A Functional Phosphatidylinositol 3,4,5-Trisphosphate/Phosphoinositide Binding Domain in the Clathrin Adaptor AP-2 α Subunit

IMPLICATIONS FOR THE ENDOCYTIC PATHWAY*

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Clathrin-coated pits are sites of concentration of ligand-bound signaling receptors. Several such receptors are known to recruit, bind, and activate the heterodimeric phosphatidylinositol-3-kinase, resulting in the generation of phosphatidylinositol 3,4,5-trisphosphate. We report here that dioctanoyl-phosphatidylinositol-3,4,5-P3 binds specifically and saturably to soluble AP-2 and with greater affinity to AP-2 within assembled coat structures. Soluble n-oleoyl-inositol hexakisphosphate shows converse behavior. Binding to bovine brain clathrin-coated vesicles is evident only after detergent extraction. These observations and evidence for recognition of the diacylglyceryl backbone as well as the inositol phosphate headgroup are consistent with AP-2 interaction with membrane phosphoinositides in coated vesicles and with soluble inositol phosphates in cytoplasm. A discrete binding domain is identified near the N terminus of the AP-2 α subunit, and an expressed fusion protein containing this sequence exhibits specific, high affinity binding that is virtually identical to the parent protein. This region of the AP-2 α sequence also shows the greatest conservation between a Caenorhabditis elegans homolog and mammalian α, consistent with a function in recognition of an evolutionarily unchanging low molecular weight ligand. Binding of phosphatidylinositol 3,4,5-trisphosphate to AP-2 inhibits the protein’s clathrin binding and assembly activities. These findings are discussed in the context of the potential roles of phosphoinositides and AP-2 in the internalization and trafficking of cell surface receptors.

Several distinct forms of coated membranes have been identified within eukaryotic cells. Of these, the best characterized to date is the clathrin-coated membrane, distinguished morphologically by its extremely regular polygonal structure. In addition, the coat also contains additional components in stoichiometric quantities termed APs (11–13). Two predominant and ubiquitous mammalian APs have been identified; denoted AP-1 and AP-2, they have been localized to the Golgi and plasma membrane regions, respectively. Each AP is a heterotetrameric protein containing two large subunits of about 100–115 kDa (α and β in AP-2, γ and β in AP-1) and two smaller subunits of 47–50 and 17–19 kDa (reviewed in Refs. 3, 4). Two closely related forms of the mouse brain α subunit occur, denoted αA and αC, that are 84% identical (5). These α subunits also share some homology with the γ subunit (~25% identity), but both show only a distant, although detectable, relationship to the β subunit (3).

The comparative structural complexity of the APs is mirrored in the diversity of their functional attributes. APs were initially appreciated for their ability to drive clathrin assembly under physiological solution conditions (6), and the α, γ (7), and β (8, 9) subunits can bind independently to clathrin. Plasma membrane AP-2 can also interact with the cytoplasmic domains of certain cell surface receptors (10), consistent with an adaptor role between the clathrin lattice and surface receptors. A high affinity binding site on AP-2 for polyphosphoinositols and polyphosphoinositides (collectively referred to here as PPI) was independently identified by several laboratories (11–13). This interaction is characterized by recognition of inositides such as PI-4,5-P2 in the context of a lipid micelle, and by binding of soluble IP6 with KD ~ 10–150 μM. Binding of PPI affects the properties of AP-2 including its ability to bind and assemble clathrin (11) and to undergo a specific self-association reaction (14). These observations suggest that one or more PPI species are important regulatory factors in AP-2 function and, by extension, in the membrane transport processes in which the protein is involved.

It is now well appreciated that PPIs are involved in multiple cellular functions. 4- and 5-phosphorylated inositides are generated at the plasma membrane and serve as substrates for the

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1 The abbreviations used are: AP, assembly or associated proteins; PPI, polyphosphoinositols and polyphosphoinositides; PI-P, phosphatidylinositol 3,4,5-trisphosphate; PI-3-kinases, phosphatidylinositol 3-kinases; diC8, dioctanoyl; MES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropylthiogalactoside; 8-Az-ATP, 8-azido-adenosine-5-triphosphate; PEG, polyethylene glycol; IP6, n-oleoyl-inositol hexakisphosphate; IP7, n-oleoyl-inositol 1,3,4,5-tetrakisphosphate; IP8, n-oleoyl-inositol 1,4,5-trisphosphate; IP9, n-oleoyl-inositol phosphate.
initiation of signaling cascades mediated by calcium gradients and protein kinase C-mediated phosphorylation events (reviewed in Refs. 15, 16). These PPIs are also thought to be involved in the modulation of cell structure through regulation of actin monomer-microrifilament dynamics (reviewed in Refs. 17, 18). Furthermore, 3-phosphorylated inositides, predominantly or exclusively phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃) in intact cells, are generated by members of a family of phosphatidylinositol 3-kinases (PI-3-kinases), the best characterized being the heterodimeric p85-p110α. These enzymes are activated in tyrosine kinase (19) and G protein-coupled receptor pathways (20). Cellular responses associated with receptor-induced PI-3-kinase activation include mitogenesis, membrane ruffling, and alterations in membrane trafficking (reviewed in Refs. 21–23). Many, if not all, of these receptors are internalized through plasma membrane-coated pits and vesicles, suggesting that PIP₃ will be generated in proximity to AP-2.

In this report, we provide direct evidence that a short chain, water-soluble form of PIP₃, di-octanoyl phosphatidylinositol 3,4,5-trisphosphate (dCyC₃PtdIns(3,4,5)P₃), binds to the high affinity PPI site on AP-2 and affect the protein’s function. We also report the identification of a discrete binding domain contained within a 75-residue sequence of the AP-2 subunit as a discrete functional binding domain.

**EXPERIMENTAL PROCEDURES**

**Materials**
DiC₈PtdIns(3,4,5)P₃ and diC₈-PtdIns(3,4,5)P₃ were synthesized as described by Kishita et al. (24). α-myo-inositol hexakisphosphate (IP₃), α-myo-inositol 1,3,4,5-tetrakisphosphate (IP₄), and α-myo-inositol 1,4,5-trisphosphate (IP₃) were obtained from Calbiochem. [α-32P]8-Azido-adenosine-5'-triphosphate (8-Az-ATP) was from ICN. [3H]IP₆, and [35S]labeled dATP was from Amersham Corp. PEG-8000 and the Sequenase version 2.0 DNA sequencing kit were obtained from United States Biochemical Corp., Biogen, and Perkin Elmer-Cetus. [3H]IP₆, and [32P]8-Azido-adenosine-5'-triphosphate (8-Az-ATP) were synthesized as described by Kiss et al. (27). [3H]IP₆ was measured in the presence of 10 μM unlaabeled IP₆.

**Photoaffinity Labeling—**Photoaffinity labeling studies were carried out essentially as described previously (21). Briefly, AP-2 was incubated with 8-Az-ATP (7-10 Ci/mmol) at 13 μM in 0.1 M sodium MES (pH 7.2), 0.5–1.0 mM EDTA, for 15 min at 4°C followed by irradiation with UV light (254 nm) for 1 min using a UVI-TS-15 Mineralight lamp (Ultra-Violet Products Inc.) at a distance of 2 cm. Samples were concentrated by precipitation with 9 volumes of methanol and 0.5 volumes of 1 M sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 1-mm thick 6% (w/v) gels using the buffer system described earlier (22). Either αA or αC was used, as previous work has shown that these two highly homologous polypeptides are labeled equivalently (11); for isolation of cDNA the resolving gel was supplemented with 6 M urea (30). Probes were visualized with 0.005% Coomassie blue, and the desired band was excised and electroeluted in 25 mM Tris base, 192 mM glycine, and 0.2% SDS using a Schleicher and Schuell Elutrap system run at 100 V overnight. Eluted protein was precipitated by addition of 10 volumes of ice-cold methanol. The pellet was sequentially washed with methanol, twice with 10% trichloroacetic acid, and finally with acetone/ether (4:1, v/v) followed by air drying

**Chemical Cleavage—**For cleavage at aspartyl-proline bonds (32), purified AP-2 α was dissolved in 7 M guanidine HCl in 70% formic acid (w/v). After incubation at 37°C for 24 h, 10 volumes of cold distilled water was added to stop the reaction; the mixture was concentrated to half of its volume in a Speed Vac, concentrated ammonium hydroxide was added; and evaporation was continued until dryness. The residue was then dissolved in SDS sample buffer and separated as described below.

For cleavage at methionyl residues (33), AP-2 αc polypeptide was resuspended in 70% formic acid containing cyanoan bromide at approximately 100-fold molar excess over αc methionine. The protein solution (about 1%) was incubated in the dark under argon for 24 h at room temperature, diluted with 10 volumes of water, and dried in a Speed Vac to remove the formic acid and excess reagent and resuspended for gel electrophoresis.

To determine whether 8Az-ATP could specifically label the cleaved CNBr peptides of AP-2 αc, the dried peptides were resuspended in 0.1 M MES (pH 7.2), 0.5 mM EDTA, and 0.1 mM dithiothreitol for several hours and photoaffinity labeled by 8Az-ATP as described above.

**Peptide Analysis—**Separation of peptides was performed by Tricine/SDS-PAGE (34). The gel system consisted of a 16.5% separating gel, a 10% spacer gel, and a 4% stacking gel, made with 29:1 acrylamide/bisacrylamide ratio. To prevent blockage of the N termini of peptides during SDS-PAGE, the gel was left at room temperature for at least 2 h following polymerization, and 0.2 mM thioglycolic acid was added to the cathode buffer and pre-electrophoresed into the gel for 45 min before the peptides were applied. Transfer of peptides to ProBlott membrane was performed with a Novablot electrophoretic transfer kit. It was run approximately 1 h at 0.8 mA/cm² of gel. Following transfer to Problott membrane and autoradiography or staining with 0.2% Coomassie blue, the required band was excised and placed in the reaction chamber of an Applied Biosystems 477A Protein Sequencer for N-terminal analysis. Sequence analysis was performed with the Genetics Computer Group Sequence Analysis Software Package Version 8.0-UNIX (Madison, WI).

**Construction, Expression, and Purification of Fusion Proteins—**To prepare glutathione S-transferase fusion protein with part of the AP-2 α sequence, a fragment of cDNA encoding amino acids 5–80 of the α subunit of mouse brain AP-2 was amplified by polymerase chain reaction using a 5' sense primer containing a BamHI site (5’-GCCATCAT- GCCGGCTGATCCAAAGGGC-3’), a 3’ reverse complement primer with an EcoRI site (5’-AAGCGGTATCCAGGCGTCTATGTC-3’), and a αDNA (5’-CAATGC-3’) followed by air drying. The amplification product was digested with BamHI and EcoRI and ligated into the expression vector pGEX-2T (Pharmacia Biotech Inc.) cut with the same enzymes. The resulting construct p2TαA5–80 was transformed into the Escherichia coli strain BL21 and recombinant protein induced by the addition of 0.1 mM IPTG for 1 h. Analysis of total cell proteins showed that most (>95%) of the recombinant protein was present in the particulate fraction of the cell.

Accordingly, we chose maltose binding protein as a fusion partner; plasmid p2TαA5–80 was digested with EcoRI, treated with Klenow fragment and deoxyxynucleotide triphosphate to generate blunt ends, and finally digested with BamHI. The resulting α insert was ligated into
the expression vector pMAL-c2 (New England Biolabs) that had been treated sequentially with Sall and with Klenow fragment and deoxyribonucleotide triphosphate, and finally with BamHI. The resulting construct, pMAL-A5–80, was transformed into E. coli strain BL21.

Plasmid pMAL-Lin, with a linker containing a stop codon inserted in the BamHI site of the pMAL-c2 polyclinker, was generated as follows: oligonucleotides 5'-AATTCGAGTACCA-3' and 5'-AGCTTAGATCATCGG-3' were hybridized to form a double-stranded adapter and ligated in the vector pMAL-c2 that had been treated with EcoRI and HindIII. The resulting plasmid encodes the protein (denoted MBP-Lin) consisting of wild type maltose binding protein plus the amino acids encoded by part of the pMAL-c2 polyclinker. In all cases, polymerase chain reaction products as well as the regions spanning the insertion sites were checked by sequencing after all subcloning procedures.

Cells carrying recombinant plasmids were induced for expression by 0.1 mM IPTG for 2 h. The expressed proteins MBP-A5–80 and MBP-Lin were purified from bacterial lysates by affinity chromatography on amylose resin following the manufacturer’s recommendations.

Other Techniques—AP-2 was extracted from purified bovine brain-coated vesicles and isolated by Superose 6B (Pharmacia) gel filtration as described previously (29). For photolabeling experiments, pure AP-2 was prepared from the fractions corresponding to the trailing half of the Superose 6B AP peak (fractions 38–40 in Fig. 2 of Ref. 29) which were pooled and fractionated by affinity chromatography on clathrin-Sepharose (29). For PPI binding and clathrin assembly assays, these steps were supplemented by ammonium sulfate concentration and a second Superose 6 FPLC gel filtration step in 0.5 M Tris-HCl (pH 7.0) at 0.25 ml/min. The resulting AP-2 preparation contained no visible contamination on Coomassie Blue-stained SDS-PAGE gels. Furthermore, immunoblotting with monoclonal anti-AP-3 antibodies (CLAP3) and comparison with hydroxylapatite-purified AP-3 standards (35) indicated that the purified AP-2 preparation contained less than 0.2% AP-3.

PNG Binding to Soluble AP-2—The work of several laboratories using a variety of ligands, experimental assays, and protein purification has contributed to defining a high affinity PPI site on AP-2 (11, 37, 38). Most of our earlier results were obtained employing inhibition of self-association of partially purified AP-2 as a functional, although indirect, assay. Here, we implemented a more direct PEG precipitation assay (25) and used it to measure competition by unlabeled ligands for [3H]IP6 binding to purified AP-2, free of other APs.

Our results confirm the presence of a single high affinity binding site for IP6 on purified AP-2, with an estimated KD ~0.10 μM and stoichiometry of ~0.6 mol/mol as determined by Scatchard analysis (Fig. 1 and inset). We also evaluated the binding of several other ligands by competition with labeled IP6 (Fig. 2 and Table I). 1,4,5-IP3 (KD ~4.7 μM) was considerably weaker than IP6 whereas 1,3,4,5-IP4 (KD ~3.0 μM) was intermediate in affinity. Collectively, these results are in good agreement with values that have been previously reported for PPI binding to AP-2 (11–13, 37, 38). Finally, ATP also displaced [3H]IP6 from AP-2 although it had much weaker binding affinity, with an estimated KD ~140 μM.

3-Phosphoinositides have been implicated in a number of pathways involving endocytosis, membrane dynamics, and cytoskeletal transformations. We were able to test two short-chain water-soluble forms of 3-phosphoinositides, diC8-P3 (PIP3) and diC8-P4, for their affinity for AP-2. Both exhibited specific and saturable binding to soluble AP-2 (Fig. 2). PIP3 had higher affinity for AP-2 than all PPIs tested except IP6. Notably, PIP3 also bound approximately 10-fold more tightly to the protein than did 1,3,4,5-IP4, the latter corresponding to the inositol phosphate moiety of the inositol. Furthermore, PI-3,4-P2 also bound more tightly to AP-2 than did 1,3,4,5-IP4 although its headgroup is not as highly charged as the latter. These observations provide evidence that the glycerol backbone and/or fatty acyl chains of the inositol contribute to recognition and binding by AP-2.

PPIs and Assembled Clathrin Coat Structures—We have previously reported that PPIs affect the clathrin coat assembly of partially purified preparations of APs (11). It has recently been demonstrated that the assembly activity of AP-3 (or AP180), another clathrin coat associated protein, is also inhibited by PPI (39, 40). It is therefore important to evaluate the effect of PPI on AP-2-mediated clathrin coat assembly using purified preparations devoid of other APs, in particular AP-3. Our pres-
ent results confirm that IP₆ inhibits pure AP-2-mediated coat assembly with an ID₅₀ ~ 20 μM; interestingly, diC₈-PIP₃ is even more effective than IP₆, with an ID₅₀ ~ 5 μM (data not shown). The greater concentrations needed to block assembly compared with reversible binding, by both AP-2 (reported here) and AP-3 (39), are likely a consequence of the assay differences; the equilibrium dissociation constants are derived from freely reversible binding assays, whereas the inhibition constants are end point measurements of essentially irreversible coat assembly reactions.

We also investigated binding of PPIs to AP-2 in assembled clathrin structures, whose organization is similar to the lattice structure of clathrin-coated pits and vesicles in cells. Coat structures prepared in the presence of clathrin and AP-2, containing stoichiometric amounts of each protein (6), bound IP₆ specifically and saturably (Fig. 3, A and B). Cage structures containing equivalent amounts of clathrin but lacking AP-2 exhibited essentially no binding, as expected (Fig. 3B). Interestingly, evaluation of the binding properties of AP-2 in assembled coat structures revealed the affinity to be greatest for ATP bound specifically and saturably to AP-2, although with much lower affinity than phosphoinositides. To begin to identify the PPI binding site, we had therefore utilized [α-³²P]-azido-adenosine-5'-triphosphate [8-Az-ATP], a readily available photoaffinity probe. Only the α subunit of the heterotetrameric AP-2 complex was specifically labeled by this reagent, and the reaction could be blocked by chelation of the polyphosphate with magnesium. Unlabeled ATP, and lesser concentrations of unlabeled glycerophospho-4,5-IP₂, also inhibited labeling, consistent with the relative affinities of the two molecules for AP-2. Upon limited tryptic digestion under non-denaturing conditions, photolabel was associated solely with the ~69-kDa N-terminal core domain of the αA subunit of the AP-2 C polypeptide, corresponding approximately to residues 1–620. The two sequences are highly homologous in this region, with 92% amino acid identity (5). In contrast, the 30–39-kDa C-terminal ear and hinge domains did not incorporate label (11).

We have now further refined localization of the incorporated label within the primary sequence of the protein. Photoaffinity labeled and electroeluted bovine brain AP-2 αA subunit was incubated in 70% formic acid in the presence of 7 M guanidine HCl (as described under "Methods") to yield hydrolysate as aspartyl-proline bonds (32). Based on predicted amino acid sequence, this treatment should yield peptides of 540 Da (residues 722–727), 6393 Da (921–977), and 21,576 Da (728–920), and 6393 Da (921–977), 21,576 Da (728–920), 21,743 Da (515–721), and 57,481 Da (1–514). Only one major photoaffinity labeled band of about 55 kDa was observed (Fig. 4A). Although no radioactivity was detected in the 620 Da region, a major labeled fragment with apparent molecular weight of 30–39 kDa contained the C-terminal ear and hinge domains. This treatment did not result in any significant change in the mobility of the labeled fragment compared with the unlabeled fragment (Fig. 4A).

We next utilized cyanogen bromide to cleave a mixture of photoaffinity labeled and carrier bovine brain αC subunit. CNBr cleavage of the αC polypeptide is predicted to yield 16 peptides ranging in size from 149 to 21,803 Da; of these, those greater than ~2000 Da could be detected as distinct or partially overlapping Coomassie Blue-stained bands on high resolution polyacrylamide gels (Fig. 4A). When αC preparations that had been photoaffinity tagged by 8-Az-ATP were analyzed in this manner, a major labeled fragment with apparent Mr ~7.7 kDa was observed (Fig. 4B). This radioabeled band migrated just slightly slower than a distinct Coomassie Blue-stained peptide, presumably due to anomalous migration induced by incorporation of the polyphosphate label (Fig. 4A).

### Table I

| Ligand | AP-2 (dissociated) | AP-2 (coats) | MBP-α5–80 |
|--------|--------------------|--------------|-----------|
| IP₆    | 0.10 μM            | 0.40 μM      | 0.3 μM    |
| diC₈-PIP₃ | 1.3 μM          | 3.2 μM       | 0.34 μM   |
| (1,3,4,5)-IP₄ | 3.0 μM        | 5.2 μM       | 0.4 μM    |
| (1,4,5)-IP₃ | 4.7 μM          | 30 μM        | 4.7 μM    |
| ATP    | 140 μM             | 350 μM       | 140 μM    |

**Fig. 3.** Inhibition of specific binding of [³H]IP₆ to AP-2 in assembled clathrin structures. Panel A, inhibition of [³H]IP₆ binding to clathrin coats by diC₈-PIP₃ (closed squares), IP₆ (open circles), and diC₈-PIP₃ (closed triangles). Curves are representative of two or more independent experiments in which data points are average of duplicate measurements. Panel B, specific [³H]IP₆ binding to pure clathrin cages (open circles), assembeled coat structures containing both clathrin and AP-2 (closed triangles), intact clathrin-coated vesicles (CVs), coated vesicles depleted of clathrin (C'CVs) by mild Tris-HCl treatment (see "Methods") and Triton X-100-extracted and coated vesicles (T-CVs), normalized per μg of protein.
The N-terminal sequence of this carrier peptide was found to be (M)RGLAVFISDIRN(64% identity overall) reveal the highest region of homology to a homolog of mammalian Caenorhabditis elegans homolog of mammalian αA that has recently become available (GenBank accession U28742) with mouse αA (64% identity overall) reveals the highest region of homology to be precisely within the PPI binding domain. This part of the AP-2 α sequence has several clusters of basic residues, a relatively generic hallmark of PPI binding sites in both cytoskeleton- and membrane-associated proteins (17). Interestingly, comparison at high stringency of the sequence of a 903-residue Caenorhabditis elegans homolog of mammalian αA that has recently become available (GenBank accession U28742) with mouse αA (64% identity overall) reveals the highest region of homology to be precisely within the PPI binding domain that we identify here, exhibiting 95% identity (Fig. 8). This can be interpreted to indicate that strong pressure has maintained a functional binding interaction for PPI, an evolutionarilyunchanging low molecular weight ligand.

Changes in the properties of AP-2 between the dissociated and assembled (coat) state have been observed previously in limited proteolysis studies (41). Such effects, presumably a consequence of conformational changes, are consistent with our observation here that the assembly state of AP-2 affects PPI binding. Conversely, we find that occupancy of the PPI site also affects the protein’s properties. Dissociated AP-2 binds IP₆ with high affinity, and physiological concentrations of IP₆ block AP-2 self-association and clathrin binding (11, 14), and this report). Thus, although pure AP-2 placed in physiological salt and pH will either readily aggregate or co-assemble with clathrin into membrane-free coats, such empty lattices have generally been observed in intact cells only under toxic conditions (42). We suggest that cytosolic inositol phosphates in the cytoplasm may normally be responsible for preventing these phenomena in vivo.

**DISCUSSION**

In this study we have sought to identify the locus of the binding site for PPI in the heterotetrameric AP-2 protein. Direct confirmation that a small portion of the N-terminal region of the AP-2 α subunit is responsible for binding was provided by the finding that a bacterial fusion protein containing α residues 5–80 (MBP-α5-80) binds PPIs specifically and saturably. Both the affinity and rank order of binding of various PPIs by native AP-2 were similar or identical to that of MBP-α5-80, suggesting that this portion of the α sequence comprises essentially all of the functional PPI binding domain. This part of the AP-2 α sequence has several clusters of basic residues, a relatively generic hallmark of PPI binding sites in both cytoskeleton- and membrane-associated proteins (17). Interestingly, comparison at high stringency of the sequence of a 903-residue Caenorhabditis elegans homolog of mammalian αA that has recently become available (GenBank accession U28742) with mouse αA (64% identity overall) reveals the highest region of homology to be precisely within the PPI binding domain that we identify here, exhibiting 95% identity (Fig. 8). This can be interpreted to indicate that strong pressure has maintained a functional binding interaction for PPI, an evolutionarilyunchanging low molecular weight ligand.

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There is strong evidence to suggest that AP-2 in the coated pit and vesicle interacts with membrane phosphoinositides. Direct binding of purified AP-2 to PI-4,5-P$_2$ (11), PI-3,4-P$_2$, and PIP$_3$ (this report) has been observed, and comparison with the appropriate inositol phosphate suggests that both the diacylglycerol backbone and inositol phosphate headgroup of the inositide contribute to binding (Figs. 2 and 7). Furthermore, PPI binding to intact clathrin-coated vesicles or clathrin-depleted vesicles is cryptic, being unmasked by lipid extraction with nonionic detergent. As there are relatively high concentrations of PI-4,5-P$_2$ at the plasma membrane, under basal conditions this species must be considered a likely ligand for coated pit AP-2.

Interestingly, AP-2 in the assembled coat structure, which is morphologically similar to a clathrin-coated pit, bound a short-chain form of PIP$_3$ with highest affinity of the ligands tested. In this context, it is noteworthy that PI-3-kinase enzymes are bound and activated by receptors that are internalized through clathrin-coated pits within 1–10 min after ligand binding (43–45), including tyrosine kinase (19) and G protein-linked receptors (20, 46) (reviewed in (22, 47)). Physical association of PI-3-kinase with receptor occurs within seconds of ligand binding and can be shown to persist for more than 30 min by immunoprecipitation studies (48–50). Enzyme activation and PIP$_3$ production generally commence within 30 s of ligand binding and continue for at least 3–5 min (51, 52), or even longer than 30 min (19, 48–50). The overlapping kinetics of these events lead to the inference that the clathrin-coated pit and internalized endosome will likely contain some activated PI-3-kinase and that PIP$_3$ will be locally generated at these sites. Indeed, Corvera and colleagues (53) have directly demonstrated that clathrin-coated vesicles isolated from stimu-

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lated cells contain elevated levels of both p85 protein and PI-3-kinase enzyme activity during the first 10 min after hormonal stimulation, temporarily consistent with transient passage of the activated enzyme through the coated pit during endocytosis.

Is the affinity of AP-2 for PIP$_3$ reported here consistent with the existence of an interaction in the intact cell? Recent experiments by Rameh and co-workers (54) suggest that this should indeed be the case. Their study showed that the SH2 domain of the p85 subunit of PI-3-kinase bound PIP$_3$ in competition with protein phosphotyrosyl residues. In intact cells, physiological levels of PIP$_3$ dramatically reduced the amount of p85 recruited to activated receptor complexes, indicating substantial binding of PIP$_3$ to p85. As AP-2 has 10–50-fold greater affinity for PIP$_3$ (K$_D$ $\approx$ 100–500 nM) than does p85 ($\approx 5 \mu M$), these results indicate that PIP$_3$ generated in proximity to a plasma membrane-coated pit will very likely bind to AP-2. Conversely, they also raise the interesting possibility that this site may mediate AP-2 interactions with other phosphoproteins in the absence of elevated levels of PIP$_3$.

Several proteins implicated in membrane trafficking have recently been shown to bind PPIs, although most have not been studied with as extensive an array of substrates as AP-2. Examples include coat-associated proteins such as mammalian AP-3 and yeast coatomer (26, 39, 40, 55), as well as membrane proteins such as synaptotagmin (56). In view of the results presented here, it will be important to determine whether PIP$_3$ also serves as a high affinity ligand for these proteins. For at least one protein this is not the case as the SH2 domain of the activated enzyme through the coated pit during PI-3-kinase enzyme activity during the first 10 min after hormonal stimulation, temporarily consistent with transient passage of the activated enzyme through the coated pit during endocytosis.

A very recent study of the mechanisms are not yet clear. The identification of a distinct binding domain for PIP$_3$ and PPI suggests that AP-2 is one possible target in these processes and highlights the importance of exploring the nature and consequences of the PPI-AP-2 interaction in cells.
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