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Down-regulation of Active ACK1 Is Mediated by Association with the E3 Ubiquitin Ligase Nedd4-2*[S]

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ACK1 (activated Cdc42-associated kinase 1) is a cytoplasmic tyrosine kinase implicated in trafficking through binding to epidermal growth factor (EGF) receptor and clathrin. Here, we have identified a new ACK1-binding partner, the E3 ubiquitin ligase Nedd4-2, which binds ACK1 via a conserved PP*X*Y-containing region. We show that this motif also binds Nedd4-related proteins and several other WW domain-containing proteins, including the tumor suppressor oxidoreductase Wwox. In HeLa cells ACK1 colocalizes with Nedd4-2 in clathrin-rich vesicles, requiring this PP*X*Y motif. Nedd4-2 strongly down-regulates ACK1 levels when coexpressed, and this process can be blocked by proteasome inhibitor MG132. ACK1 degradation via Nedd4 requires their mutual interaction and a functional E3 ligase; it is also driven by ACK1 activity. ACK1 is polyubiquitinated *in vivo*, and dominant inhibitory Nedd4 blocks endogenous ACK1 turnover in response to acute EGF treatment. Because EGF stimulation activates ACK1 (Galisteo, M., Y., Y., Urena, J., and Schlessinger, J. (2006) *Proc. Natl. Acad. Sci. U. S. A.* 103, 9796–9801), our result suggest that EGF receptor-mediated ACK1 activation allows Nedd4-2 to drive kinase degradation. Thus the interplay between Nedd4-2-related E3 ligases that regulate ACK1 levels and Cbl that modifies EGF receptor impinges on cell receptor dynamics. These processes are particularly pertinent given the report of genomic amplification of the ACK1 locus in metastatic tumors.

ACK1 was identified as a cytoplasmic tyrosine kinase effector that binds to Cdc42·GTP but not Rac1 or RhoA (1). Human ACK1 and a protein referred to as ACK2 (recently proved to be a truncated form of ACK1) have been described (1–3). ACK2 activity *in vivo* (but not *in vitro*) is enhanced by active Cdc42, as assessed by increased tyrosine autophosphorylation (2). Recombinant ACK1 (comprising the kinase domain, SH3 domain, and Cdc42/Rac interactive binding domain) can undergo autophosphorylation at Tyr^{284} in the activation loop, resulting in a modest increase in its activity toward a peptide substrate (4). Crystal structures of phosphorylated and unphosphorylated ACK1 indicate that the kinase adopts an "active" conformation independent of such phosphorylation (5), although its catalytic activity is very low. *In vivo*, ACK1 may act as a mediator of EGF[2] signals to phosphorylate the guanine nucleotide exchange factor Dbl and can interact with integrin complexes and modulate cell adhesion (6, 7).

ACK1 contains an N-terminal tyrosine kinase domain flanked by SH3 and Cdc42/Rac interactive binding domains and a long proline-rich C-terminal region downstream of the clathrin-binding box (see Fig. 1*A*). The clathrin-binding motif LIDFG drives an interaction thought to be involved in receptor-mediated endocytosis (8, 9). Recently, the proline-rich region of ACK1 has been shown to contain multiple specific binding sites for SH3 partners including Grb2 and SNX9 (10). The SNX9·ACK2·clathrin complex has been invoked as promoting degradation of EGF receptor (11), although no specific mechanism was suggested. Interestingly ACK belongs to the class of proteins referred to as Hsp90 clients, where the chaperone is required for *in vivo* kinase activity (12).

ACK1 has been found in various proteomic studies as complexed to receptor tyrosine kinases such as epidermal growth factor receptor (23). ACK1 only binds to EGFR after ligand stimulation (13). Such an interaction can regulate EGFR degradation via its UBA domain binding to the C-terminal domain of ACK1. Further, ACK1 has recently been identified as a gene amplified in a number of human tumors (14). A different study found that ACK1 stimulated prostate tumorigenesis perhaps by negatively regulating the proapoptotic tumor suppressor, Wwox (15).

In this report, we have identified several additional binding partners to ACK1 using a single step affinity purification protocol. Some of these participate in clathrin-mediated endocytosis, including amphiphysin 1/2 and AP2. Based on the clathrin-binding profile of the various ACK1 domains used (including the clathrin-binding domain previously reported (8), we conclude that these proteins represent direct ACK1 binders. Most interestingly, an E3 ubiquitin ligase Nedd4-2 (neural precursor cell expressed and developmentally down-regulated) protein was found to bind to a specific ACK1 PP*X*Y motif close to the clathrin box, which required the WW domain of Nedd4-2. ACK1 and Nedd4-2 colocalized to clathrin-coated vesicles in HeLa cells. A number of other E3 ligases with WW domains have the potential to bind ACK1, indicating ACK1 may be under tight proteolytic regulation. In support of this,

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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[2] The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; E3, ubiquitin-protein isopeptide ligase; GST, glutathione *S*-transferase; HA, hemagglutinin; PBS, phosphate-buffered saline.

ACK1 is down-regulated when coexpressed with Nedd4-2, with ACK1 kinase activity being required for this turnover. In addition, ACK1 can inhibit EGFR ubiquitination without affecting receptor activation and endocytosis. Our results suggest that Nedd4-2 may regulate EGFR activity through its down-regulation of ACK1.

EXPERIMENTAL PROCEDURES

Materials—Glutathione-Sepharose beads were from Amersham Biosciences. Anti-clathrin heavy chain antibody was from Transduction Laboratory. Anti-ACK1 (A11 and H172), anti-EGFR and anti-Myc antibodies were from Santa Cruz. Anti-AP2 antibody was from Affinity Bioreagents. Anti-FLAG (M2), anti-GST (monoclonal), anti-actin, and anti-Amphiphysin (BIN1) antibodies were from Sigma. Antibody against Nedd4 WW domain was from Upstate Biotechnology Inc. Anti-HA antibody (12CA5) was from Roche Applied Science. Anti-mouse or rabbit Alexa 488, 546, and 647 secondary antibodies were from Molecular Probes.

Cell Culture—COS7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, whereas HeLa cells were maintained in minimal essential medium supplemented with L-glutamine, minimum essential amino acids, and 10% fetal bovine serum to 80% confluency before transfection.

Construction ACK1, Ubiquitin, Nedd4-2 (KIAA0439), and Amphiphysin Constructs—All of the ACK1 constructs (designated with amino acid number in Fig. 1*A*) were constructed by polymerase chain reaction using oligonucleotides flanked by BamHI and NotI restriction sites at the 5′ and 3′ ends, respectively. The PCR products were then digested with BamHI and NotI restriction enzymes and cloned into pXJ GFP-FLAG and pGEX 4T-1 vectors (Amersham Biosciences). Human ubiquitin was obtained from BamHI/NotI-digested PCR product with primers 5′-CATGGATTCATGCAGATCTTCGTGAAGACT-3′ and 5′-CATGCGGCCGCTCACCCACCTCTGAGACGGAGTAC-3′ in the pXJ-HA vector. Full-length KIAA0439 (Nedd4-2) obtained from the Kazusa DNA Research institute (Japan) was subcloned into pXJ-HA, pXJ-GST, and pXJ-Myc vectors using the HindIII and NotI sites.

In Vitro Binding Assay—GST fusion proteins were expressed in *Escherichia coli* BL21 and purified as described previously (1). GST fusion proteins were loaded onto a glutathione-Sepharose column (~200 μl; final concentration, ~2–3 mg/ml). Rat brain lysates (10 mg/ml) were prepared in lysis buffer (40 mM HEPES, pH 7.3, 0.1 M NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM sodium vanadate, 25 mM sodium fluoride 5% glycerol, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture) were passed through the GST fusion protein-Sepharose column several times. The columns were washed extensively with GST buffer (PBS, 50 mM Tris, pH 8.0, 0.1% Triton X-100), and the bound proteins were released by 10 mM glutathione in GST buffer.

Transfection and Coimmunoprecipitation—Subconfluent COS7, 293, or HeLa cells grown to 80% confluency were transfected with various DNA constructs (1–2 μg) using Lipofectamine reagent (5 μl; Invitrogen) as previously described (16). For immunoprecipitation experiment, 24 or 48 h after transfection, the cells were harvested in radioimmune precipitation assay lysis buffer (0.1 M Tris, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture). The lysed cell mixture was sonicated for 10 s followed by centrifugation at 13,000 rpm in the microcentrifuge for 20 min. Clarified cell lysates were incubated with anti-FLAG-conjugated agarose beads (M2; Sigma) for 2 h at 4 °C and then loaded onto a yellow tip column. After extensive washing with buffer (PBS containing 2 mM vanadate, 20 mM β-glycerolphosphate, and 0.1% Triton X-100), the bound proteins were eluted with 2× PAGE sample buffer by boiling for 10 min.

Western Blotting and Peptide Scan Blotting—Eluted proteins samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk in PBS for 1 h at room temperature and incubated with primary antibody (1:1000 dilutions) for 2 h at room temperature or

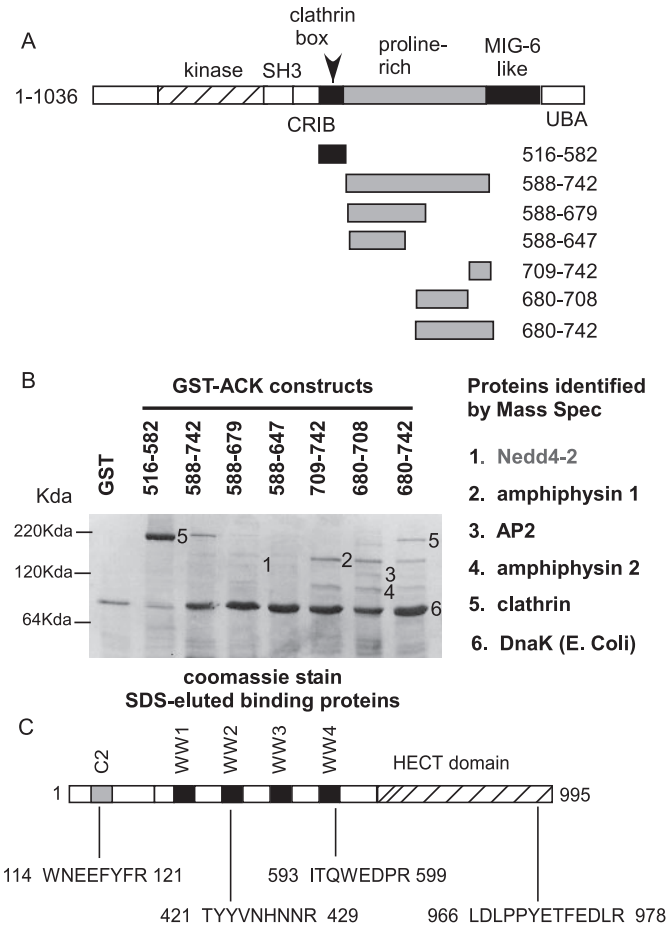

FIGURE 1. **Identification of ACK1 binding proteins from rat brain lysates.** *A*, GST-ACK1 constructs shown schematically were expressed as GST fusion proteins in *E. coli* BL21 and immobilized to Sepharose beads as described (1). *B*, the GST-ACK1 fusion proteins were used as affinity matrices to bind proteins from rat brain soluble lysates prepared as described under "Experimental Procedures." Proteins eluted by heating in 1% SDS were resolved on 9% SDS-polyacrylamide gel followed by Coomassie Blue staining. Specific protein bands (numbered *1–6*) were excised and subjected to standard in-gel tryptic digest and peptide sequence analysis by matrix-assisted laser desorption ionization time-of-flight. *C*, Nedd4-2 peptide sequences occurring in different domains of the protein. The *numbers* indicate the amino acid positions to the human Nedd4-2.

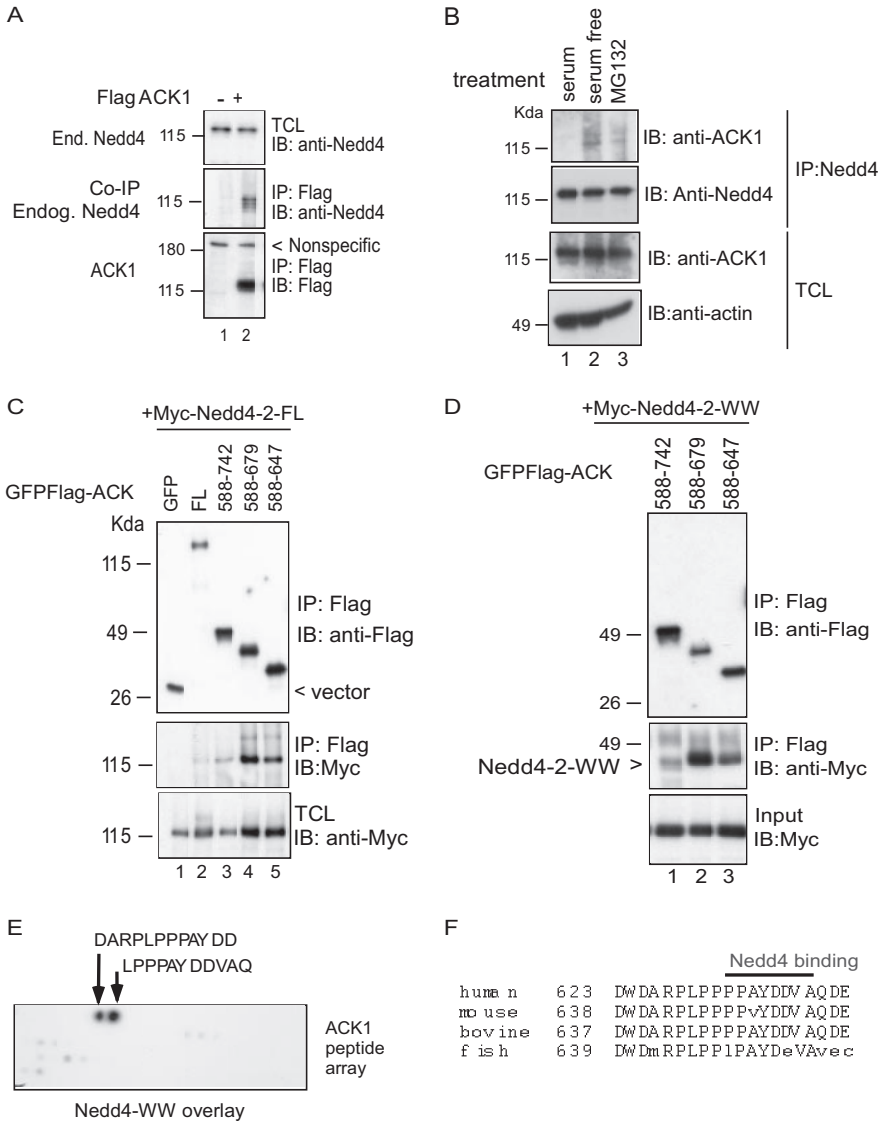

FIGURE 2. **Direct binding of ACK1 to Nedd4-2 WW domains through a PP*X*Y motif.** *A*, association of overexpressed ACK1 with endogenous Nedd4. Full-length FLAG-tagged ACK1 was overexpressed in COS7 cells and immunoprecipitated by anti-FLAG M2-agarose beads. Associated endogenous Nedd4 proteins were detected by anti-Nedd4 antibody, which was raised against its WW2 domain. *Lane 1* represents the vector (pXJ-FLAG) control. *B*, association of endogenous Nedd4 with endogenous ACK1. COS7 cells were maintained in serum (*lane 1*), serum-free medium (*lane 2*), or treated with proteasomal inhibitor MG132 (5 μM) overnight. Endogenous Nedd4 was immunoprecipitated from these cell lysates using an anti-Nedd4 antibody. Associated endogenous ACK1 was detected as described under "Experimental Procedures." *C*, mapping ACK1 binding sequence to Nedd4-2. GFP-FLAG-tagged of full-length (*FL*) ACK1 and deletion constructs (residues indicated) were coexpressed with Myc-tagged full-length Nedd4-2-FL in HEK293 cells. GFP-FLAG vector alone was used as control (indicated as GFP in *lane 1*). ACK1 constructs were recovered on M2-agarose beads, and associated Nedd4-2 was detected by anti-Myc antibody. *D*, WW domains of Nedd4-2 binds to ACK1. GFP-FLAG-tagged of ACK1 deletion constructs (as in *B*) were coexpressed with Myc-tagged Nedd4-2 WW domains (residues 235–599) in HEK293 cells. Nedd4-2 WW proteins recovered on anti-FLAG beads was detected by probing with anti-Myc. *E*, mapping of Nedd4-2 WW domain-binding motif in ACK1 using 13-mer peptides corresponding to ACK1 residues 621–940. Immobilized peptides on cellulose membrane (Jerini Pepspots) were overlaid with biotin-labeled recombinant GST-Nedd4-2 WW (amino acids 235–599) protein (5 μg/μl in PBS 0.1% Triton X-100). Binding was detected by streptavidin-horseradish peroxidase (Amersham Biosciences). Two positive peptides (sequences at top) were detected. *F*, sequence comparison of the conserved Nedd4-binding region among human (GenBank™ accession number 37999491), mouse (accession number 7948995), bovine (accession number 37999468), and fish (accession number 68404336). Nonmatching amino acids are shown as *lowercase letters*.

overnight at 4 °C. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:4000 dilutions) for 1 h at room temperature. The signals were visualized with ECL detection reagents (Amersham Biosciences). For peptide scan overlay assay, cellulose membrane with 13-mer peptides displaced by 4 amino acids, which covers the ACK sequence from residues 621 to 940, was ordered form (Jerini AG). The peptide membrane was blocked with 1% bovine serum albumin in 0.1% Triton X-100 for 30 min at room temperature and incubated with biotinylated Nedd4-2-WW domains (5 μg/ml) in 1% bovine serum albumin for 1 h at room temperature. After washing, the membrane was incubated with streptavidin-horseradish peroxidase for 1 h at room temperature.

Overlays with [γ-^{32}P]GTP-labeled Proteins—The WW domain array filter was purchased from Panomics. The GST-Ras fusion proteins (ACK1 517–647) were labeled as follows. The fusion proteins were incubated for 4 min with 10 μCi of [γ-^{32}P]GTP in 50 μl of exchange buffer (25 mM HEPES, pH 7.3, 50 mM KCl, 2.5 mM EDTA). This mixture was immediately added to 3 ml of binding and wash buffer (PBS containing 25 mM HEPES, pH 7.3, 5 mM $MgCl_2$, and 0.05% Triton X-100) containing 0.1 mM GTP and added to a roller bottle containing the array filter. Following 1 h of incubation at 4 °C, the filters were washed (three times for 10 min each time) with binding and wash buffer and exposed to PhosphorImager plates (Molecular Dynamics) for quantification or to x-ray film.

Immunofluorescence and Imaging—Transfected HeLa or COS7 cells grown on coverslip were fixed with 4% paraformaldehyde in PBS for 20 min and washed with PBS twice for 10 min. After permeabilization with 0.2% Triton X-100 for 10 min and blocking with 10% goat serum, the cells were incubated with primary antibody (1:100 dilution) in 0.5% Triton X-100/PBS for 2 h at 37 °C. The cells were then washed twice and incubated with secondary antibody (Alexa 488 or 546; 1:100 or Alexa 647; 1:50) for 1 h at room temperature. To visualize nucleus, the cells on coverslips were incubated with fluorescent dye H-33342 for 10 min and then washed with PBS. The cells were mounted and viewed by Radiance 2000 confocal micro-

scope (Bio-Rad) or Zeiss Axioplan2 linked to a cooled CCD camera (Coolsnap HQ, Roper Scientific) using a 60× oil lens. Images were collected using Lasersharp or MetaVue and processed by Adobe Photoshop.

EGF, Cell Adhesion, and Cell Cycle Effect on Endogenous ACK1 Level—For EGF stimulation, COS7 cells were serum-starved overnight before stimulation with EGF (100 ng/ml) for the time indicated. For proteasome inhibitor treatment, the cells were pretreated with MG132 (50 μM) for 1 h prior to EGF stimulation. Total cell lysates were immunoblotted with anti-ACK1 antibody. For cell adhesion, the plastic dish was coated with 10 μg/ml fibronectin (in PBS) overnight at 4 °C. The dish was washed a few times with PBS. COS7 cells were trypsinized, washed with PBS two times, and resuspended in serum-free media. The cells were then plated onto fibronectin-coated dish for 2 h before lysis. For cell cycle effect, a few plates of HeLa cells were treated with 40 ng/ml nocodazole for 16 h. Round-up cells were rinsed off the plates and concentrated before lysis.

Endocytosis of EGFR to Early Endosomes—ACK1(727–915) was transfected to COS7 cells for 6 h and serum-starved overnight. The cells were stimulated with EGF (100 ng/ml) for 15 min before fixed with 4% paraformaldehyde. The cells were then immunostained with appropriate antibodies and visualized with Alexa-conjugated secondary antibodies. H33342 (Sigma) was used for nuclear staining.

Small Interfering RNA-mediated Knockdown of ACK1—COS7 cells were treated with control and ACK small interfering RNA (synthesized from Invitrogen) for 48 h using Lipofectamine 2000 according to the manufacturer's instruction. The ACK1 small interfering RNA sequence was described previously (17).

RESULTS

Identification of E3 Ubiquitin Ligase Nedd4-2 as a New ACK1-binding Protein from Brain Lysates—Several proteins including clathrin, SH3PX1, and HSP90 have been identified as binding partners of ACK1 (10–12). We previously identified a clathrin-binding box in ACK1 (amino acids 570–575) that is similar to those in a number of clathrin-associated proteins (8). Nevertheless we have observed that ACK1 protein lacking this box or ACK1 (amino acids 588–1031) whose sequence lies C-terminal to the clathrin box, also colocalized with clathrin (data not shown). To identify sequences that might represent another "clathrin-associated domain," we examined the sequences that lie between the clathrin box and the MIG-6 homology domain (proline-rich; Fig. 1*A*). GST fusion proteins encoding fragments of ACK1 were used as affinity matrices to recover proteins from rat brain lysates. Among the affinity-purified proteins, five were unequivocally identified by tryptic mass fingerprinting: namely clathrin, amphiphysin-1, amphiphysin-2, AP2, and Nedd4-2 (marked on Fig. 1*B*). Amphiphysin binding is likely mediated by their SH3 domain. These lipid-binding proteins and AP2 participate in clathrin-mediated endocytosis. The identification of these proteins was confirmed by Western blot analysis using appropriate antibodies (supplemental Fig. S1). Association of ACK1 with amphiphysin-2 was then confirmed by coimmunoprecipitation (supplemental Fig. S1). Nedd4-2 was identified by appropriate molecular mass (and 10 matching peptide fragments, which included peptided sequences from the alternate spliced C2, WW2, and WW4 domains (Fig. 1*C*).

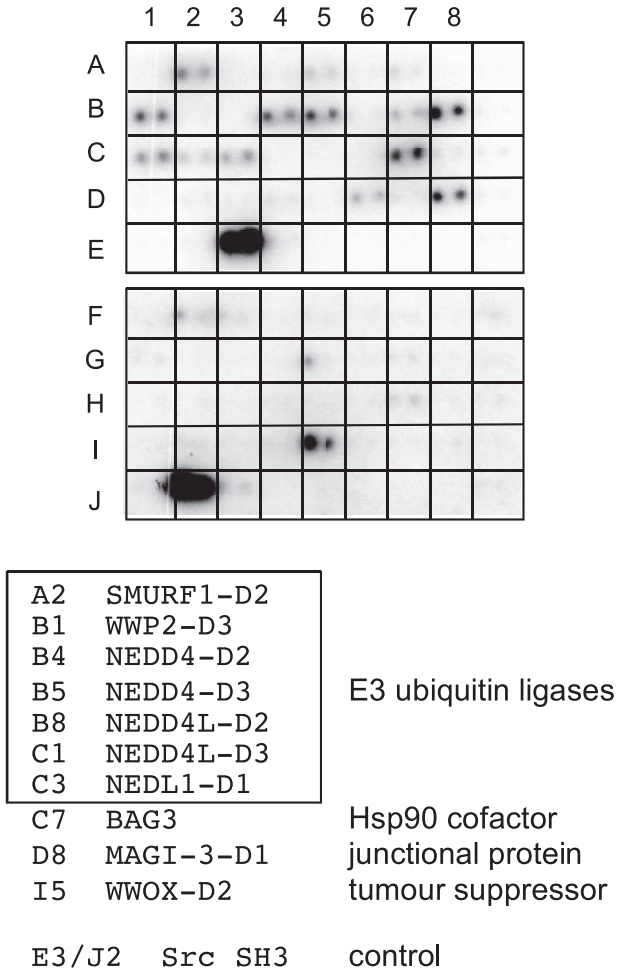

FIGURE 3. **Detection of ACK1 proline-rich sequences interact with WW domain of Nedd4 family of E3 ubiquitin ligases and others.** Arrays of WW domain immobilized on polyvinylidene difluoride (Panomics) were incubated with γ-^{32}P-labeled GST/Ras-ACK1(517–647) fusion protein as described under "Experimental Procedures." The *top panels* show the images obtained on the phosphorus image storage screen after 3 h of exposure. Positive controls at positions E3 and J2 correspond to Src SH3 domain (which also binds the proline-rich ACK1 sequence). Positive interactors are classified as E3 ubiquitin ligases (*boxed*) or other targets.

ACK1 Associates with Nedd4-2 in Vivo—Nedd4-2 is a member of a family of related E3 ubiquitin ligases consisting of a C2-like domain, four WW domains, and a HECT ubiquitin-ligase domain (Fig. 1*C*). To establish the interaction of Nedd4-2 and ACK1 *in vivo*, we first immunoprecipitated overexpressed FLAG-tagged ACK1 from COS7 cell lysates, and endogenous Nedd4 proteins were detected in the immunoprecipitates (Fig. 2*A*). Bands corresponding to COS7 Nedd4 isoforms were detected (*lane 2*). To confirm the presence of an endogenous ACK1·Nedd4-2 complex, we immunoprecipitated endogenous Nedd4 from these cells under three conditions. No ACK1 was detected in immunoprecipitates from cells incubated in serum (Fig. 2*B*, *lane 1*), but overnight incubation under serum-free or with proteasomal inhibitor MG132 (*lanes 2* and *3*) allowed us to detect the complex. This suggests the Nedd4·ACK1 complex is

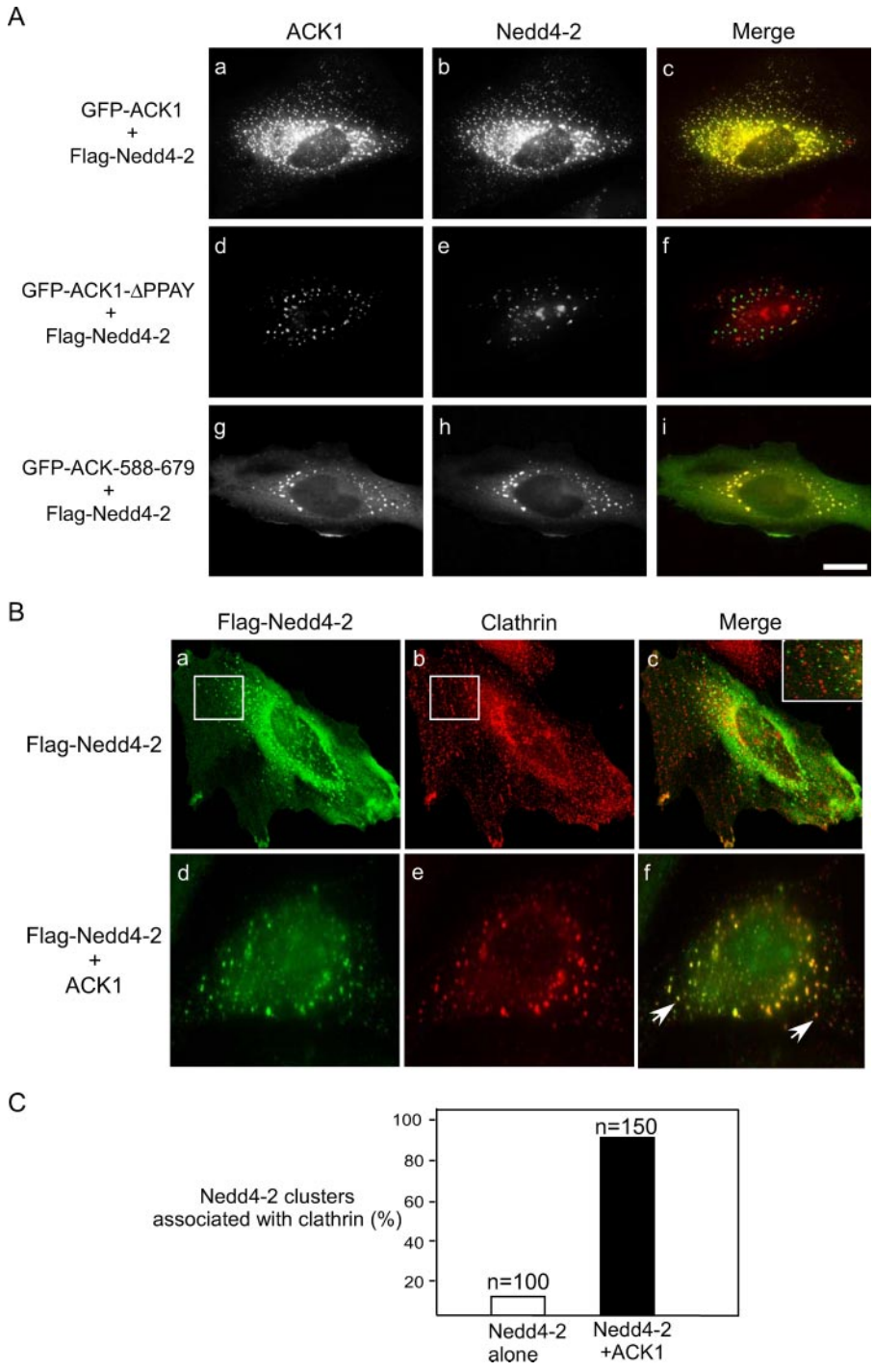

FIGURE 4. **Colocalization of ACK1 with Nedd4-2 in HeLa cells.** *A*, plasmids encoding GFP-tagged ACK1 and FLAG-tagged Nedd4-2 as indicated were microinjected into the nuclei of HeLa cells and analyzed by immunofluorescence 2 h post-injection. The Nedd4-2 is clearly "recruited" to the typical reorganized vesicles where ACK1 was localized (*panels a–c*). The ACK1 lacking the PP*X*Y motif (ACK1-ΔPPAY) does not colocalize with Nedd4-2 (*panels d–f*). Nedd4-2 was redistributed to ACK1 vesicles using a short ACK1 construct that includes the PPAY motif (residues 589–679) but lacking most characterized domains (*panels h–i*). In the merged figure, *green* is ACK1, *red* is Nedd4-2, and *yellow* is the colocalized vesicles. *B*, colocalization of ACK1 and Nedd4-2 in clathrin vesicles. Plasmids were introduced by liposome-mediated transfection overnight. *Panels a–c* show Nedd4-2 clusters are not colocalized with clathrin which is particularly clear in thin areas or lamella regions of the cell. The *inset* in *panel c* shows the magnified merge image of the boxed regions. Nedd4-2 is recruited to the clathrin containing vesicles when coexpressed with ACK1 (*panels d–f*). ACK1 was colocalized with these vesicles (channel not shown). The *arrow* in *panel f* represents typical vesicles that contain all three proteins. *C*, quantification of Nedd4-2 positive vesicles that is associated clathrin when Nedd4-2 is expressed alone or coexpressed with ACK1 as shown in *B*.

less stable in serum because there was no apparent difference in the total level of ACK1, total cell lysate (TCL).

Next we examined the ACK1 sequences responsible for interaction with Nedd4-2 using various ACK1 deletion constructs. A small region (amino acids 588–679) was sufficient to bind Nedd4-2 (Fig. 2*C*, *lane 4*). With full-length ACK1 (Fig. 2*C*, *lane 2*) there is much less soluble ACK1 protein recovered from the lysates and coexpression of Nedd4-2 reduces ACK1 levels (discussed later) and thus the apparently weaker interaction. By contrast the smaller ACK1(588–679) bound robustly to a region encompassing the four WW domains of Nedd4-2 (Fig. 2*D*, *lane 2*).

WW domains can interact with proline-rich targets including the prototype PP*X*Y motif such as those found in sodium channels (18). ACK1 contains a PP*X*Y motif (residues 632–635) that appears to be the unique site for binding for the Nedd4-2 WW domains as assessed by using a peptide scan analysis of the entire ACK1 proline-rich region (Fig. 2*E*). The membrane array containing 13-mer overlapping synthetic peptides covering ACK1 residues 621–940 was overlaid with biotinylated GST-Nedd4-2 WW1–4 (residues 234–599). Only two adjacent peptides containing the PP*X*Y motif were detected. We note that this Nedd4-binding site is conserved among vertebrate ACK1 orthologues (Fig. 2*F*) despite low overall identity (<40%) in the C-terminal half of ACK1 (say comparing human and zebra fish versions).

Another ACK1 partner Wwox has been described (15); likely Wwox binding involves the same PP*X*Y site. To look at WW binding across a spectrum of proteins, we employed recombinant WW domain arrays (Panomics) overlaid with labeled ACK1(517–647). Fig. 3 illustrates the signals generated with these arrays. Src SH3 domain serves as a positive control (strongest signals). These results suggest that Nedd4-2 can bind ACK1 via both WW2 and WW3 domains. A

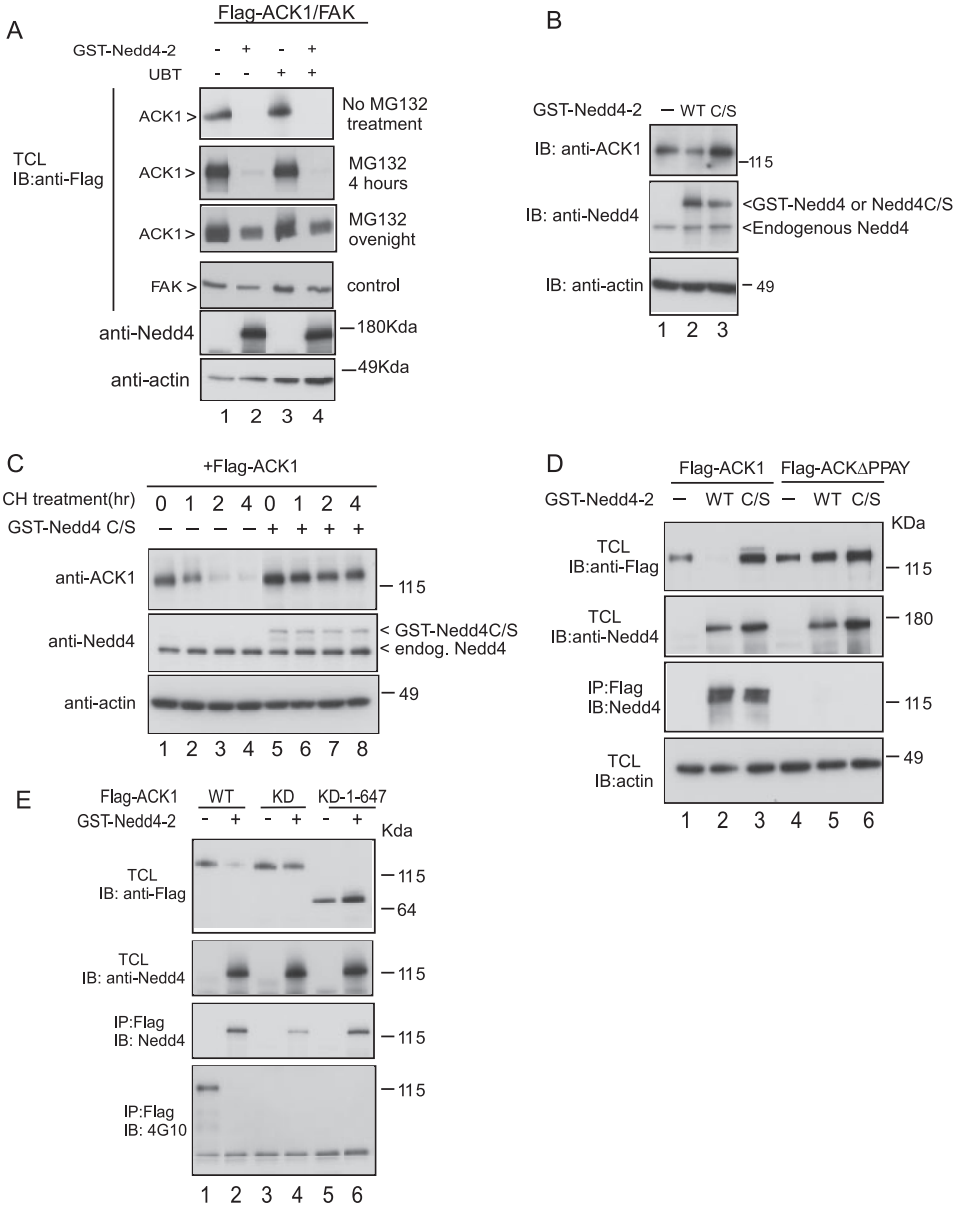

FIGURE 5. **Nedd4-2 down-regulates ACK1 in COS7 cells.** *A*, Nedd4-2 coexpression causes loss of ACK1. FLAG-tagged full-length ACK1 or FAK (as control) was expressed with GST-tagged Nedd4-2 in the absence (*lanes 1* and *2*) or presence (*lanes 3* and *4*) of ubiquitin (*UBT*) in COS7 cells. Cells transfected with FLAG-ACK1 were treated without or with proteasome inhibitor MG132 (20 μM) for either 4 h or overnight (16 h). Levels of transfected proteins are assessed by anti-FLAG antibody. Anti-actin is used as a loading control. *TCL*, total cell level. *B*, Nedd4-2 C/S mutant blocks endogenous ACK1 turnover. Wild type Nedd4 or ligase-deficient Nedd4C/S mutant were transfected to COS7 cells. 10 h after transfection, total endogenous ACK1 was detected by anti-ACK1 antibody, and both endogenous Nedd4 and overexpressed Nedd4-2C/S mutant were detected by anti-Nedd4 antibody. *C*, Nedd4C/S mutant blocks ACK1 turnover. After cotransfection of FLAG-ACK1 with or without GST-Nedd4C/S for 10 h, the cells (COS7) were treated with cycloheximide (*CH*) at concentration 100 μg/ml as indicated. *D*, ACK1 turnover requires binding to Nedd4-2 and ligase activity of Nedd4-2. FLAG-tagged full-length ACK1 or an Nedd4-2 binding-deficient ACK1 construct (ACKΔPPAY) was cotransfected with full-length GST-tagged Nedd4-2 or ligase-deficient Nedd4-2C/S. 40 h after transfection, total ACK1 level was detected with anti-FLAG antibody, and Nedd4 level was detected with anti-Nedd4 antibody. *E*, ACK1 turnover depends on its activity. Wild type (*WT*), kinase-dead (*KD*) ACK1, and C-terminal deleted kinase-dead ACK1 constructs (KD-1–647) were cotransfected with full-length Nedd4-2. Phosphorylated ACK1 was detected with antiphosphotyrosine antibody 4G10. *IB*, immunoblot; *IP*, immunoprecipitation.

majority of targets are WW domains from Nedd4-related E3 ligases (*boxed*), whereas the only other binders were BAG3, Wwox, and MAGI-3 with a single WW domain. Thus E3 and non-E3 ligases may compete for ACK1 binding. BAG3 is of interest because inhibition of BAG3 can lead to an accumulation of polyubiquitinated Hsp90 client proteins (19), and ACK1 itself is such a client protein (12).

Nedd4-2 Colocalizes to Clathrin-containing Vesicles in the Presence of ACK1—Nedd4 is a cytoplasmic protein in human cultured cells (20). It was important to establish whether full-length ACK1 colocalizes with Nedd4-2 *in vivo*. Nedd4-2 coexpressed with ACK1 in HeLa colocalized in puncta, indicating that these proteins bind *in vivo* (Fig. 4*A*, *panels a–c*). Further the Nedd4-2 puncta (without ACK1) only rarely (~10%) contained clathrin (Fig. 4, *B*, *panels a–c*, *insets*, and *C*). However when ACK1 and Nedd4-2 were cotransfected, essentially all of the Nedd4-2 was associated with ACK1-associated clathrin-containing vesicles (Fig. 4, *A*, *panels a–c*; *B*, *panels d--f*, *arrowheads*; and *C*). To test the role of the PP*X*Y motif, full-length ACK1 with or without the PP*X*Y motif were tested (Fig. 4*A*, *panels d–i*). The redistribution of Nedd4-2 to ACK1-enriched vesicles could be driven by ACK1(588–679), containing the PP*X*Y motif and clathrin-binding region (Fig. 4*A*, *panels h–i*) but not when the PP*X*Y was deleted (Fig. 4*A*, *panels d–f*). These results indicated that full-length ACK1 localizes with Nedd4-2 *in vivo* and that the PP*X*Y motif in ACK1 mediates their association.

Cdc42 may promote tyrosine auto-phosphorylation of ACK *in vivo* (4). Cdc42 can also negatively regulate the interaction of ACK2 with clathrin perhaps through a related mechanism (21). Therefore we examined whether the association of ACK1 and Nedd4-2 was influenced by activated Cdc42. We transiently coexpressed ACK1 and Nedd4-2 with wild type Cdc42, active Cdc42V12 or dominant inhibitory Cdc42N17. 1The colocalization of ACK1 and Nedd4-2 in puncta remained similar to those in Fig. 4*A* (*panels a–c*) in the presence of Cdc42WT (supplemental Fig. S2). These punctate structures were larger when active Cdc42 was present and much smaller with Cdc42N17 (supplemental Fig. S2). We conclude that although Cdc42·GTP influences the morphology of the ACK1-associated structures, Nedd4-2 binds to ACK1 independent of Cdc42.

Nedd4-2 Promotes ACK1 Turnover—In our immunofluorescence analysis (Fig. 4), we often found that coexpression of ACK1 with GST-Nedd4 strongly reduced ACK1 levels across the field *versus* expressing the kinase alone. This suggested that Nedd4-2 down-regulates ACK1 levels. FLAG-tagged ACK1 and GST-tagged Nedd4-2 were cotransfected into COS7 cells (with or without the cofactor ubiquitin), and the total ACK1 level was assessed 48 h after transfection. FLAG-ACK1 levels were dramatically reduced with GST-tagged Nedd4-2 (Fig. 5*A*, *top panel*, *lanes 2* and *4*), even after 4 h of treatment with proteasome inhibitor MG132 (*second panel*). Overnight treatment with MG132 could protect ACK1 levels (Fig. 5*A*, *third panel*). Overexpression of ubiquitin alone did not alter levels of ACK1 (*lane 3*, *top panel*). The effect of Nedd4-2 was not seen with another nonreceptor tyrosine kinase FAK (Fig. 5*A*, *fourth panel*). We also noted that Myc-tagged Nedd4-2 affected ACK1 turnover less than GST-tagged Nedd4-2 (supplemental Fig. S3), which is likely related to the dimeric nature of GST fusion proteins. To test whether Nedd4-2 affects endogenous ACK1 turnover, we overexpressed wild type GST-Nedd4-2 and a mutant that is deficient in ligase activity (C/S mutant in ligase domain). Our results indicate the ligase-deficient Nedd4-2 can block this turnover (Fig. 5*B*) and conclude that Nedd4-like E3 ligases target ACK1 for degradation.

To look at the kinetics of ACK1 turnover, we introduced expression plasmids encoding FLAG-ACK1 with and without Nedd4-2 C/S and used cycloheximide to then block protein synthesis after 4 h as shown in Fig. 5*C*. The ACK1 levels declined over 2 h following cycloheximide treatment (Fig. 5*C*, *lane 3*), an effect that could be blocked by the Nedd4-2C/S mutant (Fig. 5*C*, *lanes 5–8*).

ACK1 Turnover Depends on Binding to Nedd4, a Functional Nedd4 Ligase Activity and ACK1 Activity—We then examined whether the Nedd4-2 effect on ACK1 turnover required their direct association. The Nedd4-2 binding-deficient mutant ACK1ΔPPAY was unaffected, unlike wild type ACK1 (Fig. 5*D*, compare *lanes 2* and *5*, *top panel*), indicating that binding is required for such turnover. Similarly Nedd4-2C/S can bind ACK1 but does not promote turnover (compare *lanes 2* and *3*). However, Nedd4-2 (Fig. 5*E*, *lane 5* and *6*, *top panel*). Our results indicate that kinase activity of ACK1 is required for ACK1 turnover *in vivo*.

ACK1 Ubiquitination and the Role of Its UBA Domains—ACK1 degradation on its association with Nedd4-2 suggested that this E3 ligase mediates ACK1 ubiquitination. Not surprisingly, coexpression with HA-tagged ubiquitin resulted in its incorporation into ACK1 when the proteasome was inhibited (Fig. 6*A*, *lane 2*). Additional Nedd4-2 did not increase ACK1 ubiquitination, indicating that endogenous E3 ligase can efficiently drive this process. ACK1 contains two UBA domains of ~40 residues (within residues 955–1031) that occur among diverse proteins linked to ubiquitination (22). The smaller ~70-kDa TNK1 kinase, (Thirty-eight Negative kinase 1), the only other member of the ACK1 family, also contains two such related UBA domains at the C terminus although lacking all other protein interactions domains described here (data not shown). We examined the effect of deleting both UBAs (Fig. 6*A*, Δ*C*) on ACK1 ubiquitination: comparing *lane 2 versus lane 5*, it

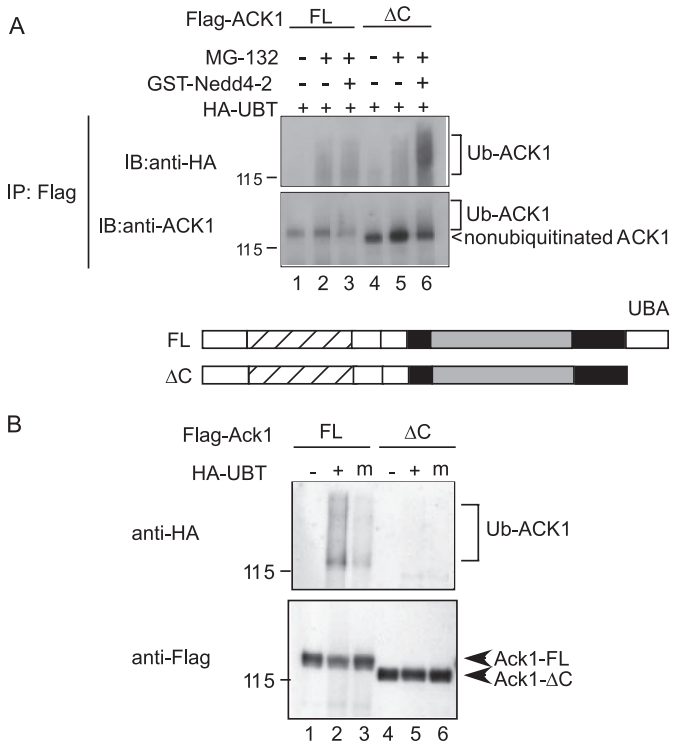

FIGURE 6. **Following ACK1 ubiquitination *in vivo*.** *A*, FLAG-tagged ACK1 full-length (*FL*) or a construct lacking the UBA domains (from residues 955–1036 (Δ*C*)), was cotransfected with HA-tagged ubiquitin (*UBT*) or together with GST Nedd4-2 in COS7 cells. 16 h after transfection, cells (*lanes 2*, *3*, *5*, and *6*) were treated 50 μM proteasome inhibitor MG132 for 6 h. ACK1 proteins were immunoprecipitated with M2-agarose beads and analyzed by Western blotting. Ubiquitinated ACK1 was detected by anti-HA antibody. Immunoprecipitated ACK1 was detected with anti-ACK1 antibody. *B*, ACK1 is polyubiquitinated. Either full-length ACK1 or ACK1-ΔC were cotransfected with normal HA-tagged ubiquitin or a mutant ubiquitin construct that was mutated at three lysine residues K29K48K63 (*m*). Ubiquitinated ACK1 was detected with anti-HA antibody.

is clear deleting these two domains elevates ACK1 levels considerably; the presence of the UBA domains clearly promotes ACK1 ubiquitination (13). Nedd4 can nonetheless increase ubiquitination of ACK1ΔC (Fig. 6*A*, *lane 6*, *top panel*). To check whether ACK1 is mono- or polyubiquitinated, we used an ubiquitin mutant with lysine-to-arginine substitutions at three major sites for chain branching (Lys^{29}, Lys^{48}, and Lys^{63} (23)). This ubiquitin mutant was poorly incorporated (Fig. 6*B*, *lanes 2* and *3*), indicating that ACK1 is polyubiquitinated as previously suggested (13).

Regulation of ACK1 Turnover by EGF—The EGFR can recruit ACK1 upon ligand binding (13), and ACK1 is ~2-fold enriched in the activated EGFR complex (24). Given that ACK1 turnover depends on its activity (Fig. 5*E*) and the kinase is activated by EGF, based on increased phosphotyrosine incorporation (3), we therefore examined whether endogenous ACK1 levels were affected by EGF stimulation. Following acute EGF stimulation, both ACK1 and EGFR were largely degraded by 3 h (Fig. 7*A*, *lane 4*). The time course of ACK1 turnover induced by EGF showed a prominent loss 2 h after stimulation (Fig. 7*B*), which is faster than EGFR turnover. Pretreatment with the proteasome inhibitor MG132 essentially prevented this (Fig. 7*B*, *lane 3*). Although ACK1 and ACK2 are reported to be tyrosine-phosphorylated upon cell adhesion (3, 7), cell plating on fibronectin did not decrease ACK1 levels (supplemental Fig.

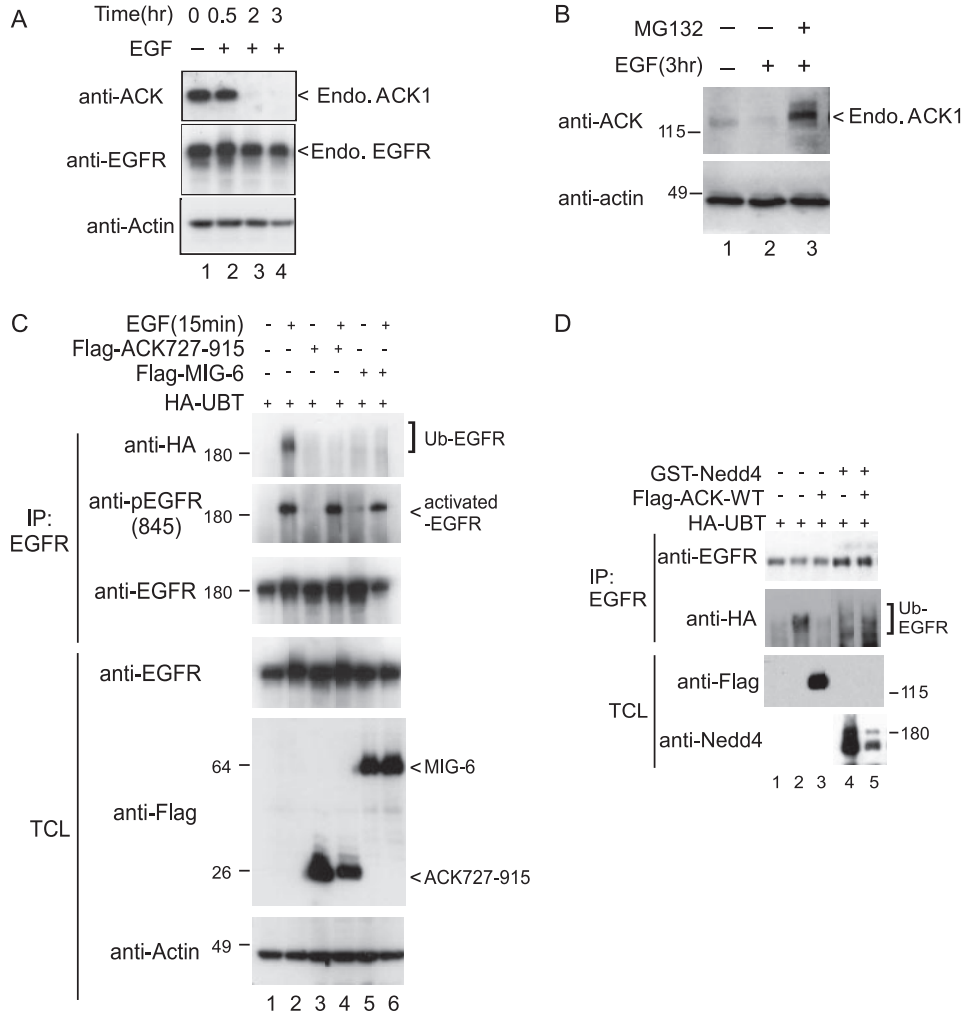

FIGURE 7. **ACK turnover can be initiated by EGF treatment.** *A*, acute EGF treatment leads to loss of endogenous ACK1. Serum-starved COS7 cells (16 h) were stimulated by EGF (100 ng/ml) for the time indicated. Total level of endogenous ACK1 and EGFR were analyzed by Western blotting. EGF stimulation causes a dramatic loss of endogenous ACK1. *B*, loss of endogenous ACK1 by EGF stimulation can be blocked by proteasome inhibitor MG132. COS7 were serum-starved overnight and pretreated with proteasome inhibitor MG132 (1 h 50 μM; *lane 3*) followed by EGF stimulation (100 ng/ml) for 3 h (*lanes 2* and *3*). *C*, ACK1 EGFR binding domain suppresses EGFR ubiquitination. FLAG-tagged ACK1(727–915) and full-length MIG-6 were coexpressed with HA-tagged ubiquitin in COS7 cells for 6 h. After serum-starved overnight, the cells were stimulated with or without EGF (100 ng/ml) for 15 min. EGFR were immunoprecipitated (*IP*) and blotted with anti-HA or anti-pEGFR (Tyr^{845}) antibodies to check the extent of EGFR ubiquitination and activation. *D*, effect of Nedd4-2 on ACK1-mediated reduction on EGFR ubiquitination. GST-tagged Nedd4-2 and FLAG-tagged ACK1 were coexpressed with HA-ubiquitin in COS7 cells. Transfected cells were serum-starved overnight and stimulated with EGF before harvesting. Ubiquitinated EGFR were detected as in C. *Lane 2* shows a normal level of EGFR ubiquitination after EGF stimulation, and *lane 3* shows inhibition of EGFR ubiquitination by ACK1. Lane 4 indicates Nedd4-2 alone did not change EGFR ubiquitination.

S4). Neither was there a significant change in ACK1 levels in metaphase (supplemental Fig. S4).

Regulation of EGFR Ubiquitination by ACK1—Because ACK1 affects EGFR turnover (13), we wondered whether binding of ACK1 affected ubiquitination of the EGFR. ACK1 contains a sequence that is conserved between ACK1 and MIG-6 and is known to directly bind to the catalytic domain of the EGF receptor (25). To assess what may occur as a direct consequence of this binding (without the complication of clathrin aggregation), this ACK1 region (727–915) or full-length MIG-6 were introduced, and endogenous EGFR modification was examined (Fig. 7*C*). In both cases there was a substantial inhibition of ubiquitination (Fig. 7*C*, *top panel*, *lanes 3* and *5*). The level of EGFR and extent of receptor auto-phosphorylation (anti-pEGFR845) in response to EGF (15 min) was indistinguishable, indicating immediate ligand-receptor responses were normal (*lanes 2* and *3*). Phosphorylation at Tyr^{845} in the kinase domain maintains the activity of EGFR (26) and is unaffected by MIG-6 or ACK1(727–915) overexpression (Fig. 7*C*). Hence binding of ACK1 to EGFR seems not to affect receptor activation but rather blocks ubiquitination.

Nedd4-2 itself has no effect on EGFR ubiquitination (Fig. 7*D*, *lane 4*), but when Nedd4-2 was cotransfected with ACK1, EGFR ubiquitination was restored to normal (Fig. 7*D*, compare *lane 3* with *lane 5*), likely by down-regulating the levels of ACK1. Cbl is probably the most important player in ubiquitination of EGFR (27), and Nedd4 has been suggested to indirectly regulate EGFR turnover by targeting Cbl for proteasomal degradation (28). Our study shows that Nedd4 also regulates ACK1 stability.

Ubiquitination of EGFR drives receptor endocytosis where ubiquitin serves as sorting signal that targets the activated receptor to endosomes and eventually to lysosomes for degradation (29). Ubiquitination can affect receptor sorting to the inner vesicles of MVB because ubiquitin serves as a signal for the sorting of cargos to these vesicular bodies (30). A recent study showed that ACK1 knockdown retained EGFR in early endosomes and inhibits the receptor sorting to inner vesicles of the MVB (17). We propose that upon ACK1 activation by EGFR (3), Nedd4-2 mediates ubiquitination and ACK1 turnover. Competition for binding to ACK1 by other WW-containing proteins may well locally modulate this, as illustrated in Fig. 8.

DISCUSSION

ACK1 is a nonreceptor kinase amplified in a some human tumors (14), and the kinase can negatively regulate the tumor suppressor Wwox to promote prostate metastasis (15) or be involved with the activation of androgen receptor (31). The functional role of ACK1 clearly relates to proteins that bind to the kinase including Cdc42, Hsp90, clathrin, and SH3 domain containing Grb2 and SNX9 (10). ACK1 is linked by SNX9 to

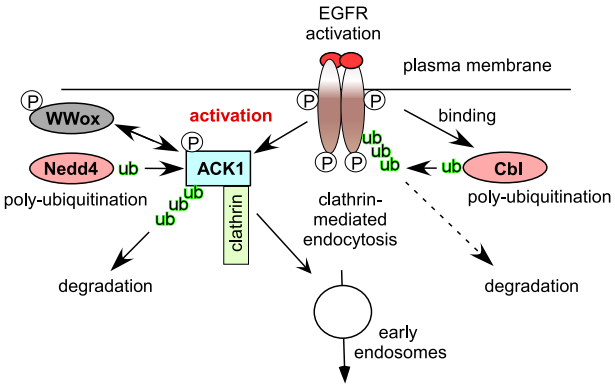

FIGURE 8. **Schematic diagram showing the inter-relationship of Nedd4-2, ACK1, and EGFR signaling and events involving ubiquitination pathways.** Ligand binding to EGFR leads to ACK1 activation (3) through a mechanism that is not yet defined. This increases ACK1 association with the receptor and increases Nedd4-2 interaction with the activated ACK1, which in turn promotes ACK1 turnover via the proteasome. Nedd4 may also regulate EGFR (47, 48); however, for simplicity we show that EGFR ubiquitination and turnover is mainly driven through the E3 ubiquitin ligase Cbl (as previously reviewed in Ref. 27). Clathrin-mediated endocytosis of both EGFR and ACK1 to endosomes and subsequent sorting within multi-vesicular bodies likely underlies some of these processes. The tumor suppressor WWox binds to ACK1 at the same site as Nedd4 (Fig. 3 and Ref. 15), and thus both proteins may compete for binding.

synaptojanin-1 (10) and with clathrin participates in membrane receptor endocytosis. Here we document additional interactions for ACK1: (i) to Nedd4-like proteins, (ii) the presence of an additional clathrin interaction sequence, and (iii) potential to directly bind amphiphysins.

Our study demonstrates that E3 ubiquitin ligase Nedd4 (and likely other family members) target ACK1 and suppress its protein level. We demonstrate that a PP*XY* motif allows interaction with the Nedd4 WW2 and WW3 domains. We are able to colocalize tagged ACK1 with full-length Nedd4-2 in mammalian cells (Fig. 4) and the endogenous proteins by coimmunoprecipitation (Fig. 2*B*). Both Nedd4-1 and Nedd4-2 are present as multiple isoforms in human cells. Most contain four WW domains that are regulated in a complex manner as exemplified by studies of the epithelial sodium channel ENaC (32). Disruption of their interaction results in Liddle syndrome, an autosomal dominant form of hypertension (33). The Nedd4-interacting protein, N4WBP5A binds two of these ENaC PP*XY* motifs, to compete with Nedd4 and increase surface expression of ENaC (34). Thus it may well be that ACK1 binding to other proteins (*cf.* Wwox) prevents its down-regulation by Nedd4.

Interestingly ACK1 down-regulation by Nedd4 requires the kinase activity of ACK1 (Fig. 5, *B* and *C*). We suspect that the active kinase is conformationally favorable for ligase activity. Nedd4-2 is known to be phosphorylated by kinases such as the serum and glucocorticoid kinase (35), decreasing its activity by promoting 14-3-3 binding (36). G-protein-coupled receptor 2 also phosphorylates Nedd4 and Nedd4-2 at a different site, which affects sodium channel function (37).

Ubiquitination plays roles other than tagging proteins for proteasomal degradation (38). Monoubiquitination can act as a reversible nonproteolytic modification that controls other functions such as endocytic trafficking, histone activity, DNA repair, and virus budding (39) and thus can be considered a general modulator along side other post-translational modifications. For example, the monoubiquitination of Eps15 (epsin-interacting protein) and CIN85 (Cbl-interacting p85) regulates interaction with their binding partners (40, 41). The two UBA domains present at the C terminus of ACK1 appear to be directly involved in its own ubiquitination. To date UBA domains are known to carry out three types of functions: (i) as target sites for protein ubiquitination; (ii) as binding sites for mono- or multi-ubiquitin chains (42, 43), to block further chain elongation (44, 45); and (iii) mediating protein-protein interactions, for example between Rad23 and Ddi1 (46). The exact role of the ACK1 UBA domains warrants further investigation.

In conclusion, we have demonstrated here that ACK1 can link through a variety of partners to proteins involved both in the endocytic and ubiquitination pathways. Upon activation ACK1 undergoes Nedd4-mediated turnover, which could well reflect the low levels of kinase reported in normal cell lines to date. Observed increased expression of ACK1 by genomic amplification and perhaps other mechanisms that promote invasive behavior *in vivo* (14) points to this negative regulation as being critical for homeostasis. One of the targets of ACK1 appears to be Wwox, whose phosphorylation by ACK1 then promotes Wwox ubiquitination (15). Clearly the reciprocal relationship between WW proteins that bind ACK1 and the stability and activity of ACK1 with respect to metastatic potential requires further investigation.

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