AP-1 binding to sorting signals and release from clathrin-coated vesicles is regulated by phosphorylation

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The adaptor protein complex-1 (AP-1) sorts and packages membrane proteins into clathrin-coated vesicles (CCVs) at the TGN and endosomes. Here we show that this process is highly regulated by phosphorylation of AP-1 subunits. Cell fractionation studies revealed that membrane-associated AP-1 differs from cytosolic AP-1 in the phosphorylation status of its β1 and μ1 subunits. AP-1 recruitment onto the membrane is associated with protein phosphatase 2A (PP2A)–mediated dephosphorylation of its β1 subunit, which enables clathrin assembly. This Golgi-associated isoform of PP2A exhibits specificity for phosphorylated β1 compared with phosphorylated μ1. Once on the membrane, the μ1 subunit undergoes phosphorylation, which results in a conformation change, as revealed by increased sensitivity to trypsin. This conformational change is associated with increased binding to sorting signals on the cytoplasmic tails of cargo molecules. Dephosphorylation of μ1 (and μ2) by another PP2A-like phosphatase reversed the effect and resulted in adaptor release from CCVs. Immunodepletion and okadaic acid inhibition studies demonstrate that PP2A is the cytosolic cofactor for Hsc-70–mediated adaptor uncoating. A model is proposed where cyclical phosphorylation/dephosphorylation of the subunits of AP-1 regulate its function from membrane recruitment until its release into cytosol.

Introduction

Adaptor protein complex-1 (AP-1)* plays a central role in the sorting and packaging of membrane proteins into clathrin-coated vesicles (CCVs) at the TGN and endosomes (Klumperman et al., 1993; Meyer et al., 2000, 2001). These vesicles transport mannose 6-phosphate receptors (MPRs) (Klumperman et al., 1993; Meyer et al., 2000; Umeda et al., 2000). The formation of CCVs is a complex process involving interactions of AP-1 with cargo molecules, clathrin, and a variety of accessory proteins. Once the CCVs bud from the donor compartment, the clathrin is disassembled and the AP-1 is subsequently released into the cytoplasm (Huang et al., 2001). This “uncoating” process is known to involve Hsc-70, cyclin G–associated kinase (GAK/auxilin 2), and an unidentified cytoplasmic factor (Hannan et al., 1998; Greener et al., 2000; Umeda et al., 2000).

AP-1 is a heterotetrameric complex composed of two ~100-kD subunits (termed γ and β1), an ~47-kD subunit (μ1), and an ~20-kD subunit (σ1). The hinge regions of the β1 and γ subunits bind clathrin via clathrin box motifs (Shih et al., 1995; Doray and Kornfeld, 2001), and the appendage of the γ subunit binds a number of accessory proteins, including γ synergin (Page et al., 1999); Rabaptin-5 (Shiba et al., 2002); Golgi-localized, γ ear–containing, adenosine 5′ diphosphate-ribosylation factor–binding proteins (GGAs) (Doray et al., 2002b); and enthoprotin/clint (Kalthoff et al., 2002; Wasiak et al., 2002). The μ1 subunit interacts with tyrosine-based sorting motifs present on the cytoplasmic tails of the MPRs and other cargo molecules (Bremnes et al., 1998; Ohno et al., 1998; Owen and Evans, 1998), and either it or the β1 subunit binds dileucine-based sorting motifs (Bremnes et al., 1998; Rapoport et al., 1998). These various protein–protein interactions must be highly coordinated if the assembly/disassembly of AP-1–containing CCVs is to proceed efficiently. Several reports have indi-
cated that this process may be regulated by phosphorylation/dephosphorylation events. To date, clathrin (Hill et al., 1988), β1 and μ1 subunits of AP-1 (Wilde and Brodsky, 1996), GGAs 1 and 3 (Doray et al., 2002a), and the cytoplasmic tails of MPRs (Meresse et al., 1990) have been shown to be phosphorylated. Further, it has been established that phosphorylation of the β1 hinge impairs clathrin binding and assembly (Wilde and Brodsky, 1996). Although the effect of μ1 phosphorylation on ligand binding has not been examined, it has been found that phosphorylation of the μ2 subunit of AP-2 greatly enhances the binding of sorting signals at the plasma membrane (Fingerhut et al., 2001; Ricotta et al., 2002). Finally, the phosphorylation of casein kinase-2 (CK2) sites in the cytoplasmic tails of the MPRs enhances binding to AP-1 (Le Borgne et al., 1993; Mauxion et al., 1996; Dittie et al., 1997).

We now report that phosphorylation of μ1 and β1 is differentially regulated and that phosphorylation of μ1 strongly enhances ligand binding, whereas dephosphorylation of this subunit by protein phosphatase 2A (PP2A) is an essential step in the CCV uncoating process. These data, together with the previous findings of others, are incorporated into a model whereby cycles of AP-1 phosphorylation/dephosphorylation regulate CCV assembly and disassembly.

**Results**

**In vivo phosphorylation of AP-1**

Wilde and Brodsky (1996) have reported that the μ1 and β1 subunits of AP-1 are phosphoproteins, and that the phosphorylated form of β1 is predominantly cytosolic. The subcellular distribution of phosphorylated μ1 was not examined. To pursue this point, membrane and cytosolic fractions were prepared from mouse L cells (LSS) that had been incubated with 32Porthophosphate for 4 h. Then the AP-1 in each fraction was immunoprecipitated and analyzed by SDS-PAGE. As shown in Fig. 1 A, β1 was the only subunit labeled in the cytosolic AP-1, whereas μ1 was selectively labeled in the membrane-associated AP-1. Western blotting of the fractions showed an equal distribution of AP-1 between the membranes and the cytosol. The addition of 10 nM okadaic acid to the labeling media resulted in phosphorylation of the β1 subunit isolated from both locations as well as a shift of AP-1 distribution toward the membranes. Because this concentration of okadaic acid has been reported to selectively inhibit PP2A (Mumbay and Walter, 1993; Bertrand et al., 1997), this result suggests that the β1 subunit is dephosphorylated by PP2A upon recruitment of AP-1 onto the membrane, whereas μ1 becomes phosphorylated.

**Phosphorylation of μ1 enhances ligand binding**

AP-2 with a phosphorylated μ2 subunit exhibits a 15–30-fold enhanced avidity for tyrosine- and dileucine-based sorting motifs as compared with the nonphosphorylated species in binding studies (Fingerhut et al., 2001; Ricotta et al., 2002). It has also been established that the phosphorylation of the μ2 subunit is essential for endocytosis (Olusanya et al., 2001; Conner and Schmid, 2002). To determine whether phosphorylation of μ1 also enhances ligand bind-

![Figure 1. In vivo phosphorylation of AP-1 subunits.](image-url)
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One possible explanation for the enhanced binding to the CK2 acidic sorting motifs is that phosphorylation of μ1 induces a conformational change that exposes basic surfaces that are buried in the nonphosphorylated state. It is also apparent that μ1 phosphorylation is not the only factor that regulates the interaction of AP-1 with the MPR cytoplasmic tails, as phosphorylation of the CK2 sites enhances binding as well.

We next tested whether dephosphorylation of CCV-derived AP-1 impaired ligand binding. In a preliminary experiment, we showed that in vitro–phosphorylated μ1 (and μ2) was dephosphorylated by low nanogram amounts of PP2A, and this dephosphorylation was completely prevented by okadaic acid (Fig. 3 A). As seen in Fig. 3 B, the PP2A treatment decreased the ability of the CCV-derived AP-1 to bind to the CD-MPR tail. Because μ1 is the only subunit of the CCV-derived AP-1 that is phosphorylated, this finding confirms that μ1 phosphorylation enhances ligand binding.

One mechanism whereby the phosphorylation of μ1 could affect ligand binding is by inducing a conformation...
change in this subunit that favors interaction with the ligand. As a way to explore this, we looked for an alteration in trypsin sensitivity of phosphorylated and nonphosphorylated AP-1. Fig. 4 A shows that the AP-1 subunit of cytosol-derived AP-1 is completely resistant to concentrations of trypsin that totally cleave the AP-1 subunit. In contrast, the AP-1 subunit of CCV-derived AP-1 is readily cleaved by low concentrations of trypsin (Fig. 4 B). This finding suggests that upon phosphorylation, the AP-1 subunit undergoes a conformational change that exposes a trypsin cleavage site, as observed in the case of AP-2 (Matsui and Kirchhausen, 1990). This change in conformation could expose regions of AP-1 that are involved in ligand binding, perhaps similar to the “jack knife” conformational change proposed by Collins et al. (2002) for AP-2. It should also be noted that the AP-1 subunit of AP-1 derived from the two sources showed a modest difference in susceptibility to trypsin. This suggests that phosphorylation of this subunit also results in a conformational change or that phosphorylation of AP-1 induces a change in AP-1 conformation.

**PP2A is the cytosolic cofactor for adaptor release from CCVs**

Hannan et al. (1998) have reported that AP-1 and AP-2 release from CCVs requires ATP, Hsc-70, and a cytosolic factor with an apparent size of ~100 kD on gel filtration. Because PP2A can exist as a heterotrimer (or dimer) of ~100–125 kD, we hypothesized that it could be the cytosolic factor of Hannan et al. (1998) and function via its ability to dephosphorylate AP-1. This would be predicted to impair AP-1 binding to its membrane ligands and thereby facilitate its release into the cytosol. To test this hypothesis, CCVs were treated with cytosol, or with Hsc-70 and PP2A, either alone or together, in the presence of an ATP-regenerating system, and the release of AP-1 was determined. As shown in Fig. 5 A, cytosol plus ATP induced AP-1 release as expected. Importantly, the combina-

![Figure 4. Phosphorylated AP-1 α1 has increased sensitivity to trypsin. 2.5 μg of immunopurified AP-1 from bovine liver cytosol (A) or CCVs (B) in 100 μl buffer E was incubated with varying amounts of trypsin as indicated. The reaction was performed at 37°C for 8 min and immediately terminated by the addition of an excess of soybean trypsin inhibitor at 4°C. 25% of the reaction was subjected to SDS-PAGE and Western blotting for the γ, β1, and α1 subunits of AP-1 with their respective antibodies, as mentioned under the Materials and methods. The bands were quantitated using a densitometer. The amount of uncleaved (trypsin resistant) subunits expressed as a percentage of the starting amount was plotted against trypsin concentration using KaleidaGraph. The inset shows the blots of the three subunits, γ, β1, and α1.](http://jcb.rupress.org/content/160/5/702/F4.large.jpg)

![Figure 5. PP2A mediates adaptor release in the presence of Hsc-70 and an ATP-regenerating system. (A and B) 5 μg of purified bovine liver CCVs was incubated with varying combinations of bovine brain cytosol, Hsc-70, PP2A, and ATP-regenerating system at 25°C for 3 min (A and B) or 5 min (A) in a final volume of 50 μl. At the indicated time, the reaction was centrifuged at 100,000 × g in a Beckman Coulter ultracentrifuge. 40 μl of the supernatant was collected and subjected to SDS-PAGE, followed by immunoblotting for released AP-1 (A) and AP-2 (B). (C) 5 μg of CCVs and 0.5 μg Hsc-70 were incubated with varying concentrations of PP2A in the presence of the ATP-regenerating system for 5 min and worked up as above. The bands corresponding to the released AP-1 were quantitated using a densitometer and expressed as a percentage of the input. The figures represent 1 of 5–10 independent experiments.](http://jcb.rupress.org/content/160/5/702/F5.large.jpg)
tion of recombinant Hsc-70 and purified PP2A in the same conditions was even more effective in stimulating AP-1 release. No enhancement of AP-1 release was noted when these proteins were tested alone. The combination of Hsc-70 and PP2A also induced AP-2 release from the CCVs (Fig. 5 B). Half-maximal release of AP-1 occurred at a PP2A concentration of 2.2 ng/reaction (~44 ng/ml) (Fig. 5 C).

Two additional experiments were performed to confirm that PP2A was indeed the cytosolic factor involved in AP-1 uncoating from CCVs. First, we established that okadaic acid inhibited the cytosol-mediated AP-1 release from CCVs as well as release catalyzed by the Hsc-70–PP2A combination (Fig. 6 A). In a second experiment, it was shown that immunodepletion of PP2A from cytosol (Fig. 6 B) resulted in the loss of AP-1–releasing activity, and the addition of PP2A restored this function (Fig. 6 C). These experiments indicate that PP2A is the cytosolic factor that acts in conjunction with Hsc-70 to catalyze adaptor release from CCVs. The observation that okadaic acid causes increased, nonspecific membrane localization of AP-2 (Lauritsen et al., 2000) supports the notion that μ2 dephosphorylation is also essential for release from membranes. In addition, overexpression of GAK, the kinase implicated in μ1/2 phosphorylation and clathrin uncoating, resulted in the sustained association of AP-1 with TGN budding profiles and of AP-2 with plasma membrane pits independent of clathrin (Umeda et al., 2000; Zhao et al., 2001).

Hsc-70 is known to be recruited onto CCVs via binding to the DnaJ homologues auxilin 2 (GAK) and auxilin 1, which are present on the vesicles (Jiang et al., 1997; Ungewickell et al., 1997). Because phosphatases have been reported to associate with heat shock proteins (Gear et al., 1997; Polanowska-Grabowska et al., 1997), we tested whether PP2A interacts with Hsc-70. A GST fusion protein containing the common A subunit of the PP2A family was immobilized on glutathione-Sepharose beads and incubated with bovine liver cytosol. 20% of the pellet was subjected to SDS-PAGE and subsequent immunoblotting with anti–Hsc-70 mAB. The “smile” in the bound Hsc-70 is caused by the GST–PP2A-A fusion protein, which runs at a similar molecular weight as Hsc-70, i.e., ~70 kD. (B) Aliquots (2.5 μg) of recombinant bovine Hsc-70 were incubated at 25°C for 15 min in the presence or absence of 800 μM ATP and 5 mM magnesium acetate in 25 μl of buffer A. The reactions were then used for binding to 100 μg of GST or GST–PP2A-A fusion proteins prebound to glutathione-Sepharose beads at 4°C for 4 h. 20% of the pellet was subjected to SDS-PAGE and subsequent immunoblotting with anti–Hsc-70 mAB. (C) Bovine liver cytosol (2.5 mg) was subjected to immunoprecipitation with either rabbit preimmune sera or rabbit polyclonal antibody toward PP2A-Aα/β. The immune complexes were captured onto BSA-blocked protein A–agarose beads (Sigma-Aldrich). After four washes with PBS, the bound proteins were eluted by boiling in SDS sample buffer and subjected to SDS-PAGE. Immunoblotting was performed using mAb against Hsc-70.
phosphoprotein phosphatases (Mivechi et al., 1993), possibly providing another means for cooperative interactions in the uncoating process.

**AP-1 released from CCVs by PP2A and Hsc-70 displays low-avidity ligand binding**

If PP2A dephosphorylates the \( \mu_1 \) subunit of AP-1 during the uncoating process, then the released AP-1 should exhibit lower-avidity binding to sorting signals relative to AP-1 with a phosphorylated \( \mu_1 \) subunit. This was tested by comparing ligand binding of AP-1 released from CCVs by Tris treatment versus PP2A–Hsc-70 (Fig. 8). Whereas AP-1 recovered by both procedures bound GST–CI-MPR full-length cytoplasmic tail approximately equally, the AP-1 released by PP2A–Hsc-70 bound much less well to the CD-MPR cytoplasmic tail. This result provides further evidence that PP2A-catalyzed release of AP-1 from CCVs is associated with dephosphorylation of \( \mu_1 \).

**Golgi-associated phosphatase has a preference for phosphorylated \( \beta_1 \) versus phosphorylated \( \mu_1 \)**

The in vivo \(^{32}\)P labeling experiment showed that the \( \beta_1 \) subunit of AP-1 is phosphorylated in the cytosol but not on the membrane, whereas the reverse was true for \( \mu_1 \) (Fig. 1). This indicated that the Golgi apparatus contains a phosphatase that acts efficiently on \( \beta_1 \) and less well on \( \mu_1 \). On the other hand, the PP2A recruited onto CCVs would be predicted to act efficiently on \( \mu_1 \) while sparing \( \beta_1 \) from dephosphorylation. To test this directly, we compared dephosphorylation of \( \beta_1 \) and \( \mu_1 \) mediated by Golgi membranes versus purified PP2A from bovine kidney (Fig. 9). The kidney PP2A dephosphorylated \( \mu_1 \) much more rapidly than \( \beta_1 \), whereas the Golgi membrane extract acted on \( \beta_1 \) more readily than \( \mu_1 \). Dephosphorylation catalyzed by Golgi membranes was inhibited by 50 nM okadaic acid, indicating that it is mediated by a member of the PP2A family (unpublished data). Thus, we conclude that different isoforms of PP2A residing in specific subcellular compartments display differential substrate specificity toward \( \beta_1 \) and \( \mu_1 \).

**Discussion**

These results, combined with previous findings of others, lead us to propose a model whereby cycles of phosphorylation and dephosphorylation regulate AP-1 function (Fig. 10). AP-1 in the cytosol is selectively phosphorylated on the hinge of its \( \beta_1 \) subunit, which impairs its binding to clathrin (Wilde and Brodsky, 1996). Upon recruitment of AP-1 onto Golgi membranes, the \( \beta_1 \) subunit becomes dephosphorylated, most likely by a PP2A family member. The \( \mu_1 \) subunit, in contrast, becomes phosphorylated at this time. This phosphorylation is likely to be catalyzed by GAK (auxilin-2) (Kanaoka et al., 1997; Kimura et al., 1997), which binds to the \( \gamma \)-appendage of AP-1 (Umeda et al., 2000) and copurifies with AP-1 on a hydroxyapatite column (Ricotta et al., 2002). Although adaptor-associated kinase-1 (AAK-1) has also been shown to phosphorylate \( \mu_1 \) in vitro, it is primarily associated with AP-2 at the plasma membrane (Connor and Schmid, 2002) and copurifies with AP-2 on a hydroxyapatite column (Ricotta et al., 2002). Clearly, the rate of \( \mu_1 \) phosphorylation must exceed the rate of dephosphorylation, because at steady-state, the \( \mu_1 \) subunit of membrane-associated AP-1 is phosphorylated (Fig. 1). In this regard, it is relevant that the Golgi-associated PP2A has a strong preference for the \( \beta_1 \) subunit over the \( \mu_1 \) subunit (Fig. 9). Phosphorylation of the \( \mu_1 \) subunit enhances binding to sorting signals on the cytoplasmic tails of the MPR cargo molecules, which should facilitate their capture and retention in the forming CCVs. This was most evident with the CD-MPR, but was also observed with the COOH-terminal CK2 site on the CI-MPR. This is similar to the effect of \( \mu_2 \) phosphorylation on binding to sorting signals on cargo proteins at the plasma membrane (Fingerhut et al., 2001; Olusanya et al., 2001; Ricotta et al., 2002).
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Recently, Collins et al. (2002) have determined the structure of the AP-2 “core” complex and find that the binding site for the YXX∅ (where ∅ is a bulky hydrophobic residue) endocytic motif in μ2 is blocked by portions of the β2 subunit. These authors propose that a conformational change must occur to allow binding to this sorting signal, and they postulate that this is triggered by phosphorylation of μ2. Phosphorylation of μ1 at the Golgi membrane may also induce a conformational change that exposes the binding site for phosphorylated CK2 sites and possibly other binding motifs present in the MPR cytoplasmic tails. Our finding that μ1 phosphorylation increases sensitivity to trypsin digestion is consistent with this hypothesis. The likely source of the kinase that phosphorylates the MPRs is the CK2 associated with AP-1 (Meresse et al., 1990, Doray et al., 2002b).

It has recently been demonstrated that the MPRs also bind to the GGA family of proteins (Puertollano et al., 2001; Takatsu et al., 2001; Zhu et al., 2001) and that this interaction is required for packaging of the MPRs into AP-1 CCVs (Doray et al., 2002b). We found that the GGAs with their bound MPRs interact with AP-1 and that the CK2 associated with AP-1 phosphorylates GGAs 1 and 3 (Doray et al., 2002b). This results in autoinhibition of cargo binding, providing a mechanism for the transfer of the MPRs from these GGAs to AP-1. The CK2 would also be expected to phosphorylate the CK2 sites in the cytoplasmic tails of the MPRs, thereby enhancing their binding to AP-1.

The membrane-associated AP-1 also binds clathrin and facilitates clathrin assembly, which proceeds to the formation of a CCV that eventually buds off from the membrane. The vesicle carries within itself a dormant CK2 (associated with AP-1) and an active GAK (Korolchuk and Banting, 2002). After budding, clathrin disassembly occurs, most likely triggered by GAK and Hsc-70 (Braell et al., 1984; Schlossman et al., 1984; Prasad et al., 1993; Barouch et al., 1994; Ungewickell et al., 1995; Umeda et al., 2000; Morgan et al., 2001). During this process, the β1 subunit of AP-1 becomes phosphorylated, but the kinase that mediates this event is currently unknown. However, it is tempting to speculate that the phosphorylation of the β1 hinge could additionally contribute to clathrin dissociation.

After clathrin release, the adaptors remain on the membrane, possibly to mediate “postbudding” roles (Huang et al., 2001), such as the interaction with motor kinesins like KIF13A (Nakagawa et al., 2000) or with Rabaptin-5 (Shiba et al., 2002). At some point, presumably before vesicle fusion with the acceptor compartment, the adaptors are released back to the cytosol. This event, in our model, is mediated by Hsc-70 in concert with PP2A, which is recruited via its interaction with Hsc-70 (Figs. 5–7). PP2A-mediated dephosphorylation would reverse the conformation change in the phosphorylated μ1 subunit that was induced by its phosphorylation. This would be expected to decrease the avidity for cargo sorting signals, thereby facilitating dissociation from the membrane. AP-1 dissociation may be further stimulated by PP2A-mediated dephosphorylation of the CK2 sites in the cytoplasmic tails of the cargo molecules (Mollov et al., 1998). In addition, dephosphorylation of μ1 could decrease surface charge interactions between the μ1 subunit and the lipid bilayer of the membrane, as speculated for μ2 (Collins et al., 2002). The released AP-1 will have a phosphorylated β1 subunit and a dephosphorylated μ1 subunit, as found in the cytosol of mouse L cells (Fig. 1). A similar mechanism appears to account for AP-2 release from CCVs (Fig. 5 B). This requirement for μ2 dephosphorylation in CCV recycling could explain why maintaining μ2 in a phosphorylated state eventually results in inhibition of CCV-mediated transferrin uptake (Conner and Schmid, 2002).

This model explains the critical role of cycles of phosphorylation and dephosphorylation in regulating clathrin-mediated cargo transport at the TGN. In future studies, it will be necessary to identify the PP2A family member associated

Figure 10. Working model for phosphoregulation of AP-1. (1) Cytosolic AP-1 has a phosphorylated β1 hinge that prevents its association with clathrin and a dephosphorylated μ1 subunit, which presumably is in its inactive “closed” conformation. AP-1 is recruited onto the Golgi membranes via ARF-GTP (gray circle) where the β1 hinge gets dephosphorylated by a Golgi-associated isofrom of PP2A. (2) Dephosphorylation of the β1 hinge allows AP-1 to initiate clathrin assembly. Concurrently, μ1 is phosphorylated, most likely by GAK (auxilin 2) (Umeda et al., 2000). This results in a conformational change that exposes ligand binding sites on μ1, thereby enhancing interactions with cargo sorting signals at the TGN. (3) During the maturation of the clathrin-coated bud, the β1 is maintained in a dephosphorylated state and μ1 in a phosphorylated state, presumably by GAK, which is incorporated into the forming CCV as an active enzyme (Korolchuk and Banting, 2002). After budding, clathrin disassembly occurs, most likely triggered by GAK and Hsc-70 (Braell et al., 1984; Schlossman et al., 1984; Prasad et al., 1993; Barouch et al., 1994; Ungewickell et al., 1995; Umeda et al., 2000; Morgan et al., 2001). During this process, the β1 subunit of AP-1 becomes phosphorylated, but the kinase that mediates this event is currently unknown. However, it is tempting to speculate that the phosphorylation of the β1 hinge could additionally contribute to clathrin dissociation.

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This model explains the critical role of cycles of phosphorylation and dephosphorylation in regulating clathrin-mediated cargo transport at the TGN. In future studies, it will be necessary to identify the PP2A family member associated
with the TGN and the kinase that phosphorylates the β1 subunit. In addition, it will be important to gain an understanding of how the kinases and phosphatases are themselves regulated so that they act at the correct time to coordinate this complex series of events.

Materials and methods

Materials

Rabbit skeletal muscle creatine phosphokinase, recombinant bovine Hsc70 expressed in Escherichia coli, and PPAZ from bovine kidney were obtained from Calbiochem. Phosphocreatine, okadaic acid, protein G-agarose beads, and adenosine 5’ triphosphate were from Sigma-Aldrich, which also supplied the AP-1 monoclonal antibody TD.1 was from BAbCo. Anti–AP-1 antibodies were prepared as described earlier (Traub et al., 1995). Anti-clathrin monoclonal antibody 100/2 was obtained from Sigma-Aldrich. Rabbit anti–Hsc-70 monoclonal antibody was obtained from Santa Cruz Biotechnology, Inc. AP-1 was probed with anti–AP-1 γ-hinge monoclonal 100/3 antibody and anti–β1/2 monoclonal 100/1 antibody; AP-2 was probed with anti–AP-2α monoclonal 100/2 antibody from Sigma-Aldrich. Rabbit anti-μ (RyV)1 was prepared as described earlier (Traub et al., 1995). Anti-clathrin monoclonal antibody TD.1 was from BabCo. Anti–AP-1 γ monoclonal antibody from Transduction Laboratories was used for immunoprecipitation of AP-1. Species-specific HRP-conjugated secondary antibodies used for Western blotting were from Amersham Biosciences.

Methods

Bovine liver was harvested immediately after slaughter and was used for CCV preparation as previously described (Meresse et al., 1990). Sucrose gradient–purified CCVs were stored at 4°C for 4 h at 30°C in 20 mM Hepes, pH 7.0, 2 mM magnesium acetate, 125 mM potassium acetate, 0.1% Triton X-100, 1 mM DTT; buffer C (1 M Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM PMSE); buffer D (0.1 M Tris, pH 8.0, 0.1 M NaCl, 0.5% sodium deoxycholate, 0.2% SDS, 1% Triton X-100, 10 mM β-mercaptoethanol), and buffer E (25 mM Tris-Cl, pH 7.5, 125 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT). All chemicals were reagent grade and obtained from Sigma-Aldrich.

Buffers.

The following buffers were used: buffer A (10 mM [NH4]2SO4, 20 mM Hepes, pH 7.0, 2 mM magnesium acetate, 25 mM KCl, 1 mM PMSE); buffer B (20 mM Hepes, pH 7.2, 5 mM magnesium acetate, 125 mM potassium acetate, 0.1% Triton X-100, 1 mM DTT); buffer C (1 M Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM PMSE); buffer D (0.1 M Tris, pH 8.0, 0.1 M NaCl, 0.5% sodium deoxycholate, 0.2% SDS, 1% Triton X-100, 10 mM β-mercaptoethanol), and buffer E (25 mM Tris-Cl, pH 7.5, 125 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT). All chemicals were reagent grade and obtained from Sigma-Aldrich.

Antibodies.

Rat anti–Hsc-70 monoclonal antibody was obtained from Calbiochem. Rabbit anti–PPA-2α/β was from Santa Cruz Biotechnology, Inc. AP-1 was probed with anti–AP-1 γ-hinge monoclonal 100/3 antibody and anti–β1/2 monoclonal 100/1 antibody; AP-2 was probed with anti–AP-2α monoclonal 100/2 antibody from Sigma-Aldrich. Rabbit anti-μ (RyV)1 was prepared as described earlier (Traub et al., 1995). Anti-clathrin monoclonal antibody TD.1 was from BabCo. Anti–AP-1 γ monoclonal antibody from Transduction Laboratories was used for immunoprecipitation of AP-1. Species-specific HRP-conjugated secondary antibodies used for Western blotting were from Amersham Biosciences.

Dephosphorylation assays.

Dephosphorylation assays were performed on phosphorylated AP-1 prepared both in vivo and in vitro. AP-1 was phosphorylated in vivo in the presence of okadaic acid and immunoprecipitated from whole cell extracts using whole cell extracts prepared from NIH/3T3 cells as lysates as above. The washed beads were then subjected to dephosphorylation in buffer A with either commercially obtained bovine kidney PPA2 or Golgi-enriched membranes. The duration of incubations and the amount of PPA2 and Golgi membrane used are indicated in the figure legends. CCVs were used to phosphorylate the μ1 subunit of AP-1 in vitro because they contain AP-1 as well as GAK kinase, which is known to act on μ1 (Umeda et al., 2000). 25 μg of CCVs was incubated for 4 h at 30°C in buffer A supplemented with 1.5 mM ATP γ32P (ICN Biomedicals; specific activity 12.5 μCi/mmol) ATP in a final volume of 40 μl. After incubation, PPA2 was added, as indicated, for an additional 15 min, and the entire mixture was subjected to SDS-PAGE using a 12% gel and autoradiography after drying. The phosphorylated 50-kD and 47-kD bands were the only detectable products of the reaction. The use of a 12% gel was required to resolve the μ1 and μ2 subunits (Loeb et al., 1989), which otherwise run together (Korolchuk and Banting, 2002).

Limited proteolysis of purified AP-1.

Aliquots (2.5 μg) of purified AP-1, of either cytoplasmic or CCV source, were incubated with various amounts of bovine pancreatic trypsin (Sigma-Aldrich), as indicated in the figure legend for Fig. 4, in a final volume of 100 μl of buffer E at 37°C for 8 min. The tubes were then placed on ice, and the reactions were arrested by the addition of an excess of soybean trypsin inhibitor (Sigma-Aldrich). Aliquots of the reactions were subjected to SDS-PAGE and immunoblotting. The bands were quantitated using an Eastman Kodak Co. densitometer and plotted using KaleidaGraph.

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