The interaction of coatomer with inositol polyphosphates is conserved in *Saccharomyces cerevisiae*

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Coatomer is an oligomeric complex of coat proteins that regulates vesicular traffic through the Golgi complex and from the Golgi to the endoplasmic reticulum [Pelham (1994) Cell **79**, 1125–1127]. We have investigated whether the binding of InsP_6 to mammalian coatomer [Fleischer, Xie, Mayrleitner, Shears and Fleischer (1994) J. Biol. Chem. **269**, 17826–17832] is conserved in the genetically amenable model *Saccharomyces cerevisiae*. We have isolated coatomer from *S. cerevisiae* and found it to bind InsP_6 at two apparent classes of binding sites (K_{D1} = 0.8 ± 0.2 nM; $K_{D2} = 361 \pm 102$ nM). Ligand specificity was studied by displacing 4.5 nM [^{3}H]InsP_6 from coatomer with various Ins derivatives. The following IC_{50} values (nM) were obtained: *myo*-InsP_6 = 6; bis(diphospho)inositol tetrakisphosphate = 6; diphosphoinositol pentakisphosphate = 6; *scyllo*-InsP_6 = 12; Ins(1,3,4,5,6)P_5 = 13; Ins(1,2,4,5,6)P_5 = 22; Ins(1,3,4,5)P_4 = 22; 1-*O*-(1,2-di-*O*-octanoyl-*sn*-glycero-3-phospho)-D-Ins(3,4,5)P_3 = 290. Less than 10% of the ^{3}H label was displaced by 1 μM of either Ins(1,4,5)P_3 or inositol hexakisulphate. A cell-free lysate of *S. cerevisiae* synthesized diphosphoinositol polyphosphates (PP-InsP_n) from InsP_6, but our binding data, plus measurements of the relative levels of inositol polyphosphates in intact yeast [Hawkins, Stephens and Piggott (1993) J. Biol. Chem. **268**, 3374–3383], indicate that InsP_6 is the major physiologically relevant ligand. Thus a reconstituted vesicle trafficking system using coatomer and other functionally related components isolated from yeast should be a useful model for elucidating the functional significance of the binding of InsP_6 by coatomer.

INTRODUCTION

A fundamental problem in cell biology is to understand how proteins are directed towards specific cellular sites. The elucidation of this issue is central to our understanding the molecular mechanisms that underly processes such as the accumulation of exogenous macromolecules, secretion, synaptic transmission, organelle biogenesis and cell polarity. Transport of cellular proteins is mediated by vesicles that bud from a donor compartment and fuse with an acceptor compartment (see [1] for a review). Several independent lines of enquiry have converged on the possibility of direct links between vesicle traffic and inositol-containing compounds. For example, VPS34p is a protein of *Saccharomyces cerevisiae* that participates in targeting soluble hydrolases to the yeast vacuole and is also involved in vacuole morphogenesis during budding; VPS34p shows sequence similarity to the catalytic subunit of PtdIns 3-kinase [2]. In addition, two cytosolic brain proteins that prime exocytosis, PEP1 and PEP3, have been respectively identified as a PtdIns4*P* 5-kinase, and a PtdIns-transport protein [3]. Thirdly, at least in mammalian systems, some of the proteins that control the formation and fusion of transport vesicles may be grouped together into a subclass by virtue of their ability to bind inositol polyphosphates with high affinity; this is known to be a characteristic of AP-2 [4], synaptotagmin [5], AP-3 [6,7] and coatomer [8].

The latter group of proteins have been of particular interest to our laboratory. We, and others, have shown that the degree of phosphorylation of the inositol polyphosphates has dictated the rank order with which they bind to these proteins (i.e. InsP_6 > InsP_5 > InsP_4 > InsP_3 [4,6–8]), except for the slight variation shown by synaptotagmin (InsP_5 > InsP_4 > InsP_6 [5]). Since InsP_6 is the compound in these series that is the most abundant (see [9]), it therefore has the strongest case for being the most general, physiologically relevant, ligand for this family of proteins. The pursuit of the significance of this binding of inositol polyphosphates has led to the observation that InsP_6 inhibits the ability of both AP-2 and AP-3 to promote clathrin assembly [4,6,7]. It has been proposed [7] that InsP_6 acts as a constitutive inhibitor of the endocytic pathways that these proteins help to control, by analogy with the putative 'fusion clamps' [1] that are believed to regulate exocytic processes. Perhaps this inhibition could be relieved if an appropriate regulatory stimulus were to modify the free cellular concentration of InsP_6. Another proposal is that covalent modification of proteins such as AP-3 might alleviate constitutive inhibition of their function by decreasing their affinity for inositol polyphosphates [7]. In contrast with these data, which provide some scope for hypothesizing on the physiological relevance of inositol polyphosphates binding to AP-2 and AP-3, studies with coatomer have not yielded as much useful information. Coatomer (the name given to an oligomeric complex of about 700–800 kDa [10]) consist of several individual coat proteins (COPs). COPs play important roles in controlling vesicle traffic through the Golgi complex, and retrograde transport from the Golgi to the endoplasmic reticulum [11–15]. The only known physiological consequence of these particular proteins binding inositol polyphosphates is the puzzling inhibition of the inherent K^+-channel activity of mammalian coatomer [8]. One strategy that might provide more information would be to test the effects of exogenous Ins*P*s in a reconstituted, coatomer-regulated, vesicle-trafficking assay. Such an approach could be particularly

Abbreviations used: Ins, *myo*-inositol, except where the *scyllo*- epimer is designated; PP-InsP_5 and InsP_5-PP, two distinct diphosphoinositol pentakisphosphate isomers; PP-InsP_4-PP, a bis(diphospho)inositol tetrakisphosphate (the absolute positions of the diphosphate groups are not known for any of the compounds studied here); DiC_8PtdIns(3,4,5)P_3, 1-*O*-(1,2-di-*O*-octanoyl-*sn*-glycero-3-phospho)-D-inositol 3,4,5-trisphosphate; InsS_6, inositol hexakis-sulphate; COPs, **co**at **p**roteins; DTT, dithiothreitol.

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useful if it were to utilize yeast, since this organism is genetically tractable and it contains COPs that are very similar to those in mammalian cells [14,15]. It was with this ultimate goal in mind that we have now investigated whether the binding of InsP_6 by coatomer is conserved between mammals and yeast. We concentrated upon this particular polyphosphate because of previous experiments where intact cells of *S. cerevisiae* were labeled with 20 μCi/ml [^{3}H]inositol for 96 h [16]. In the latter study, InsP_6 was the only inositol polyphosphate to incorporate significant amounts of [^{3}H]inositol (about 1200 d.p.m.); levels of an uncharacterized InsP_5 were only slightly greater than background, and no other inositol polyphosphates were detected [16]. We have also compared the affinity of InsP_6 with that of several other ligands in order to gain further insight into the determinants of ligand specificity.

The discovery of more phosphorylated derivatives of InsP_6, namely diphosphoinositol pentakisphosphate (PP-InsP_5) and bis(diphospho)inositol tetrakisphosphate (PP-InsP_4-PP) [17,18], has added a new level of complexity to this field of research. PP-InsP_5 has been shown to bind to AP-2, AP-3 and mammalian coatomer 5–10-fold more strongly than InsP_6 [7,8]. While the total amounts of InsP_6 in intact mammalian cells are about 20 times greater than those of PP-InsP_5 [17–20], there is some uncertainty concerning what proportions of each ligand are free in the cytosol [21]. Thus, it is unclear whether there is any functional significance to the property of proteins such as coatomer to bind PP-InsP_5 tightly *in vitro*. We have therefore also considered whether yeast might be a useful model that could provide some insight into this particular question. Diphosphoinositol polyphosphates have not previously been observed in intact yeast cells [16]. We therefore investigated whether cell-free lysates of *S. cerevisiae* can synthesize PP-InsP_5 and PP-InsP_4-PP, and we have also studied the affinity of these compounds for yeast coatomer.

MATERIALS AND METHODS

Materials

The strain of *S. cerevisiae* used in the present study was RSY607 (MATα, ura3-52, leu2-3,-112, Δpep4::URA3). Cell lysates were prepared as described in [22] and were stored at −70 °C. Coatomer was isolated by Dr. Midori Hosobuchi (Department of Molecular and Cellular Biology, Howard Hughes Medical Institute, University of California, Berkeley, CA, USA) as previously described [14] and was stored between −70 °C and −90 °C. The nominal concentration of coatomer was determined with Bradford's colorimetric assay [23] using BSA as a standard. NEN–du Pont supplied non-radioactive PP-InsP_5 (NLP051), non-radioactive PP-InsP_4-PP (NLP052), [β-^{32}P]PP-InsP_5 (NEG214), [β-^{32}P]PP-InsP_4-PP (NEG215) and PP-[^{3}H]InsP_4-PP (NET1098); these were prepared enzymically by phosphorylation of InsP_6 using partly purified extracts from rabbit brain. [^{3}H]InsP_6 was also supplied by NEN–du Pont. Ins(1,2,4,5,6)P_5 was prepared as previously described [7]. Ins(1,3,4,5,6)P_5 and InsP_6 were purchased from Calbiochem, Ins(1,3,4,5)P_4 was purchased from the University of Rhode Island Foundation (Kingston, RI, U.S.A.), and Ins(1,4,5)P_3 was supplied by LC Laboratories (Woburn, MA, U.S.A.). Inositol hexakis-sulphate (InsS_6) was purchased from Sigma, and *scyllo*-InsP_6 was generously given by Dr. Max Tate, University of Adelaide, Adelaide, Australia. 1-*O*-(1,2-Di-*O*-octanoyl-*sn*-glycero-3-phospho)-D-Ins(3,4,5)P_3 [DiC$_8$PtdIns(3,4,5)P_3] was synthesized as previously described [24] and was kindly provided by Dr. J. R. Falck (Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.).

Methods

Inositol polyphosphate binding assay

The binding of InsP_6 to coatomer was studied at 0–4 °C in 50–100 ul incubations containing 25 mM Tris/HCl, pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 5 mg/ml bovine γ-globulin, 2500–10000 d.p.m. of [^{3}H]InsP_6 and nominally 0.5–0.9 μg of coatomer. The total concentration of InsP_6 was adjusted by the addition of non-radioactive InsP_6. After 30 min, the relative amounts of bound and free ligand were determined by a poly(ethylene glycol) precipitation procedure as described previously [7]. Non-specific binding of [^{3}H]InsP_6 to coatomer was defined as the amount bound in the presence of 5 μM InsP_6. Binding parameters were determined by non-linear regression using the GraphPad Prism program (GraphPad Software Inc., San Diego, CA 92121, U.S.A.). In some instances the data were transformed into Scatchard plots, but these were not used for the calculation of binding parameters. Displacement curves were also obtained, and where indicated the non-radioactive InsP_6 was replaced by the designated competing ligand.

Inositol polyphosphate metabolism.

The pathways of [^{3}H]InsP_6 metabolism were studied by incubating approx. 40000 d.p.m. at 25 °C in 0.25 ml of medium containing 100 mM KCl, 20 mM Hepes (adjusted to pH 7.2 with KOH), 7 mM $MgSO_4$, 1 mM Na_2EDTA, 1 mM DTT, 5 mM Na_2ATP, 10 mM NaF, 10 mM phosphocreatine and 0.05 mg/ml phosphocreatine kinase. Reactions were quenched, and the reaction mixtures deproteinized and analysed by HPLC as previously described [19].

RESULTS

InsP_6 binds to yeast coatomer with high affinity

We have previously demonstrated that bovine liver coatomer binds InsP_6 with high affinity (K_D = 0.2 nM) at a single class of binding sites [8]. We have now found that this property of coatomer is conserved in yeast (Figure 1). An analysis of the data using non-linear regression indicated that yeast coatomer has two classes of InsP_6-binding sites (see the legend to Figure 1). One of these sites is remarkably similar to that of mammalian coatomer in its high affinity for InsP_6 ($K_D = 0.8 \pm 0.2$ nM; $B_{max.} = 0.13 \pm 0.02$; mean ± S.E.M. for four experiments, including that shown in Figure 1). The second binding site had much lower affinity for InsP_6 ($K_D = 361 \pm 102$ nM; $B_{max.} = 0.41 \pm 0.015$). The $B_{max.}$ determinations for both binding sites are nominal, because of some uncertainty in the absolute amount of coatomer protein added to each assay (see the under 'Materials' in the Materials and methods section). However, a comparison of the relative $B_{max.}$ values indicates that yeast coatomer has three times more low-affinity sites than high-affinity sites.

Synthesis of diphosphoinositol polyphosphates by *S. cerevisiae*

Mammalian coatomer binds PP-InsP_5 five times more avidly than InsP_6 [8]. Before making this comparison with yeast coatomer, we investigated whether this organism could synthesize diphosphoinositol polyphosphates. We have approached this issue by incubating [^{3}H]InsP_6 with cell-free lysates of *S. cerevisiae*. Our incubations contained ATP, which serves as a phosphate donor for the synthesis of diphosphoinositol polyphosphates [17,18] and also competitively inhibits phytase-mediated hydrolysis of InsP_6 [25,26]; NaF was also added to our incubations

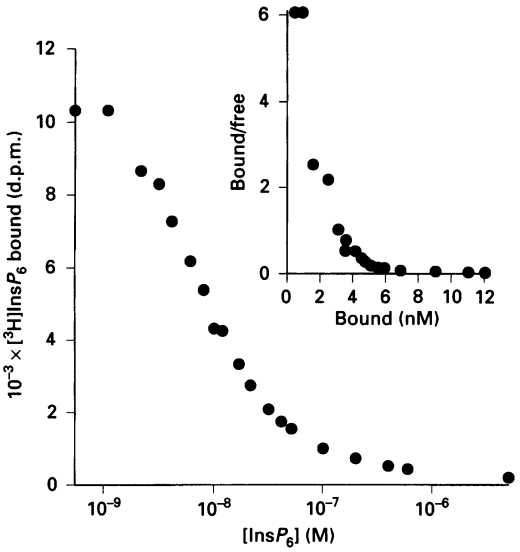

Figure 1 Binding of InsP_6 to yeast coatomer

Coatomer was incubated with [^{3}H]InsP_6 and the indicated concentrations of non-radioactive InsP_6; the amounts of bound and free ligand were estimated as described in the Materials and methods section. The inset shows a Scatchard transformation of the data. Analysis of the binding isotherm by non-linear regression (see the Materials and methods section) indicated that there were two classes of binding sites ($P < 0.001$ compared with a one-site fit) with K_D values of 1 ± 0.14 nM and 257 ± 38 nM. Three further experiments gave similar results (see the text for mean values for all four experiments).

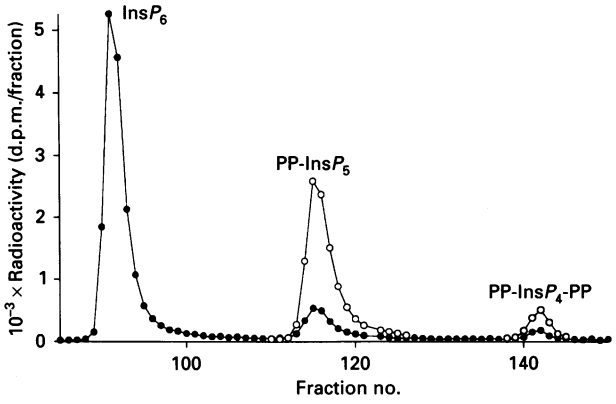

Figure 2 HPLC analysis of [^{3}H]InsP_6 metabolism by cell lysate of *S. cerevisiae*

[^{3}H]InsP_6 was incubated with cell lysate (0.46 mg of protein/ml) for 2 min as described in the Materials and methods section. Reactions were then quenched, and the mixture deproteinized and 'spiked' with approx. 10000 d.p.m. of [β-^{32}P]PP-InsP_5 and 1500 d.p.m. of [β-^{32}P]PP-InsP_4-PP and chromatographed by HPLC (see the Materials and methods section). Both ^{3}H d.p.m./fraction (●) and ^{32}P d.p.m./fraction (○) are shown. The chromatograph begins at fraction 85; earlier fractions did not contain significant amounts of radioactivity. Data are representative of three experiments.

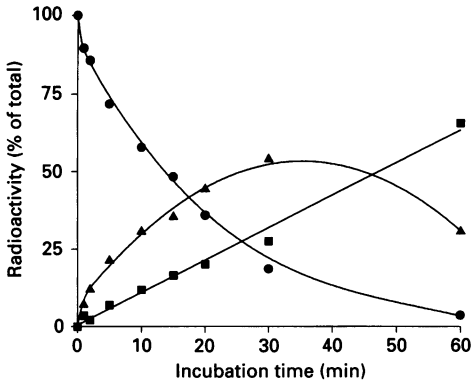

Figure 3 Time course of InsP_6 metabolism by a cell lysate of *S. cerevisiae*

Assays were performed as decribed in the legend to Figure 2 and were analysed by HPLC. ●, d.p.m. of [^{3}H]InsP_6; ▲, d.p.m. of ^{3}H-labelled material eluted close to (but not exactly with) an internal [β-^{32}P]PP-InsP_5 standard (peak A; see the text for details); ■, d.p.m. of ^{3}H-labelled material that was co-eluted with an internal [β-^{32}P]PP-InsP_4-PP standard. Data are representative of three experiments.

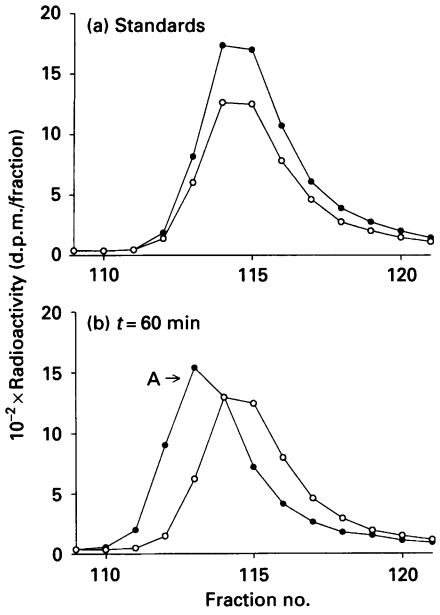

Figure 4 Comparison of the HPLC elution of a product of [^{3}H]InsP_6 metabolism with standards of PP-InsP_5

InsP_6 was incubated for 60 min as described in the legend to Figure 2. Reactions were quenched, and the mixtures was deproteinized, spiked with approx. 5000 d.p.m. of [β-^{32}P]PP-InsP_5 and subjected to HPLC (see the Materials and methods section). The region of the chromatograph containing peak A (see Figure 3) is shown in (**b**): ●,^{3}H d.p.m. ; ○, ^{32}P d.p.m. The latter HPLC run was immediately preceeded by an HPLC analysis of a mixture of standards of PP-[^{3}H]PP-InsP_5 (●, approx. 7000 d.p.m.) and [β-^{32}P]PP-InsP_4-PP (○, approx. 5000 d.p.m.) and these results appear in (**a**).

to inhibit phytases [25,26]. Indeed, in our incubations there was no significant dephosphorylation of [^{3}H]InsP_6 in incubations of up to 60 min duration (results not shown). Instead, HPLC analysis demonstrated that, at 25 °C, InsP_6 was metabolized to two diphosphoinositol polyphosphates (Figure 2). At early time points (i.e. $t = 2$ min) two such metabolites were observed, and they were co-eluted with internal standards of [β-^{32}P]PP-InsP_5 and [β-^{32}P]PP-InsP_4-PP (Figure 2).

When [^{3}H]InsP_6 was metabolized by the yeast cell extracts, its levels decreased exponentially over a 60 min time course (Figure 3). Throughout this time there was a complete co-elution of the [β-^{32}P]PP-InsP_4-PP standard with the accompanying ^{3}H-labelled

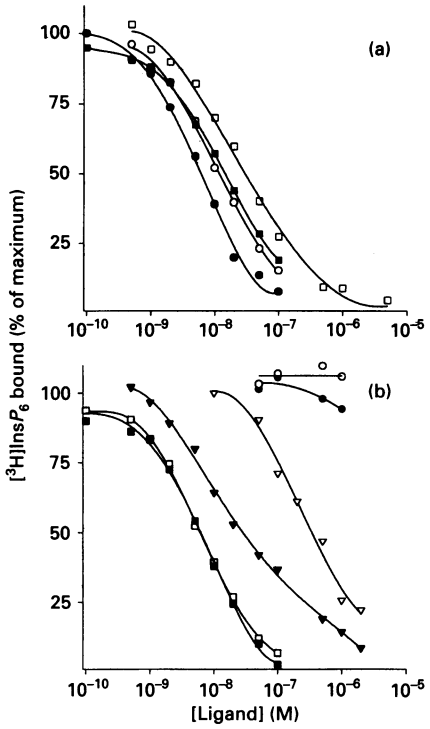

Figure 5 Displacement of [^{3}H]InsP_6 from coatomer by various ligands

Coatomer was incubated with approx. 10000 d.p.m. of [^{3}H]InsP_6 (4.5 nM) and the indicated amounts of various inositol derivatives. The percentage of [^{3}H]InsP_6 bound was determined as described in the Materials and methods section. (**a**) Shows curves describing the displacement of [^{3}H]InsP_6 by *myo*-InsP_6 (●), *scyllo*-InsP_6 (○), Ins(1,3,4,5,6)P_5 (■) and Ins(1,2,4,5,6)P_5 (□). (**b**) Displays curves which describe the displacement of [^{3}H]InsP_6 by PP-InsP_4-PP (■), PP-InsP_5 (□), Ins(1,3,4,5)P_4 (▼), DiC$_8$PtdIns(3,4,5)P_3 (▽), Ins(1,4,5)P_3 (●) and InsS_6(○). Data for each curve (which is representative of two to four experiments) are means of duplicate determinations.

Table 1 IC_{50} values for the displacement of InsP_6 from coatomer by various ligands

IC_{50} values for yeast coatomer were determined from data shown in Figure 5. The relative IC_{50} values for coatomer prepared from bovine liver were calculated from data described in [8]. –, ligand was not tested. >, those experiments where there was < 10% displacement of [^{3}H]InsP_6 from coatomer by 1 μM of the indicated ligand.

Ligand	IC_{50} (yeast) (nM)	Relative IC_{50}	
		Yeast	Bovine liver
PP-InsP_4-PP	6	1	–
PP-InsP_5	6	1	0.2
InsP_6	6	1	1
scyllo-InsP_6	12	2	–
Ins(1,3,4,5,6)P_5	13	2.1	34
Ins(1,2,4,5,6)P_5	22	3.7	–
Ins(1,3,4,5)P_4	22	3.7	1
DiC$_8$PtdIns(3,4,5)P_3	290	48	–
Ins(1,4,5)P_3	> 1000	> 200	> 200
InsS_6	> 1000	> 200	–

peak (Figure 2 and results not shown), the levels of which increased linearly (Figure 3). In contrast, at the later time points (e.g. t = 60 min) the [β-^{32}P]PP-InsP_5 standard was not co-eluted with the accompanying ^{3}H-labelled peak (which is designated as peak A in Figure 4). Instead, the peak fraction of the^{3}H-labelled material eluted 1 min before the [β-^{32}P]PP-InsP_5 standard. We consistently found that this slight difference in elution properties developed as the incubation time increased [cf. Figure 2 (t = 2 min) with Figure 4b (t = 60 min)]. Figure 4(a) is a control HPLC chromatograph that shows the co-chromatography of isomerically identical standards of ^{3}H- and ^{32}P-labelled PP-InsP_5; the latter data were obtained immediately before the chromatograph shown in Figure 4(b). We therefore conclude that peak A consists of two compounds, one which accumulated relatively rapidly (and which co-eluted with [β-^{32}P]PP-InsP_5), plus a second ^{3}H-labelled diphosphoinositol polyphosphate that accumulated more slowly.

We have previously demonstrated that mammalian cells also have the enzymic capacity to synthesize more than one isomer of diphosphoinositol pentakisphosphate [19]. One of them, PP-InsP_5, is formed by phosphorylation of InsP_6 by an ATP-dependent kinase and also by dephosphorylation of PP-InsP_4-PP by a diphosphatase [19]. The other isomer, which was designated InsP_5-PP, can be formed by dephosphorylation of PP-InsP_4-PP by a purified multiple-inositol polyphosphate phosphatase [19]. Furthermore, under HPLC conditions similar to those of Figures 3 and 4, InsP_5-PP was eluted upon HPLC just before a [β-^{32}P]PP-InsP_5 standard [19], as did the earlier-eluted material of peak A (Figure 4). The possibility that peak A may contain both PP-InsP_5 and InsP_5-PP led us to incubate cell lysates of *S. cerevisiae* for 60 min with approx. 40000 d.p.m. of PP-[^{3}H]InsP_4-PP, under the same incubation conditions that were used to phosphorylate InsP_6 (see the Materials and methods section). Upon HPLC analysis, 20% of the ^{3}H was associated with a metabolite that was eluted with the characteristics of peak A, that is, the peak of this ^{3}H-labelled peak was eluted 1 min before a [β-^{32}P]PP-InsP_5 standard (results not shown). Thus peak A may contain the same isomers of [^{3}H]InsP_5-PP and PP-[^{3}H]InsP_5 that can be produced by mammalian enzymes. In any case, having shown that yeast can synthesize diphosphoinositol polyphosphates, it was worthwhile to include them in our study of the specificity of InsP_6 binding to yeast coatomer.

The specificity of InsP_6 binding to coatomer

Some important information on ligand specificity was provided by examining the relative abilities of a number of inositol derivatives to displace [^{3}H]InsP_6 from coatomer (Figure 5; Table 1). For example, up to 1 μM InsS_6 was completely ineffective at displacing 4.5 nM [^{3}H]InsP_6 (Figure 5b). This observation demonstrates the specificity of coatomer for phosphates, rather than simple negative charge density.

We previously demonstrated that bovine liver coatomer has a similar affinity for both InsP_6 and Ins(1,3,4,5)P_4 (see Table 1 and [8]). The situation is not substantially different for yeast coatomer; the value of the IC_{50} calculated from the Ins(1,3,4,5)P_4 displacement curve was only 4-fold less than that for InsP_6 (Figure 5b and Table 1). When this observation is compared with the very similar IC_{50} value for displacement of [^{3}H]InsP_6 by *scyllo*-InsP_6 (in which the 2-phosphate is equatorial instead of axial), Ins(1,3,4,5,6)P_5 and Ins(1,2,4,5,6)P_5 (Figure 5; Table 1), it appears that the 2-, 3- and 6-phosphates are not, by themselves, quantitatively important determinants of ligand specificity. Ins(1,4,5)P_3 was a relatively ineffective ligand,1 μM of which displaced less than 10% of bound [^{3}H]InsP_6 from yeast coatomer (Figure 5b); again, a similar result was obtained with bovine liver coatomer (Table 1). A comparison of the IC_{50} values for Ins(1,3,4,5)P_4 (25 nM), Ins(1,4,5)P_3 (> 1 μM) and DiC$_8$Ptd-

Ins(3,4,5)P_3 (290 nM) suggests that at least four free monoester phosphates are required for high-affinity binding (Figure 5b; Table 1). The IC_{50} values for the displacement of [^{3}H]InsP_6 by InsP_6, PP-InsP_5 and PP-InsP_4-PP were identical (Figure 5). The data obtained with PP-InsP_5 underscore yet another feature of the binding of inositol polyphosphates to coatomer that is conserved between mammals and yeast. Note also that this is the first study of PP-InsP_4-PP binding to any protein.

Among the list of compounds that have been examined for their ability to bind to both yeast and mammalian coatomer, Ins(1,3,4,5,6)P_5 was the only one where there was a substantial difference; it was 16-fold less effective at displacing [^{3}H]InsP_6 from bovine liver coatomer as compared with yeast coatomer (Table 1).

DISCUSSION

A key goal of the current study was to evaluate *S. cerevisiae* as a model for studying the interactions of inositol polyphosphates with coatomer. It is therefore an important result that the ability of this multimeric protein complex to bind InsP_6, which was initially identified as a characteristic of coatomer isolated from bovine liver [8], is conserved in yeast. This observation provides us with the incentive to search for effects of InsP_6 in coatomer-regulated vesicle trafficking assays utilizing components isolated from yeast. If this approach were to utilize the library of secretion mutants that yeast has provided, and mutants of coatomer itself (e.g. [12]), then we ought to be optimistic of gaining some insight into the physiological significance of the interaction between coatomer and InsP_6.

We have also obtained additional new information on ligand specificity (see Figure 5 and Table 1). For example, the inability of high concentrations of InsS_6 to displace [^{3}H]InsP_6 demonstrates the specificity of coatomer for inositol phosphates, rather than simple negative charge density. On the other hand, we have also shown that the polyphosphate-binding site does have some flexibility, since *scyllo*-InsP_6, PP-InsP_5, PP-InsP_4-PP, Ins(1,2,4,5,6)P_5, Ins(1,3,4,5,6)P_5 and Ins(1,3,4,5)P_4 were all no more than 4-fold less as effective at displacing [^{3}H]InsP_6 from coatomer than was InsP_6 itself (Figure 5; Table 1). Once the total number of free monoester phosphates was decreased to three [as in DiC_8PtdIns(3,4,5)P_3 and Ins(1,4,5)P_3], ligand affinity was then reduced to a far greater extent (Figure 5; Table 1). While the experiments with DiC_8PtdIns(3,4,5)P_3 add to our knowledge of the determinants of ligand specificity, it should be noted that the naturally occuring PtdIns(3,4,5)P_3 has not been found to be synthesized by *S. cerevisiae* [16]. However, Norris et al. [6] speculated that 3-phosphorylated inositol lipids could be physiologically relevant ligands for another member of the family of InsP_6-binding proteins involved with vesicle traffic, namely AP-3 [6,7], but only if the lipids could bind with higher affinity than InsP_6. Our data with DiC_8PtdIns(3,4,5)P_3 do not provide a precedent that is consistent with the proposal of Norris et al. [6]. Indeed, our displacement curves, when considered in relation to the levels of inositol polyphosphates in intact yeast cells [16], together indicate that none of the competing ligands we have studied have both the necessary affinity and abundance to prevent InsP_6 from being the physiologically relevant ligand for coatomer *in vivo*.

Some differences did emerge in our comparison of yeast and bovine liver coatomer. In our earlier experiments, only a single class of high-affinity InsP_6-binding sites was observed [8]. While the latter's affinity for InsP_6 (K_D = 0.2 nM) is remarkably similar to the high-affinity binding site that we have now observed in yeast coatomer (K_D = 0.8 nM; see the Results section), our new experiments have also revealed a second class of lower-affinity binding sites (K_D 361 nM; see the Results section). Although total cellular levels of InsP_6 *in vivo* are in the 5–50 μM range [9,16], there is some doubt as to the free cytosolic concentration of this ligand [21]. Moreover, the absolute affinities of both sites *in vivo* may be somewhat different from those that we have determined *in vitro*, because the intracellular environment is rather different from the experimental conditions that are necessary for binding studies. Thus we do not have sufficient precise information to speculate on the degree to which the low-affinity binding site might be saturated *in vivo*. On the other hand, the subnanamolar value of the K_D for high-affinity binding (see above) indicates that InsP_6 has more than sufficient capacity to saturate these particular sites. This consideration in turn leads to the prediction that there is a constitutive function for InsP_6.

The comparison between bovine liver coatomer and yeast coatomer also revealed a difference in their relative affinities for Ins(1,3,4,5,6)P_5. The latter was over 30-fold less effective than InsP_6 at displacing [^{3}H]InsP_6 from mammalian coatomer, whereas Ins(1,3,4,5,6)P_5 bound only 2-fold less avidly than InsP_6 to yeast coatomer (Table 1). It is possible that evolutionary pressure has imposed this specificity upon mammalian coatomer, in order to avoid the high cellular levels of Ins(1,3,4,5,6)P_5 from competing with bound InsP_6. This competition should not arise in yeast, where InsP_6 is apparently in large excess over Ins(1,3,4,5,6)P_5 [16]. Note that among the inositol phosphates that have been tested for their abilities to bind to both yeast and mammalian coatomer, Ins(1,3,4,5,6)P_5 was the only one where a substantial difference was noted (Table 1).

Since yeast cell lysates readily converted InsP_6 into diphosphoinositol polyphosphates (Figures 2–4), it is possible that the reason these compounds were not previously detected in intact cells [16] was because they are prevented from attaining sufficiently high steady-state levels by virtue of their being rapidly dephosphorylated back to InsP_6, as is the case in mammalian systems [17–20]. Another interpretation of our *in vitro* metabolic data is that yeast contains the same polyphosphate isomers as have been detected in mammalian systems, and which have been designated as PP-InsP_5, InsP_5-PP and PP-InsP_4-PP [18–20]. However, we cannot exclude the possibility that the isomers we have observed yeast to synthesize are actually different from those produced by mammalian systems. For example, two isomers of PP-InsP_5 may be formed in yeast by there being more than one form of InsP_6 kinase activity, or even a PP-InsP_5 isomerase, as well as the possibility (see the Results section) of more than one route of PP-InsP_4-PP dephosphorylation. Some extensive enzyme purification will be needed in order to distinguish between these various alternatives. There is also a need for techniques that can determine the structures of individual isomers of the diphosphoinositol polyphosphates using the small amounts of material that can normally be obtained from cell cultures. Our new studies with yeast cell lysates contrast with previous work with homogenates of rat liver and intact AR4-2J pancreatoma cells [19], where there was evidence only for the formation of one isomer of diphosphoinositol pentakisphosphate. It will now be important to determine the significance of this additional complexity to the metabolism of inositol polyphosphates, and we should also consider if it is also a property of some cell types of higher organisms. However, with regards to InsP_6 and our objective of elucidating the significance of it binding to coatomer, our results indicate that a reconstituted, coatomer-regulated vesicle trafficking system isolated from yeast should prove to be a useful and generally applicable model system.

REFERENCES

1 Rothman, J. E. (1994) Nature (London) **372**, 55–63
2 Kapeller, R. and Cantley, L. C. (1994) Bioessays **16**, 565–576
3 Hay, J. C., Fisette, P.L, Jenkins, G. H. et al. (1995) Nature (London) **374**, 173–177
4 Beck, K. A. and Keen, J. H. (1991) J. Biol. Chem. **266**, 4442–4447
5 Niinobe, M., Yamaguchi, Y., Fukuda, M. and Mikoshiba, K. (1994) Biochem. Biophys. Res. Commun. **205**, 1036–1042
6 Norris, F. A., Ungewickell, E. and Majerus, P. W. (1995) J. Biol. Chem. **270**, 214–217
7 Ye, W., Ali, N., Bembenek, M. E., Shears, S. B. and Lafer, E. M. (1995) J. Biol. Chem. **270**, 1564–1568
8 Fleischer, B., Xie, J., Mayrleitner, M., Shears, S. B. and Fleischer, S. (1994) J. Biol. Chem. **269**, 17826–17832
9 Shears, S. B. (1992) Adv. Second Messenger Phosphoprotein Res. **26**, 63–92
10 Waters, M. G., Serafini, T. and Rothman, J. E. (1991) Nature (London) **349**, 248–251
11 Barlowe, C., Orci, L., Yeung, T. et al. (1994) Cell **77**, 895–907
12 Letourneur, F., Gaynor, E. C., Hennecke, S. et al. (1994) Cell **79**, 1199–1207
13 Pelham, H. R. B. (1994) Cell **79**, 1125–1127
14 Hosobuchi, M., Kreis, T. and Schekman, R. (1992) Nature (London) **360**, 603–605
15 Duden, R., Hosobuchi, M., Hamamoto, S., Winey, M., Byers, B. and Schekman, R. (1994) J. Biol. Chem. **269**, 24486–24495
16 Hawkins, P. T., Stephens, L. R. and Piggott, J. R. (1993) J. Biol. Chem. **268**, 3374–3383
17 Menniti, F. S., Miller, R. N., Putney, J. W., Jr. and Shears, S. B. (1993) J. Biol. Chem. **268**, 3850–3856
18 Stephens, L. R., Radenberg, T., Thiel, U. et al. (1993) J. Biol. Chem. **268**, 4009–4015
19 Shears, S. B., Ali, N., Craxton, A. and Bembenek, M. (1995) J. Biol. Chem. **270**, 10489–10497
20 Glennon, M. C. and Shears, S. B. (1993) Biochem. J. **293**, 583–590
21 Poyner, D. R., Cooke, F., Hanley, M. R., Reynolds, D. J. M. and Hawkins, P. T. (1993) J. Biol. Chem. **268**, 1032–1038
22 Wuestehube, L. J. and Schekman, R. (1992) Methods Enzymol. **219**, 124–136
23 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
24 Toker, A., Meyer, M., Reddy, K. K. et al. (1994) J. Biol. Chem. **269** 32358–32367
25 Gibson, D. M. and Ullah, A. H. J. (1988) Arch. Biochem. Biophys. **260** 503–513
26 Nayini, N. R. and Markakis, M. (1986) in Phytic acid: Chemistry and Applications (Graf, E., ed.), pp. 101–118, Pilatus Press, Minneapolis

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