Differential requirements for AP-2 in clathrin-mediated endocytosis

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P-2 complexes are key components in clathrin-mediated endocytosis (CME). They trigger clathrin assembly, interact directly with cargo molecules, and recruit a number of endocytic accessory factors. Adaptor-associated kinase (AAK1), an AP-2 binding partner, modulates AP-2 function by phosphorylating its \( \mu_2 \) subunit. Here, we examined the effects of adenosiviral-mediated overexpression of WT AAK1, kinase-dead, and truncation mutants in HeLa cells, and show that AAK1 also regulates AP-2 function in vivo. WT AAK1 overexpression selectively blocks transferrin (Tfn) receptor and LRP endocytosis. Inhibition was kinase independent, but required the full-length AAK1 as truncation mutants were not inhibitory. Although changes in \( \mu_2 \) phosphorylation were not detected, AAK1 overexpression significantly decreased the phosphorylation of large adaptin subunits and the normally punctate AP-2 distribution was dispersed, suggesting that AAK1 overexpression inhibited Tfn endocytosis by functionally sequestering AP-2. Surprisingly, clathrin distribution and EGF uptake were unaffected by AAK1 overexpression. Thus, AP-2 may not be stoichiometrically required for coat assembly, and may have a more cargo-selective function in CME than previously thought.

**Introduction**

Clathrin-mediated endocytosis (CME) is important for a variety of biological processes ranging from nutrient uptake to synaptic vesicle recycling. Although more than 30 accessory proteins are believed to control this internalization pathway (Slepnev and De Camilli, 2000), clathrin and the adaptor protein complex (AP-2), constitute the major coat constituents (Brodsky et al., 2001). It is thought that the assembly of clathrin into progressively curved lattices provides the driving force behind the generation of coated pits and coated vesicles. The targeting and assembly of clathrin, as well as its coupling to cargo destined for internalization, is facilitated by the action of AP-2. AP-2 is a multifunctional heterotetramer, consisting of two large subunits (\( \alpha \) and \( \beta_2 \)), a medium subunit (\( \mu_2 \)), and a small subunit (\( \sigma_2 \)). The large subunits function in plasma membrane targeting (Robinson, 1993) and act as a platform to recruit other functionally relevant accessory proteins like amphiphysin, Eps15, epsin, etc. (Traub et al., 1999). The \( \mu_2 \) subunit interacts with the cytoplasmic domain of membrane-bound receptors containing tyrosine-based internalization motifs (Kirchhausen, 1999), whereas the \( \sigma_2 \) subunit appears to stabilize the AP-2 complex (Collins et al., 2002).

Phosphorylation of AP-2 complexes regulates their recruitment to the plasma membrane (Fingerhut et al., 2001), their interaction with cargo molecules (Ricotta et al., 2002), and their assembly with clathrin (Wilde and Brodsky, 1996). Several kinases copurify with clathrin-coated vesicles (CCVs), including (1) casein kinase II, which appears to phosphorylate clathrin light chains (Korolchuk and Banting, 2002); (2) an unknown kinase(s) that phosphorylates the \( \alpha \) and \( B_2 \) adaptins and may regulate their plasma membrane recruitment (Fingerhut et al., 2001); and (3) two related kinases, GAK/auxilin 2 (Umeda et al., 2000) and the newly discovered adaptor-associated kinase (AAK1) (Conner and Schmid, 2002), that phosphorylate \( \mu_2 \). In vitro phosphorylation of \( \mu_2 \) by AAK1 increases AP-2 affinity for tyrosine-based internalization motifs roughly 25-fold (Ricotta et al., 2002). Additionally, AAK1 inhibits AP-2-stimulated transferrin (Tfn) internalization in perforated cell assays that reconstitute early steps in CCV formation (Conner and Schmid, 2002). Here, we establish that full-length AAK1 interacts with and perturbs AP-2 function in vivo. Surprisingly, the disruption of AP-2 function by AAK1 overexpression reveals a more cargo-specific role for AP-2 in clathrin-dependent receptor-mediated endocytosis.

**Abbreviations used in this paper:** AAK1, adaptor-associated kinase; CCV, clathrin-coated vesicle; CME, clathrin-mediated endocytosis; siRNA, small interfering RNA; Tfn, transferrin; TfnR, Tfn receptor; tTA, tetra-cycline transactivator.

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Results and discussion

To test the in vivo role of AAK1 in regulating AP-2 function, we generated recombinant tetracycline-regulatable adenoviruses encoding AAK1 constructs postulated to compete for endogenous AAK1 function (Fig. 1 A). Identical constructs for baculovirus protein expression were also generated for biochemical analysis to allow for correlation of in vivo and in vitro observations. Endogenous kinase(s), including AAK1, that are known to cofractionate with AP-2 (Conner and Schmid, 2002; Korolchuk and Banting, 2002) were first inactivated by pretreatment with FSBA, an ATP analogue and irreversible kinase inhibitor (Fig. 1 B; Olusanya et al., 2001). Addition of WT AAK1 to FSBA-inactivated AP-2 resulted in efficient μ2 phosphorylation when incubated in the presence of [γ-32P]ATP (Fig. 1 B; Conner and Schmid, 2002). As expected, point mutations of conserved residues within the kinase domain, either K74A or D176A, predicted to disrupt nucleotide binding and catalysis, respectively, severely inhibited AAK1 activity for either phosphorylation of μ2 or autophosphorylation. A truncated AAK1 construct (ΔAID) that lacks the α-adaptin–interacting domain (AID) was efficiently autophosphorylated, thus ΔAID AAK1 is a fully active kinase. However, 10-fold more ΔAID was required to phosphorylate μ2 (Fig. 1 B). Thus, although the majority of AP-2 interaction is supported by the AID, other lower affinity AP-2 binding domains must exist. Indeed, GST fusion protein pull-downs demonstrate that most AP-2 binding is supported by the AID fragment (Fig. 1 C); however, AP-2 binding by both the ΔAID and QPA fragments was detected when ~10-fold more AAK1 fragment–GST fusion protein was used (Fig. 1 C). We conclude that efficient recruitment of AAK1 to AP-2 and its phosphorylation of μ2 requires the AID and that each of the individual domains and truncated mutants retain their expected activities.

We next infected tetracycline transactivator (tTA) HeLa cells with adenoviruses encoding various AAK1 constructs and assayed for their ability to internalize biotinylated Tfα. Cells overexpressing WT AAK1 showed a significant, concentration-dependent inhibition in Tfα endocytosis compared with controls (Fig. 2, A and C), a result consistent with in vitro observations (Conner and Schmid, 2002). Although somewhat less potent, inhibition was also observed in cells overexpressing either of the kinase-inactive AAK1 mutants. This is also consistent with the weaker, but observable inhibition of FSBA-treated AAK1 on endocytosis in perforated cells (Conner and Schmid, 2002). Interestingly, neither overexpression of the kinase-active ΔAID, nor the QPA or AID fragments of AAK1, which we expected to effectively compete for AAK1-interacting partners and thus perturb AAK1 function, significantly affected Tfα endocytosis (Fig. 2 B). From these observations, we conclude that the internalization defects seen with full-length AAK1 constructs do not simply reflect rampant kinase activity, a sequestration of AAK1-interacting partners, or interference with other AP-2 partners that interact through the ear domain of α adaptin.

The generality of the observed block in endocytosis resulting from full-length AAK1 overexpression was confirmed by examining another constitutively internalized receptor, LRP (low-density lipoprotein receptor-related protein) and the uptake of its ligand, RAP (receptor-associated protein), in immunofluorescence assays. Cells infected with adenovirus encoding WT AAK1 showed a significant decrease in RAP internalization and a corresponding increase in surface-associated RAP relative to controls (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200304069/DC1). Together, these data suggest that AAK1 interacts in vivo with AP-2 to regulate its function in the constitutive internalization of ligand complexes. Indeed, exogenous AAK1 coimmunoprecipitated with AP-2 complexes from cells overexpressing WT AAK1 (unpublished data). Inhibition by AAK1 occurred independently of its kinase activity, but required extended interactions between full-length AAK1 and AP-2 complexes, because neither the isolated COOH-terminal AID that binds the α-ear nor an active kinase/QPA construct inhibited endocytosis. These data establish that AAK1 can bind to and disrupt the function of AP-2 complexes in vivo.

In vitro kinase assays did not reveal any major AAK1 targets other than μ2 in either cytosolic or membrane fractions (Conner and Schmid, 2002). Moreover, μ2 phosphorylation is known to be required for endocytosis in vivo (Olusanya et al., 2001). Thus, we expected that overexpression of full-
length AAK1 constructs inhibited AP-2 function by shifting the balance of μ.2 into a phosphorylated (WT AAK1) or de-phosphorylated (K74A or D176A AAK1) state. However, immunoprecipitation of AP-2 complexes from whole cell lysates, following in vivo labeling, did not show any significant alteration in μ.2 phosphorylation in cells overexpressing either WT or kinase-dead AAK1 (Fig. 3 A). Although we cannot rule out the existence of a specifically localized subpopulation of phosphorylated μ.2, these data suggest that μ.2 phosphorylation activity of AAK1 in vivo is tightly regulated. Unexpectedly, a significant decrease in phosphorylation of the large AP-2 subunits was observed. Thus, rather than altering the μ.2 phosphorylation state, the observed receptor internalization block appears to result from the kinase activity–independent binding of full-length AAK1 to AP-2, which exerts a more global effect on AP-2 function.

To explore the mechanism of AAK1-mediated inhibition, we examined its effects on AP-2 localization. Overexpression of WT AAK1 caused a dramatic displacement of AP-2 from the normally punctate structures at the plasma membrane seen in control cells (Fig. 3 B). A similar, but less dramatic phenotype was observed in cells overexpressing either kinase-inactive AAK1 mutant (unpublished data). By comparison, overexpression of dominant–negative dynamin mutants that inhibit endocytosis resulted in the increased clustering of AP-2–containing coated pits on the plasma membrane. Consistent with their lack of effect on Tf and RAP internalization, neither the ΔAID, QPA, or AID fragments showed any significant AP-2 localization defect at the plasma membrane (Fig. 3 C; and data not depicted). To eliminate the possibility that full-length AAK1 overexpression masks the AP.6 epitope on α-adaptin, we performed ELISA assays and found that excess AAK1 did not inhibit AP.6 binding to immobilized AP-2 (unpublished data). Finally, we also probed cells with antibodies against the β2 subunit of AP-2 and identical results were obtained (unpublished data).

Postulating that AAK1 overexpression sequesters AP-2 in the cytosol, we determined the distribution of AP-2 in cytosol and membrane fractions. Surprisingly, WT AAK1 overexpression did not appear to alter the AP-2 distribution between soluble and particulate pools compared with control cells, or that of cells overexpressing K74A, D176A, AID, or ΔAID constructs (Fig. 3 D; and data not depicted). Although these fractionation experiments cannot eliminate the possibility that cytosolic AP-2 forms sedimentable structures following AAK1 overexpression, or that AAK1 overexpressions causes the mislocalization of AP-2 to other disperse membranes within the cytosol, these observations suggest that AAK1 overexpression inhibits endocytosis of the Tfn receptor (TfnR) and LRP by functionally sequestering AP-2 complexes and preventing their clustering on the plasma membrane.

To extend our analysis of AAK1 function in CME, we used small interfering RNAs (siRNAs) to knock down AAK1 expression in cells. Transfection of two different siRNAs that specifically target AAK1 reduced AAK1 expression by 80% in either A549 or HeLa cells. However, in neither case did we observe an alteration in Tf in internalization, AP-2 distribution, or μ.2 phosphorylation (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200304069/DC1).
There are several possible conclusions that can be drawn based on the inability to detect an effect of AAK1 depletion on μ2 phosphorylation. One possibility is that AAK1 is not the endogenous μ2 kinase. However, given that AAK1 copurifies with CCVs and colocalizes with endocytic clathrin-coated pits in both neuronal and nonneuronal cells (Conner and Schmid, 2002), and that the AAK1 phosphorylation site on μ2 (provided by J. Bonifacino, National Institutes of Health, Bethesda, MD) indicates equal loading. (B) Quantitation of phosphorylated large adaptin subunits from whole cell lysates relative to the tTA control. Data shown are representative of three independent experiments. (C) tTA HeLa cells, cultured in the absence of G418, were infected with the indicated AAK1 or dynamin adenovirus constructs. All cells are virally infected, but only those cells that retain tTA express the adenovirus-encoded constructs. Infected cells were fixed with ice-cold acetone and methanol extracted before further processing for immunolocalization of AAK1 using pAbs against either the ΔAID or AID fragment and the mAb AP.6 that recognizes the α-adaptin subunit of AP-2. Samples were visualized by epifluorescence microscopy using a Zeiss Axiophot with an attached Zeiss Axiocam. (D) The distribution of AP-2 in particulate (P) and soluble (S) fractions, obtained as previously described (Damke et al., 1994), was tested in adenovirus-infected cells by immunoblot analysis, using the mAb 100/2 (Sigma-Aldrich).
coated pits engaged in EGF uptake. However, our results are completely consistent with recent findings reporting that siRNA-mediated AP-2–depleted cells are capable of forming clathrin-coated pits that are competent for the internalization of the EGFR and an LDLR chimera, but defective in TfnR endocytosis (Motley et al., 2003). Thus, we conclude that the functional sequestration of AP-2 by AAK1 overexpression demonstrates an unexpected cargo-selective requirement for this coat constituent in CME.

A currently accepted paradigm for AP-2 function is that, in addition to cargo recognition, it is essential for the recruitment and assembly of clathrin. However, our results and recent results of others (Motley et al., 2003) suggest that the proper localization of AP-2 is not a prerequisite for the formation of functionally active clathrin-coated pits at the plasma membrane. Although unexpected, this observation is also consistent with the finding that overexpression of mutant epsin, incapable of binding PtdIns(4,5)P₂,
causes AP-2 to aggregate in the cytoplasm, having no apparent effect on the punctate distribution of clathrin on the plasma membrane (Ford et al., 2002). These findings are also consistent with observations in yeast where clathrin functionality is unaffected by the absence of all known heterotrimeric adaptor proteins (Huang et al., 1999). Thus, it is probable that AP-2 is not stoichiometrically required for the assembly of clathrin-coated pits at the plasma membrane and its major function is in cargo recruitment. We hypothesize that cells use a wider variety of specialized adaptors for clathrin assembly and cargo recognition than previously thought. What proteins recruit and promote clathrin assembly? It is likely that other adaptors, such as AP180/CALM (Morgan et al., 1999; Tebar et al., 1999), β-arrestin (Goodman et al., 1996; Lin et al., 1997), Da2 (Mishra et al., 2002), or HIP1R (Metzler et al., 2004) can function independently of AP-2 or selectively package cargo molecules and trigger clathrin assembly.

Materials and methods

Protein isolation and antibody production
AAK1 constructs fused to GST were purified from baculovirus-infected Tn5 cells as previously described (Conner and Schmid, 2002). APs, isolated as previously described (Smythe et al., 1992), were inactivated for endogenous kinase activity by pretreatment with 4 mM FSBA (fluorosulphonyl-benzoyladenosine; Sigma-Aldrich) for 2 h on ice. Unbound FSBA was removed from protein preparations by two sequential gel filtrations using G25 mini-spin columns (Amersham Biosciences).

Polyclonal antibodies against the COOH-terminal AID fragment of AAK1 expressed in E. coli and the NH2-terminal ΔAID fragment expressed in baculovirus-infected Tn5 cells were generated in this laboratory as previously described (Conner and Schmid, 2002).

Endocytosis assays
Except where noted, tTA HeLa cells were cultured and infected in the presence of G418 to maintain expression of the tTA that is required for protein overexpression. tTA HeLa cells were infected with recombinant adenoviruses as previously described (Altschuler et al., 1998). Viral loads showing >90% infection and uniform protein overexpression were used, as determined by immunolocalization. Internalization of biotinylated Tfn was measured by two sequential gel filtrations using G25 mini-spin columns (Amersham Biosciences).

Other assays
Kinase assays and in vitro protein interaction tests were performed essentially as described (Conner and Schmid, 2002). In vivo labeling and immunoprecipitation of AP-2 was performed as described using the mAb AP.6 (Wilde and Brodsky, 1996).

Online supplemental material
Fig. S1 shows immunofluorescence assays for RAP-GST endocytosis in control and WT AAK1-overexpressing cells. Fig. S2 shows the time course of siRNA-mediated AAK1 reduction by immunoblot analysis as well as single round Tfn internalization assays after siRNA treatment with control and AAK1-specific oligonucleotides. Supplemental materials and methods include information regarding AAK1 site-directed mutagenesis, the generalization of AAK1 adenovirus and baculovirus constructs, RAP internalization, and siRNA treatments. All supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200304069/DC1.

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References


Site-directed mutagenesis and generation of AAK1 fragments

To generate point mutations in the AAK1 cDNA, PCR was employed using the following primers.

**K74A AAK1:**
5′-GGGATGAAATGTGCCTTGGCACGCATGTTTGTCAACAATGAGC-3′
3′-GGGATGAAATGTGCCTTGGCACGCATGTTTGTCAACAATGAGC-5′

**D176A AAK1:**
5′-GCTCATTGTGACAAACATGCGTGCCAAGGCACATTTCATCCC-3′
3′-GCTCATTGTGACAAACATGCGTGCCAAGGCACATTTCATCCC-5′

HA-tagged AAK1 cDNA constructs and adenovirus production

Using either WT or point mutant AAK1 cDNA constructs as template, primers (encoding a 5′ HA tag, MYPYDVPDYA and shown below) were used to generate PCR products that were subcloned into pCR2.1 (Invitrogen), digested with SpeI/NotI (sites from pCR2.1), and then subcloned into a modified pAdlox plasmid. The constructs were then used in the generation of adenovirus as previously described (Altschuler, Y., S.M. Barbas, L.J. Terlecky, K. Tang, S. Hardy, K.E. Mostov, and S.L. Schmid. 1998. *J. Cell Biol.* 143:1871–1881).

**Full-length AAK1 and point mutants (amino acids 1–863):**
5′-CTCGAGACCATGTACCCATACGACGTGCCAGACTACGCTATGAAGAAGTTTTTCGACTCCCGG-3′
3′-GAATTCTTAAATAGCCTTGGCTTCTGGGGTGG-5′

**AID AAK1 (amino acids 1–628):**
5′-CTCGAGACCATGTACCCATACGACGTGCCAGACTACGCTATGAAGAAGTTTTTCGACTCCCGG-3′
3′-GAATTCTTAGGTTTTGGGGGATGAGG-5′

**QPA AAK1 (amino acids 313–628):**
5′-CTCGAGACCATGTACCCATACGACGTGCCAGACTACGCTATGAAGCTACTCAAGAAAGAGTG-3′
3′-GAATTCTTAGGTTTTGGGGGATGAGG-5′

**AID AAK1 (amino acids 629–863):**
5′-CTCGAGACCATGTACCCATACGACGTGCCAGACTACGCTATGAACGTGCTGGGCACAGGCGTATTC-3′
3′-GAATTCTAATAGCCTTGGCTTCTGGGGTGG-5′

GST-tagged AAK1 cDNA constructs and baculovirus-expressed protein production

Primers (see below) were used to generate PCR products, using either WT or point mutant AAK1 cDNA constructs. Products were sub cloned into pCR2.1 (Invitrogen), digested with EcoRI, and subcloned into the EcoRI site of pVL1393 in frame with GST that had been subcloned into the EcoRI/NorI site. The constructs were used to generate baculovirus, protein was expressed in TN5 cells, and isolated as previously described (Conner, S.D., and S.L. Schmid. 2002. *J. Cell Biol.* 156:921–929). cDNA constructs were sequence verified.

**Full-length AAK1 and point mutants (amino acids 1–863):**
5′-GAATTCGATGAAGAAGTTTTTCGACTCCCGG-3′
3′-GAATTCAATAGCCTTGGCTTCTGGGGTGG-5′

**AID AAK1 (amino acids 1–628):**
5′-GAATTCGATGAAGAAGTTTTTCGACTCCCGG-3′
3′-GAATTCGGTTTTGGGGGATGAGG-5′

**QPA AAK1 (amino acids 313–628):**
5′-GAATTCGATGAAGCTACTCAAGAAAGAGTG-3′
3′-GAATTCGGTTTTGGGGGATGAGG-5′

**AID-AAK1 (amino acids 629–863):**
5′-GAATTCGATGCAACGTGCTGGGCACAGGCGTATTC-3′
3′-GAATTCAATAGCCTTGGCTTCTGGGGTGG-5′

siRNA treatment

siRNA treatments were performed according to established protocols (Elbashir, S.M., J. Harborth, K. Weber, and T. Tuschl. 2002. *Methods*. 26:199–213) except that Transfectamine 2000 (Invitrogen) was used as a delivery agent. siRNA oligos were obtained from QIAGEN that target the following AAK1 sequences: AAK1-1, 5′-AATTCGATGAAGAAGTTTTTCGACTCCCGG-3′; AAK1-2, 5′-AGATGCTTCATCTGATGGACT-3′; and a control oligo, 5′-AATTCGATGAAGAAGTTTTTCGACTCCCGG-3′.

RAP internalization

For RAP-GST internalization, tTA HeLa cells were plated on coverslips and were infected with WT AAK1 or control adenoviruses for 18 h at 37°C in DMEM containing 10% fetal calf serum. Cells were plated on ice and washed with PBS, and the media was replaced with DMEM containing 4 μg/mL RAP-GST. Slides were then transferred to 37°C for 15 min to allow internalization, transferred back to ice, and immediately fixed and probed for RAP-GST using pAb against GST.