Identification of an adaptor-associated kinase, AAK1, as a regulator of clathrin-mediated endocytosis

Sean D. Conner and Sandra L. Schmid

The Scripps Research Institute, La Jolla, CA 92037

The μ2 subunit of the AP2 complex is known to be phosphorylated in vitro by a copurifying kinase, and it has been demonstrated recently that μ2 phosphorylation is required for transferrin endocytosis (Olusanya, O., P.D. Andrews, J.R. Swedlow, and E. Smythe. 2001. *Curr. Biol.* 11:896–900). However, the identity of the endogenous kinase responsible for this phosphorylation is unknown. Here we identify and characterize a novel member of the Prk/Ark family of serine/threonine kinases, adaptor-associated kinase (AAK)1. We find that AAK1 copurifies with adaptor protein (AP)2 and that it directly binds the ear domain of α-adaptin in vivo and in vitro. In neuronal cells, AAK1 is enriched at presynaptic terminals, whereas in nonneuronal cells it colocalizes with clathrin and AP2 in clathrin-coated pits and at the leading edge of migrating cells. AAK1 specifically phosphorylates the μ subunit in vitro, and stage-specific assays for endocytosis show that μ phosphorylation by AAK1 results in a decrease in AP2-stimulated transferrin internalization. Together, these results provide strong evidence that AAK1 is the endogenous μ2 kinase and plays a regulatory role in clathrin-mediated endocytosis. These results also lend support to the idea that clathrin-mediated endocytosis is controlled by cycles of phosphorylation/desphosphorylation.

Introduction

Endocytic clathrin-coated vesicle (CCV)* formation is essential for the recycling of synaptic vesicles after neurotransmission and for receptor-mediated endocytosis that controls a variety of important biological processes including nutrient uptake, immune response, and cellular response to activated signaling receptors. Two oligomeric protein complexes, clathrin and the adaptor protein (AP)2 complex, constitute the major coat proteins of these endocytic vesicles. Clathrin assembles into a polygonal lattice surrounding endocytic vesicles (for review see Kirchhausen, 2000). The heterotetrameric AP2 complex, consisting of two ∼100-kD subunits, the α and β2 adaptins, a medium subunit, μ2 of ∼50 kD, and a small ∼17-kD σ2 subunit, is multifunctional (Kirchhausen, 1999). Although the function of σ2 is unclear, the α and β2 subunits are known to mediate membrane targeting and protein–protein interactions with clathrin and other endocytic machinery components like dynamin, auxilin, Eps15, etc. The major function of

the μ2 subunit appears to be the recognition of endocytic cargo (Ohno et al., 1995).

Clathrin-mediated endocytosis is initiated by the recruitment of AP2 complexes to saturable and protease-sensitive sites on the plasma membrane (PM). AP2 complexes interact directly with tyrosine-based sorting motifs on the cytoplasmic domains of receptors to affect their concentration into coated pits and coordinate the assembly of clathrin (Schmid, 1997). The assembly of clathrin into a lattice is, in turn, thought to drive invagination of the PM and thus facilitate vesicle formation (Musacchio et al., 1999). After coat assembly, the coated pit constricts and pinches off from the PM to form a CCV. This cycle is completed by the disassembly of coat proteins and facilitated by the action of the ATPase, Hsc70 (Hannan et al., 1998; Newmyer and Schmid, 2001), which releases the coat components for another round of clathrin-mediated endocytosis. Although many components of the endocytic machinery have been identified, we lack a full understanding of the mechanisms that regulate the clathrin-mediated endocytic pathway.

Phosphorylation/dephosphorylation of proteins involved in endocytosis has long been implicated as a key regulatory step in controlling clathrin-mediated endocytosis (Pauloin et al., 1988; Robinson et al., 1994; Turner et al., 1999). For example, isolated CCVs appear to contain at least two kinase activities (Pauloin et al., 1984; Bar-Zvi and Branton, 1986). One kinase activity associated with CCVs appears

to be casein kinase II, a poly-L-lysine–activated serine/threonine (S/T) kinase, which preferentially phosphorylates the b light chain of clathrin (Bar-Zvi and Branton, 1986). Also present on CCVs is a Ca^{2+}-, calmodulin-, and cAMP-independent and poly-L-lysine–insensitive kinase activity that phosphorylates the adaptor complex component pp50, the $\mu2$ subunit, in vitro (Pauloin et al., 1982, 1984). However, the identity of this kinase is currently unknown. In vivo labeling experiments reveal that the α, $\beta2$, and $\mu2$ subunits of the AP2 complex are phosphorylated on serine and threonine residues, and these phosphorylated AP2 complexes are impaired in their ability to bind clathrin cages in vitro (Wilde and Brodsky, 1996). Phosphorylation of AP2 appears to enhance binding to membranes and tyrosine- and leucine-based sorting signals (Fingerhut et al., 2001). Most recently, it has also been found that $\mu2$ phosphorylation is essential for clathrin-mediated transferrin uptake (Olusanya et al., 2001). Moreover, phosphorylation of coat proteins by endogenous kinase(s) activated by exogenous melittin and poly-L-lysine is known to destabilize the clathrin coat of isolated CCVs (Georgieva-Hanson et al., 1988).

Phosphorylation also appears to be important in modulating the function of several other proteins implicated in endocytosis, including dynamin 1, amphiphysins 1 and 2, synaptojanin, epsin, and Eps15. Indeed, these proteins are phosphorylated in resting nerve terminals and coordinately dephosphorylated upon nerve terminal stimulation (Liu et al., 1994; Bauerfeind et al., 1997; Chen et al., 1999; Cousin et al., 2001). Additionally, in vitro evidence suggests that phosphorylation plays a central role in regulating the protein–protein interactions of amphiphysin with other endocytic components like dynamin 1, synaptojanin 1, AP2, and clathrin (Slepnev et al., 1998).

We have identified a novel S/T kinase, we now call adaptor-associated kinase (AAK)1, by using a phage display–based protein interaction screen. We find that AAK1 binds directly to α-adaptin in vivo and in vitro. Poly-L-lysine–independent kinase assays reveal that AAK1 specifically phosphorylates the μ subunit of the AP complex in vitro. Introduction of the kinase into in vitro stage-specific assays that reconstitute endocytosis shows an inhibitory effect that strongly correlates with phosphorylation of the μ subunit. These results suggest that AAK1 may play a regulatory role in clathrin-mediated endocytosis and that in combination with an as yet unidentified phosphatase(s) AAK1 regulates endocytosis through a phosphorylation/dephosphorylation cycle.

Results

Identification of a novel α-adaptin–interacting protein

To identify α-adaptin protein-interacting partners, we employed a phage display approach using the ear domain (amino acids 701–938) as a bait to screen a rat brain cDNA library. After three rounds of biopanning and phage amplification to enrich for specific interactions, we isolated several cDNAs. As expected, we isolated phage-containing insert cDNAs that encoded proteins known to interact directly with α-adaptin such as epsin and amphiphysin (unpublished data). However, nearly a third of the isolated phage contained inserts that encoded a previously uncharacterized cDNA, KIAA1048, we now call AAK1 (Fig. 1, AAK1). Sequence analysis predicts the cDNA to encode a S/T kinase of ~100 kD. The encoded protein appears to be modular with the S/T kinase domain located at its NH_2 terminus. The middle domain of the protein is rich in glutamine (28%), proline (18%), and alanine (13%), whereas the COOH-terminal domain encodes the region identified by

Figure 1. The predicted amino acid sequence of the AAK1. The S/T kinase domain is underlined, whereas the COOH-terminal domain, which was identified by phage display, is in bold. This COOH-terminal domain was also used to generate polyclonal antibodies. The clathrin, EH domain, and α-adaptin interaction motifs, DLL, NPF, and DPF, respectively, are boxed.
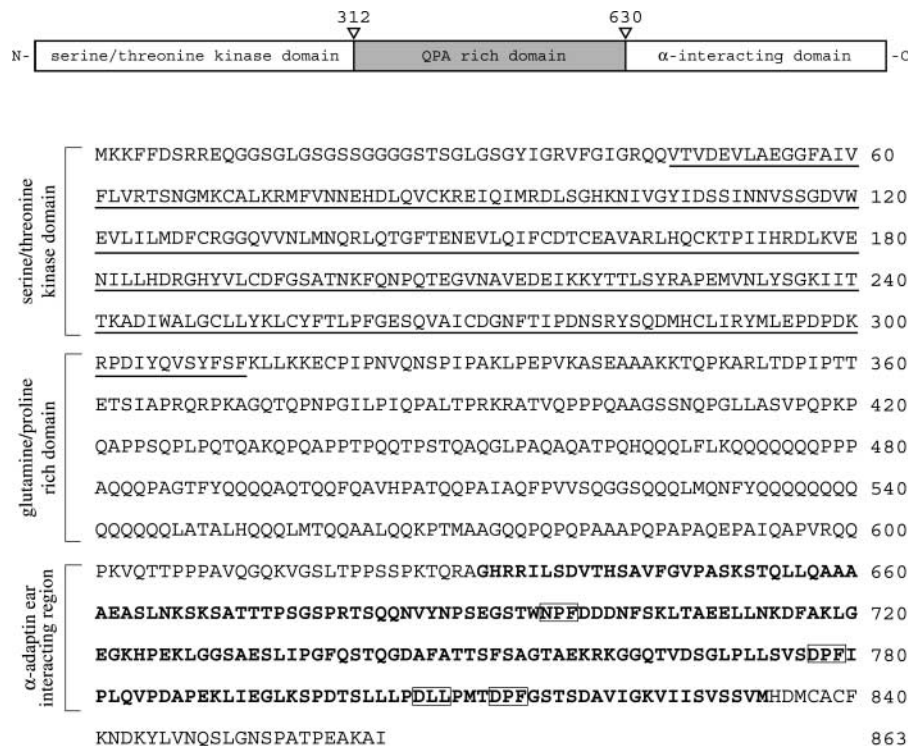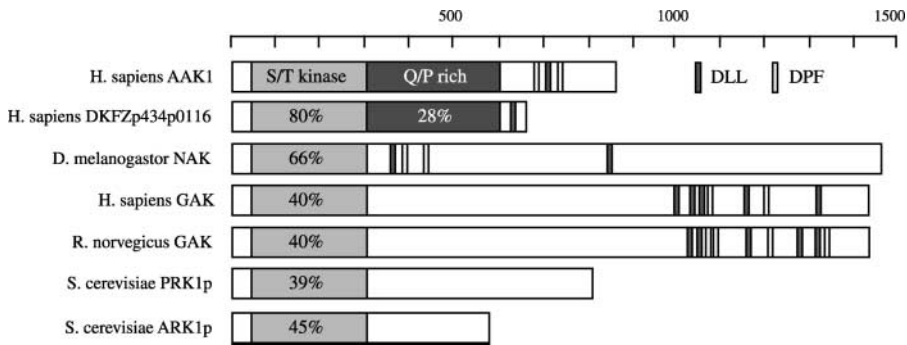
Figure 2. **AAK1 shares strong identity to the Ark family of S/T kinases in the kinase domain.** Sequence data available from GenBank/EMBL/DDBJ under accession nos.: AAK1, NP_055726; DKFZp434p0116, NP_060063; Numb-associated kinase (NAK), AF15596; human cycling-associated kinase (hGAK), NP_005246; rat cycling-associated kinase (rGAK), NP_112292; Prk1p, NP_012171; Ark1p, NP_014378. Top bar indicates amino acid number.

phage display, which we have designated the α-adaptin–interacting domain. The COOH-terminal α-adaptin–interacting domain contains three motifs demonstrated previously to be important in protein–protein interactions: an α-adaptin–interacting DPF motif (Owen et al., 1999), an NPF motif that confers binding to EH domains (Salcini et al., 1997), and a DLL motif known to mediate clathrin binding (Morgan et al., 2000).

BLAST searches indicate that AAK1 is similar to the Ark family of S/T kinases (Cope et al., 1999). The greatest identity in this family of S/T kinases is within the kinase domain (Fig. 2); however, other Ark family members appear to lack the QPA-rich middle domain of AAK1. AAK1 is most similar to another human cDNA, DKFZp434P0116.1, having ~80% identity in the kinase domain. DKFZp434P0116.1 also contains a middle QPA-rich domain that is 28% identical to AAK1. AAK1 also has a *Drosophila melanogaster* homologue, the Numb-associated kinase, which shares strong identity in the kinase domain, as well as sharing DLL and DPF motif features.

AAK1 interacts directly with α-adaptin

To independently test the direct interaction of AAK1 with α-adaptin, we generated a glutathione *S*-transferase (GST) fusion protein with the COOH-terminal domain of AAK1 (amino acids 679–893) for a GST pull-down experiment. We found that AAK1 (679–893), immobilized on glutathione-agarose beads, was able to specifically pull-down the isolated ear domain (amino acids 701–938) of α-adaptin (Fig. 3, lane 2) as detected by immunoblot analysis probing for α-adaptin. Likewise, both COOH-terminal domain and full-length AAK1–GST fusion proteins were capable of pulling down α-adaptin from rat brain cytosol (Fig. 3, lanes 1 and 5), whereas the GST alone control was not found to interact with α-adaptin (Fig. 3, lanes 3 and 4). Thus, these results demonstrate that AAK1 interacts directly with α-adaptin in vitro.

AAK1 cofractionates with APs and clathrin-coated vesicles

To determine the subcellular localization of endogenous AAK1, we generated antibodies against the COOH-terminal α-adaptin–interacting domain and found them to recognize, by immunoblot analysis, a single polypeptide in lysates from rat brain, bovine brain, and rat liver (Fig. 4; unpublished data). Interestingly, the polypeptide migrates as an ~145-kD species in rat or bovine brain lysates and at the predicted molecular weight of ~100 kD in rat liver lysates. Although the reason for these MW differences was not explored, it is likely that they are accounted for by tissue-specific splice variants.

Since AAK1 is capable of interacting directly with APs in vitro, we postulated that if these interactions occur in vivo AAK1 should share a similar subcellular fractionation pattern. Bovine brain fractionation experiments reveal that the majority of AAK1 is associated with membranes and that it cofractionates with AP complexes and clathrin (Fig. 4 A). When CCVs, clathrin, and APs isolated from bovine brain and CCVs from rat liver were tested for the presence of AAK1, we found that it was present in all three protein samples (Fig. 4 B, lanes 1–4). Moreover, AAK1 was present in immunoprecipitated AP2 samples (unpublished data). These combined observations strongly argue that AAK1 interacts directly with AP complexes in vivo and suggests that AAK1 in these fractions might regulate clathrin and AP function.

AAK1 localizes to regions active in endocytosis

AAK1 binds directly to AP2. Thus, if AAK1 plays a role in regulating aspects of clathrin-mediated endocytosis we would expect it to be enriched in regions of the cell that are active in endocytosis. To test this idea, rat hippo-
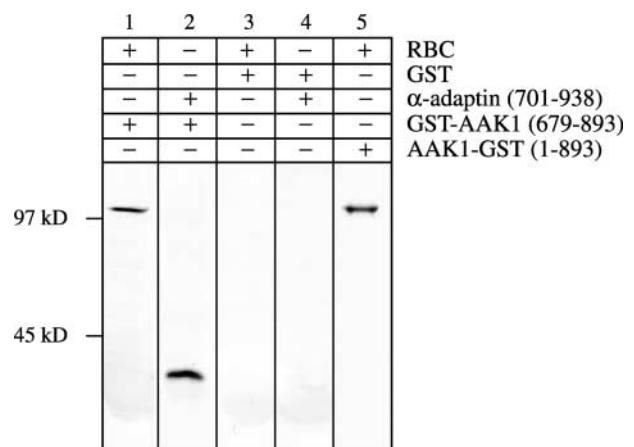

Figure 3. **AAK1 interacts directly with α-adaptin in vitro.** AAK1–GST fusion proteins, COOH-terminal domain, or full-length AAK1 consisting of amino acids 679–893 and 1–893, respectively, immobilized on glutathione-agarose beads were incubated with the COOH-terminal domain of α-adaptin (amino acids 701–938, lane 2) or rat brain cytosol (RBC, lanes 1 and 5). Immunoblot analysis probing for the presence of α-adaptin reveals that AAK1 is capable of interacting directly with the COOH-terminal domain of α-adaptin (lane 2) and the full-length protein in rat brain cytosol (lanes 1 and 5). This interaction is likely through AAK1 DPF motifs. pAb 0927 and mAb AP.6 were used to detect full-length α-adaptin and its COOH-terminal domain, respectively.
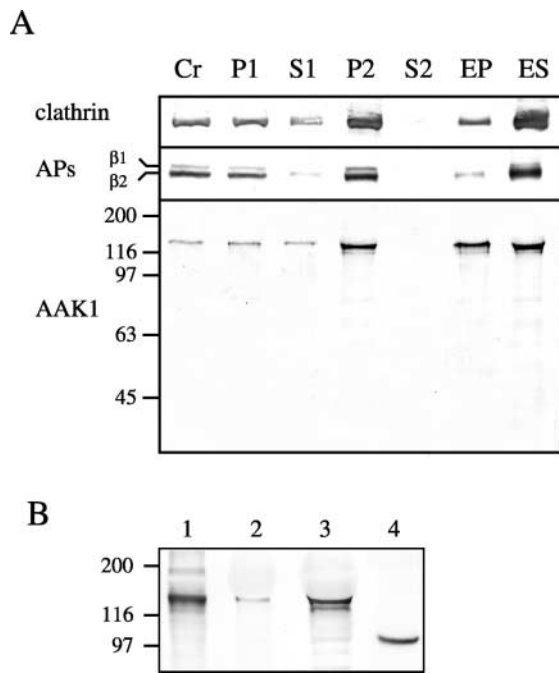
Figure 4. **AAK1 is enriched in bovine brain membrane fractions and associated with isolated CCV and AP2 preparations.** (A) Immunoblot analysis reveals that AAK1 (visualized with pAb 5366) cofractionates with both clathrin (visualized with mAb TD.1) and APs (visualized with mAb 100-1 that recognizes both β1 and β2 subunits), which are found in the low (P1) and high (P2) speed membrane pellets of homogenized bovine brain. Treatment of the high speed membrane pellet, which is highly enriched for clathrin-coated vesicles, with 0.75 M Tris, pH 7, extracts the majority of AAK1 to a soluble pool (ES); however, a fraction of AAK1 remains membrane associated when the extracted membranes are repelleted (EP). Each lane represents equivalent fractions of each sample. (B) AAK1 is found associated with isolated APs (lane 1), clathrin (lane 2), and CCVs (lane 3), and CCVs from rat liver (lane 4). Cr, crude bovine brain homogenate; S1, low speed supernatant; S2, high speed supernatant (cytosol).

Figure 5. **Immunolocalization of AAK1 in rat hippocampal neurons.** Rat hippocampal neurons were incubated with rhodamine-dextran (A), which marks sites of endocytosis, and then fixed and stained with pAb 5366 to detect AAK1 (B). AAK1 colocalizes with sites active in endocytosis (C, yellow overlay). Likewise, AAK1 immunolabel (E and H) is found to colocalize with regions that are highly enriched in AP2 (D, mAb AP.6 immunolabel) and dynamin 1 (G, mAb hudy1 immunolabel) as revealed by their overlays (F and I). Images were visualized by confocal microscopy.

campal neurons were cultured in the presence of a fluid phase endocytic marker, rhodamine-dextran (Fig. 5 A), and then probed for AAK1 (Fig. 5 B). We found good colocalization of AAK1 with the endocytosed marker in these cells (Fig. 5 C). Indeed, AAK1 colocalized with a variety of components of the endocytic machinery, including clathrin (unpublished data), AP2 (Fig. 5 F), and dynamin 1 (Fig. 5 I), all proteins known to be highly enriched at presynaptic terminals. AAK1 also colocalized to endocytic structures in nonneuronal cells. Immunolocalization of AAK1 in HeLa cells revealed a punctate immunolabeling pattern throughout the cell (Fig. 6 A) that colocalized with AP2 (Fig. 6 C). Interestingly, in migrating cells AAK1 was highly enriched at the leading edge (Fig. 6 D), where it was also found to colocalize with AP2 and clathrin (Fig. 6, F and I, respectively). The localization of AAK1 to sites active in endocytosis and its colocalization with known components of the endocytic machinery is consistent with the hypothesis that AAK1 plays a functional role in regulating clathrin-mediated endocytosis in these regions of the cell.
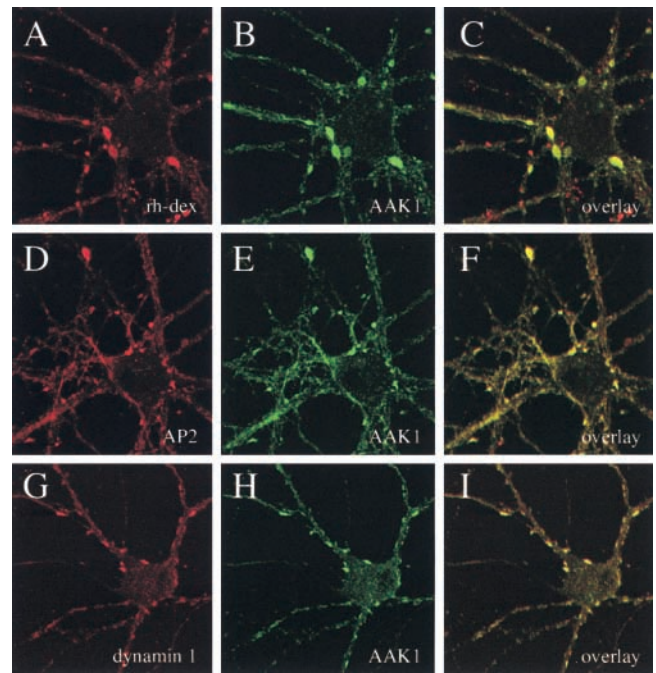
AAK1 specifically phosphorylates the μ subunit of APs

AAK1 encodes a S/T kinase, and thus we were interested in identifying phosphorylation substrates. We find that when isolated baculovirus-expressed AAK1 is incubated alone in the presence of $[\gamma^{32}\text{-P}]$ATP, it is capable of autophosphorylation (Fig. 7 A, lane 1). However, since AAK1 cofractionates and interacts directly with APs we next asked if AAK1 was capable of phosphorylating proteins contained in these fractions. Previously, it has been observed that fractions enriched in either APs or CCVs contain an endogenous kinase(s), and incubation of these fractions with $[\gamma^{32}\text{-P}]$ATP results in phosphorylation of several proteins, the predominant being μ, which migrates at ~50 kD (Pauloin and Thurieau, 1993) (Fig. 5 A, lane 2). Addition of recombinant AAK1 to AP fractions results in an increased phosphorylation of the 50-kD protein (Fig. 7 A, lane 3, arrowhead), in addition to the AAK1 autophosphorylation band. We observe this same enriched 50-kD phosphorylation pattern when AAK1 is incubated with clathrin-coated vesicles and $[\gamma^{32}\text{-P}]$ATP (unpublished data). No other phosphorylation products appear to be generated by the presence of recombinant AAK1.

To verify that the enriched phosphorylated 50-kD product that results from AAK1 addition to AP protein preparations was indeed μ, kinase assays were performed with APs and the μ subunits were immunoprecipitated (after dissociating the AP2 complex) using antibodies specific for either
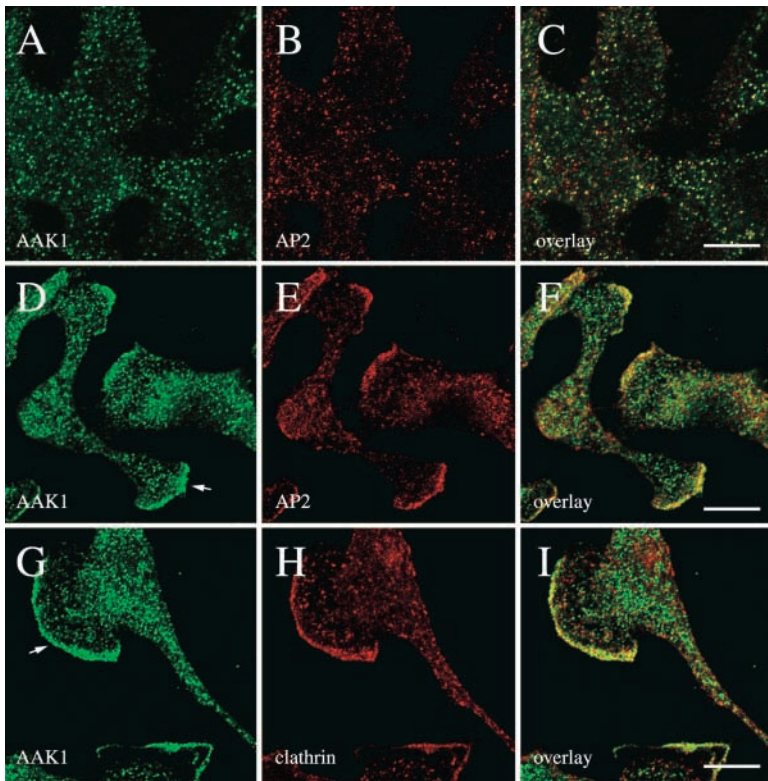
Figure 6.   **AAK1 associates with endocytic structures in HeLa cells.** AAK1 (A) shows good colocalization with AP2 (B, mAb AP.6 immunolabel) in nonmigrating HeLa cells as observed in their overlay (C, yellow). In migrating HeLa cells, AAK1 is highly enriched at the leading edge (D and G, arrow) where it colocalizes with AP2 (E) and clathrin (H, mAb X22 immunolabel). Immunolocalizations visualized by confocal microscopy. Bars, 5 μm.

$\mu 1$ or $\mu 2$. We find that both $\mu 1$ and $\mu 2$ phosphorylation was increased as a result of AAK1 addition over the endogenous kinase activity (Fig. 7 B). Given that AAK1 is capable of autophosphorylation and that it migrates in a similar region of the gel as the α and $\beta 2$ subunits of the adaptor complex, we also immunoprecipitated α and $\beta 2$ subunits with specific antibodies to test for their phosphorylation. However, neither of these proteins was phosphorylated as a result of AAK1 addition to APs (unpublished data). These results provide strong evidence that μ is a specific target of AAK1 phosphorylation in CCVs and suggests that the observed phosphorylation of μ can be attributed to the presence of endogenous AAK1 in these samples (Fig. 4). Nevertheless, these results do not exclude the possibility of other unidentified kinases with μ as their substrate.

AAK1 inhibits AP2-dependent transferrin endocytosis

To test the potential function of AAK1 in clathrin-mediated endocytosis, we employed an in vitro stage-specific perforated cell assay that faithfully reconstitutes early and late stages of endocytosis. This assay measures the ATP- and cytosol-dependent sequestration of receptor-bound biotinylated transferrin (BTfn) into constricted coated pits and coated vesicles (Carter et al., 1993). The addition of purified AP2 complexes, in a concentration-dependent manner, stimulates the formation of coated pits and increases the efficiency of BTfn sequestration into constricted coated pits (Fig. 8 A) (Smythe et al., 1992; McLauchlan et al., 1998). However, supplementing each reaction with 30 μg/ml recombinant AAK1–GST fusion protein results in a decrease in AP2-stimulated BTfn sequestration with maximal inhibition at substoichiometric levels of AAK1 relative to APs (Fig.

8 A). From these results, we postulated that the observed inhibition was due to the additional kinase activity of AAK1 in these reaction mixtures. To test this idea, we inactivated AAK1–GST fusion protein by preincubation with 1 mM fluorosulphonylbenzoyladenosine (FSBA), an ATP analogue that irreversibly binds to the ATP-binding site and has been demonstrated recently to inactivate the endogenous kinase activity found in AP preparations (Olusanya et al., 2001). Supplementing reactions with 30 μg/ml FSBA-treated AAK1 (free FSBA removed by gel filtration) had little effect on AP2-stimulated BTfn sequestration at AP concentrations up to 173 μg/ml. However, at higher AP concentrations we
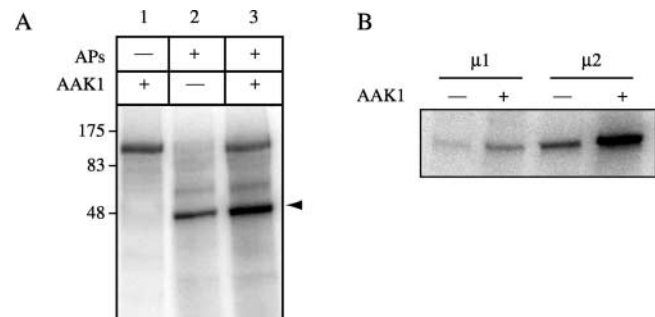

Figure 7.   **AAK1 preferentially phosphorylates the μ subunit.** (A) Isolated baculovirus-expressed AAK1–GST fusion protein is capable of autophosphorylation in vitro when incubated with $[\gamma\text{-}^{32}P]$ATP (lane 1). Likewise, incubation of AAK1–GST with fractions enriched in APs shows an increase in phosphorylation of a $\sim$50-kD protein over APs alone (arrowhead, lane 2 compared with 3). (B) The $\sim$50-kD band, which is preferentially phosphorylated by AAK1, was identified as μ by immunoprecipitation with pAbs against $\mu 1$ and $\mu 2$.
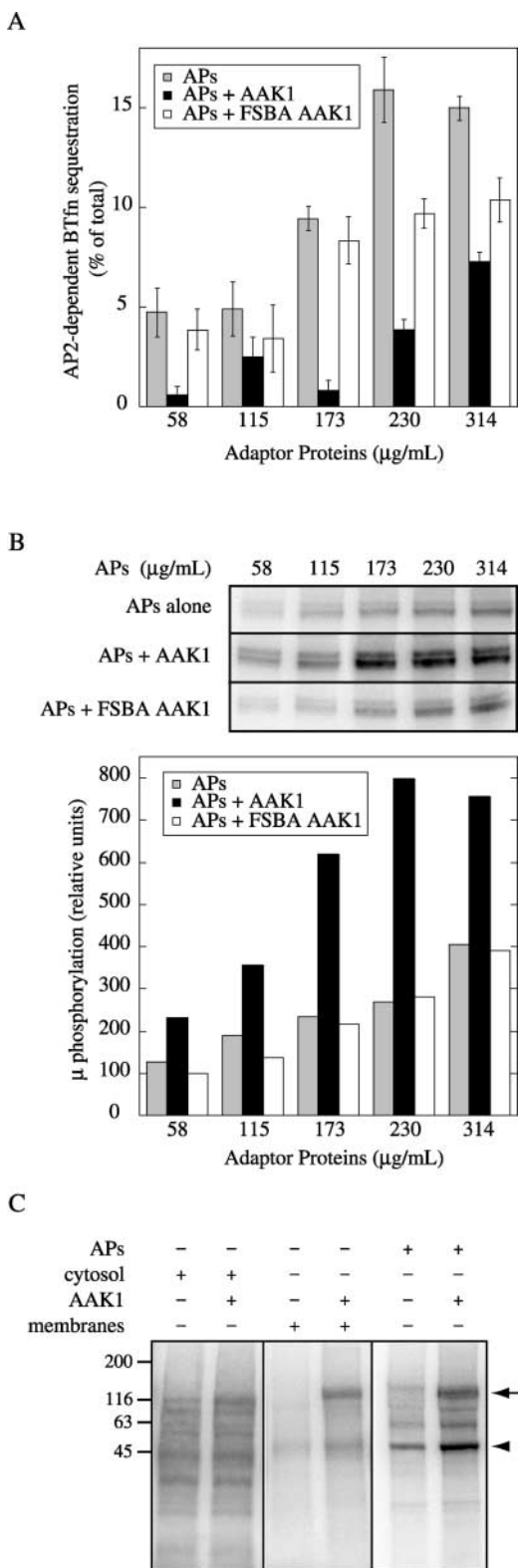
A

B

C

Figure 8.   Phosphorylation of μ by AAK1 inhibits AP2-stimulated BTfn sequestration. (A) APs stimulate the sequestration of BTfn in a concentration-dependent manner in stage-specific in vitro endocytosis assays that measure AP2-stimulated constricted coated pit formation (grey bars). However, the addition of 30 μg/ml recombinant AAK1–GST fusion protein results in a decrease in AP2-dependent stimulation (black bars). In contrast, addition of 30 μg/ml FSBA-treated AAK1–GST fusion protein has little effect on AP2 stimulation up to an AP concentration of 173 μg/ml (white bars). Results are the mean ±

begin to observe an inhibitory effect, though not to the same magnitude as the addition of untreated AAK1. GST alone was not found to have any effect on AP-stimulated endocytosis (unpublished data). These observations suggest that AAK1 plays a role in AP2-stimulated endocytosis and that the majority of the observed inhibition results from the additional AAK1 kinase activity.

AAK1 phosphorylates the μ subunit of the AP complex (Fig. 5); thus, we postulated that the observed decrease in AP2-stimulated BTfn internalization was the result of μ phosphorylation. Not surprisingly, kinase assays reveal increased μ phosphorylation with an increase in AP concentration (Fig. 8 B). Likewise, the addition of 30 μg/ml of AAK1 resulted in an increase in μ phosphorylation over the endogenous kinase activity. No additional μ phosphorylation was observed by the addition of 30 μg/ml FSBA-treated AAK1. These experiments reveal a strong correlation between increased μ phosphorylation and inhibition of AP2-stimulated BTfn sequestration. However, we also wanted to test the possibility that another AAK1 target might be present in either cytosol or on A431 membrane preparations. Aside from AAK1 autophosphorylation, kinase assays incorporating membrane or cytosol preparations reveal no other substantial phosphorylation products resulting from AAK1 addition (Fig. 8 C), although we cannot exclude other minor phosphorylation events. Collectively, these data provide functional evidence that AAK1 plays a role in AP2-stimulated endocytosis at an early step by specifically phosphorylating the μ subunit of the AP complex.

Discussion

We have identified a previously uncharacterized S/T kinase, AAK1, that specifically interacts with APs and phosphorylates the μ subunit of AP complexes. Although it is clear that μ2 phosphorylation occurs in vitro and in vivo (Pauloin and Thurieau, 1993; Wilde and Brodsky, 1996), the consequence of this phosphorylation has not been elucidated. Accumulating evidence suggests several possible roles for the phosphorylation of μ2. Since the μ2 subunit can interact directly with tyrosine-based internalization motifs (Ohno et al., 1995, 1996; Rapoport et al., 1997), it has been postulated to function in cargo recognition. Thus, it is possible that phosphorylation of the μ2 subunit might modulate the

SEM of six experiments. (B) AAK1-dependent inhibition directly correlates with the phosphorylation of μ. The autoradiogram shows that increased μ phosphorylation is observed when increasing concentrations of APs are incubated with [γ-³²P]ATP. Likewise, the addition of 30 μg/ml of AAK1–GST fusion protein results in a significant increase in μ phosphorylation, whereas 30 μg/ml FSBA-treated AAK1–GST fusion protein shows only endogenous levels of μ phosphorylation. Note that we sometimes resolve a μ doublet on gradient gels in our kinase assays. Phosphorylated μ was quantitated (graph below) using the ImageQuant Software package from Molecular Dynamics, and the graph represents the mean of two kinase experiments. (C) AAK1 specifically phosphorylates the μ subunit of APs. APs, K562 cytosol, and A431 membrane preparations were each incubated with [γ-³²P]ATP in the presence or absence of AAK1–GST. Only the μ subunit (arrowhead) of APs is significantly phosphorylated as a result of additional AAK1–GST (AAK1 autophosphorylation, arrow).

ability of APs to be recruited to sorting signals or vice versa. Indeed, it has been observed that phosphorylation of the $\mu2$, α, and $\beta2$ subunits enhances AP2 association with the tyrosine- and leucine-based sorting motifs of the mannose-6-phosphate receptor and lysosomal acid phosphatase precursor, respectively (Fingerhut et al., 2001). Moreover, recent results from Höning and colleagues (Ricotta et al., 2002) demonstrate that phosphorylation of $\mu2$ enhances the affinity of AP2 for internalization motifs, independent of the influence of the large AP subunits.

$\mu2$ phosphorylation may also be important in regulating interactions between AP2 and clathrin. Yeast two-hybrid screens have defined a direct interaction between $\beta2$-adaptin and $\mu2$ (Page and Robinson, 1995). It has also been found that the AP2 interaction with clathrin is mediated through the hinge region of $\beta2$ (Shih et al., 1995) and phosphorylated APs are impaired in their ability to bind clathrin cages in vitro (Wilde and Brodsky, 1996). Although the phosphorylated AP subunits responsible for preventing interaction with clathrin have not been defined, it is reasonable to postulate that conformational changes in $\mu2$ and/or $\beta2$ that result from $\mu2$ phosphorylation might play a regulatory role in AP2–clathrin interaction.

Recently, $\mu2$ phosphorylation has been shown to be required for clathrin-mediated endocytosis of transferrin (Olusanya et al., 2001). Olusanya and colleagues found that by preventing the phosphorylation of $\mu2$, either by inhibiting the endogenous kinase activity found in AP2 preparations with the ATP analogue, FSBA, or by site-directed mutagenesis of the $\mu2$ phosphorylation site, transferrin uptake was inhibited in vitro and in vivo, respectively. In contrast, we find that supplementing endocytosis assays with AAK1, an endogenous kinase in AP2 preparations, and thus increasing the phosphorylation state of $\mu2$, results in an inhibitory effect. However, we do not believe these two results are contradictory. The experiments of Olusanya et al. (2001) allow for a single round of $\mu2$ phosphorylation, whereas the addition of AAK1 in our experiments allows for multiple rounds of phosphorylation. Together, these results suggest that, although $\mu2$ phosphorylation is essential for endocytosis, maintaining $\mu2$ in a phosphorylated state, by the addition of exogenous AAK1, results in an inhibition of transferrin uptake. This suggests that a protein phosphatase is required in clathrin-mediated endocytosis to dephosphorylate $\mu2$. Interestingly, AP2 preparations are known to copurify with a phosphatase activity that efficiently dephosphorylates APs during AP complex purification (Morris et al., 1990). Similarly, analyses of coated membrane proteins in intact neurons failed to detect $\mu2$ phosphorylation (Keen and Black, 1986), which hints at the presence of an active $\mu2$ phosphatase in these cells. Although this is in contrast with the observed $\mu2$ phosphorylation in nonneuronal cells (Wilde and Brodsky, 1996), this difference may reflect the relative activities of the clathrin-mediated endocytic pathway in neuronal verses nonneuronal tissues.

Given these observations, we hypothesize that AAK1 functions to phosphorylate $\mu2$ and facilitate AP2 recruitment to tyrosine- and leucine-containing sorting signals. The binding of AP2 to sorting signals could then facilitate the concentration of cargo molecules through AP self-assembly properties. Once concentration of cargo has occurred, an

as yet unidentified phosphatase would be required to dephosphorylate $\mu2$ and allow AP2 to interact with clathrin and progress through the process of vesicle formation. This simple model predicts that endocytosis is tightly regulated by cycles of phosphorylation/dephosphorylation. However, it is most likely an oversimplification given that several other components of the endocytic machinery are known to be coordinately phosphorylated/dephosphorylated during the cycle of clathrin-mediated endocytosis. Whether AAK1 phosphorylates other components of the endocytic machinery awaits determination.

It is interesting to note that Prk1p, an Ark kinase family member, regulates Pan1p activity by phosphorylation. Pan1p is a yeast homologue of Eps15, a protein known to function in endocytosis (Wendland and Emr, 1998), and is marked for phosphorylation on the threonine residue of LxxQxTG motifs (Zeng and Cai, 1999). The phosphorylation site of $\mu2$ has been mapped to threonine 156 (Pauloin and Thurieau, 1993; Olusanya et al., 2001), and it shares a similar motif [IxxQxTG] with isoleucine being substituted for leucine in the first position. The AAK1, that copurifies with AP2, has been shown recently to phosphorylate $\mu2$ at this same site (Ricotta et al., 2002), and thus it is likely that $\mu2$ is targeted for AAK1 phosphorylation through its IxxQxTG motif.

An important next step will be to test the in vivo consequences of perturbing AAK1 function on endocytosis. Since AAK1 is localized to the leading edge of migrating cells, a region active in actin turnover and remodeling, and is related to Ark1p and Prk1p, proteins shown to be involved in regulating actin organization in yeast (Cope et al., 1999) and endocytosis (Zeng and Cai, 1999), there is a strong possibility that AAK1 may also function to coordinate cytoskeletal actin dynamics and endocytosis. Additionally, it will be important to identify AAK1-interacting partners that potentially modulate its activity. The identification of AAK1 partners and potentially other phosphorylation substrates and the identification of the endogenous phosphatase found in AP preparations will help to understand how the cycle of phosphorylation/dephosphorylation regulates clathrin-mediated endocytosis.

Materials and methods

Phage display library production and biopanning

To construct the rat brain phage display library, mRNA was isolated from rat brain according to established protocols (Schibler et al., 1980) and used for cDNA production using a random primed OrientExpress cDNA synthesis kit (Novagen). The resulting cDNA was then used in the construction of a phage display library using the T7Select System (Novagen). The derived rat brain phage display library was then used for biopanning according the T7Select System manual.

For biopanning, a GST fusion protein with the ear domain of α-adaptin (amino acids 701–938; a gift from L. Traub, University of Pittsburgh, Pittsburgh, PA) was immobilized on a glutathione-agarose matrix (Amersham Pharmacia Biotech). The phage display library was incubated with the immobilized α-adaptin ear in the presence of a panning buffer (0.5% BSA, 25 μg/ml sheared salmon sperm DNA, 0.5% NP-40, 25 μg/ml heparin, 0.05% sodium azide, all in PBS, pH 7.4) for 1 h at room temperature. The α-adaptin ear immobilized ligand with bound phage was then washed with 10 column vol of PBS, pH 7.4. Bound phage were then amplified by inoculating BLT5403 bacterial cells ($OD_{600} = 0.75$), and incubating with constant shaking at 37°C until cell lysis was observed ($\sim$2.5 h). Bacterial debris was removed by centrifugation, and the supernatant was used for further rounds of biopanning with new immobilized α-adaptin ear ligand. After three rounds

of biopanning and phage amplification to enrich for specific protein interactions, phage were plated on BLT5403 cell lawns, plaques were isolated, and cDNA inserts were amplified by PCR using the T7Select UP (5′-GGAGCT-GTCGTATTCCAGTC-3′) and DN (5′-AACCCCTCAAGACCCGTTTAG-3′) primers and used for DNA sequencing.

Preparation of cDNA constructs

The COOH-terminal domain of AAK1 (amino acids 679–893) was obtained from isolated phage by PCR using the T7Select UP and DN primers (see above). The PCR product was cloned into pCR2.1 using the Topo cloning kit (Invitrogen). The COOH-terminal cDNA fragment was then excised by an EcoRI and XhoI digest for subcloning into pGEX4T-3 (Amersham Pharmacia Biotech) for the generation of a GST fusion protein. Human full-length AAK1 (clone KIAA1048; GenBank/EMBL/DDBJ under accession no. BC002695) cDNA constructs in pBluescript were provided by the Kazusa Research Institute (Kisarazu, Chiba, Japan). This full-length cDNA insert was subcloned into pVL1393 (PharMingen) for baculovirus expression by PCR using primers 5′-TCTAGACATATGCAGCCAAAGGC-CAGACTG-3′ and 5′-GAATTCTTGGGTTTTGGGGGATGAGG-3′ to introduce XbaI and EcoRI cloning sites, respectively. A COOH-terminal GST tag was introduced into the pVL1393 AAK1 construct by subcloning GST from pGEX4T-3 (Amersham Pharmacia Biotech) by PCR with 5′-GGAAT-TCCTGGTTCCGCGTGGGTCCATGTCCCCTATACTAGGTTATTG-3′ and 5′-GCGGCCGCTTACAGATCCGATTTTGGAGGATGG-3′ primers containing EcoRI and NotI restriction sites, respectively, into the appropriate pVL1393 restriction sites.

Fusion protein expression and isolation

The pGEX4T-3 GST fusion construct containing the COOH-terminal domain of AAK1 (amino acids 679–893) was transformed into BL21(DE3) cells. Expression of the fusion protein was induced by the addition of 0.1 mM IPTG for 3 h at 25°C. Bacterial cells were lysed by nitrogen cavitation, cellular debris was removed by centrifugation, and the soluble fraction containing the expressed fusion protein was isolated using glutathione-agarose beads. Bound protein was eluted from beads using 20 mM reduced glutathione in TBST (50 mM Tris, pH 7.5, 0.9% NaCl, 0.05% Tween-20, and 0.5% BSA) and used for in vitro binding experiments or as an immunogen for antibody production (see below). The full-length AAK1–GST fusion construct in pVL1393 was used for protein expression using the BaculoGold Expression System following manufacturer protocols (PharMingen). After infection with generated baculovirus and expression in TN5 cells, cells were pelleted, processed, and fusion protein was isolated following identical procedures as those followed for bacterially expressed GST fusion proteins above.

In vitro protein interaction tests

Affinity matrices made with GST fusion proteins immobilized on glutathione-agarose beads were incubated for 1 h at room temperature with isolated protein or bovine brain cytosol in TBST. Matrices were then washed with 10 column vol of TBST, and bound protein was analyzed by SDS-PAGE.

Antibodies

Polyclonal antibodies against μ1 and μ2 were gifts from M. Robinson (University of Cambridge, Cambridge, UK). The monoclonal antibodies, AP.6 (Chin et al., 1989), against α-adaptin, and the monoclonal antibodies, TD.1 (Nathke et al., 1992) and X22 (Brodsky, 1985), against clathrin were gifts from F. Brodsky (University of California, San Francisco, CA). The monoclonal antibody against β-adaptin, 100-1 (Ahle et al., 1988), was a gift from E. Ungewickell (Hanover Medical School, Hanover, Germany). The monoclonal (Warnock et al., 1995), against dynamin, hudy1, and the polyclonal antibody against AP2, 0927 (Hannan et al., 1998), were generated in this lab.

To generate antibodies to AAK1 (designated #5366 and #5367), the isolated COOH-terminal domain (amino acids 679–893) fused to GST within a pGEX4T-3 vector was purified from *Escherichia coli* and used as an immunogen. The immunogen was resuspended in Freund's adjuvant, injected subcutaneously into New Zealand white rabbits every 4 wk for 3 mo, and sera was collected from the central ear artery according to described protocols (Harlow and Lane, 1988).

Electrophoresis and immunoblot analysis

Cell fractions were subjected to SDS-PAGE and immunoblot analysis as described (Towbin et al., 1979). Cell fractions were pelleted, resuspended in SDS-PAGE sample buffer, and denatured for 2 min at 100°C. The proteins were resolved on an acrylamide gel and transferred to nitrocellulose for immunolabeling as described (Towbin et al., 1979) or stained with Ponceau S (Sigma-Aldrich). For immunoblots, blots were washed twice for a total of 30 min in TBST and then incubated for 30 min in TBST containing diluted antibody (1:2,000). The blots were then washed three times in TBST over 30 min and incubated in TBST with goat anti–rabbit antibodies conjugated to alkaline phosphatase (Sigma-Aldrich) diluted 1:10,000. Blots were washed in TBST three more times over 30 min. Immunolabel signals were detected by BCIP/NBT colorimetric development as described (Harlow and Lane, 1988). Controls used in this experiment included blots incubated with preimmune antisera and secondary antibody alone. Each of these immunoblots showed no signal (unpublished data).

Bovine brain fractionation

Bovine brains were harvested immediately after slaughter, frozen on dry ice, and stored at −70°C for later use. Frozen brains were defrosted rapidly using room temperature MES buffer (100 mM MES, pH 6.5, 0.5 mM MgCl$_2$, 1 mM EGTA, 1 mM PMSF, 0.8 mM DTT, 0.02% NaN$_3$). Brain tissue (700 g) was rinsed in MES buffer and homogenized with an equal volume of buffer in a Waring Blender. The homogenate (Cr) was centrifuged at 15,000 g for 30 min at 4°C. This low speed pellet (P1) and supernatant (S1) were collected, and the supernatant was then centrifuged at 100,000 g for 1 h at 4°C in a Ti45 rotor (Beckman Coulter). The crude microsomal pellet (P2) containing clathrin and APs) was resuspended in extraction buffer (0.75 M Tris, pH 7.0, 0.1 mM PMSF, 0.1 mM DTT) using a dounce homogenizer and allowed to incubate for 20 min at 4°C. The sample was then centrifuged at 100,000 g for 1 h at 4°C in a Ti45 rotor and the supernatant (ES, containing released clathrin and APs) and pellet (EP) were collected. Collected samples were then analyzed by SDS-PAGE and immunoblotting. Clathrin-coated vesicles and AP complexes were isolated as described previously (Hannan et al., 1998; Smythe et al., 1992).

Kinase assays

Kinase assays were performed essentially as described (Wilde and Brodsky, 1996) with minor modification. Briefly, isolated baculovirus expressed AAK1–GST and cell fractions were inoculated in kinase buffer (150 mM KCl, 5 mM MgCl$_2$, 100 μM [γ-^{32}P]ATP) for 15 min at room temperature. The kinase reaction was stopped by precipitating the protein with the addition of TCA to a final concentration of 10%. Precipitated proteins were pelleted by centrifugation, the supernatant containing free [γ-^{32}P]ATP was aspirated, and the pellet was resuspended in 0.1 M NaOH and SDS-PAGE protein sample buffer and boiled for 2 min at 100°C. Proteins were resolved on a 10–20% gradient polyacrylamide gel (Bio-Rad Laboratories), transferred to nitrocellulose, exposed to a phosphorimaging plate, and analyzed using the ImageQuant Software package (Molecular Dynamics). To inactivate AAK1–GST fusion proteins, isolated protein was treated with 1 mM FSBA (Sigma-Aldrich) for 30 min at room temperature. Unbound FSBA was removed from protein preparations by gel filtration using G25 minispin column (Amersham Pharmacia Biotech), and eluted protein was used in both kinase and in vitro endocytosis assays.

Immunoprecipitation of phosphorylated proteins

After the kinase assay, the phosphorylation reaction was stopped by the addition of 20% SDS in H$_2$0 to a final concentration of 1% and boiled for 2 min at 100°C. The reaction was then resuspended in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) and proteins preabsorbed with protein G beads (Amersham Pharmacia Biotech) for 1 h at room temperature. Beads were pelleted, and samples were transferred to a new Eppendorf tube containing diluted antibodies (1:1000) specific for either μ1, μ2, α-adaptin, or β2-adaptin and incubated with constant rocking for 1 h at room temperature. Protein G beads were then added to each sample and incubated for 1 h at room temperature, beads were pelleted, washed with 10 bead vol of RIPA buffer, and pelleted protein G beads were resuspended in SDS-PAGE sample buffer, boiled for 2 min at 100°C and resolved by SDS-PAGE, transferred to nitrocellulose, and placed on a phosphoimaging plate for analysis.

Immunolocalizations and rhodamine-dextran uptake

For Immunofluorescence experiments, HeLa cells were grown on coverslips and then fixed with ice cold 4% formaldehyde for 25 min. Cells were washed three times with TBST and then incubated with the designated antibodies for 1 h at room temperature. Samples were washed three times with TBST, and the appropriate secondary antibodies were added at 1:5,000 dilution and incubated for 1 h at room temperature. For rhodamine-dextran uptake experiments, rat hippocampal neurons were incubated for 10 min at 37°C in the presence of 100 μM rhodamine-dextran (Molecular Probes), cells were washed three times with TBS to remove excess rhodamine-dextran, and then fixed and processed as described above. Samples were visualized by laser scanning confocal microscopy with a Bio-Rad Laboratories 1024.

Assays for ligand sequestration and internalization

Perforated cell assays that measure biochemically distinct stage-specific endocytic events in vitro were performed essentially as described (Carter et al., 1993) except that reaction mixtures were incubated for a shorter time of 7 min at 37°C and then processed as described.

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