

Is an ATPase involved in uncoating of plasma membrane adaptor complex AP-2?

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Abstract The coat of clathrin-coated vesicles mostly consists of clathrin and adaptor complexes AP-1 or AP-2. Clathrin is released from the vesicles in an ATP-dependent fashion prior to their fusion with endosomes. In the present study we found that ATP strongly inhibits *in vitro* binding of cytosolic AP-2 to membranes of stripped vesicles, and promotes the release of endogenous AP-2 from clathrin-deprived coated vesicles. Both effects required hydrolysis of ATP. In contrast, binding of AP-1 to stripped vesicles was not affected by ATP, but was enhanced by GTP- γ -S. These results point to an ATPase that promotes the release of AP-2 from clathrin-coated vesicles.

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Key words: ATPase; Plasma membrane adaptor complex; Adaptin-2

1. Introduction

Clathrin-coated vesicles mediate selective transport from the *trans* Golgi network (TGN) and from the plasma membrane to endosomes [1–4]. The coat of these vesicles consists mostly of clathrin and, in addition, of two topologically distinct adaptor complexes, AP-1 and AP-2. AP-1 complex is a constituent of clathrin-coated vesicles forming at the TGN, whereas AP-2 complex is associated with the coated vesicles generated at the plasma membrane for receptor-mediated endocytosis. Coated pit formation involves binding of adaptor complexes to the cytoplasmic domain of certain transmembrane proteins (receptors) to build an inner shell of the coat [5,6]. Clathrin triskelia then bind to the adaptor complexes, and form a polygonal lattice which promotes invagination and pinching off the donor membrane. Prior to fusion with the target membrane, clathrin is released from the vesicles in an ATP-dependent reaction which is catalyzed by the cytosolic heat shock cognate protein Hsc70 together with a 100 kDa cofactor, auxilin [7–11]. Hsc70 acts directly on clathrin triskelia, but not on adaptor complexes. It is still unclear whether adaptors are (partially or totally) removed from the membranes prior to vesicle fusion.

Here we investigated the interaction of adaptor complexes with vesicles using an *in vitro* assay similar to that described by Traub et al. [12]. Our results point to an ATPase which promotes dissociation of the plasma membrane adaptors (AP-2).

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Abbreviations: AP, adaptin; ATP- γ -S, adenosine 5'-O-(3-thiotriphosphate); GTP- γ -S, guanosine 5'-O-(3-thiotriphosphate); Hsc70, 70 kDa heat shock cognate protein; TGN, *trans* Golgi network

2. Materials and methods

2.1. Reagents

Enhanced chemiluminescence reagent (ECL) was from Amersham. Drugs for polyacrylamide electrophoresis were from BioRad, and all other reagents, including nucleotides, were from Sigma Chemicals (St. Louis, MO). The monoclonal anti- α - or anti- γ -adaptin antibodies (mAB 100/2 or mAB 100/1, respectively), biotinylated anti-mouse Ig and peroxidase-conjugated extravidin were from Sigma Chemicals. Brains from freshly slaughtered bovines were obtained from the slaughterhouse La Lagunita (Mendoza, Argentina).

2.2. Preparation of coated vesicles and cytosol

Clathrin-coated vesicles were purified from bovine brain according to Campbell et al. [13] and stored in buffer A (100 mM 2-(*N*-morpholino)ethanesulfonic acid/NaOH pH 6.5, 1 mM EGTA, 0.5 mM MgCl₂, 0.2 mM dithiothreitol, 0.005% PMSF and 0.05% sodium azide) at -70°C for up to 3 months. To prepare cytosol, a post-nuclear fraction obtained from bovine brain as above [13] was subjected to centrifugation at $100\,000\times g$ for 60 min, supplemented with aprotinin (0.1 TIU/ml), leupeptin (5 $\mu\text{g/ml}$) and PMSF (1 mM), and stored at -70°C for up to 2 weeks.

2.3. Stripping of vesicles

Clathrin-coated vesicles were diluted to 1.5 mg/ml of protein with 10 mM Tris-HCl pH 8.5 and incubated overnight at 4°C . After centrifugation at $50\,000\times g$ for 30 min, the supernatant mainly consisted of clathrin lattices [14]. The pellet, referred to as clathrin-deprived vesicles, was resuspended in 1 M Tris-HCl pH 7.0, incubated for 20 min at room temperature and centrifuged at $20\,000\times g$ for 15 min. The supernatant, which is referred to as coated vesicle extract as a source of adaptors, was dialyzed against buffer B and concentrated to 2 mg/ml of protein by ultrafiltration using an Amicon PM10 membrane. The vesicle pellet was washed once with 1 M Tris-HCl pH 7.0 and once with buffer B (25 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid/KOH pH 7.0, 125 mM potassium acetate, 5 mM magnesium acetate, 1 mM dithiothreitol). The final pellet, referred to as stripped vesicles, was resuspended to 5 mg/ml of protein in buffer B, and stored in small aliquots at -70°C as a source of membranes for *in vitro* binding.

2.4. *In vitro* assay for adaptor binding

A typical binding assay was performed with 200 $\mu\text{g/ml}$ of stripped vesicles and 200 $\mu\text{g/ml}$ of cytosol in a final volume of 250 μl of buffer B. Cytosol was cleared of aggregates by centrifugation at $100\,000\times g$ for 15 min immediately before use. Coated vesicle extract was used as an alternative source of adaptors at 100 $\mu\text{g/ml}$. Nucleotides or analogues were added as indicated in the figure legends. The ATP-regenerating system consisted of 1 mM ATP, 5 mM creatine phosphate and 10 U/ml creatine kinase [12]. Binding was performed at 37°C and stopped by adding three volumes of ice-cold buffer B. Vesicles were sedimented at $10\,000\times g$ for 15 min at 4°C , and washed once with 1 ml of buffer B. (The vesicles were easily sedimentable at $10\,000\times g$, which we considered optimal, as at higher velocity the background caused by unspecifically aggregated adaptors increased.) The final pellet was dissolved by boiling in 20 μl of SDS sample buffer for subsequent SDS-PAGE analysis [15].

2.5. *In vitro* assay for release of adaptors

150 μg of clathrin-deprived coated vesicles were diluted into 750 μl

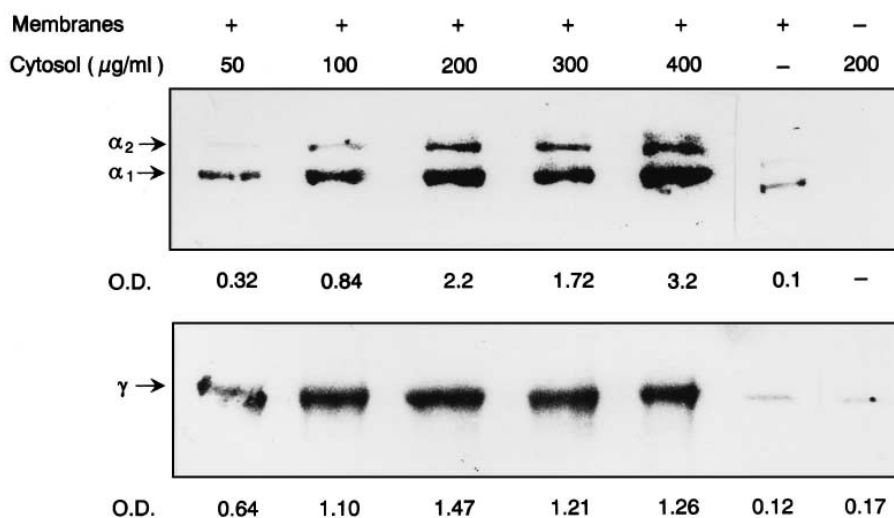


Fig. 1. Binding of cytosolic adaptors to stripped vesicles. Stripped vesicles (200 μg/ml) and the indicated concentrations of cleared cytosol were incubated at 37°C for 15 min. Reactions were stopped on ice, and the vesicles collected by centrifugation. The pellets were resolved on 8% gels and transferred onto nitrocellulose. The blots were processed for immunodetection of the α-subunit of AP-2 (top panel) and the γ-subunit of AP-1 (bottom panel). Bands were quantified by densitometry of the films. O.D.: optical density expressed as relative units.

of buffer B and incubated for 20 min at 37°C in the presence or absence of nucleotides as indicated in the figure legends. After centrifugation at 10000×*g* for 10 min the pellets were solubilized by boiling in SDS sample buffer for SDS-PAGE [15].

2.6. Analytical procedures

SDS-PAGE was carried out according to Laemmli [15] on minigels with 8% acrylamide. After electrophoresis the proteins were electrotransferred onto 0.2 μm pore diameter nitrocellulose membrane (Sartorius) [12], and processed for immunodetection with anti-adaptin Ig followed by biotin-conjugated anti-mouse Ig and peroxidase-conjugated extravidin. Chemiluminescence (ECL, Amersham) was detected using Rx-Kodakfilm and quantified by densitometry with NIH soft program 3.1. High contrast prints of the films are shown.

Protein content was measured according to Lowry [16].

3. Results

We developed an *in vitro* system for binding of adaptor proteins to membranes from purified clathrin-coated vesicles, which had been stripped to remove endogenous coat proteins. When cytosol was incubated with the stripped vesicles, cytosolic AP-1 and AP-2 were bound and sedimented together with the membranes (Fig. 1). The binding was fast, reaching within 10 min the maximum for both adaptors, and temperature dependent with an optimum at 37°C (data not shown).

Binding of cytosolic AP-2 to stripped vesicles was inhibited by ATP (Fig. 2, top panel). In the presence of an ATP-regenerating system the binding was reduced to 5% of the control.

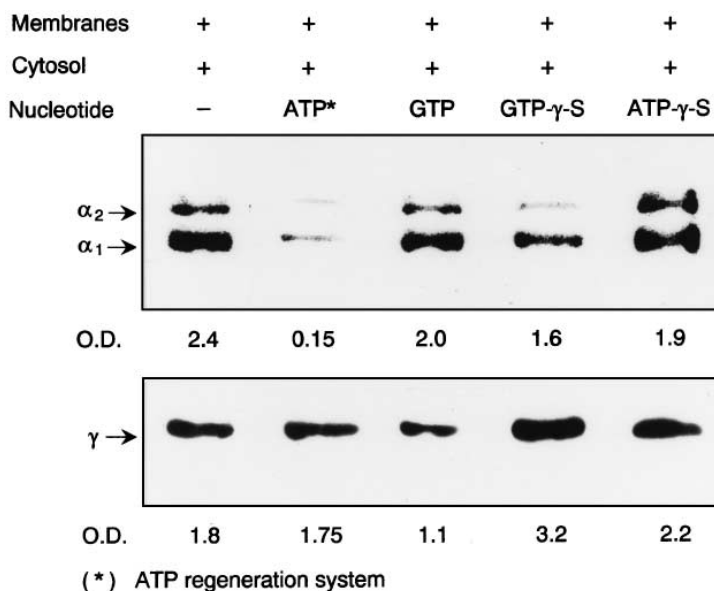


Fig. 2. ATP reduces binding of cytosolic AP-2 to stripped vesicles. Stripped vesicles (200 μg/ml) and cleared cytosol (200 μg/ml) were incubated at 37°C for 20 min in the presence of an ATP-regenerating system, 1 mM GTP, 100 μM GTPγS or 1 mM ATPγS. The reactions were stopped on ice, vesicles were collected by centrifugation and analysed for the α-subunit of AP-2 (top panel) and the γ-subunit of AP-1 (bottom panel). Bands were quantified by densitometry of the films. O.D.: optical density expressed as relative units.

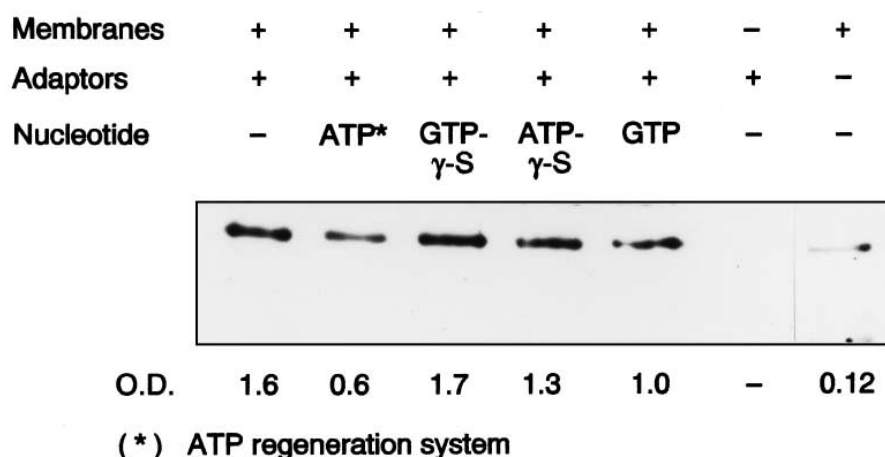


Fig. 3. Binding of AP-2 from a coated vesicle extract to stripped vesicles. Stripped vesicles (200 μ g/ml), adaptors from a coated vesicle extract (100 μ g/ml) and an ATP-regenerating system, 100 μ M GTP γ S, 1 mM ATP γ S or 1 mM GTP were incubated at 37°C for 20 min. Reactions were stopped on ice, vesicles were collected by centrifugation and analysed for the α -subunit of AP-2. Bands were quantified by densitometry of the films. O.D.: optical density expressed as relative units.

The non-hydrolyzable analogue ATP γ S did not affect the binding of AP-2 to stripped vesicles, suggesting that hydrolysis of ATP was required for inhibition. Neither GTP nor GTP γ S exerted a significant effect on the binding of AP-2.

In contrast, binding of cytosolic AP-1 to stripped vesicles was not affected by ATP, but was improved in the presence of GTP γ S (Fig. 2, bottom panel) as previously demonstrated by others [12,17].

Binding of AP-2 from an extract of clathrin-deprived coated vesicles was also inhibited by ATP (Fig. 3), although this effect was less pronounced than that observed for AP-2 from cytosol. Only the α 1-subunit of AP-2 was detectable in the bound fraction from the coated vesicle extract. As the α 2-subunit was at the limit of detection in the extract of coated vesicles, it is not possible to decide whether the lack of the α 2-subunit in the bound fraction reflects lack of binding or lim-

ited detection. When nucleotides were added to vesicles from which clathrin had been selectively stripped, ATP promoted the release of endogenous AP-2 (Fig. 4).

4. Discussion

Disassembly of clathrin from clathrin-coated vesicles in vitro is regulated by the cytosolic heat shock cognate protein Hsc70, which acts as a clathrin-dependent ATPase [7–11,18]. During this Hsc70-dependent uncoating of clathrin, the adaptor complexes remain bound to the membranes [10].

Here we show that the interaction of plasma membrane adaptor complex AP-2 with membranes can be regulated by an ATP-dependent system. Our results show that the association of cytosolic AP-2 with stripped vesicles can be inhibited by addition of an ATP-regenerating system. ATP may exert

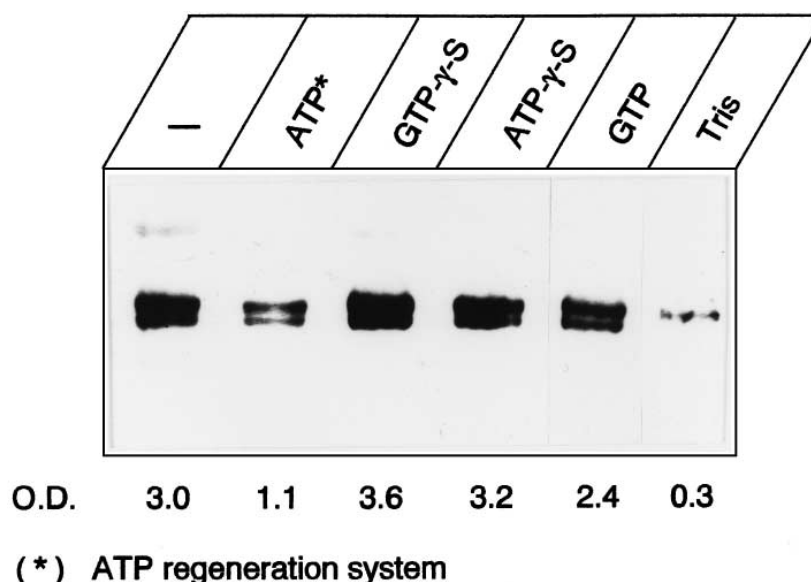


Fig. 4. Release of membrane-bound AP-2 is favoured by ATP. Clathrin-deprived coated vesicles (200 μ g/ml) were incubated for 20 min at 37°C in the presence of an ATP-regenerating system, 100 μ M GTP γ S, 1 mM ATP γ S or 1 mM GTP. A tube containing vesicles in 1 M Tris pH 7.0 was included as a control. Vesicles were collected by centrifugation and analysed for the α -subunit of AP-2. Bands were quantified by densitometry of the films. O.D.: optical density expressed as relative units.

its effect on the binding of AP-2, the dissociation of the bound AP-2, or a combination of both. As ATP promoted rapid dissociation of AP-2 from clathrin-deprived coated vesicles, we assume that the major effect of the nucleotide applies to the release of AP-2. The latter proceeds very slowly in the absence of ATP. However, we cannot exclude an additional effect of ATP on the binding, as AP-2 appeared to be associated to a different extent with the vesicles after binding (Fig. 2) or release (Fig. 4) in the presence of ATP.

The effect of ATP on the interaction of AP-2 with vesicle membranes required addition of an ATP-regenerating system, suggesting that it depends on hydrolysis of the nucleotide. This event might be associated to a modification (phosphorylation) of either AP-2 or components of the membrane. The ATPase-dependent release of AP-2 resembles Hsc70-dependent uncoating of clathrin. Efficient uncoating of clathrin by substoichiometric amounts of Hsc70 depends on hydrolysis of ATP, whereas under conditions of molar excess over clathrin Hsc70 can dissociate clathrin without hydrolysis of ATP [19]. As we observed the effect of ATP on the interaction of AP-2 with clathrin-deprived vesicles (Fig. 4) or stripped vesicles (Fig. 3) in the absence of cytosol, it seems unlikely that ATP-induced release of AP-2 is related to the Hsc70-dependent disassembly of clathrin from coated vesicles.

The putative AP-2-releasing ATPase may be either an integral membrane protein or a cytosolic protein firmly attached to the membrane and resistant to stripping. In support of this, the release of AP-2 from the clathrin-coated vesicles by ATP did not depend on addition of cytosol (data not shown).

It has been found that ATP and cytosol are required for assembly of coated pits at the plasma membrane and for budding of coated vesicles [20]. The seemingly contradictory effects of ATP may be explained by differences in the experimental system; while the abovementioned studies described the effect of ATP on the binding of AP-2 to newly forming coated pits, we used pre-formed coated vesicles as acceptor membranes. Therefore, in early steps of endocytosis ATP may favour the binding of AP-2 complexes to coated pits during their formation at the plasma membrane [20], while the same nucleotide may promote dissociation of AP-2 after coated vesicles have pinched off the plasma membrane.

Plasma membrane AP-2 was released selectively from the coated vesicles by ATP, and no effect on release of the Golgi-specific AP-1 was observed (results not shown). The translocation of cytosolic AP-1 onto purified Golgi membranes re-

quires binding of GTP or GTP analogues bound to the small GTPase ARF1 [12,17]. In agreement with other authors we found that GTP γ S enhanced the binding of cytosolic AP-1 to stripped vesicles, but no effect on the binding of AP-2 was observed. All these data clearly demonstrate that the mechanism of assembly and disassembly of AP-2 differs from that of AP-1.

Analysing the characteristics of the ATPase for AP-2 release or answering the question whether this protein is related to Hsc70 should be the aim of further studies.

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