Phosphoinositide–AP-2 Interactions Required for Targeting to Plasma Membrane Clathrin-coated Pits

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Abstract. The clathrin-associated AP-2 adaptor protein is a major polyphosphoinositide-binding protein in mammalian cells. A high affinity binding site has previously been localized to the NH_2-terminal region of the AP-2 α subunit (Gaidarov et al. 1996. *J. Biol. Chem.* 271:20922–20929). Here we used deletion and site-directed mutagenesis to determine that α residues 21–80 comprise a discrete folding and inositide-binding domain. Further, positively charged residues located within this region are involved in binding, with a lysine triad at positions 55–57 particularly critical. Mutant peptides and protein in which these residues were changed to glutamine retained wild-type structural and functional characteristics by several criteria including circular dichroism spectra, resistance to limited proteol-ysis, and clathrin binding activity. When expressed in intact cells, mutated α subunit showed defective localization to clathrin-coated pits; at high expression levels, the appearance of endogenous AP-2 in coated pits was also blocked consistent with a dominant-negative phenotype. These results, together with recent work indicating that phosphoinositides are also critical to ligand-dependent recruitment of arrestin-receptor complexes to coated pits (Gaidarov et al. 1999. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:871–881), suggest that phosphoinositides play a critical and general role in adaptor incorporation into plasma membrane clathrin-coated pits.

Key words: clathrin • adaptor • phosphatidylinositols • endocytosis • adaptins

RECEPTOR-MEDIATED endocytosis is a multistep process by which certain cell surface proteins are specifically and efficiently internalized into cells through plasma membrane coated pits. Clathrin, the major structural component of the cell surface coated pit, is a triskelion-shaped protein that forms the regular polygonal surface lattice of the coat and provides its structural integrity. Another stoichiometric component of the coat is the multimeric protein complex termed adaptor or AP[1], for assembly or associated proteins. Most of the APs are heterotetrameric proteins and multiple forms have been identified (reviewed in Schmid, 1997). The best characterized of these are the AP-1 and AP-2 proteins, which are involved in clathrin coat formation and sorting at the Golgi and plasma membrane, respectively. These AP molecules consist of two large subunits (γ and β' in AP-1 and α and β in AP-2), a medium subunit ($\mu1$ in AP-1 and $\mu2$ in AP-2), and a small subunit ($\sigma1$ in AP-1 and $\sigma2$ in AP-2). The α subunits have some homology to the γ subunit and both are very distantly related to the β/β' subunits (reviewed in Keen, 1990; Schmid, 1997).

AP-2 is critical for two of the key functions of the early steps of the endocytosis pathway: the formation of the clathrin lattice and selection of specific cargo proteins for internalization. AP-2 interacts with clathrin through the α and β subunits (Goodman and Keen, 1995; Shih et al., 1995) and promotes coat formation (Prasad and Keen, 1991). Interaction of the AP-2 μ subunit with receptors containing tyrosine-based internalization motifs contributes to their localization to coated pits (Ohno et al., 1995; Sorkin et al., 1995; Boll et al., 1996; Heilker et al., 1996; Shiratori et al., 1997), and there is evidence that AP-2 may interact with other classes of internalization signals as well (Rapoport, 1998; Rodionov and Bakke, 1998).

The function of AP-2 in endocytosis is probably modulated by multiple factors. Protein–protein interactions of AP-2 with other macromolecules implicated in the endocytosis pathway such as dynamin (Wang et al., 1995), synaptotagmin (Zhang et al., 1994), amphiphysin (David et al., 1996), Eps15 (Benmerah et al., 1996; Iannolo et al.,

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1. *Abbreviations used in this paper:* AP, assembly or associated protein; CD, circular dichroism; MBP, maltose-binding protein; PH, pleckstrin homology; PPI, polyphosphoinositide or inositol polyphosphate.

1997), and Shc (Okabayashi et al., 1996) have been identified. In addition, AP-2 has also been shown to bind with high affinity to certain polyphosphoinositides and inositol polyphosphates (collectively referred to here as PPI) (Beck and Keen, 1991; Timerman et al., 1992; Voglmaier et al., 1992; Gaidarov et al., 1996). In recent years polyphosphoinositides have been implicated in different vesicular trafficking events. In yeast, the VPS34 protein, a homologue of a catalytic subunit of a mammalian PI-3 kinase, has been shown to be required for the sorting of newly synthesized vacuolar enzymes from the Golgi to the vacuole (Schu et al., 1993; Stack et al., 1993; Stack and Emr, 1994). In mammalian cells, a similar trafficking route from the Golgi to lysosomes was blocked by wortmannin, a potent inhibitor of PI-3 kinase (Brown et al., 1995; Davidson, 1995). PI-3 kinase has also been implicated in the endocytosis (Gommerman et al., 1997) or postendocytic trafficking (Joly et al., 1994, 1995) of growth factor receptors and in the formation of constitutive exocytic vesicles at the Golgi (Jones and Howell, 1997). Additionally, phosphoinositides were shown to be involved in endocytic coated vesicle formation and endosomal membrane targeting of AP-2 in an in vitro system (West et al., 1997; Jost et al., 1998). Finally, many proteins involved in endocytosis either bind PPIs (AP-2 [Beck and Keen, 1991; Gaidarov et al., 1996]; synaptotagmin [Niinobe et al., 1994; Schiavo et al., 1996]; AP180 [Ye et al., 1995; Hao et al., 1997]; dynamin [Salim et al., 1996; Zheng et al., 1996; Barylko et al., 1998]), or are involved in their metabolism (synaptojanin [McPherson et al., 1996; Haffner et al., 1997; Woscholski et al., 1997]).

In previous reports we identified and characterized high affinity, stoichiometric binding of various PPIs to AP-2 (Beck and Keen, 1991), and determined that AP-2 in an assembled coat structure had unique PPI binding properties, exhibiting a preference for phosphoinositides over inositol polyphosphates (Gaidarov et al., 1996). Initial studies also indicated that comparatively high concentrations of PPIs (~20–50 μM) could inhibit AP-mediated coat assembly (Beck and Keen, 1991). Related effects have been observed in studies of the arrestin–clathrin interaction (Gaidarov et al., 1999). In both cases, a high affinity PPI binding site ($K_D \leq 0.1$ μM), which is the focus of this report, appears not be involved. We were subsequently able to localize the PPI binding site to the NH_2-terminal amino acids 5 to 80 of the α subunit of AP-2 (Gaidarov et al., 1996), a region that is identical at the amino acid level in the cognate α_A and α_C genes (Robinson, 1989). Taking into account this inositide specificity and the findings described above, we speculated that the interaction of PPIs with AP-2 could be important in its role in formation of plasma membrane clathrin-coated pits and/or in the recruitment of receptors. In this study we investigated this hypothesis in intact cells using a mutagenesis and intact cell expression approach. Our results indicate that a functional PPI binding site is indeed necessary for AP-2 recruitment to sites of clathrin-coated pit formation.

Materials and Methods

Materials

Clathrin was purified from bovine brain–coated vesicles as described

(Keen, 1987). D-myo-Inositol hexakiphosphate (IP_6) was obtained from Calbiochem. [³H]IP_6 was from DuPont NEN, ³⁵S-Translabel was from ICN. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-trypsin was from Worthington Biochemical, Inc. Restriction and modification enzymes were purchased from Boehringer Mannheim. S-Sepharose and Sepharose 2B were from Sigma Chemical Co. TnT rabbit reticulocyte transcription-translation system was from Promega. All other chemicals were reagent grade or better and were purchased from Sigma Chemical Co. or Fisher.

Construction of Deletion and Site Mutants in Maltose-binding Protein α5-80

Deletion and site-directed mutations in the α5-80 insert in the plasmid pMAL αA5-80 (Gaidarov et al., 1996) were performed using a combination of subcloning procedures and polymerase chain reaction. Details of these procedures are available upon request. The fusion proteins were expressed and purified as described previously (Gaidarov et al., 1996).

Preparation of Wild-type and Mutant α5-80 Peptides

Purified maltose-binding protein chimera were dialyzed into 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 2 mM $CaCl_2$, and digested with the Factor Xa (1 mg enzyme/200 mg fusion protein) for 24–36 h at 4°C. The digestion mixture was then incubated with amylose beads to remove MBP and undigested fusion protein. The supernatant was then applied to an S-Sepharose column (Pharmacia) and the column was washed with 20 mM sodium phosphate, pH 7.3, 200 mM NaF. The bound peptide was eluted with 20 mM sodium phosphate and 1 M NaF. The eluate was dialyzed into 10 mM sodium phosphate, pH 7.3, and 100 mM NaF, and used for circular dichroism spectroscopy. Purity of the peptide was checked by SDS-electrophoresis on an 8–25% gradient gel using a PhastSystem (Pharmacia).

Inositol Polyphosphate–binding Assay

The binding of [³H]IP_6 to recombinant fusion proteins was determined by a polyethylene glycol precipitation procedure as described previously (Gaidarov et al., 1996).

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy was performed on an Aviv-62DS instrument at ambient temperature under nitrogen atmosphere with peptides at 0.1–0.4 mg/ml in 10 mM sodium phosphate, 100 mM NaF. The CD spectra were analyzed using secondary structure prediction software based on the method described by Andrade et al. (1993).

In Vitro Transcription-Translation of Wild-type and Mutant AP-2α_A

Wild-type and mutant α_A subunits were expressed in vitro using a TnT rabbit reticulocyte lysate transcription-translation system. First, plasmid pSP65α_A (Goodman and Keen, 1995) was modified to optimize expression by removing most of the 5′ untranslated region beyond 10 bp upstream of the initiation codon of α_A cDNA. Analysis showed that the resulting plasmid pSP65α_A2 gave 5–10-fold higher expression than pSP65α_A. Mutant α_A cDNA fragments were cloned into the pSP65α_A2 by a series of subcloning procedures, the details of which are available on request. The resulting plasmid pSP65 α_AKKK-Q and the plasmid pSP65α_A2 were used for in vitro expression of mutant and wild-type α_A.

Transcription-translation reactions were performed according to the manufacturer's recommendations in the presence of ³⁵S-Translabel. After incubation for 2 h at 30°C, translation reactions were centrifuged at 100,000 rpm for 20 min at 4°C in a TLA100 rotor (Beckman).

Proteolysis and Clathrin Cage Binding Assays of In Vitro Translated α Polypeptides

Limited tryptic proteolysis and clathrin cage binding experiments with in vitro–translated wild-type and mutant AP-2α polypeptides were performed essentially as described previously (Goodman and Keen, 1995).

Expression of Wild-type and Mutant Adaptin Constructs in Mammalian Cells

Plasmid containing the αγα construct (derived from bovine AP-2α_C) in

pBluescript SK− (Robinson, 1993) was digested with EcoRI, treated with Klenow fragment and dNTP, and subsequently digested with SacI. Excised fragments were ligated into the plasmid pSP65 that had been digested with SacI and SmaI, resulting in the plasmid pSP65-dN-αγα. This plasmid was cut with SacI and SalI, and the excised fragment was ligated together with the linker connecting NdeI and SacI sites into the plasmids pSP65$_A$2 and pSP65 α$_A$KKK-Q from which NdeI-SalI fragments were excised. The resultant plasmids pSP65-WTαγα and pSP65-M18αγα contained wild-type and mutant αγα chimeric inserts. These plasmids were consecutively treated with SalI, blunted with Klenow fragment and dNTP, and then digested with EcoRI. The excised inserts were ligated into eukaryotic expression vector pcDNA3 that had been cut with EcoRI and EcoRV, resulting in plasmid constructs pcDNA3αγαWT and pcDNA3-αγαKKK-Q.

For transient expression, BALB/c-3T3 cells were grown in T-75 flasks in a humidified atmosphere with 5% CO_2 in DME supplemented with 5% fetal bovine serum, 5% calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were grown to 60–70% confluence and transfected with expression constructs using Lipofectamine reagent. In brief, 15 μg of pcDNA3αγαWT or pcDNA3αγαKKK-Q were incubated with 80 μl of Lipofectamine in 5 ml of DMEM for 30 min at room temperature. After the incubation 5 ml of DME was added and the mixture was transferred to a flask with DMEM-rinsed BALB/c-3T3 cells. After 4 h of incubation the mixture was substituted with complete media and the cells were incubated for 15–18 h. Cells were then trypsinized and plated on 12-mm round glass coverslips in a 24-well plate. Immunofluorescence analysis was performed 48 h after transfection.

Coimmunoprecipitation of β-Adaptin with αγα Polypeptides

MOP8 cells transiently transfected for 48 h with the wild-type αγα or KKK/Q mutant αγα constructs (3 T-75 flasks) were washed with PBS and scraped into 5 mM Tris-HCl, pH 7.0, supplemented with protease inhibitors. After homogenization, 1 M Tris-HCl, pH 7.0, was added to the broken cells to a final concentration of 0.5 M, the suspension was incubated for 30 min on ice and centrifuged at 100,000 rpm for 30 min in TLA100 rotor (Beckman). The supernatant was diluted to yield a concentration of 125 mM Tris-HCl and supplemented with protease inhibitors and Triton X-100 (0.05%). The AP-2 αγα protein was immunoprecipitated with the antibody 100/3 (Ahle et al., 1988) and resolved by SDS-PAGE. Western blot analysis was performed using antibodies R2 and 100/3, specific for the β- (Voglmaier et al., 1992) and γ-adaptins, respectively, as well as affinity-purified antibodies against μ2 and σ2 (Page and Robinson, 1995).

Immunofluorescence Microscopy Analysis

Immunofluorescence analysis was performed as described previously (Santini and Keen, 1996). In brief, cells were washed, fixed, permeabilized, and exposed to mouse monoclonal antibody 100/3 (50 μg/ml) for detection of αγα polypeptide and rabbit Ab31 (1:150) for detection of endogenous AP-2 (Sorkin et al., 1995). We have found that Ab31, though it was made against a fragment containing both the hinge and ear sequences of rat brain α$_C$, reacts only with α hinge and not with the ear domain (data not shown). Treatment of the α subunit with trypsin is known to generate two major fragments corresponding to the core and the intact hinge + ear domains, while elastase cleaves the ear fragment leaving intact core + hinge (Wilde and Brodsky, 1996). On immunoblotting, Ab31 recognized the intact hinge + ear domain generated by trypsin, but failed to recognize the separate ear domain produced by elastase. Antibody 100/2, specific for the α ear, recognized both the hinge + ear fragment generated by trypsin and the ear domain generated by elastase. This allowed us to use Ab 31 to detect endogenous α but not αγα, which lacks the hinge region of α.

Clathrin was detected using rabbit polyclonal antibody 27004 (1:150 dilution) (Willingham et al., 1981). Primary antibodies were detected with fluorescein-conjugated donkey anti–mouse IgG or rhodamine-lissamine–conjugated donkey anti–rabbit IgG secondary antibodies (Jackson ImmunoResearch). Confocal microscopy was performed on a Bio-Rad MRC-600 laser scanning confocal microscope, using a Zeiss Plan-Apo 63× 1.40 NA oil immersion objective. Images were collected sequentially in the photon counting mode using single line excitation.

Results

Dissection of the AP-2 α PPI Binding Site by Truncation Mutagenesis

In our previous study, using photoaffinity labeling and bacterially expressed fusion proteins, we localized the high affinity PPI binding site on the clathrin adaptor protein AP-2 to the region between residues 5 and 80 at the NH_2-terminal of the α subunit. To determine whether PPI binding could be localized to a shorter sequence within this region, we produced several maltose-binding protein (MBP) fusion proteins containing smaller fragments (Fig. 1). Among fusion proteins containing either residues 5–21, 21–80, 5–49, or 50–80, only those containing residues 21–80 retained specific IP_6 binding, with affinity similar to that of the full fragment 5–80 (Fig. 1); the other fusion proteins did not display any detectable binding. This suggested that the PPI binding site in the AP-2 α subunit may not be represented by a short stretch of residues, but that a relatively large portion of the sequence between amino acids 21–80 may be required to form a discrete domain with proper tertiary structure.

Site-directed Mutagenesis of the AP-2 α PPI Binding Domain

The AP-2 α sequence between residues 5 and 80 is a fairly basic region with several clusters of cationic residues. As it is likely that positive charges are involved in the interaction with the negatively charged phosphate groups of PPIs, we investigated more closely the role of these basic residues in IP_6 binding. Accordingly, we produced a series of fusion proteins of MBP with the AP-2 α5-80 fragment (denoted MBP-α5-80) in which each basic amino acid (10 lysines and 4 arginines) was changed to a glutaminyl residue. Glutamine was chosen because it contains a substantial side chain, similar to lysyl and arginyl residues, but is uncharged. Each of these fusion proteins was purified by affinity chromatography and tested for IP_6 binding.

We found that residues scattered throughout the α 5–80 region affected IP_6 binding, though to differing extents (Fig. 2). The mutations could be divided into several groups in terms of their effects on IP_6 binding: no reduc-
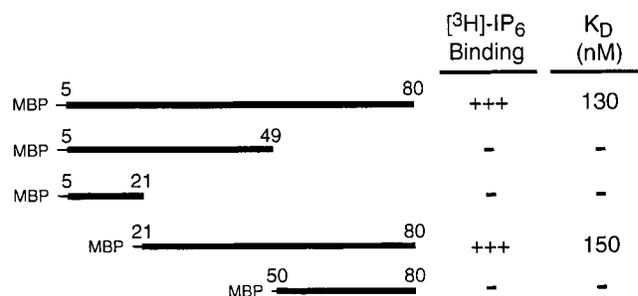

	[³H]-IP₆ Binding	K_D (nM)
MBP 5 ——————— 80	+++	130
MBP 5 ——— 49	−	−
MBP 5 — 21	−	−
MBP 21 ——————— 80	+++	150
MBP 50 — 80	−	−

Figure 1. A PPI binding site in the NH_2-terminal region of AP-2 α. Indicated segments of the AP-2α sequence coupled at their NH_2 terminus to MBP were analyzed for [³H]IP_6 binding and approximate binding affinity. A high affinity site is present within residues 21–80, while shorter segments do not retain specific binding activity.
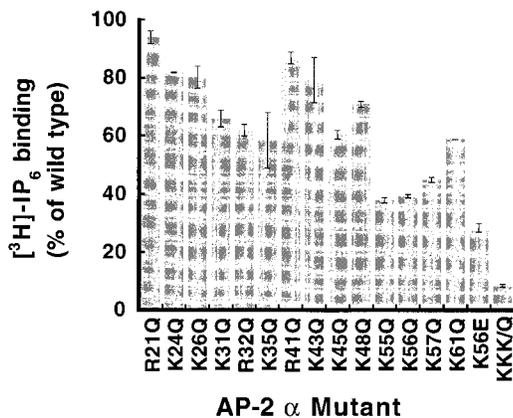
Figure 2. Site-directed mutagenesis of the PPI binding domain of AP-2 α MBP fusion proteins containing the indicated residue alterations were expressed and tested for retention of [³H]IP₆ binding. The lysine triad at positions 55–57 was most critical, and alteration to glutaminyl residues virtually abolished binding.

Figure 3. Secondary structure of the PPI binding domain of AP-2 α. (a) Circular dichroism spectrum in aqueous solution (solid line) of the isolated wild-type AP-2 α5-80 fragment cleaved from MBP reveals substantial secondary structure. In 50% trifluoroethanol (dashed line) it exhibits almost complete α-helical folding. (b) The circular dichroism spectra of the wild-type (solid line) and KKK/Q mutant (dashed line) AP-2 α5-80 polypeptides are virtually identical, consistent with retention of the secondary structure present in the native structure.

tion in IP$_6$ binding activity (R21); slight ($\sim$20%) reduction (K24, K26, R41, K43, K48); substantial ($\sim$40%) reduction (K31, R32, K35, K45, K61); and large ($>$60%) inhibition (K55, K56, K57). To investigate further the role of lysyl residues 55–57 whose alteration to glutamines had the most pronounced effect on IP$_6$ binding, we generated an additional mutant in which residue K56 was changed to glutamic acid with reversal of charge. The IP$_6$ binding ability of this mutant was even more greatly diminished, to $\sim$30% of the wild-type protein, compared with substitution with glutamine. When all three lysyl residues were changed to glutamines in a single mutant, denoted KKK/Q, the IP$_6$ binding ability was decreased to $<$10% of the wild-type protein. This mutant, essentially devoid of IP$_6$ binding, was characterized further using biophysical methods and functional assays described below to determine whether these residues are directly involved in PPI binding, or whether the decrease in PPI binding is the result of gross conformational change in the structure of the protein.

Structural Analysis of the Wild-type and Mutant AP-2 α PPI Binding Domains by Circular Dichroism Spectroscopy

CD spectroscopy is a very useful method for the rapid determination of secondary structures of peptides and proteins. We employed CD spectroscopy to characterize the secondary structure of the AP-2 PPI binding domain and to monitor any changes resulting from the mutations introduced in its sequence. The AP-2 α5-80 fragments of the wild-type and KKK/Q mutant fusion proteins were cleaved with Factor Xa and purified from MBP by consecutive affinity and ion-exchange chromatography steps as described in Materials and Methods. The purification procedure resulted in peptide preparations that were uniformly $>$95% homogeneous (data not shown). A CD spectrum of the isolated wild-type AP-2 α5-80 fragment is shown in Fig. 3 a. The positive absorption peak at 195 nm and two negative peaks at 207 and 222 nm indicate that
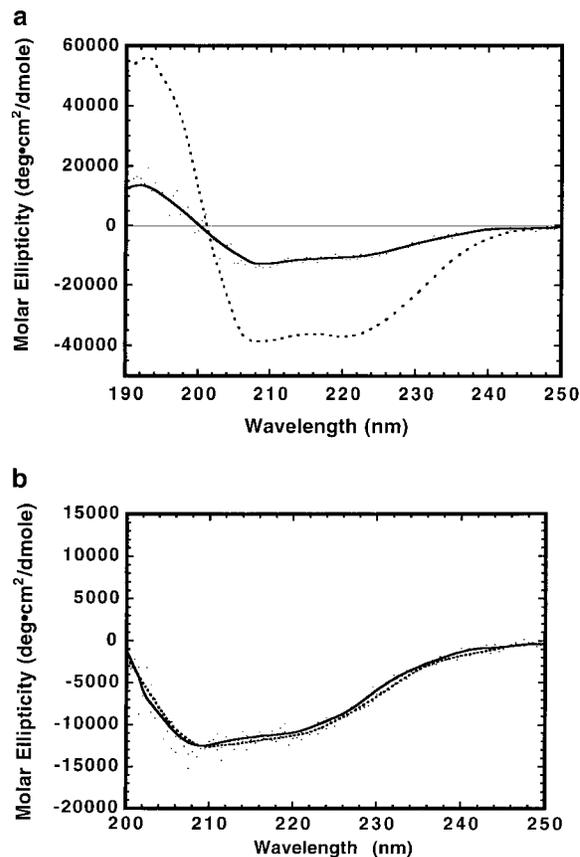
the conformation of the α5-80 peptide has substantial α-helical content. Secondary structure calculated by the method of Andrade et al. (1993) yielded estimates of $\sim$37% α-helix and 26% β sheet. Also shown in Fig. 3 a is the CD spectra of the wild-type AP-2 α5-80 fragment in 50% trifluoroethanol, known to induce an α-helical conformation in oligopeptides (Sonnichsen et al., 1992). Under these conditions, the spectrum of the wild-type fragment exhibited much more pronounced maximum and minima, corresponding to almost 100% α-helicity.

The CD spectrum of the mutant AP-2 α5-80-KKK/Q fragment is presented in Fig. 3 b, along with the spectrum of the wild-type peptide. The mutant α5-80-KKK/Q has secondary structure content practically identical to that of the wild-type protein. The CD spectrum of the purified peptide derived from the charge inversion mutant AP-2 α5-80-K56E was also indistinguishable from that of the wild-type (data not shown). These results argue that the decrease in PPI binding observed with these mutants is not the result of gross conformational changes induced by the

amino acid substitutions, but rather results from disruption of direct interactions between the ligand and basic residues on the protein.

We tried to determine whether ligand binding induces any conformational change in the PPI binding domain α5-80. Unfortunately, in the presence of IP_6 at concentrations as low as 1 μM the peptide aggregated. This problem could not be overcome by the addition of salt and/or nonionic detergents.

Structural and Functional Characterization of the KKK/Q Mutant in the Context of the Full-length In Vitro Translated AP-2 α Subunit

To further evaluate the effects of the KKK/Q mutation on the overall properties of the AP-2α subunit, we performed structural and functional assays on full-length wild-type and mutant AP-2 α polypeptides generated in a rabbit reticulocyte in vitro translation system. We have previously shown that the in vitro translated AP-2 α polypeptide is folded similarly to that in the native AP-2 complex isolated from bovine brain (Goodman and Keen, 1995). For example, limited tryptic proteolysis of in vitro–translated AP-2α generates fragments of ~55–66 kD and 40 kD, corresponding to the NH$_2$-terminal core and COOH-terminal appendage domain generated on similar treatment of the native bovine brain AP-2 (Zaremba and Keen, 1985). Similar limited proteolysis of in vitro translated mutant KKK/Q α_A polypeptide produced a pattern with the characteristic core and appendage domains, virtually identical to that of the wild-type polypeptide (Fig. 4 a). This result demonstrates that alteration of the K(55-57) residues in the NH$_2$-terminal region of the AP α polypeptide does not cause gross misfolding of the entire subunit on synthesis.

The isolated AP-2 α subunit generated by in vitro translation has also been shown to bind specifically to clathrin (Goodman and Keen, 1995). This provided a useful assay to ask whether lysyl residues 55–57 were directly involved in clathrin binding, or whether their substitution with glutamines altered structural properties of the isolated AP-2 α polypeptide required for this interaction. We found that binding of the in vitro–translated mutant KKK/Q α polypeptide to clathrin cages was indistinguishable from that of the wild-type polypeptide (Fig. 4 b). Collectively, these results indicate that the mutant KKK/Q AP-2 α polypeptide retained the native tertiary structure and function of the wild-type protein, but that it is essentially devoid of PPI binding ability.

Functional Characterization of Mutant KKK/Q AP-2 α in Intact Cells

To investigate the functional role of the AP-2 PPI binding site in intact cells, we employed an $\alpha\gamma\alpha$ construct described previously (Robinson, 1993) and kindly provided by Dr. M.S. Robinson (University of Cambridge). The $\alpha\gamma\alpha$ construct encodes mouse α_C polypeptide in which the hinge region between the core and appendage domains, corresponding to α_C residues 620–700, has been substituted with the hinge region of the bovine Golgi-specific AP-1 γ subunit. This enabled us to specifically localize the expressed $\alpha\gamma\alpha$ polypeptides in transiently transfected
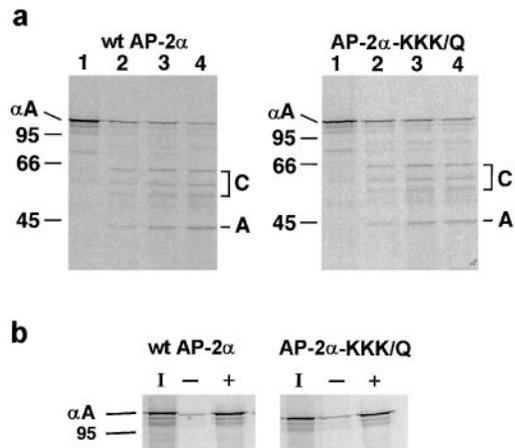
Figure 4. AP-2 α and the KKK/Q mutant lacking PPI binding have similar domain structure and clathrin binding activity. (a) In vitro translated wild-type AP-2 α subjected to limited proteolysis with 0, 50, 100, or 150 ng/ml trypsin (lanes 1–4, respectively) for 15 min at room temperature results in generation of fragments corresponding to the 60–66-kD core (C) and 40-kD appendage (A) domains, following preferential cleavage in the hinge region as previously described (Goodman and Keen, 1995). The cleavage pattern of the mutant AP-2 α KKK/Q translation product is virtually identical in terms of both protease sensitivity and fragments generated, indicating the presence of similar folded domains. (b) Most of input (I) of in vitro–translated wild-type AP-2 α binds to clathrin cages (30 μg) and becomes sedimentable in their presence (+), while little sediments in their absence (−), as previously reported (Goodman and Keen, 1995). The mutant AP-2 α KKK/Q translation product shows essentially identical behavior.

mouse fibroblasts using a γ-specific monoclonal antibody (mAb 100/3) which does not recognize the endogenous (mouse) protein (Ahle et al., 1988). Additionally, the endogenous AP-2 α polypeptide could also be uniquely localized using Ab31, a rabbit polyclonal anti-α antibody kindly provided by Dr. A. Sorkin (University of Colorado). We found that though Ab31 was produced by inoculation with a fragment consisting of the hinge and appendage domains of the rat brain α_C subunit (Sorkin et al., 1995), it reacts only with the α hinge region and not with the α appendage (see Materials and Methods). Thus, the endogenous and the transiently expressed exogenous α polypeptides could be detected independently, providing important tools for the study of mutant α subunits.

First, we asked whether wild-type and KKK/Q mutant AP-2 $\alpha\gamma\alpha$ polypeptides expressed after transfection are incorporated into AP-2 adaptor complexes in intact cells. Lysates of mock, wild-type $\alpha\gamma\alpha$, and KKK/Q mutant $\alpha\gamma\alpha$–transfected MOP8 mouse fibroblasts were challenged with monoclonal antibody 100/3, and the resultant immunoprecipitates were fractionated by SDS-PAGE and analyzed by immunoblotting with antibodies to the other AP-2 subunits. As shown in Fig. 5, reactivity with the anti-γ 100/3 antibody was detected only in immunoprecipitates from cells transfected with the wild-type or mutant $\alpha\gamma\alpha$ constructs, consistent with the inability of this antibody to recognize the endogenous mouse AP-2 γ polypeptide. On im-
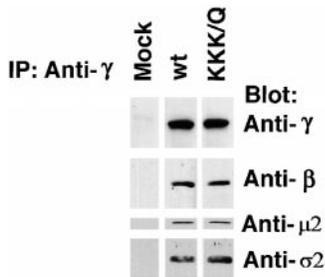
Figure 5. Wild-type and mutant KKK/Q AP-2 α polypeptides are associated with AP-2 subunits in intact cells. Equal amounts of lysates prepared under nondenaturing conditions from untransfected mouse MOP8 cells (mock) or cells transfected with either wild-type AP-2 αγα (wt) or mutant αγα (KKK/Q) cDNAs were immunoprecipitated (IP) with antibody 100/3, which recognizes only the bovine γ hinge insert present in the exogenous transiently expressed protein. Both the wild-type and PPI binding defective KKK/Q mutant AP-2 α polypeptides (revealed by blotting with anti-γ antibodies) are incorporated into AP-2 complexes as revealed by immunoblotting of the immunoprecipitates with anti-β, anti-μ2, and anti-σ2 antibodies.

munoblotting with antibodies to the β, μ2, or σ2 subunits of AP-2, no signal was detected in the immunoprecipitates from mock-transfected cell lysates demonstrating that recovery of the endogenous AP-2 subunits were dependent on their incorporation into complexes containing exogenous αγα polypeptide. However, anti-γ immunoprecipitates of cells transfected with either the wild-type or the KKK/Q αγα constructs contained the endogenous β2, μ2, and σ2 subunits in similar amounts. These findings confirm the results of Page and Robinson (1995) in indicating that the wild-type αγα polypeptide becomes incorporated into AP-2 complexes, which we denote AP-2$_{WT}$. Furthermore, the results presented here demonstrate that the mutant KKK/Q αγα polypeptide behaves indistinguishably from the wild-type, associating with the other AP-2 subunits and forming complexes (which we denote AP-2$_{PPI-}$) in the transiently transfected cells.

To investigate the cellular phenotype resulting from knockout of the PPI binding site of AP-2, we analyzed transfected BALB/c-3T3 cells by confocal fluorescence microscopy. Though the transfection efficiency of BALB/c-3T3 cells was lower than that of MOP-8 cells in our hands, the former were chosen for this experiment because their morphology after fixation is much more amenable to immunofluorescence analysis of plasma membrane coated pits. Cells transfected with wild-type (AP-2$_{WT}$) or mutant KKK/Q (AP-2$_{PPI-}$) αγα constructs were double-labeled with mouse monoclonal antibody 100/3 to reveal the localization of the exogenous αγα product, and with either rabbit Ab31 or 27004 to localize endogenous α-adaptin or clathrin, respectively.

Fig. 6 a shows the localization of the AP-2$_{WT}$ αγα product at several different expression levels in transiently transfected cells. The vast majority of the expressed AP-2$_{WT}$ protein (upper panels) had a punctate distribution in the plane of the plasma membrane, with very little diffuse signal detectable. Comparison with the distribution of endogenous AP-2 α (lower panels) indicated almost complete colocalization (Fig. 6 a). The images also show that the presence of the γ hinge did not misdirect the protein to the Golgi region. Consistent with this finding, the AP-2$_{WT}$ distribution was also largely coincident with the localization of plasma membrane coated pits stained with anti-clathrin antibody, but did not colocalize with anti-clathrin staining in the trans-Golgi network (data not shown). Similar observations were made by Robinson (1993) on her initial use of the αγα construct for expression in Rat1 cells.

At low expression levels, AP-2$_{WT}$ had no detectable effect on the distribution of the endogenous α adaptin (Fig. 6 a, left panels). Interestingly, in cells with higher levels of expression there is an apparent dominant-negative effect in that the level of endogenous α adaptin in clathrin-coated pits is decreased compared with untransfected cells in the same field (Fig. 6 a, center and right panels). Only at
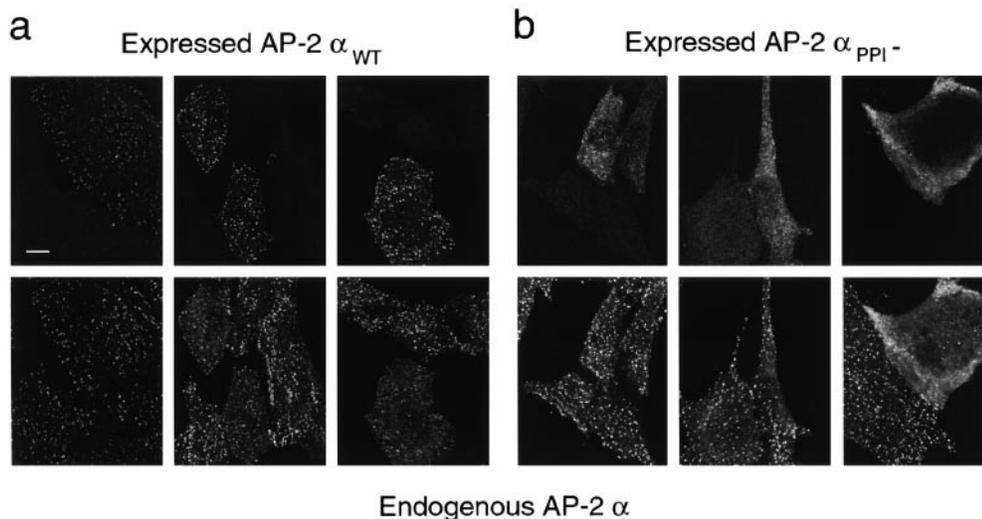

Figure 6. AP-2$_{WT}$, but not AP-2$_{PPI-}$, is targeted normally to clathrin-coated pits at the plasma membrane. (a) AP-2$_{WT}$ expressed in BALB/c-3T3 cells exhibits a punctate pattern entirely coincident with that of endogenous AP-2 α and is localized to plasma membrane clathrin-coated pits. With increasing levels of αγα expression (right panels), the incorporation of the endogenous AP-2 α polypeptide into coated pits is diminished. (b) Expressed AP-2$_{PPI-}$ does not colocalize with endogenous AP-2 α in coated pits. With increasing expression of AP-2$_{PPI-}$, correct localization of endogenous AP-2 to plasma membrane clathrin–coated pits is also impaired (right panels). Bar, 10 μm.

unphysiologically elevated levels of expression is there any evidence for significant accumulation of soluble AP-2$_{WT}$ protein (data not shown), and there is no detectable effect on the normal distribution of clathrin at the plasma membrane or in the Golgi region.

The localization of the mutant AP-2$_{PPI−}$ protein at several different levels of expression are shown in Figs. 6 b and 7. The distribution of the mutant protein differed radically from that of the wild-type protein. Generally, most of the AP-2$_{PPI−}$ localization was diffuse and at any level of expression, no significant amount of the mutant polypeptide could be detected in clathrin-coated pits at the plasma membrane. In some cells a small amount of finely punctate signal was detectable, most of which was intracellular. With few exceptions this signal did not coincide with that of endogenous AP-2 α (Fig. 6 b), nor did it colocalize with either early or recycling endosomes (labeled with endocytosed fluorescent transferrin), or with the late endosome/lysosomal compartment (labeled with endocytosed fluorescent dextran) (data not shown). Interestingly, with increasing expression levels of the mutant AP-2$_{PPI−}$ protein, the proper localization of AP-2 to discrete plasma membrane sites was diminished (Fig. 6 b, right panels).

Similarly, at low levels of mutant AP-2$_{PPI−}$ expression, the localization of clathrin to plasma membrane was not noticeably affected (Fig. 7, left and middle panels). However, clathrin localization was clearly abnormal at higher levels of mutant expression with a reduced number of plasma membrane coated pits present in comparison to adjacent, nonexpressing cells (Fig. 7, right panels). Interestingly, the clathrin signal in the Golgi region also seemed to be affected by elevated levels of AP-2$_{PPI−}$ expression, consistent with continuity between the plasma membrane and Golgi pools of clathrin.

Finally, we evaluated the internalization of the fluorescently tagged transferrin by cells expressing αγα constructs. In cells expressing low levels of either the AP-2$_{WT}$ or AP-2$_{PPI−}$ mutant proteins, internalization of transferrin was indistinguishable from that in neighboring cells that were not expressing either product (data not shown), consistent with the absence of an effect on coated pit distribution in these cells. Interestingly, transferrin internalization was greatly diminished in cells expressing moderate levels of the mutant AP-2$_{PPI−}$ protein, consistent with the disruption of clathrin-coated pits in that population. In contrast, AP-2$_{WT}$ did not detectably affect transferrin uptake until very high levels of expression were attained.

Discussion

In this study, we have sought to determine the importance of the high affinity PPI binding site located in the NH$_2$-terminal region of the AP-2α subunit in the process of receptor-mediated endocytosis. In previous reports we identified the polypeptide region involved in binding (Beck and Keen, 1991; Gaidarov et al., 1996) and provided in vitro evidence that PI-3,4,5-P$_3$, a product of phosphatidylinositol 3-kinase, is the ligand of highest known affinity for AP-2 in assembled coat structures (Gaidarov et al., 1996). Though these results suggested that PIP$_3$ is a physiologically relevant ligand for AP-2, in the absence of definitive data from intact cells this conjecture remains uncertain, as
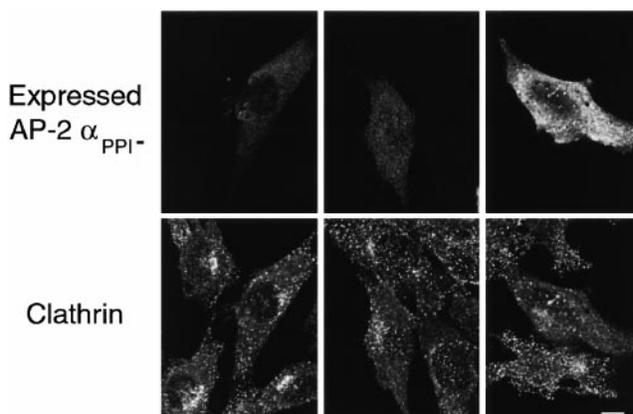

Figure 7. Clathrin distribution is normal at low expression levels of AP-2$_{PPI−}$, but is perturbed at higher levels. Bar, 10 μm.

does the precise identity of other ligand(s) that may interact with AP-2. Accordingly, we undertook a complementary approach to study the physiological function of the AP-2 PPI binding site. We sought to identify and alter amino acid residues critical for PPI binding to AP-2, and to evaluate the effects of expression of this mutant AP-2 in intact cells. The results of these efforts lead to the conclusion that an active PPI site is indeed required for AP-2 function in receptor-mediated endocytosis.

In some PPI-binding proteins short peptides (8–20 residues) have been found to be sufficient for high affinity binding of inositol phosphates or phosphoinositides. Examples include certain actin-associated proteins such as gelsolin (Janmey et al., 1992; Yu et al., 1992) and profilin (Raghunathan et al., 1992; Sohn et al., 1995) and some C2 domain–containing proteins such as synaptotagmin (Fukuda et al., 1994, 1995). Pleckstrin homology (PH) domains, which are found in a number of proteins involved in signal transduction and are believed to function in membrane recruitment and regulation of enzymatic activity (Shaw, 1996; Fukuda and Mikoshiba, 1997; Lemmon and Ferguson, 1998), provide a contrasting pattern. In these proteins essentially the entire ~100 amino acid module is necessary for high affinity ligand binding.

The mutagenesis analysis reported here suggests that the latter characterization is more applicable to the PPI binding site in AP-2 α. It has a highly organized secondary structure and seems to require a ≥60 residue region for full binding activity, from which we infer that this portion of the α structure comprises a distinct structural and functional domain. Positively charged amino acids throughout the region contribute to the binding interaction (Fig. 2), with two clusters of basic residues toward each end (a lysine triad at 55–57 and K31/R32/K35) appearing to be most important. In parallel with the PH domains whose tertiary structure in complex with ligands has been determined (e.g., that in β-spectrin; Hyvonen et al., 1995), the PPI binding region in AP-2 may be a large positively charged surface with some residues in direct contact with the bound ligand, while others may be responsible for the initial electrostatic recruitment of the PPI to the binding pocket or the formation of the charged surface. It is in-

creasingly appreciated that protein domains may have remarkably similar three-dimensional structure but share very limited sequence homology. Consequently, the relationship of the AP-2 binding site to other PPI binding domains with which it does not share detectable sequence identity will probably only be answered after determination of its tertiary structure.

This region of the AP-2 α sequence, and the basic residues in particular, are virtually entirely conserved in both *Drosophila* and *C. elegans* homologues of mammalian α_A. Furthermore, although the overall identity of two recently identified yeast α homologues with the mammalian protein in this region is 30–40%, most of the basic residues required for inositide binding in the mammalian protein, in particular the lysine triad, are also conserved (Fig. 8). This extends the inference of a functional PPI binding domain to these lower eukaryotes. Interestingly, the mammalian AP-1 γ, AP-3 δ, and recently identified ε subunit of a novel AP-4 complex show distinct but considerably less conservation of several of these basic residues (Fig. 8): to the best of our knowledge the PPI binding properties of these proteins have not been reported. Finally, the COPI coatomer (Chaudhary et al., 1998) and AP180 (Ye et al., 1995) also bind PPIs but have no discernible sequence similarity to AP-2 α. Collectively, these observations suggest that PPI binding by coat subunits involved in membrane transport is a ubiquitous phenomenon, and that the nature of specific residues in this binding domain may impart inositide binding specificity.

There is increasing evidence for an essential role of phosphoinositides in transport vesicle function at different locations in mammalian cells. Phosphoinositides, particularly PIP_2, formed secondarily to ARF activation of phospholipase D, have been implicated in the recruitment of COPI coat proteins onto the membranes of the Golgi stacks (Donaldson et al., 1992; Palmer et al., 1993; Ktistakis et al., 1996). The specific interaction between these acidic phospholipids and coatomer, which has been shown to bind PPIs and particularly PIP_3 with high affinity (Fleischer et al., 1994; Chaudhary et al., 1998), could contribute to recruitment of the coatomer to a specific membrane location, though this is controversial (Stamnes et al., 1998). With regard to AP-2, broken cell assays have shown that PIP_2 sequestration, accomplished either pharmacologically with neomycin or biochemically using the PH domain of PLCδ, had an inhibitory effect on AP-2 recruitment to the plasma membrane, indirectly implicating phospho-

inositides in AP-2 targeting (West et al., 1997; Jost et al., 1998).

Our in vitro binding data indicated that the (assembled) coat form of AP-2 shows the highest affinity for phosphoinositides, as compared with inositol phosphates, and that the converse is true for the soluble (disassembled) AP-2 protein (Beck and Keen, 1991; Gaidarov et al., 1996). These observations suggest that the presence of phosphoinositides will drive the AP-2 molecule toward its higher affinity, assembled form. This conjecture is supported by the observation reported here that AP-2 lacking a functional PPI site is not incorporated into coated pits. Further, it has been reported that the receptor cytoplasmic tail interaction of AP-2 with bound phosphoinositide is comparable to that with AP-2 in an assembled coat structure, and that both are of higher affinity than that with free AP-2 (Rapoport et al., 1998), again suggesting that inositide binding drives AP-2 toward an assembled conformation thereby promoting its ability to interact with clathrin.

The results reported here provide direct support for the notion that PPIs play a physiologically important role in membrane recruitment of AP-2. The mutant AP-2$_{PPI-}$ assayed in vitro is almost totally defective in PPI binding, but otherwise indistinguishable from the wild-type protein by multiple structural and functional criteria. However, in intact cells AP-2$_{PPI-}$ is almost completely defective in incorporation into plasma membrane clathrin-coated pits. Unlike the wild-type protein, it tends to have a diffuse distribution throughout the cell (Figs. 6 a and 7). At high expression levels, a small amount of punctate signal is also detectable which may reflect the inability of AP-2$_{PPI-}$ to bind PPIs and resist self-association (Beck and Keen, 1991). These observations are generally consistent with earlier results of Page and Robinson (Page and Robinson, 1995). Using α/γ chimeras, their results indicated that the plasma membrane/Golgi targeting signals are localized primarily between residues 130 and 330–350 in the α and γ sequences, respectively. Interestingly, chimeric proteins in which 132, or even 36, residues from the NH₂-terminal region of the AP-2α subunit had been replaced by corresponding γ sequences gave substantial diffuse signal and considerably reduced, though still detectable, recruitment to plasma membrane coated pits (for example see Page and Robinson, 1995; Fig. 3, A and B). This may reflect cooperation of the plasma membrane targeting signal, localized by these workers to the distal α sequence, with the action of a hypothetical PPI binding domain in the NH₂-
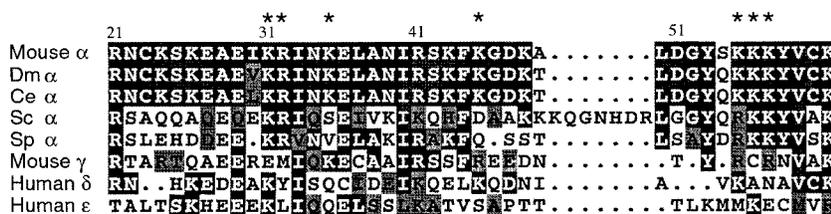
Figure 8. Basic residues involved in PPI binding in mammalian AP-2 α are highly conserved. Sequence alignments of mouse AP-2 α (Mouse α, accession number P17426) with *Drosophila melanogaster* (Dm α, accession number Y13092), *Caenorhabditis elegans* (Ce α, accession number U28742), *Saccharomyces cerevisiae* (Sc α, accession number P38065), *Schizosaccharomyces pombe* (Sp α, accession number AB004535), mouse γ adaptin (mouse γ, accession number X54424), human δ (accession number AF002163), and human ε (I.M.A.G.E. Clone ID 1031294; Dell'Angelica, E., personal communication). Sequence comparisons were performed with PILEUP, with numbering according to the mouse AP-2 α sequence. Residues identical to the mouse AP-2 α protein in four or more other sequences are highlighted on a black background; nonidentical but conserved residues in three or more sequences are shaded. Asterisks denote basic residues critical to PPI binding in the AP-2 α sequence.

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terminal region of the γ sequence (see above and Fig. 8). According to this reasoning, the AP-2$_{PPI-}$ is not detectably recruited to coated pits despite presence of a plasma membrane targeting signal because it lacks PPI binding.

Interestingly, AP-2$_{PPI-}$ also acts as a dominant-negative inhibitor of coated pit formation. This suggests that excess inactive AP-2$_{PPI-}$ complexes effectively sequester the other AP-2 subunits and/or occupy the limited sites that must be available for coat formation (Moore et al., 1987; Santini et al., 1998; Gaidarov et al., 1999; Subtil et al., 1999), indicating in either case that the binding to AP-2 of PIP$_3$ or another specific PPI ligand in the membrane is important early during the receptor mediated endocytosis process, i.e., at the stage of clathrin-coated pit formation. We have recently demonstrated that clathrin-coated pits form at specific and defined sites on the plasma membrane, and that a cytoskeletal framework in tight association with the membrane likely plays a major organizational role in this process (Gaidarov et al., 1999). Together, these results suggest a model in which coat formation is initiated and anchored by interactions oriented both outward toward the plasma membrane and inward toward a neighboring skeletal structure.

This general model is supported by our recent demonstration that PPIs are also involved in the ligand-dependent internalization of another class of receptors, the G-protein–coupled receptors. Nonvisual arrestins, which have been shown to act as adaptors in the internalization of β$_2$-adrenergic receptors (Ferguson et al., 1996; Goodman et al., 1996), bind PPIs with high affinity. We found that soluble PPIs and phosphoinositides differentially modulate arrestin interaction with clathrin and receptor. Furthermore, as in the case of the AP-2 adaptor reported here, a functional PPI binding site is critical to the ligand-dependent recruitment of the receptor-arrestin complex to clathrin-coated pits (Gaidarov et al., 1999).

Together, these findings point to common themes of phosphoinositide action in membrane trafficking events: they may serve either as recruitment signals for coat components and/or to modulate the interaction of coat components with receptor complexes. The presence in clathrin-coated pits and vesicles of synaptojanin (Haffne et al., 1997), a phosphoinositide 5-phosphatase, suggests that adaptor functions may be regulated by a complex interplay of different enzymes involved in site-specific phosphoinositide metabolism. Additional enzymes involved in adaptor/coat regulation, and the factors, which modulate their activity, are yet to be discovered.

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