The EMBO Journal Vol.16 No.9 pp.2240–2250, 1997

Regulatory interactions in the recognition of endocytic sorting signals by AP-2 complexes

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Many plasma membrane proteins destined for endocytosis are concentrated into clathrin-coated pits through the recognition of a tyrosine-based motif in their cytosolic domains by an adaptor (AP-2) complex. The μ2 subunit of isolated AP-2 complexes binds specifically, but rather weakly, to proteins bearing the tyrosine-based signal. We now demonstrate, using peptides with a photoreactive probe, that this binding is strengthened significantly when the AP-2 complex is present in clathrin coats, indicating that there is cooperativity between receptor–AP-2 interactions and coat formation. Phosphoinositides with a phosphate at the D-3 position of the inositol ring, but not other isomers, also increase the affinity of the AP-2 complex for the tyrosine-based motif. AP-2 is the first protein known (in any context) to interact with phosphatidylinositol 3-phosphate. Our findings indicate that receptor recruitment can be coupled to clathrin coat assembly and suggest a mechanism for regulation of membrane traffic by lipid products of phosphoinositide 3-kinases.

Keywords: adaptors/clathrin/coated pits/membrane traffic/protein sorting

Introduction

Clathrin-coated pits are sorting engines for protein traffic to the endosomal compartment. They recruit their cargo, consisting of membrane-bound proteins often with their ligands, which are engulfed in coated vesicles upon budding of the donor membrane. Specificity of recruitment involves recognition by coat components of signal sequences in the cytosolic domains of the membrane proteins. Coated vesicles always contain cargo (Carpentier *et al.*, 1982; Geuze *et al.*, 1984), raising the intriguing question of how coupling of coat formation and protein sorting is achieved.

Important components of clathrin-coated structures are the so-called 'AP' or 'adaptor' complexes, which participate in sorting steps at the plasma membrane (AP-2) or in the *trans*-Golgi network (AP-1) (reviewed in Pearse and Robinson, 1990; Robinson, 1992; Kirchhausen, 1993). The heterotetrameric AP complexes bind selectively to the appropriate membrane and interact both with clathrin (Zaremba and Keen, 1983; Pearse and Robinson, 1984; Ahle and Ungewickell, 1989; Keen and Beck, 1989; Matsui and Kirchhausen, 1990; Keen *et al.*, 1991; Prasad and Keen, 1991; Schroder and Ungewickell, 1991; Gallusser and Kirchhausen, 1993; Shih *et al.*, 1995; Traub *et al.*, 1995) and with the cytoplasmic domain of receptors (Pearse, 1988; Glickman *et al.*, 1989; Beltzer and Spiess, 1991; Sosa *et al.*, 1993; Boll *et al.*, 1995; Gilboa *et al.*, 1995; Nesterov *et al.*, 1995a; Ohno *et al.*, 1995; Heilker *et al.*, 1996). The α chain of the AP-2 heterotetramer can bind clathrin (Goodman and Keen, 1995), synaptotagmin (Zhang *et al.*, 1994), Eps15 (Benmerah *et al.*, 1995; Tebar *et al.*, 1996), Grb2 (Okabayashi *et al.*, 1996) and small phosphorylated molecules like inositol phosphates and phosphoinositides (Beck and Keen, 1991a; Timerman *et al.*, 1992; Voglmaier *et al.*, 1992; Gaidarov *et al.*, 1996). The physiological significance of these interactions is under investigation. The β chain contacts clathrin and drives coat assembly (Ahle and Ungewickell, 1989; Schroder and Ungewickell, 1991; Gallusser and Kirchhausen, 1993; Shih *et al.*, 1995). The μ2 chain recognizes the tyrosine-based endocytic motif (Ohno *et al.*, 1995; Boll *et al.*, 1996), a sequence of four amino acids of the form tyrosine–polar–polar–large hydrophobic (YppØ), which is used for sorting proteins from the plasma membrane to the endosome (Trowbridge *et al.*, 1993; Thomas and Roth, 1994).

The interactions just described do not by themselves explain how cargo recruitment and coat formation are coupled and how concentration of cargo into coated structures is achieved. Previous work has shown that the isolated AP-2 complex, or even its isolated μ2 subunit, can recognize the tyrosine-based motif (Ohno *et al*., 1995; Boll *et al*., 1996). This interaction reflects the specificity seen *in vivo*, but it is rather weak (dissociation constant ~10 μM). Are there regulatory mechanisms that enhance the interaction? One obvious candidate for such modulation is the clathrin coat itself. If the coat increased affinity for the sorting signal, there would be a simple explanation for the coupling of cargo recruitment to coat formation. A distinct increase in proteolytic sensitivity indeed suggests that the μ2 subunit undergoes a conformational change when AP-2 enters a clathrin coat (Matsui and Kirchhausen, 1990).

Recent evidence indicates that vesicle formation is likely to be regulated in an even more complex manner. In particular, there appears to be an important role for phosphatidylinositol 3-kinases (PtdIns 3-kinases). In yeast cells, for example, genetic elimination of Vps34, a PI 3-kinase, results in defective membrane traffic from the Golgi to the vacuolar compartment (reviewed in DeCamilli *et al*., 1996). Similarly, in mammalian cells, Wortmannin,

an inhibitor of PtdIns 3-kinases, affects the post-endosomal constitutive traffic of receptors such as those for transferrin or mannose 6-phosphate (Davidson, 1995; Martys *et al*., 1996; Spiro *et al*., 1996). PtdIns 3-kinase activity is also required for the trafficking of some receptors from the plasma membrane to the lysosome. In the case of the platelet-derived growth factor (PDGF) receptor, ligand binding results in recruitment of PtdIns 3 kinase to the receptor cytosolic tail. Mutations in the receptor tail that prevent this recruitment block subsequent lysosomal targeting of the receptor (Joly *et al*., 1994, 1995). A significant increase in PtdIns 3 kinase activity has been detected in coated vesicles from activated cells (Kapeller *et al*., 1993), raising the possibility that the products of this enzymatic activity could affect the ability of AP-2 complexes to recognize receptor tails. Two possible mechanisms may be imagined. One is that the phosphoinositides may be concentrated locally to provide a special membrane site for vesicle formation or coat assembly; the other is that they serve as local signaling molecules to somehow enhance recruitment of receptors and coats.

We now present evidence for both the simple cooperative mechanism, by which clathrin increases AP-2 affinity for sorting motifs, and the signaling mechanism, by which phosphoinositides likewise enhance AP-2–receptor interactions. We have developed an assay for the AP-2–receptor tail interaction, based on the use of cross-linkable peptides containing tyrosine-based endocytic motifs. Photoreactive peptides corresponding to the cytoplasmic tail of the *trans*-Golgi network integral protein TGN38 can be cross-linked in a specific manner to the μ2 subunit of an intact AP-2 complex. We show that formation of AP-2–clathrin coats *in vitro* increases the affinity of μ2 for the tyrosine motif, probably through a conformational change in the AP-2 complex. This switch in the state of the complex provides a mechanism for coupling assembly of clathrin coats with selective enrichment of receptors in coated pits. We argue that this coupling can ensure that clathrin-coated vesicles are only formed when they contain 'cargo'. Moreover, we show that binding of phosphoinositides to free AP-2 complexes also increases the affinity of their μ2 chain for the tyrosine endocytic signal and that this effect requires a phosphate group in the third position of the inositol ring. We propose that in cells the ability of AP-2 complexes to recognize tyrosine-based sorting signals depends on at least two factors: binding of AP-2 to phosphoinositides of defined structure and incorporation into the clathrin coat.

Results

The μ2 in intact AP-2 complexes is the specific target of peptides containing a tyrosine-based endocytic motif

In our earlier studies, we demonstrated that isolated μ2 chains recognize peptides containing endocytic motifs of the form YppØ and that the same specificity is maintained when μ2 is part of an AP-2 complex (Ohno *et al.*, 1995; Boll *et al.*, 1996). We used surface plasmon resonance to determine the affinity of short peptides derived from receptor tails for immobilized AP-2 complexes (Boll *et al.*, 1995, 1996; Ohno *et al.*, 1995). It is not practical, however, to use this technique to study the interaction between

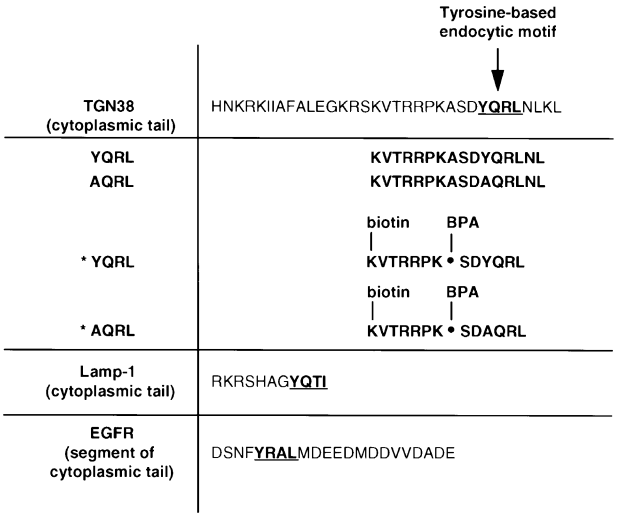

Fig. 1. Schematic representation of peptides used for the cross-linking studies. The figure compares the sequence of the complete 34 amino acid cytoplasmic tail of TGN38 containing the Y-motif 'YQRL' with the sequence of some of the synthetic 16mer peptides used in this study. The cross-linker photoreactive peptides, named *YQRL and *AQRL, were modified by replacement of A with BPA at Y-3, by addition of biotin to the N-terminus and by truncation of the last two residues. YQRL and AQRL refer to the comparable synthetic peptides with no modifications. YRAL refers to the 22 amino acid peptide corresponding to the segment of the cytoplasmic tail of the EGFR known to bind AP-2 and which contains the Y-motif 'YRAL'. YQTI corresponds to the sequence of the complete 11 amino acid cytoplasmic tail of Lamp-1 containing the Y-motif 'YQTI' and also known to interact with AP-2. The tyrosine-based endocytic motifs are underlined.

coat-associated AP-2 complexes and receptor tails. To examine the interaction of receptor tails with AP complexes directly, we therefore designed and synthesized a receptor tail-like peptide (*YQRL, Figure 1) containing the UV-activatable cross-linker benzoylphenylalanine (BPA). We determined the ability of the peptide to label μ2 in isolation and within AP-2 complexes. The sequence of the peptide corresponds to the wild-type TGN38 cytoplasmic tail (Luzio *et al.*, 1990) which mediates the endocytosis of TGN38 via its tyrosine motif *in vivo* and which is known to interact with isolated μ2 and with intact AP-2 (Ohno *et al.*, 1995; Boll *et al.*, 1996). The 14 residue peptide is biotin labeled at the ε position of the N-terminal lysine to facilitate identification of the cross-linked products by membrane protein blot analysis. The cross-linking BPA reagent is inserted at position Y-3, which is known not to be important for recognition by the μ2 chain (Ohno *et al.*, 1995; Boll *et al.*, 1996).

We first established that the photoreactive peptide specifically recognizes μ2 (Figure 2). The *YQRL peptide cross-links to a GST fusion protein containing the segment of μ2 that binds the tyrosine motif (lane 9), but poorly to a GST fusion protein containing the equivalent segment of μ1 (lane 8), and not at all to GST alone (lane 7). A peptide with a sequence known not to bind to μ2 (Ohno *et al.*, 1995; Boll *et al.*, 1996), *AQRL, in which the anchor tyrosine of *YQRL has been replaced with alanine, fails to cross-link to μ2 (lane 6), indicating that the interaction detected in this way is specific.

We then showed that the *YQRL peptide also binds selectively to μ2 in intact AP-2 complexes (Figure 3, lane

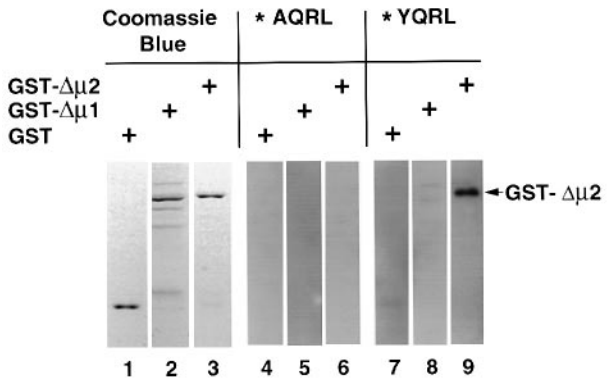

Fig. 2. Specificity of cross-linking to isolated μ2. Similar amounts of recombinant GST, GST–Δμ1 and GST–Δμ2 (0.1 mg/ml) in coat assembly buffer were incubated with the synthetic cross-linking photoreactive peptides *YQRL or *AQRL (0.2 μM final concentration) and subjected to UV irradiation for 3 min followed by SDS–PAGE (12.5% gel). The samples were transferred to nitrocellulose which was then incubated with streptavidin–HRP. The presence of cross-linked products was determined by the appearance of bands upon development of the membranes by enhanced chemiluminescence (ECL). Lanes 1–3 correspond to the Coomassie blue staining of the samples used in the experiment. Lanes 4–6 show the ECL staining pattern obtained with the mutant cross-linking peptide *AQRL, and lanes 7–9 to the pattern obtained with the wild-type cross-linking peptide *YQRL.

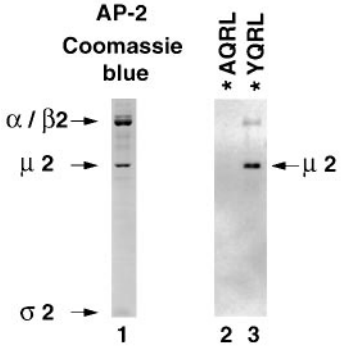

Fig. 3. μ2 is the specific target of *YQRL in free AP-2 complexes. AP-2 complexes purified from bovine brain coated vesicles were incubated with the cross-linking peptides *AQRL (lane 2) or *YQRL (lane 3) and tested for the appearance of cross-linked species upon UV irradiation after fractionation using SDS–PAGE (12.5% gel). Lane 1, Coomassie blue staining to indicate the electrophoretic mobility of the subunits of AP-2 (α, β2, μ2 and σ2). Lanes 2–3, analysis by ECL of AP-2 samples transferred to nitrocellulose and tested for the appearance of cross-linked products. The cross-linking was performed in coat assembly buffer using a final concentration of 0.5 mg/ml of AP-2 and 0.2 μM of photoreactive peptides.

3). Substitution of alanine for tyrosine (*AQRL) abolishes the interaction (lane 2). A weaker labeled band, corresponding in electrophoretic mobility to the large chains, was often detected. The intensity of this band varied between experiments and in general could not be reduced by simultaneous incubation with an excess (up to a concentration of 200 μM) of unlabeled peptides YQRL or AQRL, lacking BPA and biotin. We conclude that it probably represents a non-specific interaction, independent of the Y-motif. In contrast, the unmodified peptide YQRL competes with *YQRL for binding to μ2 (Figure 4). The concentration for half-maximal competition is ~10–50 μM, in excellent agreement with affinities measured in a related analysis by surface plasmon resonance (Boll *et al.*,

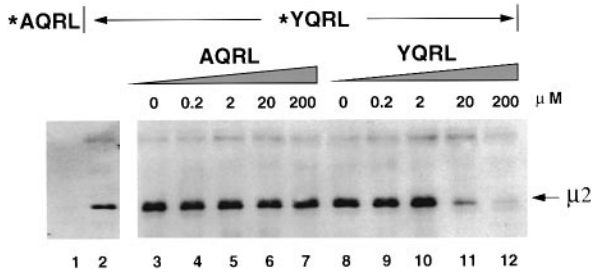

Fig. 4. The specificity of *YQRL for μ2 is not affected by the BPA cross-linking reagent. Similar amounts of AP-2 (0.5 mg/ml) in coat assembly buffer were incubated with 0.2 μM of the cross-linking peptides *AQRL (lane 1) or *YQRL (lane 2) to demonstrate that μ2 interacts preferentially with the peptide containing the native YQRL tyrosine motif sequence but not with the peptide containing the AQRL mutant motif. In the remaining samples from another experiment (lanes 3–12), equivalent amounts of AP-2 were incubated with a mixture of *YQRL and increasing amounts (0–200 μM) of unmodified mutant AQRL peptide (lanes 3–7) or unmodified native YQRL peptide (lanes 8–12). Competition by YQRL and not by AQRL confirms that the interaction of the cross-linking peptide *YQRL with μ2 is specific and that incorporation of the photoreactive BPA reagent and of biotin into *YQRL does not affect the selectivity of the tyrosine motif.

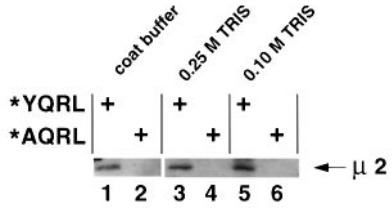

Fig. 5. Effect of Tris-containing buffers on the cross-linking efficiency of *YQRL in AP-2 complexes. AP-2 complexes (0.5 mg/ml) were subjected to a cross-linking reaction with 0.2 μM of *YQRL (lanes 1, 3 and 5) or with *AQRL (lanes 2, 4 and 6). AP-2 was suspended in coat assembly buffer (lanes 1 and 2) or in Tris-containing buffer (0.25 M Tris; lanes 3 and 4 or 0.1 M Tris; lanes 5 and 6).

1996). The same extent of μ2 labeling with *YQRL was obtained when AP-2 was suspended in Tris-containing buffer (Figure 5, lanes 3–6), a condition known to prevent AP aggregation (Keen *et al.*, 1979), instead of coat assembly buffer (lanes 1 and 2).

Specific peptide cross-linking to μ2 is enhanced in AP-2-containing coats

We next compared the binding affinities of *YQRL for free AP-2 complexes and for AP-2 complexes reconstituted into membrane- and receptor-free coats (Figure 6). Cross-linking of *YQRL to free AP-2 and to AP-2 in clathrin coats was carried out in coat assembly buffer under identical experimental conditions, using similar amounts of APs in each reaction. The cross-linking efficiency for μ2 in the AP-2-containing coats (lane 6) was 8- to 10-fold higher than that for free AP-2 (lane 4). This increase in cross-linking efficiency was observed in a total of eight independent experiments and ranged between eight and 60 times. The increased efficiency was not simply due to the presence of clathrin in the solution, since the efficiency of cross-linking of *YQRL to μ2 in free AP-2 was unchanged when the same amount of clathrin and AP-2 present in the coat sample was incubated in AP buffer containing 150 mM NaCl, a condition used to inhibit coat formation (lanes 1 and 2). The increased efficiency is also

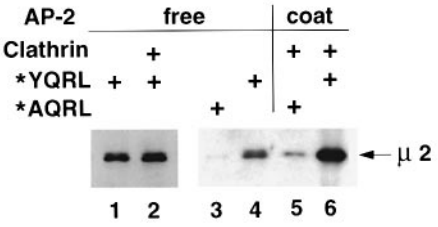

Fig. 6. Enhancement of the efficiency of *YQRL cross-linking to μ2 in AP-2s that are forming clathrin coats. Similar amounts of AP-2 (0.2 mg/ml), free and in the absence of clathrin (lanes 1, 3 and 4), free and in the presence of clathrin (0.6 mg/ml) (lane 2), or as part of clathrin coats (lanes 5 and 6) were subjected to cross-linking with peptide *AQRL (lanes 3 and 5) or with peptide *YQRL (lanes 1, 2, 4 and 6). The cross-linking reactions were performed in two different experiments containing AP buffer (lanes 1 and 2) or coat assembly buffer (lanes 3, 4, 5 and 6). The efficiency of *YQRL cross-linking to μ2 is substantially enhanced when AP-2 is co-assembled with clathrin (lane 6).

not accounted for by a change in the solubility properties of AP-2, since, as noted earlier, μ2 labeled equally well when free AP-2 was suspended in coat assembly buffer (used to drive coat formation; Figure 5, lane 1) or in AP buffer containing Tris (to ensure AP solubility; Figure 5, lanes 3 and 5). The specificity of the interaction between the tyrosine-based motif and μ2 in AP-2-containing coat was confirmed by the minimal amount of a cross-linked product using *AQRL (Figure 6, lane 3) instead of *YQRL as the photoreactive probe (Figure 6, lane 4). A similar coat-specific stimulatory effect on AP-2 was observed using coated vesicles isolated from bovine brains instead of using reconstituted AP-2–clathrin coats (data not shown). A possible way to explain this result is that the receptor tails are displaced from AP-2 by the cross-linker peptide *YQRL because it might have an affinity for μ2 that is higher than the affinity displayed by most tails.

Even though the extent of TGN38 tail cross-linking monitored with *YQRL was increased in the coats, the reaction was still specific to the μ2 chain. Thus, peptide contacts to other AP-2 chains do not appear to allow for the observed enhancement. Competition experiments, performed as in Figure 5, were used to show that the apparent affinity of unlabeled peptides for AP-2, as determined by their ability to block *YQRL cross-linking, was also enhanced in coats, in this case, by ~8-fold (Figure 7A). That is, the concentration for half-maximal competition was lowered from ~10–50 μM (n = 5) with free AP-2 to ~1–5 μM (n = 3) with AP-2 in clathrin-containing coats. The 8- to 10-fold effect corresponds to the observed increase in cross-linking efficiency determined in the absence of competitor, suggesting a similar effect on the interaction of the modified and unmodified peptides with μ2 chain. We therefore conclude that the increased cross-linking reflects a comparable increase in binding affinity of the YQRL-containing peptide for the μ2 chain in AP-2-containing coats.

Does this increase in affinity involve an enhanced recognition for other receptor tails known to interact with free AP-2? To answer this question, we used two other synthetic peptides containing Y-motifs as competitors for the *YQRL photoreactive peptide. The peptides were YQTI, corresponding to the complete cytosolic tail of the lysosomal membrane protein Lamp-1, and the peptide

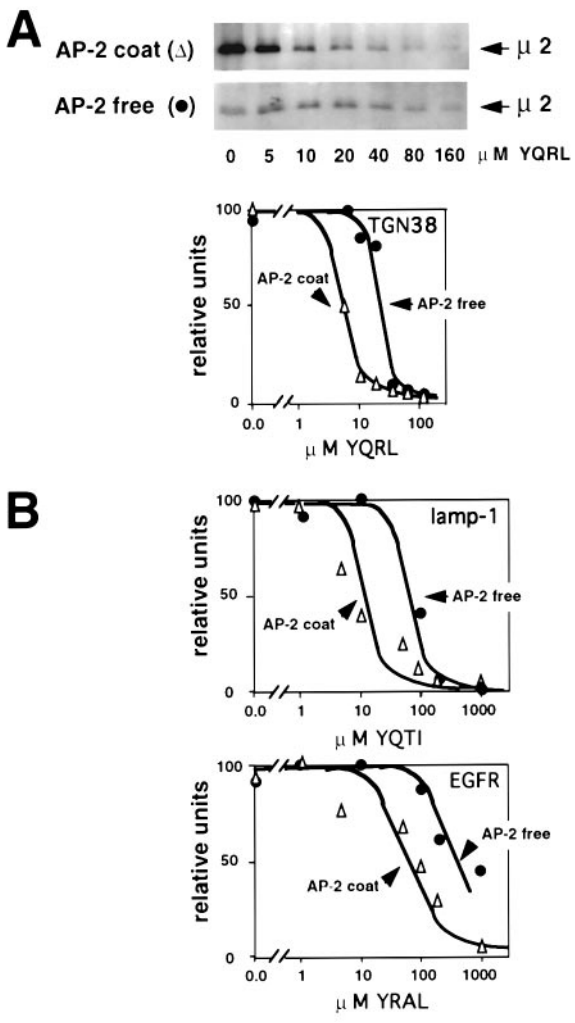

Fig. 7. Increased affinity of μ2 for the tyrosine-motif in AP-2 contained in clathrin coats. (**A**) Equivalent amounts of AP-2 (0.5 mg/ml), either free or contained in clathrin coats, were incubated with *YQRL (0.2 μM) and increasing amounts of the competitor peptide YQRL (0, 5, 10, 20, 40, 80 and 160 μM) and subjected to cross-linking. The relative intensity of the labeled μ2 band as a function of competitor is shown in the bottom part of the figure. These results correspond to a single experiment and are representative of three independent experiments performed with different preparations of AP-2 and clathrin. (**B**) Similar experiments were performed using the competitor peptide YQTI corresponding to the complete cytoplasmic tail of Lamp-1 and the competitor peptide YRAL corresponding to a segment of the EGFR. The plots correspond to the relative intensity of μ2 chain from AP-2 free or as part of coats labeled with *YQRL as a function of increasing amounts of the competitor peptides.

YRAL, corresponding to a portion of the cytoplasmic tail of the epidermal growth factor receptor (EGFR). As indicated in Figure 7B, the relative affinity of free AP-2 for the Lamp-1 peptide was slightly lower (~70 μM) than for the TGN38 peptide (10–50 μM range) whereas the affinity for the EGFR peptide was substantially lower (~400 μM). Nevertheless, the concentration of the YQTI and YRAL peptides required for half-maximal competition was 7–10 times higher when AP-2 formed coats than when AP-2 were free. From these results, we conclude that the increase in the affinity state of AP-2 complexes elicited by their interaction with clathrin involves a general

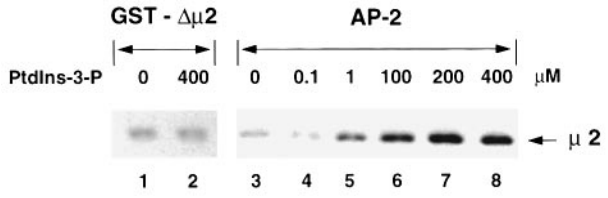

Fig. 8. Enhancement of the efficiency of *YQRL cross-linking to μ2 in AP-2 by PtdIns 3-P. GST–Δμ2 (0.13 mg/ml) or AP-2 complexes (0.13 mg/ml) were incubated in AP buffer with the indicated amounts of PtdIns 3-P (0–400 μM) and subjected to a cross-linking reaction with *YQRL (0.2 μM).

recognition of receptor tails carrying Y-motif sorting signals.

The increased efficiency of cross-linking in coats might in principle be due to non-equilibrium effects, for example by rapid rebinding of dissociating peptide by APs within a coat during the 3 min cross-linking interval. The competition experiment rules out this alternative. Moreover, other observations show that small proteins have free access to coat interiors. For example, μ2 has enhanced accessibility to proteases when AP-2 is incorporated into a clathrin coat (Matsui and Kirchhausen, 1990). This observation shows not only that proteases can enter a coat readily but also that μ2 is likely to undergo an assembly-dependent conformational change.

Peptides in which the cross-linking reagent was inserted at positions Y-5 (rather than Y-3) gave similar results (data not shown). That is, these peptides labeled the μ2 chain in AP-2 coats more efficiently than in free AP-2, but labeling was somewhat less intensive than with the cross-linker at Y-3. Moreover, no other chains were labeled in these reactions. We could not detect any cross-linking to μ2 or to other AP-2 subunits using peptides with BPA replacing L at Y+5, suggesting that the side chain at this position probably projects away and is not in direct contact with the μ2-binding domain. In fact, the amino acid identity at this position does not affect recognition of peptides by the fusion protein GST–Δμ2 in selection experiments from combinatorial peptide libraries (Boll *et al.*, 1996).

Binding of phosphoinositides to AP-2 increases the affinity of μ2 for the peptide containing the tyrosine-based endocytic motif

We have also investigated the effects of inositol polyphosphates and phosphoinositides on the ability of AP-2 complexes to recognize tyrosine-based endocytic signals. Co-incubation of free AP-2 and *YQRL with micelles of phosphatidylinositol 3-phosphate (PtdIns 3-P) shows that the amount of *YQRL cross-linked to μ2 increases 8- to 40-fold (measured in four different experiments) in proportion to the amount of PtdIns 3-P, reaching saturation at ~100 μM PtdIns 3-P (Figure 8, lanes 3–8). As shown above for the stimulatory effect on AP-2 by clathrin, the enhancement by PtdIns 3-P represents an 8- to 10-fold increase in affinity, as measured by competition with the non-modified YQRL peptide (data not shown). A cross-linking experiment mixing up to 400 μM PtdIns 3-P with GST–Δμ2 did not reveal any significant effects on the cross-linking efficiency of *YQRL to the fusion protein. We note, however, that this GST fusion protein lacks the first 122 residues of μ2 and, because full-length GST–Δμ2 is not soluble, it was not possible to ascertain if the amino-terminal part of μ2 or the intact AP-2 complex is required for the enhanced affinity in response to PtdIns 3-P.

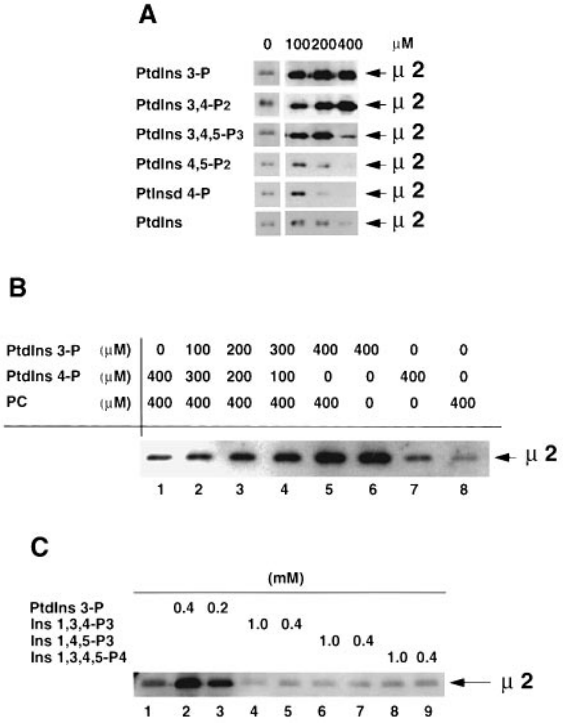

Fig. 9. Enhancement of cross-linking efficiency depends on the phosphorylation pattern of the inositol ring of the phosphoinositides. (**A**) Similar amounts of AP-2 (0.13 mg/ml) in AP buffer were incubated with 0, 100, 200 or 400 μM of various phosphoinositides and subjected in independent experiments to a cross-linking reaction with *YQRL (0.2 μM). Phosphoinositides containing a phosphate at position D-3 of the inositol ring were most effective in increasing the efficiency of the cross-linking reaction. (**B**) AP-2 (0.13 mg/ml) in AP buffer was incubated with different mixtures containing a constant amount of phosphatidylcholine (PC) (400 μM; lanes 1–5) and various amounts of PtdIns 3-P and PtdIns 4-P (0–400 μM; lanes 1–5) and then processed for a cross-linking reaction with *YQRL (0.2 μM). Controls included incubations with PtdIns 3-P only (400 μM; lane 6), PtdIns 4-P only (400 μM; lane 7) and PC only (400 μM; lane 8). (**C**) AP-2 (0.13 mg/ml) was incubated without (lane 1) or with 0.4 or 0.2 mM PtdIns 3-P (lanes 2 and 3) or with 1 or 0.4 mM of the inositol phosphates Ins 1,3,4-P_3 (lanes 4 and 5), Ins 1,4,5-P_3 (lanes 6 and 7) and Ins 1,3,4,5-P_4 (lanes 8 and 9) and then processed for a cross-linking reaction with *YQRL (0.2 μM). Inositol phosphates do not enhance the affinity of AP-2 for the tyrosine-sorting motif.

We then tested the phosphorylation specificity on the cross-linking reaction by using phosphoinositides modified with phosphate groups present at different positions in the inositol ring and at various concentrations up to 400 μM (Figure 9A). It was found that the efficiency of cross-linking of *YQRL to μ2 was greatest when the inositol ring was phosphorylated at position D-3 (e.g. PtdIns 3-P) and that addition of phosphate groups to other ring positions reduced [e.g. phosphatidylinositol 3,4-bisphosphate (PtdIns 3,4-P_2)] or even inhibited [phosphatidylinositol 3,4,5-trisphosphate (PtdIns 3,4,5-P_3)] the enhancement effect. The effect of the lipids was also explored at concentrations in the 0.1–100 μM range (data not shown). It was found that the enhancement effect of PtdIns 3-P was proportionally less than at 100 μM; in contrast, no

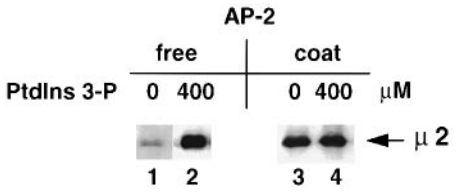

Fig. 10. PtdIns 3-P has no effect on the recognition of the tyrosine motif when AP-2 is part of clathrin coats. Similar amounts (0.13 mg/ml) of free AP-2 or AP-2 assembled into coats were subjected to the cross-linking reaction with *YQRL (0.2 mM) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of PtdIns 3-P (400 μM). The cross-linking reactions were carried out in coat assembly buffer. No additional stimulatory effect of PtdIns 3-P is detected with coats.

stimulatory effect was detected with the other phosphatidylinositides. Non-phosphorylated inositols had no effect and phosphoinositides with a phosphate group at D-4 or D-5 (e.g. PtdIns 4-P or PtdIns 4,5-P_2) had mostly an inhibitory effect. To differentiate whether the stimulatory effect was due to the glycerol–phospholipid backbone or to the phosphorylated inositol portion, AP-2s were incubated with micelles containing the same amount of glycerol–phospholipid backbone by using a constant mixture of phosphatidylcholine (PC) and phosphatidylinositide, but where the relative amounts of the stimulatory PtdIns 3-P and the non-stimulatory PtdIns 4-P were varied. As indicated by the results in Figure 9B, the cross-linking signal reflecting the association of *YQRL with μ2 increased in proportion to the content of PtdIns 3-P, confirming that the stimulatory effect elicited by the phosphoinositides can be accounted for by the pattern of phosphorylation in their inositol rings. It was found further that incubation of AP-2 with up to 1 mM of the corresponding inositol phosphates Ins 1,3,4-P_3, Ins 1,4,5-P_3 and Ins 1,3,4,5-P_4, which lack the fatty acid on the glycerol backbone, did not have an effect on the efficiency of the cross-linking reaction (Figure 9C). In addition, co-incubation of Ins 1,2,3,4,5,6-P_6 (400 μM) with PtdIns 3,4-P_2 (100 μM) did not compete the stimulation detected with PtdIns 3,4-P_2 alone (data not shown). Taken together, these results suggest that the interaction of AP-2 with phosphoinositides leading to the enhanced affinity of μ2 for *YQRL is constrained by the stereospecific requirements imparted by the presence of a phosphate group at position D-3 of the inositol ring and by presentation of the inositol ring in the context of a fatty acid and glycerol backbone.

Finally, we found that the stimulatory effects on AP-2 by PtdIns 3-P and by clathrin are not additive. As indicated by the cross-linking results depicted in Figure 10, the incubation of free AP-2 with 400 μM PtdIns 3-P elicited the expected 8- to 10-fold increase in the cross-linking signal with *YQRL, whereas the same amount of PtdIns 3-P had no noticeable effects in a parallel experiment carried out with AP-2/clathrin coats. There are at least two possible explanations that can account for the non-additive results. The first possibility that we favor is that AP complexes can be driven onto the same high affinity state by interactions either with 3′-phosphoinositides or with clathrin. Alternatively, the lipid micelles might be too large to reach the AP-2 complexes located underneath the clathrin coat, therefore preventing their stimulatory activity. To test this second possibility, we included 1% (v/v) Triton X-100 in an experiment carried out with PtdIns 3,4-P_2. It was found, however, that this mixture did not elicit a stimulatory effect with AP-2–clathrin coats even though the normal stimulatory effect was detected with free AP-2 (not shown).

Discussion

APs interact with the cytoplasmic tails of the membrane proteins that traffic through the clathrin pathway (Glickman *et al.*, 1989; Sorkin and Carpenter, 1993; Sosa *et al.*, 1993; Boll *et al.*, 1995; Gilboa *et al.*, 1995; Nesterov *et al.*, 1995a; Sorkin *et al.*, 1995, 1996; Heilker *et al.*, 1996; Honing *et al.*, 1996). Is the interaction between APs and sorting signals regulated by changes in the properties of the AP complex in response to contacts with other molecules? We demonstrate in the experiments described in this study that the interaction of AP-2 with clathrin or with specific phosphoinositides enhances the ability of the μ2 subunit of AP-2 to recognize a peptide containing the tyrosine-based endocytic motif. We propose that these enhancements represent significant *in vivo* regulatory interactions.

The effect of clathrin on receptor tail recognition

To detect the effect of clathrin on the affinity of μ2 for a receptor tail, we have compared the cross-linking of a photoreactive probe to μ2 in coats and in free APs. We have found a substantial increase in affinity on incorporation into coats by two independent methods. In one, we directly measured labeling of μ2 with *YQRL; in the other, we used increasing amounts of a non-photoreactive, but otherwise equivalent, YQRL peptide in a competition assay. The 10-fold increase in affinity is substantial, when compared with physiologically significant changes in affinity between interacting proteins in signal transduction cascades (Mauxion *et al.*, 1996) or in genetic switches (Heilker *et al.*, 1996). This increase in affinity is not restricted to the TGN38 tail but seems to be a general effect, since the augmented affinity was also detected using peptides derived from the cytosolic tail of the EGFR and from Lamp-1. These peptides contain their specific Y-motifs but their sequences are otherwise completely unrelated. We therefore believe that the modulation of AP affinity by clathrin is likely to be physiologically relevant.

Affinity changes in protein–ligand interactions usually result from conformational transitions. An indication that incorporation into coats produces a conformational change in AP-2 comes from studies showing an enhanced susceptibility of μ2 to proteases after coat assembly (Matsui and Kirchhausen, 1990). A conformational transition that would couple clathrin binding with receptor interactions would need to extend over a significant distance because AP complexes span a gap of ~10 nm between the clathrin lattice and the surface of the membrane bilayer in a coated vesicle (Heuser and Kirchhausen, 1985; Vigers *et al.*, 1986). This separation is likely to be the distance between the β chain hinge, which bears a clathrin-binding site (Shih *et al.*, 1995), and the membrane surface, which holds the often rather short cytoplasmic tails of receptors.

Does the assembly of AP complexes with clathrin

precede cargo binding by the μ chain or do the complexes that associate with clathrin already bear their cargo? The results presented here are consistent with either order. We have shown here that when AP-2 and clathrin co-assemble, there is an increase in the affinity of the μ2 chain for receptor tails. Assuming equilibrium, binding of receptor by the μ2 chain will reciprocally increase the affinity of APs for clathrin and hence enhance the likelihood that the receptor-loaded AP complex will be incorporated into a coated pit. In other words, linkage between clathrin association and endocytic signal binding implies that AP complexes already incorporated into coated pits will have a higher affinity for tyrosine motifs than will isolated APs. However, this linkage equally implies that APs with bound receptors will have a higher affinity for clathrin than will unliganded APs.

Two lines of evidence have been cited to support the model that receptors are captured by pre-formed coated pits. In one set of experiments, measurements of the lateral mobility in the plasma membrane of molecules bearing internalization signals showed that the proteins encountered regions of constrained diffusion (Fire *et al.*, 1995). It was suggested that these regions might be pre-existing coated pits containing immobilized APs, which first become loaded with cargo molecules and then complete coat assembly. However, these results are equally consistent with a model in which mobile receptors already bearing APs become constrained when they encounter clathrin at the edge of a coated pit. In another set of experiments (Santini and Keen, 1996), it was found that exposure of the high affinity FceRI receptor to IgE, a condition that leads to activation and rapid internalization of the receptor, did not change the number of coated pits nor the extent of clathrin and AP-2 recruitment to the membrane in the activated cells. Based on this study, it was concluded that, at least in the case of receptors that internalize in response to ligand binding, endocytosis does not involve formation of new coated pits. The observations do not, however, distinguish between capture of receptors by a clathrin–AP lattice or addition of receptor–AP complexes to the growing edge of a coated pit.

The suggestion that receptors associate with AP complexes before entry into coated pits comes from two groups of experiments. In one, recruitment of AP-1 to *trans*-Golgi network membranes was shown to be enhanced by the presence of the mannose 6-phosphate receptor in these membranes (Mëresse *et al.*, 1990; Le Borgne *et al.*, 1993, 1996). In the other, activation with EGF was shown to induce the association of a fraction of cell surface EGFRs with AP-2 complexes (Sorkin and Carpenter, 1993; Boll *et al.*, 1995). No association with clathrin was detected (Sorkin and Carpenter, 1993; Boll *et al.*, 1995; Sorkin *et al.*, 1995). It was therefore proposed that the AP-2–receptor association might precede coated pit formation. It has been difficult, however, to demonstrate in cells a direct association of AP complexes with receptors other than with EGFR, even for those like the low density lipoprotein (LDL) and the transferrin receptor, known to be transported efficiently via the clathrin pathway (our unpublished observations) (Nesterov *et al.*, 1995a).

We propose that the coupling of membrane-bound receptor binding and clathrin assembly has an important role in coat formation (Figure 11), whatever the order of receptor–AP association. Receptor-loaded APs at the membrane, switched by ligand occupancy into the high affinity state for clathrin, can recruit soluble clathrin trimers from the cytosol or can be captured by available clathrin legs at the edge of a partly formed coated pit. As the lattice grows, additional APs will co-assemble with it, and those already bound to receptor tails will have a greater likelihood of entering. At the same time, unloaded APs that are already trapped by clathrin on a partially formed coated pit could capture more weakly interacting membrane-bound receptors, by virtue of having been switched to the clathrin-induced high affinity state for tyrosine motifs.

The linkage between clathrin binding to APs and their affinity for cargo helps ensure that coated pit assembly is coupled to cargo loading. Indeed, electron micrographs of

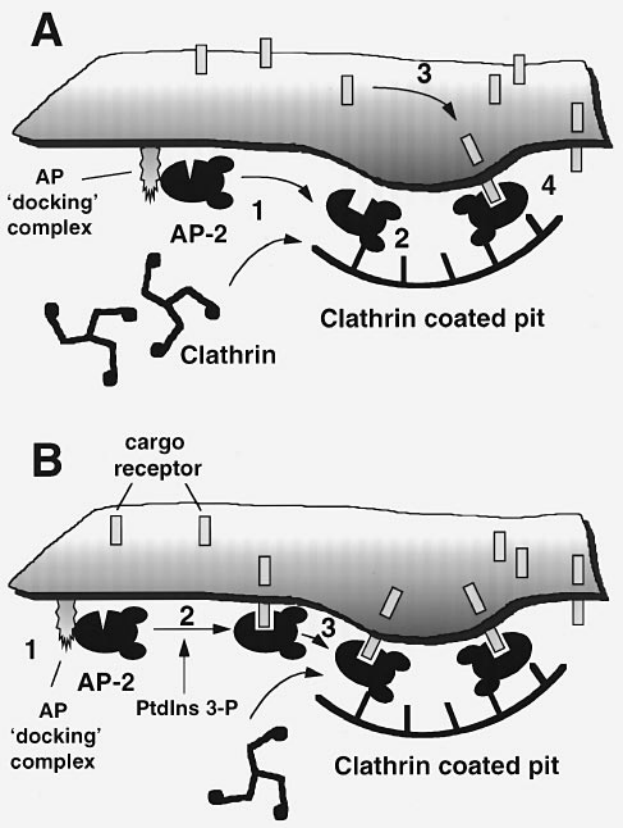

Fig. 11. Recognition of tyrosine-based endocytic signals by AP-2 complexes is enhanced by clathrin coats and by 3′-phosphoinositides. Proposed models for the capture of membrane cargo receptors by clathrin-coated pits. (**A**) AP-2 complexes are first targeted from the cytosol to the plasma membrane by interaction with its putative membrane-bound docking complex where it interacts with cytosolic clathrin to form the lattice of coated pits (1). AP-2 complexes located in partially formed coated pits (2) have a clathrin-dependent increase in the affinity of the μ2 chain for receptor tails. This leads to the capture of mobile receptors (3) into the coated pit (4). It is proposed that the linkage between clathrin binding to APs and their increased affinity for receptors ensures that coated pit assembly is coupled to receptor sorting. (**B**) AP-2 complexes targeted to the plasma membrane by its putative membrane-bound docking complex can interact with membrane-bound 3′-phosphoinositides (2) leading to an increase in the affinity of the μ2 chain for the tyrosine-based endocytic signal located in the cytoplasmic tail of membrane receptors. The AP–receptor complex (3) recruits cytosolic clathrin to form a coated pit or can be captured by available clathrin already located at the edge of a coated pit.

coated pits forming at the plasma membrane of cells bearing the receptors for LDL, EGF, asialoglycoproteins, mannose 6-phosphate and IgA generally show significant cargo occupancy of most coated structures (Carpentier *et al.*, 1982; Geuze *et al.*, 1984; Leser *et al.*, 1996). The problem of ensuring that vesicles only form when they contain cargo is a general one, pertaining not just to AP-2-mediated traffic, but also to the related clathrin–AP-1 system in the *trans*-Golgi network, to other systems like the novel non-clathrin AP-3 complex, whose subunits have sequences that are highly related to AP-1 and AP-2 (Pevsner *et al.*, 1994; Newman *et al.*, 1995; Stepp *et al.*, 1995) but whose cellular targets remain as yet unidentified, and to the coatomers that control traffic in the ER–Golgi system.

The effect of phosphoinositides on receptor tail recognition

There is a growing body of evidence indicating that changes in the phosphorylation state of the inositol ring of phosphoinositides regulate key steps in vesicular membrane traffic (see the recent reviews by DeCamilli *et al.*, 1996 and Veithen *et al.*, 1996). Proposals for the mechanism of this regulation have included: specific recruitment of coat proteins such as AP-2, AP180 and dynamin to membranes; activation of ARF1, a small GTPase known to promote recruitment of the coatomer COPI or the clathrin AP-1 complex to *trans*-Golgi network membranes; and an increase in the local negative charge density on the cytosolic side of the membrane to facilitate curvature and membrane budding. These ideas are based on genetic studies in yeast and on pharmacological and genetic studies in mammalian cells. In yeast, elimination of Vps34p, a PtdIns 3-kinase, interferes with the vesicular traffic from the Golgi complex to the vacuole (reviewed in DeCamilli *et al.*, 1996), and elimination of a PtdIns 4-P 5-kinase appears to block the exocytic traffic from the vacuole (Ludwig *et al.*, 1995). Treatment of mammalian cells with Wortmannin, a fungal toxin that inhibits most but not all PtdIns 3-kinases (Carpenter and Cantley, 1996), leads to relatively minor effects on the endocytic step but to substantial alterations in endosomal sorting of constitutively trafficked proteins such as the transferrin or mannose 6-phosphate receptors and cathepsin D (Davidson, 1995; Martys *et al.*, 1996; Spiro *et al.*, 1996). It is important to note that treatment with Wortmannin completely blocks formation of PdtIns 3,4-P_2 and PtdIns 3,4,5-P_3 in mammalian cells but only causes a 70% decrease in the cellular content of PtdIns-3-P (B.Duckworth and L.Cantley, unpublished). In accordance with these results, a Wortmannin-insensitive phosphatidylinositol-specific PtdIns 3-kinase isolated from mammalian cells has been characterized recently (Feng and Pawson, 1994). Therefore, the absence of an effect on endocytosis does not rule out a role for PtdIns 3-P in regulating endocytic traffic. Phosphoinositides also influence the traffic of proteins that internalize in response to ligand binding. For example, a point mutation in the cytoplasmic tail of the PDGF receptor that blocks binding of p85-p110 PtdIns 3-P kinase does not affect endocytosis but does prevent sorting of the receptor from the endosomal to the lysosomal compartment (Joly *et al.*, 1994, 1995).

The observations just summarized clearly implicate phosphoinositides, particularly those phosphorylated at D-3, in the control of vesicular traffic. We have now shown that isomers of phosphoinositides phosphorylated at D-3, and most prominently PtdIns 3-P, have AP-2 as a direct target, leading to a significant enhancement in its capacity to recognize tyrosine-based endocytic signals. The only other known targets for some members of this class of phosphoinositide isomers are the calcium-independent kinase C isoform PKCε (Kauer *et al.*, 1986), SH2 domains (Kapeller *et al.*, 1993) and the serine kinase Atk (Franke *et al.*, 1997). However, these proteins bind PtdIns 3,4-P_2 or PtdIns 3,4,5-P_3 but fail to bind PtdIns 3-P. Thus, AP-2 is the first protein shown to be a specific target for PtdIns 3-P, which has hitherto been an orphan ligand.

We have not yet determined the identity of the AP subunit that makes contact with the 3′-phosphinositides. Earlier experiments have shown that inositol phosphates and phosphoinositides, even when phosphorylated at other ring positions, bind to the α chain of AP-2 (Gaidarov *et al.*, 1996). Since this interaction inhibits AP-2 self-aggregation (Beck and Keen, 1991b) and co-assembly with clathrin (Beck and Keen, 1991a), it was proposed that phosphoinositol binding could be a mechanism for maintaining APs free in the cytosol, thereby preventing formation of empty coats. The inhibitory effects, which have an apparent K_d of 0.1–5 μM, do not, however, require phosphorylation of D-3, and they are obtained with inositol phosphates as well as with phosphoinositides. In contrast, our studies indicate that AP-2 will respond only to phosphoinositides with a strict requirement for D-3 phosphorylation and with a K_d in the 20–50 μM range. Inositol phosphates do not affect the ability of AP-2 to recognize the tyrosine-based endocytic motif even when tested at high concentrations (up to 400 μM). Thus, there seem to be two different regulatory targets within the AP-2 complex, one that prevents AP aggregation and interaction with clathrin, and another that stimulates the capacity of μ2 to recognize a tyrosine-based endocytic motif.

Conclusion

We have found that clathrin enhances the capacity of AP-2 complexes to recognize an endocytic motif, and we propose that in cells, coat assembly and cargo recognition are likewise coupled. Our observations that AP-2 is a target of 3′-phosphoinositides, and that interaction with these lipids can regulate the recognition of endocytic signals, provide a distinct mechanism for increasing the specificity of sorting. The two regulatory interactions we have detected operate at complementary levels. Sensitivity to 3′-phosphoinositides is likely to be the endpoint of specific signaling pathways. Sensitivity to clathrin appears to be a more general mechanism for discriminating against budding of unloaded coated vesicles. It is possible that similar rules of regulation may apply to other AP complexes, which act in the endosomal or the *trans*-Golgi network compartment.

Materials and methods

Synthesis of peptides

Several peptides were synthesized and are represented schematically in Figure 1. The synthetic peptide YQRL (of sequence KVTRRPKAS-

DYQRLNL) corresponds to the carboxy-terminal region of the cytoplasmic tail of TGN38 and was shown previously to bind purified AP-2 complexes (Boll *et al.*, 1996). The peptide AQRL contains A instead of Y and does not bind to purified AP-2 (Boll *et al.*, 1996). This point mutation was chosen as a negative control because TGN38 bearing the modification fails to internalize (Bos *et al.*, 1993; Humphrey *et al.*, 1993). The photoreactive peptides *YQRL and *AQRL correspond in sequence to the peptides YQRL and AQRL but have A replaced by the photoreactive probe BPA at position Y-3, biotin added to the N-terminal lysine and are missing the C-terminal N and L residues. Biotin was added in order to identify the cross-linked products, as described below. A second photoreactive peptide that is similar to *YQRL but with BPA instead of P at position Y-5 was also prepared. A third photoreactive peptide was made containing BPA at Y+5, preceded by N and followed by K and L. The positions chosen for BPA replacement, Y-3, Y-5 and Y+5, are not important for recognition by purified AP-2 (Boll *et al.*, 1996). The peptide YRAL (of sequence DSNFYRALMDEED-MDDVVDADE) corresponds to the region 970–991 of the cytoplasmic tail of the EGFR that binds to AP-2 complexes (Nesterov *et al.*, 1995b; Sorkin *et al.*, 1996). The peptide YQTI (of sequence RKRSHAGYQTI) corresponds to the complete cytoplasmic tail of lamp-1 and has been shown to bind to purified AP-1 and AP-2 complexes (Honing *et al.*, 1996). The peptides were synthesized as previously described using an Applied Biosystems 430A peptide synthesizer by standard protocols using BPA (Kauer *et al.*, 1986; Shoelson *et al.*, 1993). Biotin was dissolved in dimethylsulfoxide/*N*-methylpyrrolidone (1:1) and coupled to the ε-amino group of the N-terminal lysine. The peptides were purified by gel filtration on a Biogel P2 column and analyzed by HPLC, amino acid analysis and mass spectrometry. Stock solutions of peptides were dissolved in water (2–10 mM) and kept at –20°C. Prior to use, the peptides were diluted further with water to 2 μM.

Expression vectors

μ1 and μ2 are the homologous medium chains of AP-1 and AP-2 (Ahle *et al.*, 1988; Thurieau *et al.*, 1988; Nakayama *et al.*, 1991). A truncated form of human μ2, amino acids 122–435, and a truncated form of rat μ1, amino acids 121–423, were appended to GST (GST–Δμ2 and GST–Δμ1, respectively) of the expression vector pGEX (Pharmacia, Piscataway, NJ). Expression of the GST fusion proteins in *Escherichia coli* and subsequent purification using glutathione–agarose beads (Sigma Co., St Louis, MO) were performed as described (Smith and Johnson, 1988).

Purification of clathrin and AP-2 and preparation of coats

Clathrin and AP-2 complexes were obtained from calf brain coated vesicles (Gallusser and Kirchhausen, 1993) and AP-2 complexes were purified by ion exchange chromatography on a hydroxyapatite column (Econo-Pac, Bio-Rad) (Pearse and Robinson, 1984; Boll *et al.*, 1995). Clathrin coats containing AP-2 complexes were generated by overnight dialysis against coat assembly buffer [100 mM NaMES, pH 6.6, 1 mM EDTA, 0.2 mM dithiothreitol (DTT) and 0.1% Triton X-100]. Inclusion of Triton X-100 leads to a substantial reduction in the amount of AP-2 aggregates (Gallusser and Kirchhausen, 1993; Sosa *et al.*, 1993). In the absence of clathrin, most of the AP-2 complexes eluted at the same position as AP-2 solubilized in AP buffer containing 0.5 M Tris as determined by gel filtration. For the cross-linking experiments, coats were separated from aggregates by low speed centrifugation (10 000 r.p.m. for 10 min at 4°C) and then separated from non-assembled AP-2 and clathrin by high speed centrifugation (60 000 r.p.m. for 12 min at 4°C, TLA-100 rotor, Beckman Optima TLX). Coats, contained in the high speed pellet, were resuspended at room temperature in coat assembly buffer.

Preparation of lipids

The phosphoinositides PtdIns 3-P, PtdIns 4-P, PtdIns 3,4-P_2, PtdIns 4,5-P_2 and PtdIns 3,4,5-P_3 were suspended at 1 mg/ml in 10 mM HEPES, 1 mM EGTA, pH 7.0 and sonicated in a cup water bath sonicator (Branson) at 40% full power for 5 min at room temperature. In some cases, PC (1 mg/ml) was mixed with various amounts (total of 1 mg/ml) of PtdIns 3-P and PtdIns 4-P before sonication. The micelles were kept on ice and used within 1 h after formation. The phosphoinositols Ins 1,3,4-P_3, Ins 1,4,5-P_3 and Ins 1,3,4,5-P_4 were dissolved at 10–30 mM in 10 mM HEPES, 1 mM EGTA, pH 7.0. Ins 1,2,3,4,5,6-P_6 (Sigma Co.) was dissolved at 20 mM in AP buffer titrated to pH 7.0.

UV-induced cross-linking reaction

Immediately before a cross-linking experiment, the buffer for the protein samples was changed to coat assembly buffer by gel filtration (NAP-5 columns; Pharmacia) or the samples were kept in AP buffer (100 mM NaMES, 1 mM EDTA, 150 mM NaCl, 0.02% NaN_3, 0.5 mM DTT, 0.1% Triton X-100, pH 7.0) in all the experiments with phosphoinositols and phosphoinositides. To rule out the possibility that AP-2 aggregation might affect the efficiency of cross-linking, control experiments were also performed with purified AP-2 maintained in Tris-containing buffer (0.1 or 0.25 M Tris, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.5 mM DTT, pH 7.4) in which APs are known to remain unaggregated (Keen *et al.*, 1979). Then, 10–18 μl of a solution containing the protein samples (final AP-2 concentration in the range of 0.13–0.6 mg/ml corresponding to a μ2 concentration in the range of 0.02–0.1 mg/ml) were mixed with 2 μl of the photoreactive peptides *YQRL or *AQRL (0.2 μM final concentration) and either one of the competing YQRL or AQRL peptides (final concentration in the range of 0–200 μM). When appropriate, 1–8 μl of phosphoinositols or phosphoinositides were also added to the reaction mixture. The cross-linking experiments were carried out in microtiter plates (Falcon 3911 microtest III) in a final volume of 20–22 μl. The plate containing the mixtures was maintained on top of a layer of ice for 20–30 min in total darkness. The cross-linking reaction was triggered by a 3 min exposure of the microtiter plate (still on ice) to UV radiation from a mercury lamp (H44GS-100, Osram Sylvania Inc.) placed at a distance of 10 cm from the top of the plate. Following the cross-linking reaction, 5 μl of 5× Laemmli sample buffer containing β-mercaptoethanol was added and the samples were boiled for 3 min.

Detection of cross-linked products

After a cross-linking experiment, the samples were divided into two 10 μl portions, one for protein content analysis and the other for identification of cross-linked products. We corroborated that in each experiment the samples contained equivalent amounts of protein by carrying out SDS–PAGE and Coomassie blue staining on the first aliquot. To identify the AP protein subunits cross-linked to the biotinylated photoreactive peptide, the second aliquot was also subjected to SDS–PAGE. The proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell), which was then blocked by incubation with a solution containing 10% (w/v) of non-fat dry milk (Nestle Food Co.) in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20) for 1 h at room temperature or overnight at 4°C. The membrane was washed once in TBST followed by 1 h incubation at room temperature with streptavidin conjugated with horseradish peroxidase (HRP, Zymed; 5 μl/ml) in a solution containing 5% (w/v) of the dry milk in TBST. Finally, the membrane was washed three times (10, 5 and 5 min) with TBST and immediately processed for enhanced chemiluminescence (Amersham Corp.) using photographic film for detection (X-OMAT, Kodak). Different exposure times and sample loadings were used for quantitation making sure that the ECL reaction was in the linear response range. Digitized images of the labeled bands were analyzed using MacBAS (Fuji Co.).

Acknowledgements

We thank Dr J.Setleman (Massachusetts General Hospital) for kindly providing us with the GST–Δμ2 construct and Dr Y.Chen for providing us with the lamp-1 peptide. We express our special thanks to the other members of T.K'.s lab for continuous help and discussions. This study was supported with grants from the NIH (T.K.), DK 43123 (S.S.), GM 41890 (L.C.), and from support funds from the Department of Cell Biology, Harvard Medical School (I.R.).

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Received on November 4, 1996; revised on December 30, 1996