The Cdc42 Target ACK2 Interacts with Sorting Nexin 9 (SH3PX1) to Regulate Epidermal Growth Factor Receptor Degradation*

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Activated Cdc42-associated kinase-2 (ACK2) is a non-receptor tyrosine kinase that serves as a specific effector for Cdc42, a Rho family small G-protein. Recently, we have found that ACK2 directly interacts with clathrin heavy chain through a clathrin-binding motif that is conserved in all endocytic adaptor proteins and regulates clathrin assembly, suggesting that ACK2 plays a role in clathrin-coated vesicle endocytosis (Yang, W., Lo, C. G., Dispensa, T., and Cerione, R. A. (2001) J. Biol. Chem. 276, 17468–17473). Here we report the identification of another binding partner for ACK2 that has previously been implicated in endocytosis, namely the sorting nexin protein SH3PX1 (sorting nexin 9). The interaction occurs between a proline-rich domain of ACK2 and the Src homology 3 domain (SH3) of SH3PX1. Co-immunoprecipitation studies indicate that ACK2, clathrin, and SH3PX1 form a complex in cells. Epidermal growth factor (EGF) stimulated the tyrosine phosphorylation of SH3PX1, whereas co-transfection of ACK2 with SH3PX1 resulted in the constitutive phosphorylation of SH3PX1. However, co-transfection of the kinase-dead mutant ACK2(K158R) with SH3PX1 blocked EGF-induced tyrosine phosphorylation of SH3PX1, indicating that the EGF-stimulated phosphorylation of SH3PX1 is mediated by ACK2. EGF receptor levels were significantly decreased following EGF stimulation of cells co-expressing ACK2 and SH3PX1, thus highlighting a novel role for ACK2, working together with SH3PX1 to promote the degradation of the EGF receptor.

ACK21 belongs to a family of tyrosine kinases (Activated Cdc42-associated Kinases) whose members serve as specific target/effectors for the Rho family GTP-binding protein Cdc42 (1, 2). Epidermal growth factor (EGF), bradykinin, and cell adhesion all stimulate the tyrosine phosphorylation of ACK2, suggesting that ACK2 is involved in multiple signaling pathways (1, 3). Recently, we and others have found that the ACKs directly interact with the clathrin heavy chain through a conserved clathrin-binding motif that is present in all clathrin adaptor proteins (4, 5). The interaction between ACK2 and clathrin is negatively regulated by the binding of activated Cdc42 to ACK2 as well as by mutations in the SH3 domain of ACK2 but is enhanced upon the removal of a positively charged cluster of amino acids within the amino-terminal domain of ACK2 (4). Overexpression of ACK2 increased clathrin assembly in NIH3T3 cells (4); however, the exact function of ACK2 in clathrin-coated vesicle endocytosis has not been fully determined.

Here we report that ACK2 directly interacts with the sorting nexin protein SH3PX1 (for sorting nexin 9) together with clathrin. Members of the sorting nexin family contain PX domains (6–8). SH3PX1 has previously been reported to interact with the metalloprotease disintegrins MDC9 and MDC15 (9), although the significance of this interaction is unknown. However, various lines of evidence have suggested roles for members of the family of sorting nexins in the regulation of the endocytosis and degradation of receptors (6, 7, 10–13). In this work, we show that ACK2 and SH3PX1 work together to promote the degradation of the EGF receptor.

**EXPERIMENTAL PROCEDURES**

Materials—EGF was purchased from Invitrogen. The Cy3-conjugated EGF was obtained from Molecular Probes. The pEGFP-C1 and pDsRed1-C1 vectors and the human brain cDNA were purchased from CLONTECH. Anti-clathrin light chain antibody (CON.1) was from Covance (Berkeley Antibody). Anti-clathrin heavy chain antibody was purchased from BD Transduction Laboratories and anti-phosphotyrosine antibody (4G10) was obtained from Upstate Biotechnology. Anti-ACK antibody was prepared in the animal facility at the Cornell College of Veterinary Medicine using a GST fusion protein that contains amino acid residues 1–100 of ACK2 as an antigen. All ACK2 mutants except ACK2(LI2A) were generated as described previously (4). The clathrin-binding defective mutant ACK2(LI2A) was prepared by PCR-directed mutagenesis. The mutated sequence was confirmed by DNA sequencing. The cDNA of SH3PX1 was cloned from a human brain cDNA library (CLONTECH) by PCR based on the published SH3PX1 cDNA sequence in GenBank™ accession number AF131214 (9). The SH3PX1 cDNA was subcloned into the BamHI/EcoRI sites of pcDNA3 and tagged at the amino terminus with hemagglutinin (HA).

**Cell Culture, Transfection, and Lysis**—CO87 or SK-N-DZ cells were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum. All cells were maintained in 5% CO2 at 37 °C. For transfection, the cells were cultured overnight to 90% confluency. The LipofectAmine transfection procedures were performed according to the manufacturer’s instructions (Invitrogen). Cell lysates were prepared by lysing cells at 4 °C and then rocking for 20 min with cold lysis buffer containing 40 mM Hepes (pH 7.4), 100 mM NaCl, 1% Triton X-100, 25 mM glycerol phosphate, 1 mM sodium orthovanadate, 1 mM EDTA, 10 μg/ml apro- tinin, and 10 μg/ml leupeptin. The cell lysates were cleared by centrifugation at 14,000 rpm in a microcentrifuge for 4 min at 4 °C before use.

**Identification of GST-PRD1-binding Proteins**—The PCR products of the PRD1 of ACK2 were directly subcloned into the pGEX bacterial expression vector. The expression and purification of GST fusion proteins were the same as described previously (4). The GST-PRD1 proteins, bound to glutathione beads, were incubated with precleared cell lysates (40 μg of the fusion proteins, 20–30 μl of cell lysate protein) at 4 °C for 2–5 h with rotation. The beads were washed three times with 10 ml of bacterial lysis buffer (4). The bound proteins were resolved by SDS-PAGE. The unique bands that were associated with...
ACK2 Phosphorylates SH3PX1

RESULTS

ACK2 Directly Binds to SH3PX1—To search for ACK2-binding proteins, we have prepared a number of GST fusion proteins that contained different domains from ACK2 and incubated these fusion proteins with lysates from various cell lines. When using GST-ACK2PRD1, which represents the first proline-rich domain within the carboxyl-terminal half of ACK2 (Fig. 1A), as an affinity reagent, two specific PRD1 domain-associated proteins with apparent molecular masses of 180 and 77 kDa were detected from the lysates of human neuroblastoma SK-N-DZ cell line (Fig. 1B). The 180-kDa PRD1-associated protein was clathrin heavy chain, as had been identified previously (4). Two peptide microsequences of the 77-kDa PRD1-associated protein were found to be identical to the sequence of SH3PX1 using Blast search against the GenBank™ data base (9) (Fig. 1C).

It seemed likely that the SH3 domain of SH3PX1 was responsible for its binding to the PRD1 of ACK2. To confirm this, we prepared a GST fusion protein that contained the SH3 domain of SH3PX1 and incubated it with lysates from cells that overexpressed either Myc-ACK2 or the kinase-defective Myc-ACK2(K158R). This was followed by precipitation with glutathione-agarose and Western blotting with an anti-Myc antibody. As shown in Fig. 1D, both ACK2 and ACK2(K158R) were precipitated by the GST-SH3 domain of SH3PX1 (lanes 3 and 5). Thus, the SH3 domain of SH3PX1 does in fact appear to be responsible for binding ACK2, and this binding interaction can occur independent of ACK2 tyrosine kinase activity.

We next set out to demonstrate that the ACK2-SH3PX1 interaction occurred in intact cells. The cDNA-encoding SH3PX1 was cloned from a human brain cDNA library using PCR and then inserted into the mammalian expression vector pcDNA3 for expression as an HA-tagged protein. The results presented in Fig. 1E show that HA-tagged SH3PX1 associates with endogenous ACK following its immunoprecipitation with an anti-ACK antibody. To further characterize ACK2-SH3PX1 interactions, we co-transfected Myc-tagged wild-type ACK2 or different ACK2 mutants with HA-tagged SH3PX1. The results presented in Fig. 2 compare the abilities of wild-type ACK2 and a number of ACK2 mutants to bind to SH3PX1 in cells (lanes 7–18). The results shown in the middle panel were obtained by immunoprecipitating the HA-tagged SH3PX1 and then detecting the precipitated SH3PX1 and ACK2 proteins with anti-HA and anti-Myc antibodies, respectively. The right panel shows the complementary experiments in which the Myc-tagged ACK2 proteins were precipitated and examined for associated SH3PX1, whereas the left panel shows the relative amounts of SH3PX1 and the ACK2 proteins in the cell lysates. There appeared to be some variation in the abilities of the different ACK2 mutants to bind SH3PX1 in cells. For example, we find that the kinase-dead ACK2(K158R) mutant binds SH3PX1 less effectively in cells than does wild-type ACK2 (compare lanes 7 and 11, upper panel of Fig. 2, and lanes 13 and 17, lower panel), whereas we often see that the N-terminal truncation ACK2 mutant ΔNT binds SH3PX1 with an apparent higher affinity.
ACK2PhosphorylatesSH3PX1

**ACK2 Binds to SH3PX1 in cells.** The Myc-tagged ACK2 (WT) and its mutants, including an N-terminal truncation mutant (ΔNT), an SH3 domain mutant (2W2A), a Cdc42-binding defective mutant (2H2A), a clathrin-binding defective mutant (LI2A), and a kinase-dead mutant (K158R), were co-transfected with HA-tagged SH3PX1 into COS7 cells, and the cell lysates were subjected to immunoprecipitation (IP) with an anti-Myc antibody (right panel) or an anti-HA antibody (middle panel). The precipitated proteins were resolved by SDS-PAGE and transferred onto an Immobilon membrane. The proteins were immuno-blotted with an anti-Myc antibody and an anti-HA antibody. The cell lysates that were immunoblotted with an anti-Myc antibody and an anti-HA antibody are shown in the left panel.

**ACK2 and SH3PX1 Form Complexes with Clathrin—ACK2 directly interacts with clathrin and acts as an adaptor protein (4).** We questioned whether SH3PX1 was present in ACK2-clathrin complexes. Thus, we co-transfected Myc-tagged wild-type ACK2 and various Myc-tagged ACK2 mutants with HA-tagged SH3PX1 into COS7 cells. Clathrin was immunoprecipitated from the cell lysates with an anti-clathrin light chain antibody (CON.1), and associated ACK2 and SH3PX1 were detected by Western blot analysis with anti-Myc and anti-HA antibodies, respectively. The two lower panels in Fig. 3 show the relative amounts of SH3PX1 (anti-HA) and ACK2 (anti-Myc) in the cell lysates. As shown in Fig. 3, clathrin forms ternary complexes with SH3PX1 and either wild-type ACK2 (lane 2, upper panels), the Cdc42-binding defective mutant ACK2(2H2A) (lane 4, upper panels), the kinase-dead mutant ACK2(K158R) (lane 6, upper panels), or the N-terminal truncation mutant (ΔNT) (lane 7, upper panels). Interestingly, SH3PX1 alone was also co-precipitated with clathrin (lane 1, upper panels). However, we suspect that ACK mediates the binding of SH3PX1 to clathrin, because the clathrin-binding defective mutant ACK2(LI2A) (lane 5, upper panels) and the SH3 domain-defective mutant ACK2(2W2A) (lane 3, upper panels), which show little or no binding to clathrin, caused a reduction in the amount of SH3PX1 associated with clathrin. On the other hand, the overexpression of ACKΔNT appeared to enhance the amount of SH3PX1-clathrin complexes that were formed (lane 7, upper panels).

**ACK2 Is Associated with EGF Receptor and Shows an Enhanced EGF-stimulated Tyrosine Phosphorylation.** We have shown that ACK2 is activated by EGF stimulation (1). Given that ACK2, through its interactions with clathrin and SH3PX1, may play a role in mediating EGF receptor endocytosis, we examined whether ACK2 was capable of directly binding to the EGF receptor. To examine this possibility, we transfected either the Myc-tagged wild-type ACK2 or the kinase-dead mutant ACK2(K158R) into COS7 cells and then immunoprecipitated the ACK2 proteins using an anti-Myc antibody. The EGF receptor and the ACK2 proteins were detected with an antiphosphotyrosine antibody (Fig. 4A, upper and middle panels, respectively), and the bottom panel shows the relative amounts of ACK2 immunoprecipitated as detected with an anti-Myc antibody. As shown in Fig. 4A, the EGF receptor was co-immunoprecipitated with either wild-type (lanes 2–4, upper panel) or the kinase-dead ACK2(K158R) mutant (lanes 6–8, upper panel). Thus, ACK2 is capable of associating with the EGF receptor in a manner independent of ACK2 tyrosine kinase activity. Whereas the tyrosine phosphorylation of wild-type ACK2 appeared to occur independently of EGF stimulation (Fig. 4A, lanes 1–4, middle panel), most likely reflecting ACK2 auto-phosphorylation that is activated by cell adhesion (3), the tyrosine phosphorylation of the kinase-dead ACK2(K158R) mutant was completely dependent on EGF stimulation (lanes 5–8, middle panel). This further supports the idea that ACK2 forms a complex with the EGF receptor and suggests that either the EGF receptor or an associated kinase catalyzes the EGF-stimulated tyrosine phosphorylation of ACK2.

We next examined whether SH3PX1 influences the association of ACK2 with the EGF receptor. To do this, we used an N-terminal truncation mutant of ACK2, ACK2ΔNT, which was able to form a complex with clathrin and SH3PX1 (see Fig. 3, lane 7, upper panel). This ACK2 truncation mutant was co-transfected with SH3PX1 into COS7 cells. The two upper panels in Fig. 4B show the relative amounts of EGF receptor that were co-immunoprecipitated with Myc-tagged ACK2 as detected with anti-phosphotyrosine and anti-EGF receptor antibodies. The two lower panels show the relative amounts of ACK2 (anti-Myc) and SH3PX1 (anti-HA) that were immunob-
ACK2 Phosphorylates SH3PX1

Fig. 4. ACK2 is associated with EGF receptor. A, COS7 cells were transfected with Myc-ACK2 or ACK2 kinase-dead mutant K158R for 36 h, then serum-starved for 12 h, and treated with 100 ng/ml EGF for 0, 5, 30, or 60 min. The Myc-ACK2 or the kinase-dead mutant K158R proteins were immunoprecipitated (IP) using an anti-Myc antibody. The proteins were immunoblotted either using an anti-phosphotyrosine (PY) antibody or an anti-Myc antibody. The top panel shows the tyrosine phosphorylation of EGF receptor that was co-immunoprecipitated with ACK2 or the ACK2 kinase-dead mutant ACK2(K158R). The middle panel shows the tyrosine phosphorylation of immunoprecipitated ACK2 and the kinase-dead mutant K158R corresponding to EGF stimulation. The bottom panel shows the protein amount of immunoprecipitated wild-type and kinase-dead ACK2. B, the Myc-tagged ACK2(ΔNT) was transfected or co-transfected with HA-tagged SH3PX1 into COS7 cells for 36 h, then starved for 12 h, and treated with EGF for 0, 5, 30, or 60 min. As a control, one plate of transfected cells was not serum-starved and treated with EGF (indicated as C, lanes 5 and 10). The ACK2(ΔNT) proteins were immunoprecipitated using an anti-Myc antibody. The co-precipitated EGF receptor (EGFR) and SH3PX1 were immunoblotted with an anti-EGF receptor or anti-phosphotyrosine antibody and an anti-HA antibody, respectively.

precipitated. As shown in Fig. 4B, in the absence of serum starvation and treatment with EGF (designated C, lanes 5 and 10), the ACK2ΔNT mutant stably associated with the EGF receptor with SH3PX1 having no effect on this interaction (compare lanes 5 and 10 in the second panel from the top). When cells were serum-starved overnight, ACK2ΔNT was not able to associate with the EGF receptor in the absence of SH3PX1; however, under these same conditions, but in the presence of SH3PX1, ACK2ΔNT was stably associated with the EGF receptor (compare lanes 1 and 6 in the second panel from the top). In cells expressing SH3PX1, stimulation with 100 ng/ml of EGF then caused ACK2ΔNT to dissociate from the EGF receptor (compare lanes 2–4 with lanes 7–9 in the second panel from the top), whereas in the absence of SH3PX1, the ACK2 truncation mutant was able to associate with the EGF receptor (in particular, see lanes 3 and 4 in the second panel from the top). The results shown in the top panel of Fig. 4B yield similar conclusions, although in this case, only the EGF-activated (autophosphorylated) receptor is being visualized. We suspect that the decrease in the association of ACK2ΔNT with the EGF receptor, observed in the presence of SH3PX1 and upon EGF stimulation, results from the degradation of the receptor by an ACK2ΔNT/SH3PX1 complex (see below).

SH3PX1 is an EGF-dependent Tyrosine Phosphorylation Substrate for ACK—SH3PX1 has been reported to be a phosphorylation substrate for ACK in Drosophila (15). When endogenous ACK was depleted using RNAi, the tyrosine phosphorylation of SH3PX1 was diminished (15). Likewise, we find that SH3PX1 is a tyrosine phosphorylation substrate for ACK2 in mammalian cells. Fig. 5 shows the results obtained after transfecting COS7 cells with HA-tagged SH3PX1 alone or together with Myc-tagged wild-type or kinase-dead ACK2. The upper panel shows the relative tyrosine phosphorylation of SH3PX1, whereas the middle panel shows the relative amounts of HA-tagged SH3PX1 that was immunoprecipitated with anti-HA antibody. The lower panel presents the relative amounts of ACK2 in the cell lysates. As shown in the upper panel of Fig. 5, the tyrosine phosphorylation of SH3PX1 is dependent on EGF stimulation (compare lanes 1 and 2). Co-transfection of wild-type ACK2 with SH3PX1 resulted in constitutive tyrosine phosphorylation of SH3PX1 i.e. phosphorylation was observed, even in the absence of EGF stimulation (see lanes 3 and 4, upper panel), whereas co-transfection of SH3PX1 with the kinase-dead ACK2(K158R) completely prevented the tyrosine phosphorylation of SH3PX1 (lanes 5 and 6, upper panel). Taken together, these data strongly support the conclusion that SH3PX1 is an in vivo kinase substrate for ACK and that the ACK-catalyzed phosphorylation of SH3PX1 occurs in response to an EGF signal.

ACK2 Interacts with and Phosphorylates SH3PX1 to Facilitate EGF Receptor Endocytosis and Degradation—The association of ACK2 with the EGF receptor raised the possibility that ACK2 regulates EGF receptor endocytosis and degradation. We set out to examine this by expressing wild-type ACK2 alone or together with SH3PX1 in COS7 cells. To examine the importance of the tyrosine phosphorylation of SH3PX1 in EGF receptor degradation, we also co-transfected the kinase-dead mu-
lysates with an anti-HA antibody (top panel). The expression level of Myc-ACK2 or the kinase-dead mutant ACK2(K158R) with SH3PX1 in the cells was detected by immunoblotting of the lysates (Fig. 6, upper panel). The expression level of Myc-ACK2 or the kinase-dead mutant K158R in the cells was detected by immunoblotting of the lysates with an anti-Myc antibody (middle panel). The expression level of HA-SH3PX1 in the cells was detected by immunoblotting of the lysates with an anti-HA antibody (bottom panel).

Our previous studies have shown that ACK2 contains a clathrin-binding motif and directly interacts with clathrin heavy chain in a manner similar to other endocytic adaptor proteins (4). In addition, we found that the overexpression of ACK2 enhanced clathrin assembly. Whereas these findings seemed to implicate ACK2 in receptor endocytosis, its exact function was not clear. In the present study we show that ACK2 interacts with its tyrosine kinase substrate SH3PX1, a sorting nexin, and the EGF receptor, leading to reduced levels of EGF receptor in cells. Overall, these results suggest a role for ACK2 in EGF receptor sorting and degradation during endocytosis. The fact that the tyrosine phosphorylation of SH3PX1 by ACK was required for EGF receptor degradation implies that the small GTP-binding protein, Cdc42, which activates ACK in vivo (1), may sense the EGF signal and regulate EGF receptor degradation. The tyrosine phosphorylation of SH3PX1 that we observed in the absence of ectopically expressed ACK2 was most likely mediated by endogenous ACK proteins, either ACK2 or perhaps another form of ACK. We recently obtained evidence for an ACK2-related tyrosine kinase in NIH3T3 fibroblasts that cross-reacts with specific anti-ACK antibodies (data not shown).

Sorting nexins are a family of proteins that are involved in receptor degradation and recycling. Many homologues of sorting nexins have been found in yeast and play a critical role in receptor recycling between endosomes and trans Golgi (10). Sorting nexin 1 was discovered by the yeast two-hybrid approach, using the carboxyl-terminal tail of the EGF receptor as bait (6). The sorting nexin 1 binding site on the EGF receptor is distinct from the endocytic site, with receptor degradation requiring both sites (6, 11). However, the function of sorting nexins in receptor sorting and degradation is still not completely understood. In yeast, oligomerization among sorting nexins is important for organizing the sorting and recycling of functional complexes (10). In mammalian cells, homodimerization and heterodimerization of sorting nexins have been reported, and it has been proposed that complex formation between sorting nexins may be necessary for organizing functional units for receptor sorting and degradation (12, 13). The tyrosine phosphorylation of SH3PX1 by ACKs may regulate its oligomerization with other sorting nexins to form a sorting complex. We plan to further investigate the interactions of SH3PX1 with other sorting nexins, especially with sorting nexin 1 that has been reported to facilitate degradation of the EGF receptor, and the effects of tyrosine phosphorylation by ACKs on these interactions.

Our data also imply that clathrin may be present in sorting-degradation complexes because SH3PX1 is capable of forming a complex with clathrin and ACK2. It seems that tyrosine phosphorylation of SH3PX1 is not required, as its binding to clathrin still occurs in the presence of the kinase-dead ACK2 mutant. It will be interesting to see at what stage of receptor endocytosis the sorting-degradation complexes form and what role clathrin plays in the formation of these complexes. To date, we have only determined the effects of ACK2/S4S3PX1 on EGF receptor degradation. The question of whether ACK2/S4S3PX1 plays a general role in receptor degradation or is specific for the EGF receptor will be examined in the future. It will also be interesting to determine whether the tyrosine phosphorylation of SH3PX1, as catalyzed by the ACKs, serves other cellular functions beside receptor degradation, as it has recently been reported that the Drosophila SH3PX1 is a phosphosubstrate for a Drosophila ACK homolog and that these proteins work together to influence axonal guidance (16).

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