

# Differential requirements for AP-2 in clathrin-mediated endocytosis

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**A**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 adenoviral-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  $\beta$ 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.

The online version of this article includes supplemental material.

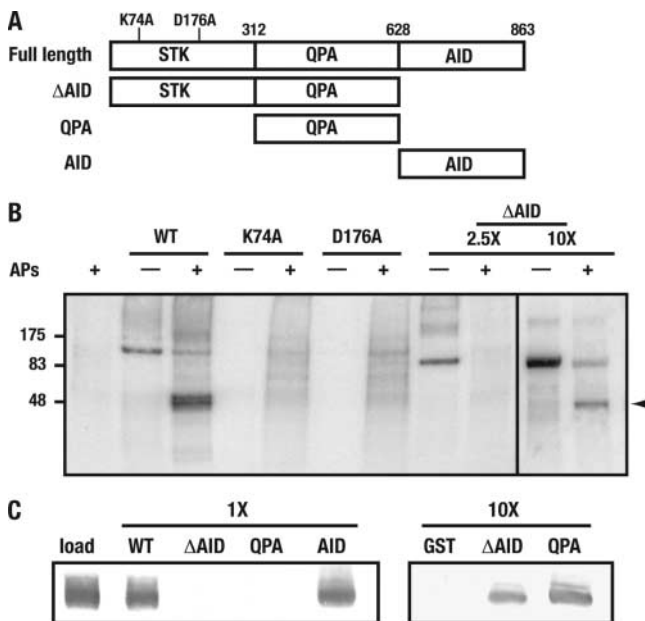
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Abbreviations used in this paper: AAK1, adaptor-associated kinase; CCV, clathrin-coated vesicle; CME, clathrin-mediated endocytosis; siRNA, small interfering RNA; Tfn, transferrin; TfnR, Tfn receptor; tTA, tetracycline transactivator.

## 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  $\mu$ 2 phosphorylation when incubated in the presence of [ $\gamma$ <sup>32</sup>P]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  $\mu$ 2 or autophosphorylation. A truncated AAK1 construct ( $\Delta$ AID) that lacks the  $\alpha$ -adaptin-interacting domain (AID) was efficiently autophosphorylated, thus  $\Delta$ AID AAK1 is a fully active kinase. However, 10-fold more  $\Delta$ AID was required to phosphorylate  $\mu$ 2 (Fig. 1 B). Thus,



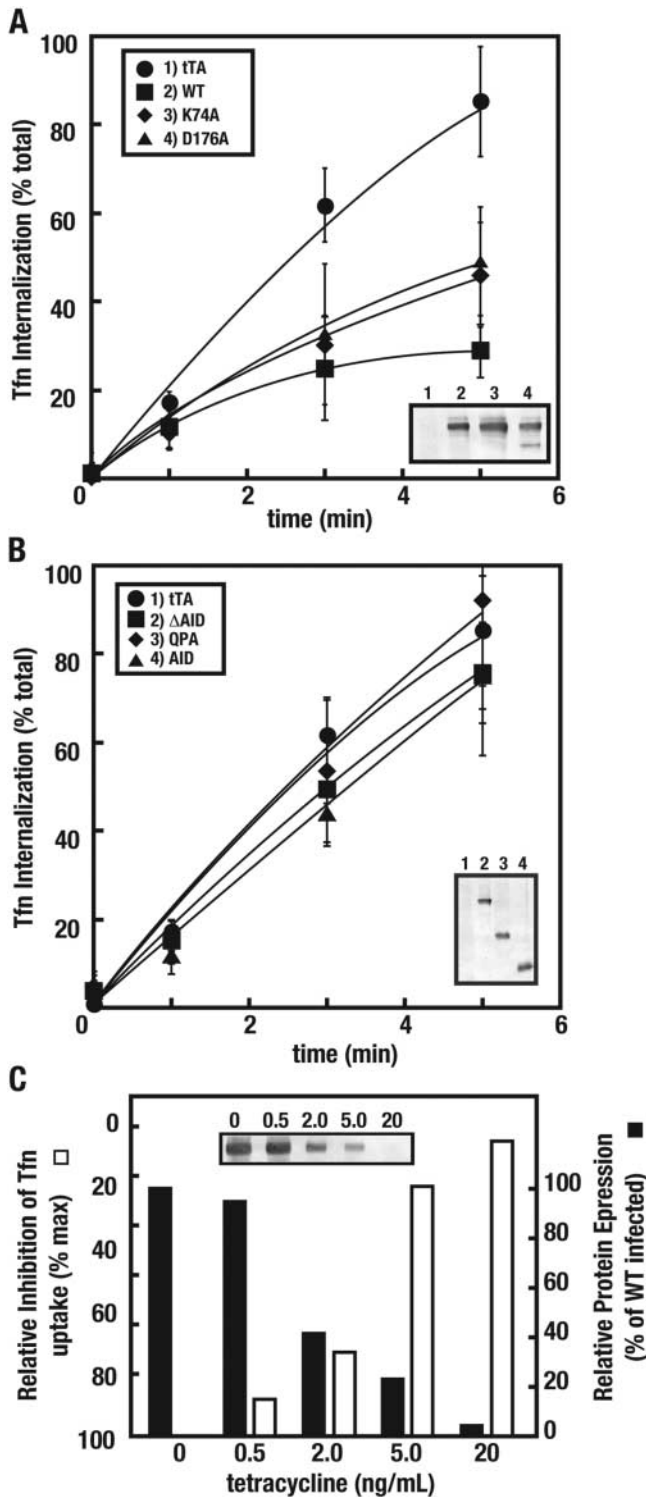
**Figure 1. Structural requirements for AAK1-mediated  $\mu$ 2 phosphorylation.** (A) Diagram illustrating the AAK1 constructs used in this study. Baculovirus constructs were GST-tagged at the COOH terminus, whereas adenovirus constructs were HA-tagged at the NH<sub>2</sub> terminus (see supplemental methods, available at <http://www.jcb.org/cgi/content/full/jcb.200304069/DC1>). (B) FSBA-inactivated APs (5.2  $\mu$ g) were incubated with AAK1-GST fusion proteins, as indicated, in the presence of [ $\gamma$ <sup>32</sup>P]ATP and phosphorylated protein detected by SDS-PAGE and autoradiography. Assays were performed with 0.25  $\mu$ M of WT, K74A, or D176A AAK1 and 0.65  $\mu$ M (2.5 $\times$ ) or 2.5  $\mu$ M (10 $\times$ ) of the  $\Delta$ AID mutant. (C) The indicated AAK1-GST fusion proteins were immobilized on glutathione-agarose beads at either  $\sim$ 0.25 mg/ml (1 $\times$ ) or  $\sim$ 2.5 mg/ml (10 $\times$ ) and incubated with isolated APs. Bound AP-2 was detected by immunoblot analysis.

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  $\Delta$ AID and QPA fragments was detected when  $\sim$ 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  $\mu$ 2 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 Tfn. Cells overexpressing WT AAK1 showed a significant, concentration-dependent inhibition in Tfn 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  $\Delta$ AID, 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 Tfn 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  $\alpha$  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  $\alpha$ -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  $\mu$ 2 in either cytosolic or membrane fractions (Conner and Schmid, 2002). Moreover,  $\mu$ 2 phosphorylation is known to be required for endocytosis *in vivo* (Olusanya et al., 2001). Thus, we expected that overexpression of full-



**Figure 2. Full-length AAK1 inhibits Tfñ uptake in vivo.** tTA-HeLa cells were infected with either control adenovirus encoding the tTA transcription activator or tetracycline-regulatable adenoviruses encoding either full-length WT AAK1 or kinase-inactive mutants (K74A or D176A) (A), or AAK1 fragments (B), as indicated in the legend, and tested for Tfñ endocytosis as described in the Materials and methods. Protein expression levels were tested by immunoblot analysis (insets), using pAbs against  $\Delta$ AID AAK1 and/or the AID AAK1 fragment. Each time point represents the average of three independent experiments  $\pm$  the standard deviation. (C) The observed inhibition is concentration dependent. Cells were infected with WT AAK1 adenovirus in the presence of increasing concentrations of tetracycline to titrate AAK1 expression, and Tfñ endocytosis was assessed as described here.

length AAK1 constructs inhibited AP-2 function by shifting the balance of  $\mu$ 2 into a phosphorylated (WT AAK1) or dephosphorylated (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  $\mu$ 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  $\mu$ 2, these data suggest that  $\mu$ 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  $\mu$ 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.

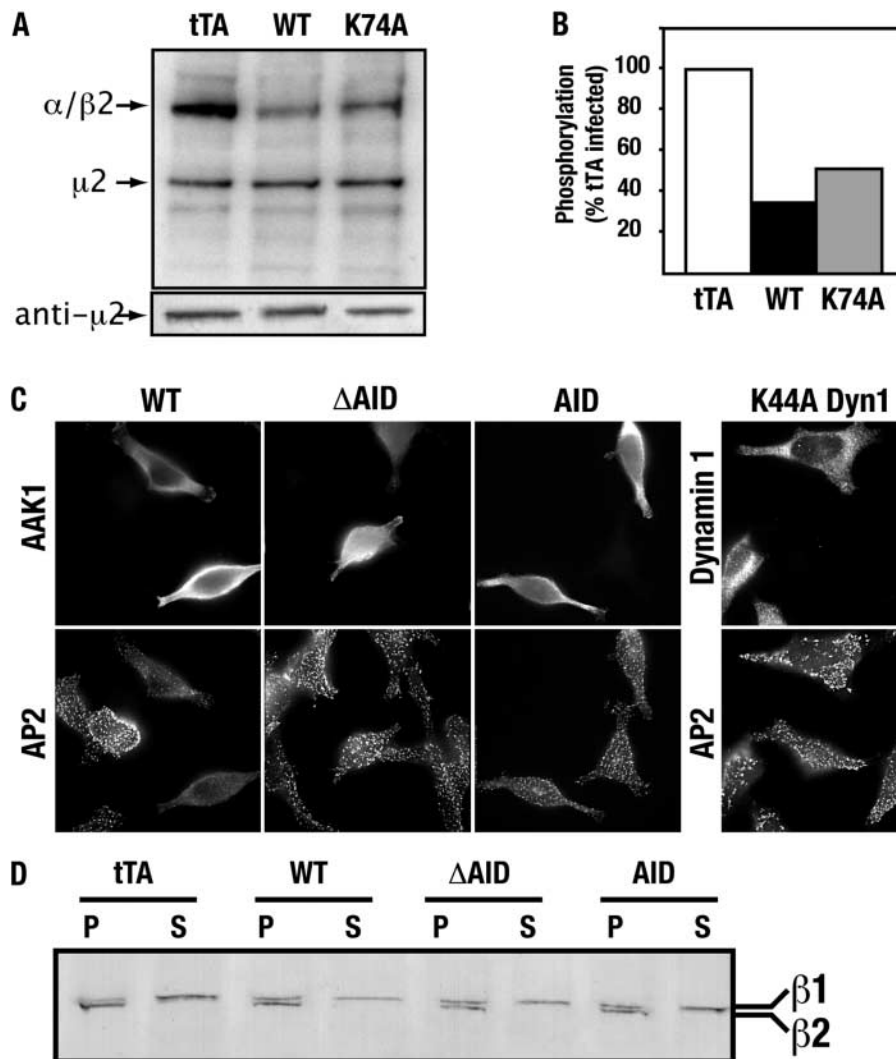
To explore the mechanism of AAK1-mediated inhibition, we examined its effects on AP-2 localization. Overexpression of WT AAK1 caused a dramatic displacement of AP-2 from the normally punctate structures at the plasma membrane seen in control cells (Fig. 3 B). A similar, but less dramatic phenotype was 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 Tfñ and RAP internalization, neither the  $\Delta$ AID, 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  $\alpha$ -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  $\beta$ 2 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  $\Delta$ AID 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 Tfñ receptor (TfñR) 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  $\sim$ 80% in either A549 or HeLa cells. However, in neither case did we observe an alteration in Tfñ internalization, AP-2 distribution, or  $\mu$ 2 phosphorylation (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200304069/DC1>).



**Figure 3. AAK1 globally disrupts AP-2 function.** (A) Adenovirally-infected tTA HeLa cells overexpressing WT AAK1, K74A AAK1, or the tTA were labeled in vivo with  $^{32}\text{P}$ -orthophosphate. AP-2 was then immunoprecipitated with the mAb AP.6 and analyzed by SDS-PAGE (top). Immunoblot (bottom) with antibodies specific for  $\mu 2$  (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  $\Delta\text{AID}$  or AID fragment and the mAb AP.6 that recognizes the  $\alpha$ -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).

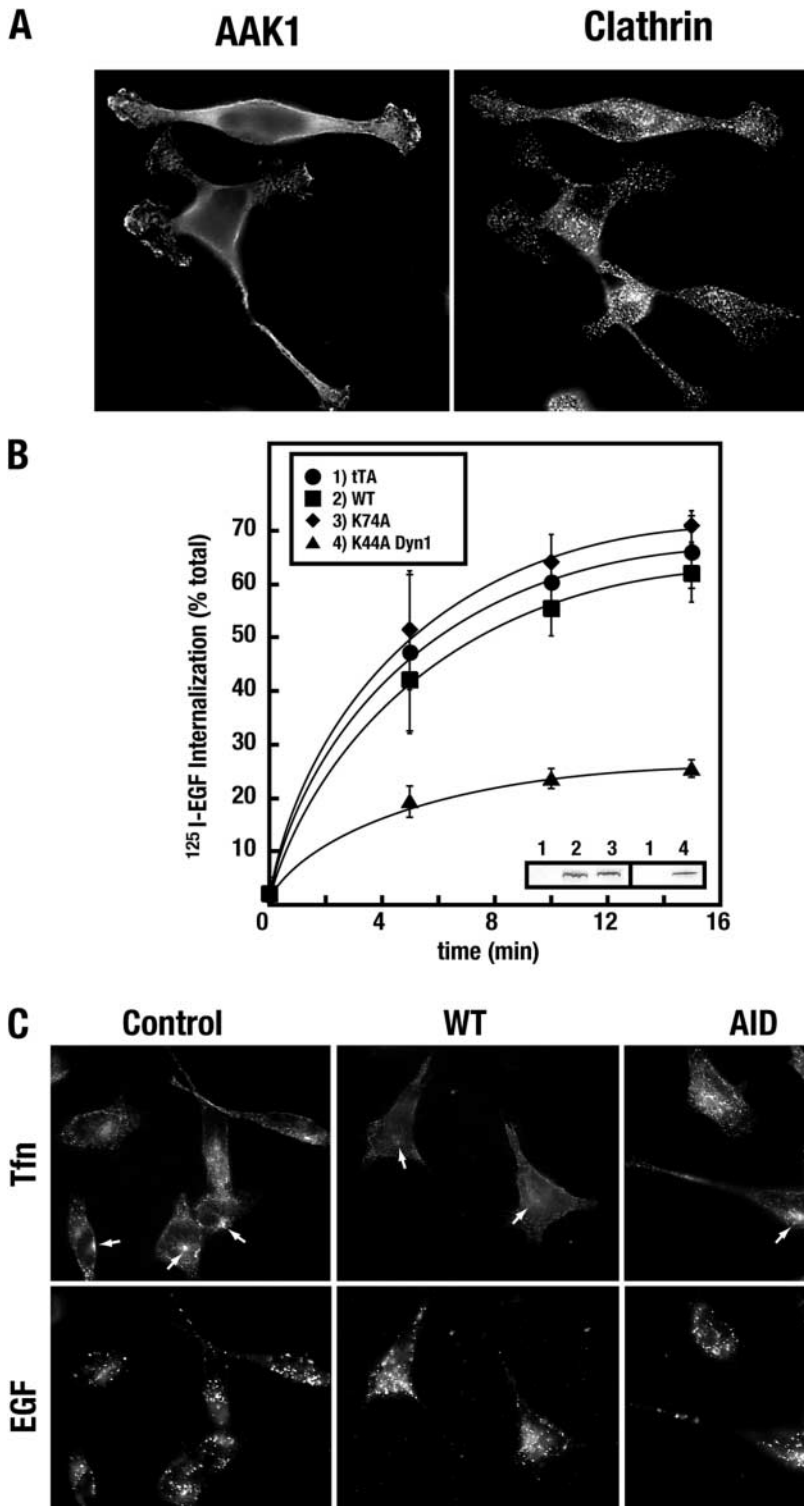


There are several possible conclusions that can be drawn based on the inability to detect an effect of AAK1 depletion on  $\mu 2$  phosphorylation. One possibility is that AAK1 is not the endogenous  $\mu 2$  kinase. However, given that AAK1 copurifies with CCVs and colocalizes with endocytic clathrin-coated pits in both neuronal and nonneuronal cells (Conner and Schmid, 2002), and that the AAK1 phosphorylation site on  $\mu 2$  is required for endocytosis in vivo (Umeda et al., 2000; Ricotta et al., 2002), we think this is unlikely. Instead, our results may reflect functional redundancy with other kinases of the Ark1/Prk1 family. This prospect would be consistent with the functional redundancy observed in yeast between the Ark1p and Prk1p proteins that regulate actin dynamics and endocytosis—a yeast phenotype is only observed following disruption of both the Ark1 and Prk1 genes (Cope et al., 1999). Indeed, GAK, another Ark1/Prk1 family member is known to be associated with CCVs and to phosphorylate  $\mu 2$  in vitro (Korolchuk and Banting, 2002; Umeda et al., 2000). Other AAK1-related kinases also exist in the mammalian genome (Conner and Schmid, 2002). Moreover, it is also possible that the levels of AAK1 that remain following siRNA treatment are sufficient to support normal levels of  $\mu 2$  phosphorylation.

The  $\mu 2$  subunit of AP-2 specifically recognizes tyrosine-based internalization motifs, whereas other AP-2 subunits are believed to function in endocytosis by directing clathrin assembly into curved lattices and by recruiting other essential cofactors to the coated pit (Kirchhausen, 1999). Therefore, we expected that clathrin-coated pit assembly would also be disrupted in cells overexpressing inhibitory AAK1 constructs that functionally sequester AP-2. Surprisingly, clathrin recruitment into coated pits was not altered in WT AAK1-overexpressing cells relative to controls (Fig. 4 A). Previous studies have established that EGF and TfnRs are internalized in the same coated pits (Lamaze et al., 1993). We therefore asked if AAK1 overexpression had any effect on EGF uptake. Surprisingly, neither WT nor K74A AAK1 overexpression had any effect on the internalization of EGF compared with controls (Fig. 4 , B and C). High concentrations of EGF are known to saturate the clathrin-mediated pathway for EGFR endocytosis (Jiang and Sorkin, 2003); therefore, care was taken to use low concentrations (2 ng/ml) of  $^{125}\text{I}$ -labeled EGF for these assays. As an additional control for CME, cells infected with recombinant K44A dynamin-1 adenovirus showed the expected EGF internalization defect (Damke et al., 1994). We cannot rule out that the small amounts of AP-2 remaining at the cell surface are selectively associated with

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)P<sub>2</sub>,



**Figure 4. AP-2-deficient clathrin-coated pits are functional for EGF uptake.**

(A) WT AAK1-infected tTA HeLa cells (cultured as described in the legend to Fig. 3 C) show no defect in the recruitment of clathrin to the plasma membrane or its ability to generate coated pits, as observed by immunolocalization using the mAb X22. (B) EGF internalization was tested in tTA HeLa cells infected with adenoviruses expressing either tTA, WT, or K74A AAK1, or K44A dynamin-1 adenoviruses, as indicated in the legend. Protein expression levels were tested by immunoblot analysis (inset) using pAbs against either the  $\Delta$ AID AAK1 fragment (lanes 1–3) or against dynamin (lanes 1 and 4). (C) tTA HeLa cells, cultured on coverslips and infected with the indicated adenoviruses, were tested for their ability to internalize rhodamine-conjugated Tfn and Alexa-488-conjugated EGF, simultaneously (Molecular Probes). Cells were incubated in the presence of 2 ng/ml EGF and 4  $\mu$ g/ml Tfn for 15 min at 37°C. Cells were then transferred to ice, washed to remove unbound ligand, fixed with acetone, and visualized by epifluorescence as described in the legend to Fig. 3. A significant decrease in Tfn accumulation in the endosome is observed in WT AAK1-infected cells relative to AID AAK1- or control-infected cells (arrows), whereas EGF uptake is unaffected.

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),  $\beta$ -arrestin (Goodman et al., 1996; Lin et al., 1997), Dab2 (Mishra et al., 2002), or HIP1R (Metzler et al., 2001; Mishra et al., 2001) can function independently of AP-2 to 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 (fluorosulphonylbenzoyladenine; 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 NH<sub>2</sub>-terminal  $\Delta$ AID 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 nearly 100% infection and uniform protein overexpression were used, as determined by immunolocalization. Internalization of biotinylated Tfn was assayed as previously described (Carter et al., 1993), assessing internalization by inaccessibility to avidin. Identical results were obtained by assessing resistance to MesNa (unpublished data). For EGF internalization, virus-infected cells were serum starved for 1.5–2 h in binding buffer (DMEM, 1% BSA) before the internalization assay. Cells were then detached from dishes in PBS/5 mM EDTA, rinsed with ice cold binding buffer, resuspended in binding buffer containing 2 ng/ml <sup>125</sup>I-EGF, and incubated on ice for 1 h. Cells were then washed with binding buffer and aliquoted. One aliquot was kept on ice to measure total ligand binding, and the rest were transferred to 37°C for the indicated times, returned to ice to stop endocytosis, and then acid washed (0.5 M NaCl, 0.2 M acetic acid, pH 2.8) for 5 min on ice to remove surface bound ligand. Cells were pelleted, the supernatant containing released ligand was aspirated, and the samples were measured for internalization with a gamma counter.

### 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 genera-

tion 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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