated with an anti-Myc antibody. The total cell lysate proteins (lanes 1, 2) and the immunoprecipitated proteins (lanes 3, 4) were immunoblotted with an anti-clathrin heavy chain antibody (>120 kDa) and an anti-Myc antibody (<120 kDa). Clathrin and Myc-ACK2 are indicated in the figure.

10 min to pellet large vesicles. The clathrin-coated vesicles were pelletted from the 8,000 × g supernatant by centrifugation at 300,000 × g for 30 min.

FIG. 2. ACK2 has a clathrin-binding motif that is present in endocytic adaptor proteins and its binding to clathrin is regulated by Cdc42 and by the SH3 domain. A, and B, mapping the clathrin-binding domain in ACK2. The GST-ACK2PRD2, ACK2PRD2-2 and ACK2CBD fusion proteins were expressed in Escherichia coli and purified with glutathione-agarose beads. The immobilized fusion proteins were incubated with SK-N-DZ cell lysates, and the precipitated proteins were resolved by SDS-PAGE, transferred to Immobilon membranes and immunoblotted with an anti-clathrin heavy chain antibody (panel B). C, the clathrin-binding motif of ACK2 is present in endocytic adaptor proteins. The amino acid residues 580–589 of ACK2 were aligned with the clathrin-binding domains of arrestin3, β-arrestin, AP-2 β2, and AP-3 β3 (15, 16). The sequence LIDF of ACK2 represents the conserved clathrin-binding motif. D, left panel, the Myc-tagged ACK2 (WT) and its mutants, including a carboxyl-terminal truncation mutant (ΔCT), an amino-terminal truncation mutant (ΔNT), an SH3 domain mutant (2W2A), and a Cdc42-binding defective mutant (2H2A) were transfected into COS-7 cells, and the cell lysates were subjected to immunoprecipitation with an anti-Myc antibody. The proteins larger than 120 kDa were immunoblotted with an anti-clathrin heavy chain antibody, and the proteins smaller than 120 kDa were immunoblotted with an anti-Myc antibody. Right panel, immobilized GST-CD42Q61L (a GTPase-defective mutant) was incubated with lysates from Myc-Aleck2NT-transfected COS-7 cells. The precipitated proteins were resolved by SDS-PAGE together with samples that were precipitated from COS-7 cells expressing wild type ACK2 (WT) or ACK2NT using an anti-Myc antibody (lanes 1 and 2), and then transferred onto an Immobilon membrane. The proteins larger than 120 kDa were blotted with an anti-clathrin heavy chain antibody, and the proteins smaller than 120 kDa were blotted with an anti-Myc antibody.

GST-ACK2 fusion proteins were prepared. The first, designated GST-ACK2PRD2-2, represented an amino-terminal deleted version of ACK2PRD2 that lacked 8 of the overlapping amino acids (i.e. residues that were also present in ACK2PRD1), whereas the second, designated ACK2CBD, essentially contained the region of overlap shared by ACK2PRD1 and ACK2PRD2 but lacked either proline-rich domain (Fig. 2A). As shown in Fig. 2B, clathrin binds to ACK2CBD as well as to ACK2PRD2, thus
indicating that the region shared between ACK2PRD1 and ACK2PRD2 does in fact contain the clathrin-binding site. Alignment of this region (amino acid residues 580–589 in ACK2) with the clathrin-binding domains from endocytic adaptor proteins (15, 16) defines a conserved clathrin-binding motif, LIDF (Fig. 2C).

Given that ACK2 is a specific target for Cdc42, an obvious question concerned whether the interaction between ACK2 and clathrin is influenced by activated Cdc42. To address this question, we transiently expressed a Myc-tagged Cdc42-binding defective mutant, ACK2-H2A (in which two histidine residues from the CRIB motif of ACK2 were mutated to alanine), in COS-7 cells. ACK2-H2A is completely incapable of binding activated Cdc42 (data not shown). However, the binding affinity of this ACK2 mutant for clathrin was increased dramatically, as indicated from co-immunoprecipitation experiments (Fig. 2D, compare lanes 2 and 6, upper left panel; the lower left panel compares the relative levels of expression of the different Myc-tagged ACK2 constructs). In addition, when we used GST-Cdc42Q61L, a GTPase-defective mutant, to precipitate ACK2, clathrin was not complexed with ACK2 (Fig. 2D, upper right panel, compare lanes 2 and 3; ΔNT designates an ACK2 construct that lacks an amino-terminal positively charged cluster but still binds Cdc42; see below). These data indicate that Cdc42 negatively regulates the interaction of ACK2 with clathrin.

We examined whether the intramolecular interaction between the SH3 and proline-rich domains of ACK2 influenced its binding to clathrin. We mutated the two conserved tryptophan residues in the SH3 domain that are critical for binding proline-rich domains (Ref. 17; designated ACK2-W2A) and tested the ability of this mutant to bind clathrin. As shown in Fig. 2D, the amount of clathrin that co-immunoprecipitated with Myc-tagged ACK2-W2A was markedly decreased compared with the case for wild-type ACK2 (upper left panel, lanes 2 and 5), indicating that perturbations of the SH3 domain of ACK2 significantly reduced its binding affinity for clathrin. Apparently, the carboxyl-terminal proline-rich domain of ACK2, upon undergoing an intramolecular interaction with the SH3 domain (11), exposes a surface that constitutes the clathrin-binding site. When Cdc42 binds to the CRIB domain between the SH3 and proline-rich domains, this intramolecular interaction would then be reversed, such that the proper binding surface is no longer accessible to clathrin.

As expected, the removal of the carboxyl-terminal 241 residues (ACK2-ΔCT) that contains the clathrin-binding site prevents clathrin from co-immunoprecipitating with Myc-tagged ACK2 (Fig. 2D, upper left panel, lane 3). However, it is interesting that the removal of the amino-terminal end of ACK2, which contains a positively charged cluster of amino acids (yielding the construct designated as ACK2-ΔNT) strongly increases the amount of clathrin that co-precipitates with Myc-tagged ACK2 (Fig. 2D, upper left panel, lane 4). This may reflect an increased accessibility of the ACK2 mutant for clathrin, because we have found that the amino-terminal truncation of ACK2 alters its cellular location and increases the amount of the protein in the soluble fraction (data not shown).

Clathrin plays a pivotal role in receptor-mediated and synaptic vesicle endocytosis as well as in trans-Golgi vesicle transport (18–24). To examine the effects of ACK2 on clathrin-dependent receptor endocytosis, we used GFP-ACK2 fusion proteins to identify cells expressing ACK2, and Cy3-conjugated transferrin to monitor receptor endocytosis by the accompanying increases in Cy3 fluorescence. As shown in Fig. 3A, the overexpression of GFP alone did not affect the endocytosis of the transferrin receptor (panels a and b; the arrows denote cells expressing GFP). However, the overexpression of GFP-ACK2 in NIH3T3 cells blocked receptor endocytosis (panels c and d; the arrows point to cells expressing GFP-ACK2). We obtained identical results in PC12 cells when overexpressing ACK2 (data not shown). The inhibition of transferrin receptor endocytosis was prevented when overexpressing a carboxyl-terminal deletion mutant of ACK2 that lacked the clathrin-binding site (panels e and f; the arrows point to the ACK2-expressing cells), thus indicating that the ability of ACK2 to bind clathrin is necessary for the inhibition of endocytosis.

The Cdc42-binding defective mutant, GFP-ACK2-2H2A, was also capable of mediating an inhibition of transferrin receptor endocytosis (Fig. 3A, panels g and h; the arrows point to cells expressing the GFP-ACK2 double mutant). This was consistent with our finding that the binding of Cdc42 is not necessary for the interaction of clathrin with ACK2. In fact, it would be expected that the expression of activated Cdc42, by interfering with the binding of ACK2 to clathrin, should reverse the ACK2-mediated inhibition of receptor endocytosis. This in fact turned out to be the case (Fig. 3B). When the GTPase-defective mutant, Cdc42Q61L, was co-expressed with GFP-ACK2, the endocytosis of transferrin receptors was essentially restored to that observed with control cells.

Adaptor protein-2 (AP-2) is a universal adaptor for plasma membrane receptor endocytosis and is necessary for the cellular uptake of transferrin receptors (25, 26). The β subunit of AP-2 contains a clathrin-binding site similar to that found on AP-2 (Fig. 2C). Thus, one plausible mechanism for the inhibition of receptor endocytosis by ACK2 is that it competes with the AP-2 β subunit for binding to clathrin. To examine this possibility, we immunoprecipitated clathrin and AP-2 separately from the lysates of cells transfected with an inducible (tetracycline-off) expression plasmid for ACK2 and then immunoblotted with either anti-AP-2 or anti-clathrin antibody to analyze the association of AP-2 and clathrin (Fig. 3C). Expression of Myc-tagged ACK2 was induced by removing tetracycline from the culture medium (lane 1, middle panel), whereas control (uninduced) cells did not show a detectable expression of ACK2 (lane 2, middle panel). Significantly less AP-2 was co-immunoprecipitated with clathrin (using an antibody against the clathrin light chain) in cells where the expression of ACK2 was induced, compared with cells showing no detectable expression of Myc-tagged ACK2 (Fig. 3C, compare lanes 3 and 4 in the lower panel). Likewise, when using an anti-AP-2 antibody, there was a significantly greater amount of clathrin co-immunoprecipitated with AP-2 in cells that did not express Myc-tagged ACK2, compared with cells that inducibly expressed ACK2 (compare lanes 5 and 6 in the upper panel). However, under the conditions where the induction of ACK2 caused a marked reduction in the amount of AP-2 associated with clathrin, a significant amount of ACK2 was co-immunoprecipitated with clathrin (Fig. 3C, compare lanes 3 and 4 in the middle panel). These results demonstrate that the overexpression of ACK2 can effectively block the interaction of AP-2 with clathrin. This appears to be translated into a change in the cellular localization of AP-2 and its association with endocytic vesicles. In control cells, the staining of AP-2 shows the typical punctate appearance expected for AP-2-containing vesicles (Fig. 3D, panel b; cell that lacks the arrow), whereas in cells overexpressing ACK2 (Fig. 3D, panel b, the arrow points to the cell), there is a marked change in AP-2 staining such that there appears to be less punctate vesicular structures and instead, AP-2 appears to be aggregated, particularly along the cell surface.

Based on the observation that overexpression of ACK2 inhibited transferrin receptor endocytosis, and competed with...
AP-2 for binding clathrin, we expected that the immuno-
staining of clathrin-coated vesicles would be significantly de-
creased in ACK2-expressing cells. However, as shown in Figs.
4A–C, this is not the case. First, it appears that the overall
staining of clathrin-associated structures is actually increased
upon overexpression of ACK2 (Fig. 4A, panels b and d, and g
and h). Note that it is possible to still detect overlap between
ACK2 and clathrin staining (Fig. 4B), as well as a high degree
of overlap between clathrin and phosphotyrosine staining in
cells expressing ACK2 (Fig. 4A, panels a and d, and c and f); the

FIG. 3. ACK2 competes with AP-2 for binding to clathrin and inhibits transferrin receptor endocytosis. A, GFP-tagged, wild-type
ACK2 (WT), the clathrin-binding defective ACK2 mutant ΔCT, and the Cdc42-binding defective mutant 2H2A were expressed in NIH3T3 cells.
After 48 h, the cells were assayed for transferrin receptor endocytosis. Cy3-labeled transferrin was used as the endocytic marker. Panels a and b,
GFP vector alone; panels c and d, GFP-ACK2; panels e and f, GFP-ACK2 ΔCT; panels g and h, GFP-ACK2-2H2A. Panels a, c, e, and g, green
fluorescence; panels b, d, f, and h, Cy3-transferrin fluorescence. B, HA-tagged Cdc42Q61L was co-transfected with GFP-tagged wild-type ACK2
into NIH3T3 cells. After 48 h, the cells were used for endocytic assays with Cy3-transferrin as the endocytic marker. The cells were then fixed and
immunostained with anti-HA antibody. Top panel, green fluorescence indicates the expression of GFP-ACK2; middle panel, anti-HA immuno-
staining indicates the expression of Cdc42Q61L; bottom panel, Cy3-transferrin endocytosis. C, the tetracycline inducible cell line expressing
Myc-tagged ACK2 (tet-off system) (12) was used for examining the competition between ACK2 and AP-2 for binding to clathrin. The cell lysates
were subjected to immunoprecipitation and immunoblotted with an anti-clathrin light chain antibody (CON.1), an anti-AP-2αC antibody and an
anti-Myc antibody. I, induced; U, uninduced. D, the expression of Myc-ACK2 was induced for 24 h and used for immunofluorescence staining.
The cells were fixed and immunostained with anti-Myc antibody (top panel, red) and anti-AP-2αC antibody (bottom panel, green). The panels shown
here are representative of the results obtained from five separate experiments, comparing greater than 500 cells.
latter is likely because of autophosphorylated ACK2 associated with clathrin based on anti-phosphotyrosine Western blot analysis (data not shown). Second, whereas roughly equivalent amounts of clathrin were found in the 1,000 \( g \) and 8,000 \( g \) supernatants and pellets, both in the absence and presence of ACK2 (Fig. 4C, lanes 1–6, upper panel), the amount of clathrin present in the 300,000 \( g \) pellet, which is enriched in clathrin-coated vesicles, was consistently increased (by at least 2-fold) in cells overexpressing ACK2 relative to control cells (compare lanes 7 and 9). Identical results were obtained when PC12 cells were used (data not shown). Fig. 4C also shows that ACK2 was predominantly present in the 300,000 \( g \) pellet. Taken together, the immunofluorescence data and the results obtained from cell fractionation experiments lead us to suspect that ACK2 may promote, rather than inhibit, clathrin assembly, as has been suggested for the adaptor proteins AP-2 and AP-3 (27).

Previous studies have shown that Rac and Rho inhibited receptor-mediated endocytosis (28); however, our findings provide, to our knowledge, the first demonstration that a Cdc42 target/effecter (ACK2) is a clathrin-binding protein and raise interesting possibilities for Cdc42 in the regulation of clathrin assembly and/or clathrin-coated vesicle endocytosis. Although ACK2 is a nonreceptor-tyrosine kinase, we have not yet found any requirement for its tyrosine kinase activity in the regulation of receptor endocytosis. This either means that the tyrosine kinase activity of ACK2 serves another function and/or has only modulatory effects on an endocytic process. Our finding that ACK2 can negatively regulate transferrin receptor endocytosis is likely a direct outcome of its ability to bind clathrin,
competitively versus AP-2 in cells, rather than a reflection of its true physiological function. It is especially attractive to consider a role for ACK2 as an adaptor of clathrin-coated vesicles in brain or neuronal cells, as it is highly expressed in these cell types (9). Whereas early work implicated Cdc42 in actin cytoskeletal rearrangements and cell shape changes, more recent studies have provided strong indications for the involvement of Cdc42 in the stimulatory regulation of membrane trafficking, exocytosis, and endocytosis (4–7). The endocytosis of neurotrophic factors has been suggested to be necessary for neurite extension and signaling that leads to differentiation (29, 30), and Cdc42 has been implicated to play positive roles in neurite extension (31, 32). ACK2 may serve to link Cdc42 effects on neuronal differentiation with the endocytosis of neurotrophic factors and/or to the assembly of neurotransmitter vesicles, which is an essential function of differentiated neuronal cells.

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REFERENCES

1. Olson, M. F., Ashworth, A., and Hall, A. (1995) Science 269, 1270–1272
2. Quo, R. G., Abo, A., McCormick, F., and Symons, M. (1997) Mol. Cell. Biol. 17, 3449–3458
3. Lin, R., Bagrodia, S., Cerione, R., and Manor, D. (1997) Curr. Biol. 7, 794–797
4. Hong-Geller, E., and Cerione, R. A. (2000) J. Cell Biol. 148, 481–494
5. Wu, W. J., Erickson, J. W., Lin, R., and Cerione, R. A. (2000) Nature 405, 800–804
6. Kroschewski, R., Hall, A., and Mellman, I. (1999) Nat. Cell Biol. 1, 8–13
7. Erickson J. W., Zhang, C. J., Kahn, R. A., Evans, T., and Cerione, R. A. (1996) J. Biol. Chem. 271, 26650–26654
8. Manser, E., Leung, T., Salihuddin, H., Tan, L., and Lim L. (1993) Nature 363, 364–367
9. Yang, W., and Cerione, R. A. (1997) J. Biol. Chem. 272, 24819–24824
10. Burbelo, P. D., Drichsel, D., and Hall, A. (1995) J. Biol. Chem. 270, 29071–29074
11. Yang, W., Lin, Q., Guan, J.-L., and Cerione, R. A. (1999) J. Biol. Chem. 274, 8524–8530
12. Zhao, J. H., Reiske, H., and Guan, J.-L. (1998) J. Cell Biol. 143, 1997–2008
13. Grimes, M. L., Zhou, J., Beattie, E. C., Yuen, E. C., Hall, D. E., Valtetta, J. S., Topp, K. S., LaVail, J. H., Bunnett, N. W., and Mobley, W. C. (1996) J. Neurosci. 16, 7950–7964
14. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
15. Krupnick, J. G., Goodman, O. B., Keen, J. H., and Benovic, J. L. (1997) J. Biol. Chem. 272, 15011–15016
16. Dell’Angelica, E. C., Klumperman, J., Stoorvogel, W., and Bonifacino, J. S. (1998) Science 280, 431–434
17. Musacchio, A., Noble, M., Paupiti, R., Wierenga, R., and Saraste, M. (1992) Nature 359, 851–855
18. Marsh, M., and McMahon, H. T. (1999) Science 285, 215–220
19. Kirchhausen, T., and Harrison, S. C. (1981) Cell 23, 755–761
20. ter Haar, E., Musacchio, A., Harrison, S. C., and Kirchhausen, T. (1998) Cell 95, 563–573
21. Hirst, J., and Robinson, M. S. (1998) Biochim. Biophys. Acta 1404, 173–193
22. Goodman, O. B. Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, B. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Nature 383, 447–450
23. Cremo, A., and De Camilli, P. (1997) Curr. Opin. Neurobiol. 7, 323–332
24. Schmid, S. L., McNiven, M. A., and De Camilli, P. (1998) Curr. Opin. Cell Biol. 10, 504–512
25. Nesterov, A., Carter, R. E., Sorkina, T., Gill, G. N., and Sorkin, A. (1999) EMBO J. 18, 2489–2499
26. Benmerah, A., Lamaze, C., Begue, B., Schmid, S. L., Dautry-Varsat, A., and Cerf-Bensussan, N. (1998) J. Cell Biol. 140, 1055–1062
27. Murphy, J. E., and Keen, J. H. (1992) J. Biol. Chem. 267, 10850–10855
28. Lamaze, C., Chuang, T. H., Terlecky, L. J., Bokoch, G. M., and Schmid, S. L. (1996) Nature 382, 177–179
29. Mundigl, O., Ochoa, G. C., David, C., Slepnev, V. I., Kabanov, A., and De Camilli, P. (1998) J. Neurosci. 18, 93–103
30. Torre, E., McNiven, M. A., and Urrutia, R. (1994) J. Biol. Chem. 269, 32411–32417
31. Crup, X. Q., Tan, I., Leung, T., and Lim, L. (1999) J. Biol. Chem. 274, 19901–19905
32. Kozma, R., Sarner, S., Ahmed, S., and Lim, L. (1997) Mol. Cell. Biol. 17, 1201–1211
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