Isolation and Characterization of a Novel Inositol Hexakisphosphate Binding Protein from Mammalian Cell Cytosol†

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ABSTRACT: Inositol hexakisphosphate (IP_6) is present in most mammalian cells, although its intracellular function is as yet undefined. We find that the total protein fraction from bovine brain cytosol contains a significant level of specific binding for IP_6 precipitable with 40% saturated ammonium sulfate. A protein complex has been isolated from this fraction that specifically binds IP_6 and is purified about 500-fold over the cytosol. The IP_6 binding protein (IP_6BP) chromatographs as a single peak of binding activity on a gel exclusion column, with a Stokes radius equivalent to 266 ± 14 kDa. The IP_6BP is a heterooligomeric complex composed of a number of subunits with molecular weights varying from 23 000 to 60 000, as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE). Scatchard analyses of IP_6 binding of both the crude ammonium sulfate fraction and the purified complex show the presence of a similar high-affinity binding site ($K_d \sim 6.0$ nM). B_{max} for the purified fraction is 1.8 nmol of IP_6/mg of protein or 0.48 mol of IP_6 bound/mol of complex. Other inositol polyphosphates, such as inositol 1,3,4,5,6-pentakisphosphate, inositol 1,3,4,5-tetrakisphosphate, and inositol 1,4,5-trisphosphate, are poor competitors for IP_6 binding to the purified complex. The purification scheme, when applied to a rat liver cytosol fraction, yields a similar IP_6BP. This complex has an apparent size of 512 000 using gel exclusion chromatography and contains an additional protein band with M_r = 97 000 by SDS–PAGE. IP_6BP exhibits no detectable inositol 1,3,4,5,6-pentakisphosphate kinase, IP_6 phosphatase, or IP_6 kinase activity. IP_6BP is distinct from coatomer and assembly protein-2 in subunit composition, immunological cross-reactivity, and inositol polyphosphate binding characteristics. The role of this unique type of IP_6 binding protein in cell function remains to be elucidated.

Inositol polyphosphates are important components of the cell cytosol that appear to be involved in cell signaling. *myo*-Inositol 1,4,5-trisphosphate (IP_3[1]) and *myo*-inositol 1,3,4,5-tetrakisphosphate (IP_4) are known to be involved in stimulus–response coupling in many types of cells. Stimulation of a large variety of cell surface receptors activates the hydrolysis of phosphatidylinositol 4,5-bisphosphate present in the plasma membrane to form intracellular IP_3 [reviewed by Berridge and Irvine (1989)]. An increase of cytosolic IP_3 opens IP_3-sensitive Ca^{2+} channels in the endoplasmic reticulum, leading to an increased cytosolic Ca^{2+} concentration (Mignery et al., 1990; Chadwick et al., 1990; Meyerleitner et al., 1991; Ferris & Snyder, 1992). In addition, IP_4 levels increase in stimulated cells via the phosphorylation of IP_3 by a specific 3-kinase (Shears, 1989; Lee et al.,1990). The mechanism of IP_4 action is not clear, but it appears to regulate Ca^{2+} homeostasis either by stimulating Ca^{2+} entry into the cell and/or equilibrating intracellular Ca^{2+} pools (Irvine, 1989; Molleman, 1991).

In contrast to IP_3 and IP_4, less is known about the functions of inositol 1,3,4,5,6-pentakisphosphate (IP_5) and inositol hexakisphosphate (IP_6). These compounds are present in cells at relatively high (micromolar) concentrations (Szwergold, 1987), but their levels do not change dramatically or transiently during agonist stimulation. However, changes in the mass ratios of IP_5 to IP_6 have been reported during cell cycle changes in rat thymocytes (Guse et al., 1993). An extracellular role for these compounds as neurotransmitters has been proposed on the basis of effects of extracellular additions of IP_6 on Ca^{2+} influx in isolated neuronal cells (Nicoletti et al., 1989) and adrenal chromaffin cells (Regunathan et al., 1992). Furthermore, the infusion of either IP_5 or IP_6 into a discrete brain stem nucleus results in dose-dependent changes in heart rate and blood pressure (Vallejo et al., 1987). It has been shown that IP_6 intracellularly injected into identified neurons of *Aplasia* also results in the opening of both Na^+ and Ca^{2+} channels (Saweda et al., 1989). IP_6 has also been reported to have antitumorigenic activity (Vusenik et al., 1993). These observations strengthen the view that the higher inositol polyphosphates may also be modulators of a variety of membrane channels.

Recent studies have shown that both IP_5 and IP_6 are further metabolized by cells to form pyrophosphates (Stephens et al., 1993; Menniti et al., 1993). Furthermore, the latter turn over rapidly in mammalian cells, suggesting that they are involved in some sort of metabolic cycling, the nature of which is as yet unknown (Menniti et al., 1993).

An important approach to elucidating the mechanism of inositol polyphosphate action on membranes has been the

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[1] Abbreviations: IP_6, inositol hexakisphosphate; IP_6BP, IP_6 binding protein; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; IP_3, *myo*-inositol 1,4,5-trisphosphate; IP_4, *myo*-inositol 1,3,4,5-tetrakisphosphate; IP_5, *myo*-inositol 1,3,4,5,6-pentakisphosphate; AP-1, clathrin assembly protein-1; AP-2, clathrin assembly protein-2; Mes, 2-morpholinoethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, poly(ethylene glycol); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; 50 KTDG, 25 mM Tris Cl, pH 7.4 adjusted at room temperature, containing 50 mM KCl, 1 mM DTT, and 10% glycerol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; TCA, trichloroacetic acid.

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isolation and biochemical characterization of intracellular membrane-bound receptors specific for these intermediates. An IP_3 receptor was first purified from brain cerebellum, a highly enriched source (Supattapone et al., 1988), and shown to be a Ca^{2+} channel after reconstitution in a lipid bilayer (Maeda et al., 1991). A similar IP_3 receptor from aortic smooth muscle was isolated (Chadwick et al., 1990) and shown to be an IP_3 gated Ca^{2+} channel (Meyerleitner et al., 1991). A distinct inositol polyphosphate receptor isolated from cerebellum microsomes has been shown to have K^+-selective channel activity modulated by IP_4 (Chadwick et al., 1992). Further studies showed this receptor to be closely related, if not identical, to clathrin assembly protein AP-2 (Timerman et al., 1992a,b; Voglmaier et al., 1992). AP-2 is a heterotetramer of 270 kDa required for assembly of the clathrin-coated vesicles involved in receptor-mediated endocytosis (Pearse & Robinson, 1990).

AP-2 binds a number of inositol polyphosphates, including IP_6, and this binding inhibits the reconstitution of clathrin cages from mixtures of purified clathrin and AP-2 (Beck & Keen, 1991). This raises the possibility that inositol polyphosphates could regulate receptor mediated endocytosis. A distinct type of coated vesicle has been identified which mediates intracisternal Golgi traffic, that is, vesicles which transport secretory products between Golgi cisternae (Malhotra et al., 1989). The protein coat of these vesicles is distinct from clathrin, and the associated adaptor proteins are homologous but distinct from both AP-1 and AP-2, adaptor proteins present in clathrin-coated vesicles (Serafini et al., 1991; Duden et al., 1991). Upon cell fractionation, the coat proteins and adaptors specific for Golgi intracisternal transport vesicles are found mostly in the cytoplasm in the form of a 600-kDa complex (Duden et al., 1991; Waters et al., 1991). We have shown in preliminary studies that coatomer binding to Golgi membranes can be correlated to increased binding of IP_6 by the membranes (Xie & Fleischer, 1993). Thus, cytoplasmic proteins may also function as IP_6 binding proteins.

The present studies were initiated to determine whether cytosolic proteins can be identified that are capable of the specific binding of inositol polyphosphates. Since bovine brain cytosol is a good source of Golgi coatomer, a putative IP_6 binding protein (Xie & Fleischer, 1993), we initially used this as our starting material. A preliminary report of a portion of this work has appeared (Xie & Fleischer, 1992).

MATERIALS AND METHODS

Materials. All non-radioactive inositol phosphates used were the potassium salts and were from Calbiochem. Labeled inositol phosphates contained [^{3}H]inositol and were obtained from NEN–DuPont. Unless specified otherwise, all other biochemicals and column packings were from Sigma.

Preparation of Bovine Brain Cytosol. Brain homogenates were prepared essentially as described by Keen et al. (1979). Bovine brains were obtained fresh from the slaughterhouse, packed in ice, and used within 2 h. All steps were carried out in a cold room at ~8 °C except centrifugations, which were at 4 °C. The meninges and medulla were discarded, and the remaining brain tissue (about 500 g) was minced and homogenized in a Waring blender with an equal volume of 0.1 M sodium 2-morpholinoethanesulfonic acid (Mes, pH 6.5) containing 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and 0.5 mM $MgCl_2$. In some experiments, the homogenizing medium also contained 0.02% NaN_3, 1 μg/mL leupeptin, and 0.5 μg/mL aprotinin. Blending was carried out using three bursts lasting 10 s each at full speed. The mixture was centrifuged for 20 min at 14 000 rpm in a Beckman JA 14 rotor. The supernatant was recentrifuged at 35 000 rpm in a Beckman Ti 45 rotor for 1 h. The final supernatant was stored in 250-mL aliquots at –80 °C before purification was carried out.

Preparation of Rat Liver Cytosol. The cytosol fraction from rat liver was prepared by our modification of the method of Malhotra et al. (1989). Male Harlan rats (300–350 g), fed ad libitum, were used. They were killed by decapitation and exsanguinated, and the livers were removed and placed in cold 0.5 M sucrose. All subsequent steps were carried out at 3–4 °C. The livers were homogenized in 0.5 M sucrose containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, pH 7.4), as described previously (Fleischer & Kervina, 1974). The homogenate was centrifuged at 3 000 rpm for 10 min in a Beckman JA 21 rotor. The supernatant was collected and filtered through two layers of cheesecloth. A step gradient was formed by layering 19 mL of supernatant over 7 mL of 1.25 M sucrose containing 10 mM Hepes (pH 7.4). The gradients were then centrifuged for 30 min in a Beckman 70 Ti rotor at 60 000 rpm. The clear supernatants above the fuzzy layer of smooth membranes at the interface were collected, pooled, and frozen at –80 °C before protein fractionation was carried out.

Assay of IP_6 Binding. Assays of [^{3}H]IP_6 binding to soluble protein were carried out using a poly(ethylene glycol) (PEG) precipitation method, essentially as described previously (Chadwick et al., 1990). Samples were incubated at 4 °C in 75 μL of solution containing 25 mM tris(hydroxymethyl)-aminomethane chloride (Tris Cl, pH 7.5, adjusted at room temperature), 100 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 5 mg/mL equine γ-globulin, and varying amounts of [^{3}H]IP_6 (≈20 000 cpm/pmol). After 5 min, 25 μL of 25% PEG was added and incubation continued for 5 min on ice. Samples were then centrifuged at 95 000 rpm for 10 min at 4 °C in a Beckman TL-100 rotor. The supernatants were aspirated, and the pellets were washed rapidly twice with 0.25 mL of incubation buffer without γ-globulin and suspended in 0.2 mL of water. The suspensions were transferred to vials containing 5 mL of EcoScint scintillation cocktail (NEN), and bound radioactivity was measured in a Beckman LS 5000TD counter. In routine binding assays of column fractions, [^{3}H]IP_6 was included at a final concentration of 10 nM. Nonspecific binding was measured in the presence of 1 μM unlabeled IP_6 and subtracted from total counts to obtain the specific binding values. In general, nonspecific binding was less than 5% of the total binding measured.

Protein Assays. Protein was measured by the procedure of Lowry et al. (1951). For column fractions, protein concentration was determined using the Bradford reagent (Bradford, 1976). In both cases, crystalline bovine serum albumin (Armour Pharmaceutical Co.) was used as a standard.

Ammonium Sulfate Fractionation of Bovine Brain Cytosol. The frozen cytosol fraction (250 mL) was placed in a water bath at room temperature with frequent mixing until it was just thawed and was then placed on ice. All of the following steps were carried out on ice or in a cold room. The fraction was diluted with 750 mL of 25 mM Tris Cl (pH 7.4 adjusted at room temperature), containing 50 mM KCl, 1 mM DTT, 10% glycerol, and 1 mM EDTA (i.e., 50 KTDG + 1 mM EDTA). Cold saturated ammonium sulfate (670 mL) was then added, with continuous stirring. After 30 min, the mixture was centrifuged at 10 000 rpm for 30 min in a Beckman JA14 rotor. The supernatant was decanted, and the insides of the

bottles were wiped to remove any remaining drops of supernatant. The pellets were resuspended in TDG (Tris–DTT–glycerol, as described above) to a final volume of 300 mL and recentrifuged at 10 000 rpm for 10 min as before to remove undissolved material.

Ammonium Sulfate Fractionation of Rat Liver Cytosol. Since the liver cytosol had about two-thirds the concentration of protein that bovine brain had, it was diluted less before precipitation of the proteins with ammonium sulfate. That is, 450 mL of supernatant was diluted with 550 mL of buffer, as above. All other steps were as described above for bovine brain cytosol.

DEAE-Cellulose Chromatography. The ammonium sulfate fraction was loaded onto a DEAE–cellulose column (44 × 2.5 cm) preequilibrated with 100 KTDG. The column was then washed with 100 mL of 100 KTDG and eluted with 340 mL of a linear gradient of 100–400 KTDG. Fractions (8 mL) were collected throughout the gradient. Fractions with high IP_6 binding (usually fractions 28–37) were pooled, 1.2 mL of 1 M potassium phosphate (pH 7.4) was added, and the fraction was diluted to a final volume of 120 mL with TDG.

Hydroxylapatite Chromatography. The sample was loaded onto a column (27 × 1.5 cm) containing 48 mL of hydroxylapatite ultragel preequilibrated with 200 KTDG containing 10 mM KH_2PO_4. The column was washed with 50 mL of the same buffer and the wash was discarded. The column was eluted with a linear gradient of 100 mL of KTDG containing 10–200 mM potassium phosphate (pH 7.4). Fractions (3 mL) were collected beginning with the start of the gradient elution. The active fractions (usually fractions 16–25) were pooled and diluted to 120 mL with TDG.

Q-Sepharose Chromatography. The sample was loaded onto a Q-Sepharose (Phamacia) column (6 × 0.75 cm) preequilibrated with 100 KTDG. It was washed with 50 mL of the same buffer, and the sample was eluted with 40 mL of a linear gradient of 100–500 KTDG buffer. Fractions (1.5 mL) were collected at the start of the gradient. The active fractions (usually fractions 20–28) were pooled and diluted to 135 mL with TDG.

Heparin-Agarose Chromatography. The sample was loaded onto a heparin–agarose (Pharmacia) column (5 × 0.75 cm) preequilibrated with TDG. It was washed with 10 mL of the same buffer, and elution was carried out with 40 mL of a linear gradient of 10–500 KTDG. Fractions (1.5 mL), beginning with the application of the gradient, were collected, and the active fractions (14–24) were pooled and made 0.5 M by the addition of 2 M KCl.

Phenyl Sepharose Chromatography. The sample was loaded onto a phenyl Sepharose (Pharmacia) column (5 × 0.75 cm) preequilibrated with 500 KTDG. The column was washed with 10 mL of TDG buffer and finally eluted using 30 mL of a linear gradient of 0–4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) in TDG buffer. Fractions (1.2 mL) were collected starting with the gradient, and the active fractions (usually 7–14) were pooled.

Batch absorption on Q-Sepharose was used to concentrate the proteins before the next step. The pooled fractions were treated with a 2.5-mL packed volume of Q-Sepharose in 100 KTDG containing 0.5% CHAPS. The mixture was allowed to stand for 1 h in the cold room with gentle mixing and was transferred to a small column made from a Pasteur pipet. The column was washed with 5 mL of the same buffer and eluted with 500 KTDG containing 0.5% CHAPS. Fractions of 0.33 mL were collected, and the protein peaks (about 1 mL total volume) were combined for further purification.

TSK-G4000 SW Chromatography. The final purification was carried out using gel exclusion chromatography on a TSK-G4000 SW (TosoHaas) column (600 × 7.5 mm) preequilibrated with 100 KTDG containing 1 mM EDTA and 0.5% (w/v) CHAPS. The column was eluted with the same buffer at a rate of 0.5 mL/min, and 0.5-mL fractions were collected and assayed for binding activity. The active fractions (usually 36–40) were checked for purity using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) before pooling. For gel electrophoresis, aliquots of 200 μL were taken, and proteins were precipitated by the addition of trichloroacetic acid (TCA) to a final concentration of 20% (w/v). After 30 min on ice, the precipitates were collected by centrifugation, washed with 0.5 mL of 6% TCA, centrifuged as before, and then washed finally with 0.5 mL of acetone and recentrifuged. The pellets were dried briefly in a vacuum and then dissolved in 15 μL of SDS–PAGE sample buffer. The composition of the sample buffer and electrophoresis conditions were as described by Laemmli (1970).

Preparation of Antiserum. Polyclonal antiserum was prepared as described previously (Gonatas et al., 1987). About 75 μg of purified IP_6BP was mixed 1:1 (v/v) with complete Freund's adjuvant to a final volume of 1 mL. One-quarter of the mixture was injected directly into each popliteal lymph node, and the remainder was injected intradermally at multiple sites in a New Zealand rabbit. About 3 weeks later, the rabbit was boosted by intramuscular injection of ~50 μg of the antigen mixed 1:1 with Freund's incomplete adjuvant. Serum collected 1 week later showed a positive reaction in Western blots versus the purified fraction. Three weeks after the last injection, the rabbit was again boosted as before, and serum was collected 2 weeks later.

Immunoprecipitation of IP_6 Binding Activity. The immunoreactivity of the antiserum prepared using purified IP_6BP from bovine brain was tested using the fraction of bovine brain cytosol precipitated in 40% saturated ammonium sulfate (ASP) as the source of IP_6BP. Before incubation with antiserum, the ASP fraction (1.7 mg of protein/mL) was centrifuged at 95 000 rpm for 10 min in a Beckman TL-100 rotor to remove any insoluble material. An aliquot of the sample was diluted with 6 vol of binding assay buffer as described above, and varying amounts of either preimmune or immune serum were added to 100 μL of diluted ASP. The samples were incubated for 1 h at room temperature and centrifuged at 95 000 rpm for 10 min as before. Duplicate aliquots of each supernatant were then assayed for IP_6 binding activity as described above.

Sources of Antibodies Used. Polyclonal antiserum against purified bovine brain IP_6BP was prepared as described above and used as detailed in the individual experiments. The affinity-purified polyclonal antibody to the α- and β-adaptins of AP-2 was kindly provided by Dr. Anthony Timerman (Timerman et al., 1992b). Anti-βCOP antibody (M3A5) (Allen & Kreis, 1986) was a gift from Dr. J. E. Rothman (Sloan-Kettering Institute for Cancer Research, New York). The monoclonal antibody to the γ-adaptin of AP-1 (M100/3) was a gift from Dr. E. Ungewickell (Max-Plank-Institute für Biochemie, Martinsreid, Germany) (Ahle et al., 1988).

SDS-PAGE and Western Blot Analysis. SDS–polyacrylamide gel electrophoresis, trans-electrophoresis, and immunostaining were carried out as described (Kijima et al., 1993).

RESULTS

A significant level of specific binding for IP_6 was found in the cytosol fraction of bovine brain (Table 1). IP_6 binding

Table 1: Purification of IP_6 Binding Protein from Bovine Brain Cytosol[a]

fraction	total protein (mg)	specific binding (pmol/mg)	purification (-fold)	recovery (%)
cytosol	4625	2.84[b]	1	100
ammonium sulfate ppt	573	21.8	7.7	95[2]
DEAE–cellulose	62	103	36.3	49
hydroxylapatite	30	237	83.4	54
heparin–agarose	10.6	374	132	30
phenyl Sepharose	2.8	521	183	11
TSK-G4000 SW	0.23	1330	468	2.3

[a] The data in this table are typical of three separate preparations. Binding assay was carried out using 10 nM [^{3}H]IP_6 in 25 mM Tris-HCl (pH 7.5) containing 100 mM KCl, 1 mM EDTA, and 1 mM DTT, essentially as described previously (Chadwick et al., 1992). Our modifications are described in Materials and Methods. [b] The measured specific binding of the cytosol was 0.45 pmol/mg protein. Ammonium sulfate precipitation of the cytosol increases the binding activity by 6-fold; an additional 5% binding activity can be obtained by further ammonium sulfate precipitation of the 40% ammonium sulfate supernatant fraction. Therefore, the inherent binding activity in the cytosol is calculated to be 2.84. $(NH_4)_2SO_4$ precipitation appears to remove an endogenous inhibitor. The final recovery of total IP_6 binding activity is 2.3%, and the protein enrichment is ~500-fold.

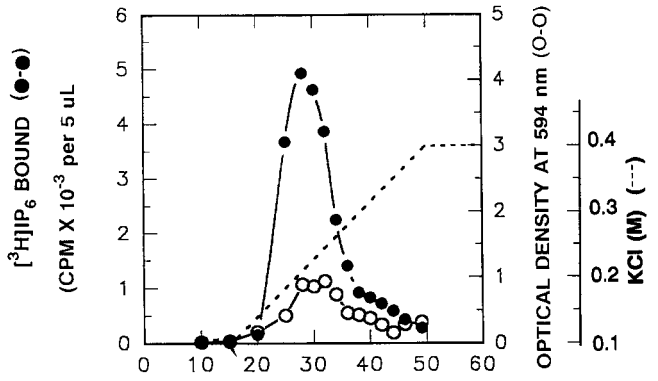

FIGURE 1: Chromatography of the 40% ammonium sulfate fraction from bovine brain cytosol on DEAE–cellulose. Details of the chromatography and assay procedures are given in Materials and Methods. IP_6 binding activity, ●; protein (by the Bradford assay), O; KCl concentration in the eluting buffer, - - -.

was partially purified by precipitation with 40% saturated ammonium sulfate. About 95% of the total precipitable binding activity was recovered in this fraction. As summarized in Table 1 (see footnote b), the yield of binding activity increased by about 6-fold after precipitation, indicating the removal of an endogenous inhibitor (possibly IP_6 itself). Further purification by DEAE–cellulose column chromatography revealed that the binding activity eluted as a major peak at about 200 mM KCl (Figure 1). About 4–5-fold purification of IP_6 binding activity was obtained in this step. The activity profile showed a tailing of the binding activity after the major peak, indicating there are other binding proteins in this fraction. Western blot analysis of the active fractions was also carried out using the monoclonal antibody M3A5, which recognizes the βCOP component of the coatomer. Coatomer was detected in tubes 33–42, with a peak at tube 37 (data not shown). Thus, the trailing peak of IP_6 binding corresponds to the coatomer elution peak.

Chromatography of the major IP_6 binding activity obtained from the DEAE column was carried out on hydroxylapatite. This step separated the binding activity from a major protein peak, leading to a further 2-fold purification (Figure 2).

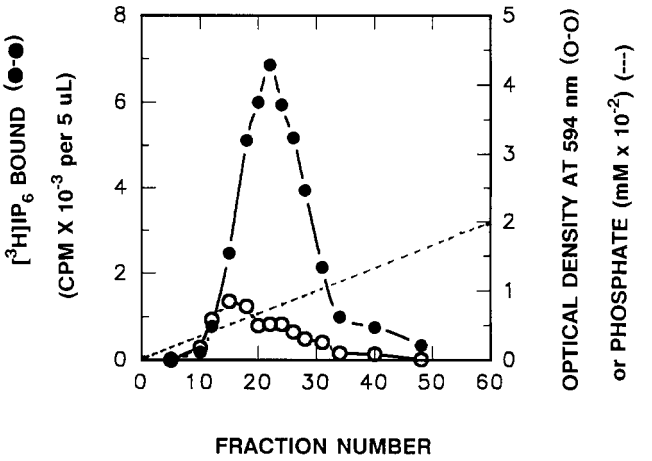

FIGURE 2: Chromatography on hydroxylapatite of the pooled IP_6 binding fractions from the DEAE–cellulose column. Details are given in Materials and Methods. IP_6 binding activity, ●; protein, O; concentration of potassium phosphate buffer (pH 7.4) in the eluting buffer, - - -.

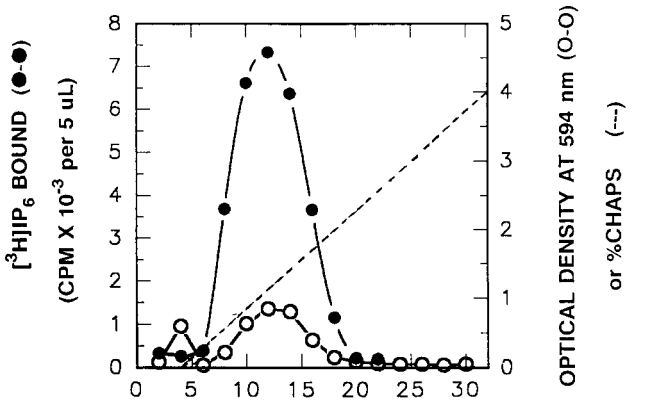

FIGURE 3: Chromatography of the pooled IP_6 binding fractions from the hydroxylapatite column on phenyl Sepharose. Details are given in Materials and Methods. IP_6 binding activity, ●; protein, O; %CHAPS used in the eluting buffer, - - -.

Chromatography on Q-Sepharose was used to concentrate the protein and remove the high level of phosphate used for elution from the hydroxylapatite column. After elution from the Q-Sepharose column, the pooled active fractions were diluted by about 10-fold to lower the salt concentration. The activity could then be adsorbed to a heparin–agarose column and eluted using a linear gradient of KCl (10–500 mM, data not shown). This step results in an additional 1.6-fold purification.

In the presence of 0.5 M KCl, the activity readily absorbed to phenyl Sepharose and was not eluted by lowering the salt concentration to zero. A single peak of activity and protein was eluted with a detergent gradient of 0–4% CHAPS (Figure 3). Purification was about 1.4-fold. A batch treatment with Q-Sepharose was used to concentrate the proteins and remove excess detergent. Final purification involved chromatography on a TSK-G4000 SW gel exclusion column. Using this procedure, we have successfully isolated a protein complex that specifically binds IP_6 and is purified by about 500-fold over the cytosol (Table 1 and Figure 4).

The IP_6 binding protein (IP_6BP) chromatographs as a single peak of binding activity on a TSK-G4000 SW gel exclusion column, with a relative size of 266 ± 14 kDa (n = 3, using three different preparations) when compared to the retention of protein standards of known size (Figure 4A), and gives a

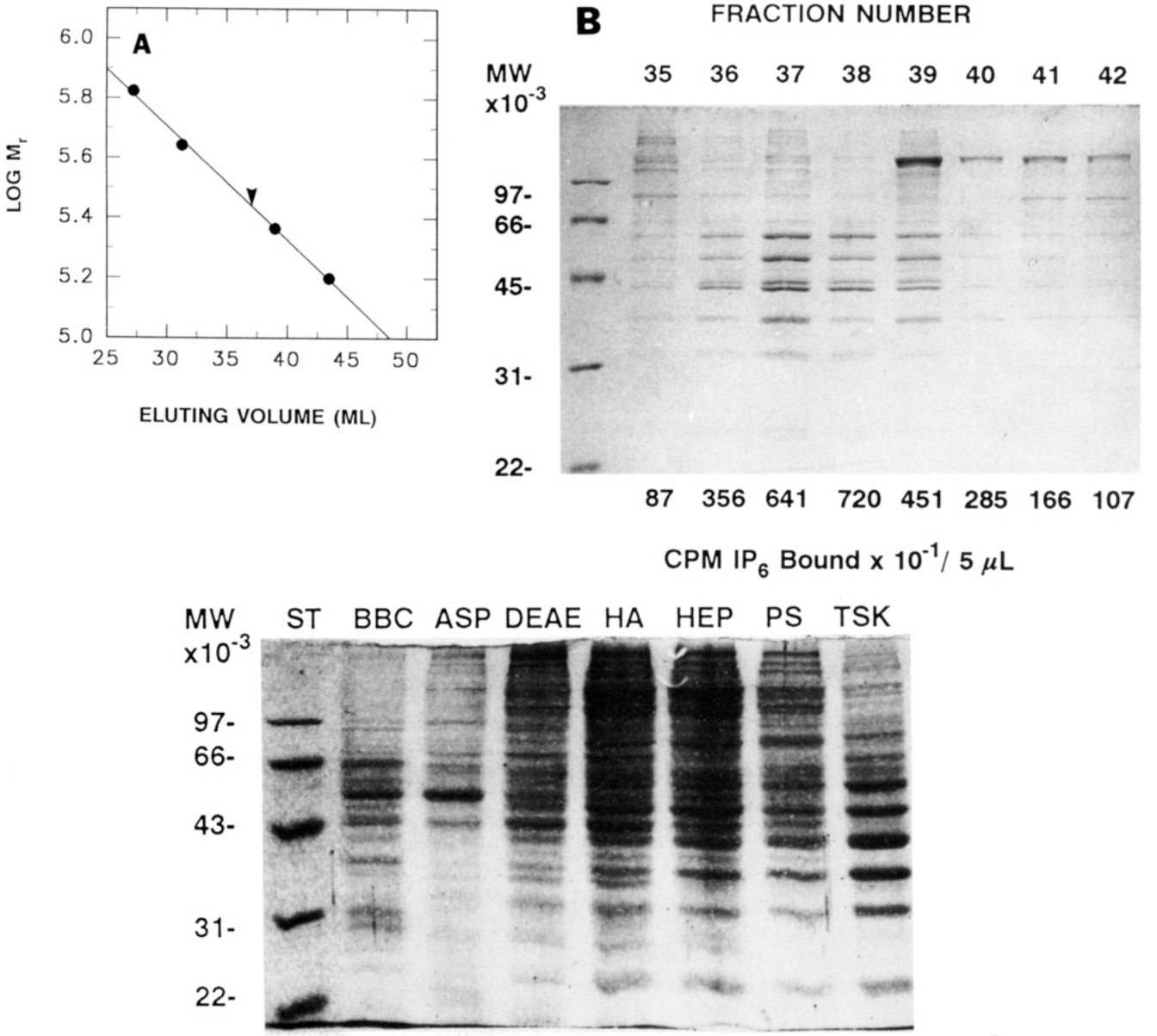

FIGURE 4: Chromatography of the pooled IP_6 binding fractions from the heparin–agarose column on TSK-G4000 SW. (A) Molecular weight determination of IP_6BP by chromatography on TSK-G4000 SW. See Materials and Methods for details of the procedure. M_r values of standard proteins run in the same buffer (●) are thyroglobulin, 669 000; ferritin, 440 000; catalase, 232 000; and aldolase, 154 000. Three different preparations of IP_6BP gave an average elution volume of 37.3 ± 0.6 mL (arrow), which corresponds to an M_r of 266 000 ± 14 000. (B) SDS–PAGE of the active fractions obtained after chromatography on TSK-G4000 SW. Electrophoresis was carried out according to Laemmli (1970) using a 12% polyacrylamide gel. The gel was stained with Coomassie Blue. Details of the chromatography and analysis of the fractions are given in Materials and Methods. Samples placed on the gel beginning from the left are as follows: lane 1, 4 μg of low molecular weight standards from Bio-Rad; lanes 2–9 are fraction numbers 35–42 from the TSK-G4000 SW gel exclusion column. The proteins in 70 μL of each fraction were concentrated by precipitation with trichloroacetic acid as described, washed with acetone to remove the acid, and dissolved in the sample buffer, and the total sample was placed on the gel. Separately, 5-μL aliquots from each fraction were assayed for IP_6 binding as described in Materials and Methods. The value obtained for each fraction is indicated below the gel. (C, bottom) SDS–PAGE of the pooled active fractions obtained from each of the purification steps summarized in Table 1. For each sample, a total of 4 μg of protein was precipitated with trichloroacetic acid as described, washed to remove the acid, and dissolved in the sample buffer, and the total sample was placed on the gel. ST: 4 μg of low molecular weight standard from Bio-Rad. The standard was run directly on the gel without prior precipitation.

characteristic and reproducible pattern of eight protein bands (~23–60 kDa) after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein profiles using SDS–PAGE of the peak activity fractions from the TSK-G4000 SW column fractionation are shown in Figure 4B.

The active fractions from the TSK-G4000 SW columns that showed the least contamination with higher molecular weight bands (usually fractions 36–39) were combined and designated as purified IP_6BP. A comparison of the fractions obtained from each of the purification steps described in Table 1 using SDS–PAGE is shown in Figure 4C. The pattern of bands that are characteristic of IP_6BP is discernible after hydroxylapatite chromatography. The pattern is enhanced after each of the next two purification steps and is prominent after gel exclusion chromatography, in which the content of contaminating larger molecular weight complexes is reduced. Rechromatography of the active fraction from the TSK column through the last three steps of the isolation does not enhance the purity of the final product. A variable level of contamination by higher molecular weight bands is present in the purified fraction, depending on how well the active bands are resolved on the gel exclusion column, how the fractions are

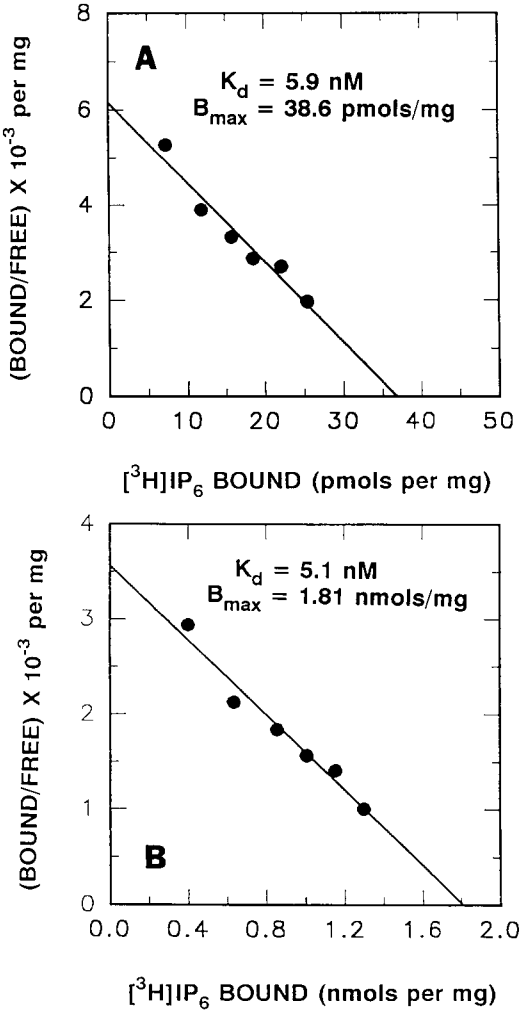

FIGURE 5: Scatchard analysis of IP_6 binding to fractions obtained from bovine brain cytosol. Specific binding was determined as described in Materials and Methods. The data shown are for one experiment, which is typical of three preparations. (A) Binding characteristics of the 40% ammonium sulfate fraction; (B) binding characteristics of purified IP_6BP.

pooled, and how heavily the gels are loaded (compare, for example, gel patterns of the different purified samples shown in Figures 4B,C and 7).

Analyses of IP_6 binding of both the ammonium sulfate fraction and the purified IP_6BP using Scatchard plots are shown in Figure 5. Single straight lines were obtained that are compatible with a single class of high-affinity binding sites in both the crude and purified fractions, with a similar K_d of 6.0 ± 0.9 nM (n=3). Maximum binding for the purified fraction is 1.8 nmol of IP_6 bound/mg of protein. Based on a molecular weight of 266 000 for the complex, maximum binding corresponds to about 0.48 mol of IP_6 bound/mol of the protein complex. The binding properties of this protein significantly differ from those of the IP_x receptor described previously by Chadwick et al. (1990). IP_6 binding to IP_6BP is more than an order of magnitude tighter (K_d of 6.0 nM compared to 120 nM), and the binding is not decreased significantly by the presence of near-physiological levels of salt (100 mM KCl) in the binding assay (data not shown). The binding of IP_6 by purified IP_6BP appears to be specific. The results of competition studies carried out with several other inositol polyphosphates are summarized in Table 2. In the presence of 10 nM [^{3}H]IP_6 (IC_{50} for IP_6 is 22 nM under these conditions), a 16-fold higher concentration of IP_5 (0.36 μM) is required to obtain 50% inhibition (IC_{50}) of IP_6 binding.

Table 2: Competition of Other Inositol Polyphosphates for IP_6 Binding to Purified IP_6BP from Bovine Brain[a]

ligand	IC_{50} (μM)	slope factor
IP_6	0.022 ± 0.0012	1.4 ± 0.1
1,4,5-IP_3	9.6 ± 1.2	1.8 ± 0.24
1,3,4,5-IP_4	0.53 ± 0.07	0.36 ± 0.05
1,3,4,5,6-IP_5	0.36 ± 0.02	0.62 ± 0.04

[a] Binding assays were carried out as described in Table 1. In all cases, the cold ligand was added to the 10 nM [^{3}H]IP_6 before addition to the assay mixture containing IP_6BP. IC_{50} values and slope factors were calculated as described (Chadwick et al., 1992). A slope factor of less than 1.0 indicates negative cooperativity, while a factor greater than 1.0 indicates positive cooperativity. A typical experiment is shown, and all assays were carried out on the same preparation on the same day. Correlation coefficients ranged from 0.99 to 0.98 for the three curves. Standard deviations were calculated from the standard deviation of the slopes obtained for each curve.

A 24-fold higher concentration of IP_4 (0.53 μM) is required, and a greater than 400-fold higher concentration of IP_3 (9.6 μM) is necessary for 50% inhibition of IP_6 binding under the same conditions.

IP_6BP appears to be widely distributed in eukaryotes. The proteins precipitated by 40% saturated ammonium sulfate from the cytoplasmic fractions from rat brain and rat liver have similar IP_6 binding capacities compared to that found for bovine brain, i.e., ~35 pmol/mg protein. The proteins precipitated from the cytoplasmic fraction of dog heart ventricle had somewhat lower binding capacities (15 pmol/mg protein). Supernatants from lobster muscle, spinach leaves, and yeast (*Saccharomyces cerevisiae*) also yielded fractions from the cytosol that bound IP_6 with K_d values in the range of 1–3 nM (data not shown).

Incubation of partially purified IP_6BP with 10 μM [^{3}H]IP_6 in the presence of 1 mM Mg^{2+} for 30 min at 37 °C resulted in no breakdown of IP_6 detectable using ion-exchange chromatography, carried out as described by Cosgrove (1969). In addition, no kinase activity was detected when the protein was incubated with 100 μM 1,3,4,5,6-IP_5 in the presence of 100 μM Mg[γ^{32}P]ATP.

Polyclonal antiserum capable of precipitating IP_6BP from bovine brain cytosol was prepared in a rabbit using purified IP_6BP. The antiserum precipitates about 70% of the total IP_6 binding capacity present in the fraction of bovine brain cytosol obtained by precipitation with 40% saturated ammonium sulfate (Figure 6). The antiserum does not inhibit IP_6 binding by IP_6BP when added to the standard assay medium (data not shown). The antiserum does not precipitate any IP_6 binding activity from rat brain or liver cytosol, but it does cross-react with purified IP_6BP from liver by Western blot analysis. The fact that our antiserum does not remove a significant amount (30%) of the total IP_6 binding proteins present in the bovine brain cytosol fraction that is insoluble in 40% saturated ammonium sulfate suggests that there may be other species of IP_6BP present.

The procedure for the purification of IP_6BP is applicable to the fractionation of rat liver cytosol as well. Using the purification steps described, a similar but not identical IP_6BP was isolated. One difference is its behavior upon gel exclusion chromatography. The activity eluted more rapidly from the TSK-G4000 SW column with a peak at about tube 30 rather than tube 37. This corresponds to a protein complex with a relative molecular size of about 512 000, which is much larger than the size obtained for IP_6 BP from bovine brain. Analysis of the protein using SDS–PAGE revealed a typical protein pattern for IP_6BP plus an additional protein band with an M_r of 97 000 (Figure 7). It appears that this protein is associated

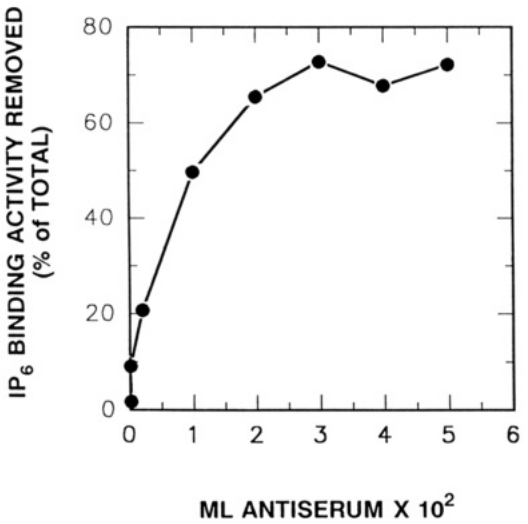

FIGURE 6: Precipitation of IP_6 binding activity in the 40% ammonium sulfate fraction of bovine brain cytosol by the addition of rabbit antiserum to IP_6BP. Details of the procedure used are given in Materials and Methods.

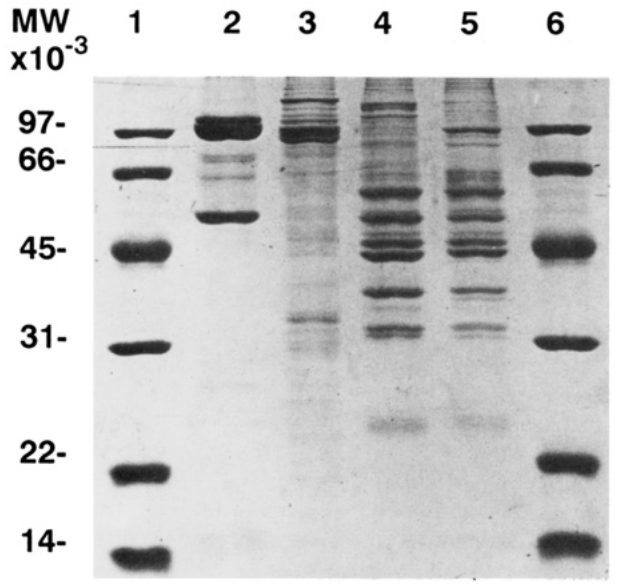

FIGURE 7: Comparison of purified IP_6BP from bovine brain and rat liver by SDS–PAGE. The purified IP_6BPs were obtained from the TSK-G4000 SW gel exclusion column. Preparation of samples and electrophoresis conditions were as described in the legend to Figure 4B. Lanes 1 and 6, 4 μg of low molecular weight standard; lane 2, 4.3 μg of adaptor protein-2 (gift of Dr. Anthony Timerman); lane 3, 2.4 μg of Golgi coatomer complex (gift of Dr. James Rothman); lane 4, 3 μg of purified IP_6BP from bovine brain cytosol; lane 5, 3 μg of purified IP_6BP from rat liver cytosol.

with IP_6BP in this preparation, possibly in the form of a dimer. Purified IP_6BP from rat liver binds a maximum of 0.72 nmol of IP_6/mg of protein or 0.37 mol/mol complex, which is similar to the value obtained for IP_6BP from bovine brain.

Immunological comparisons of AP-2, coatomer, and IP_6-BP from rat liver and bovine brain were carried out using Western blot analysis. Rabbit anti-bovine brain cytosolic IP_6-BP, which we prepared, showed a strong reaction to the 60-kDa band and the double bands at 45 and 43 kDa of IP_6BP from both rat liver and bovine brain at antisera dilutions of 1/500 to 1/2500. A weaker reaction was also seen with protein bands at 38, 24, and 23 kDa at 1/500 dilution, which was not seen at higher dilutions (data not shown). This antibody did not react with similar concentrations of purified coatomer or AP-2 run on the same gel (data not shown). Conversely,

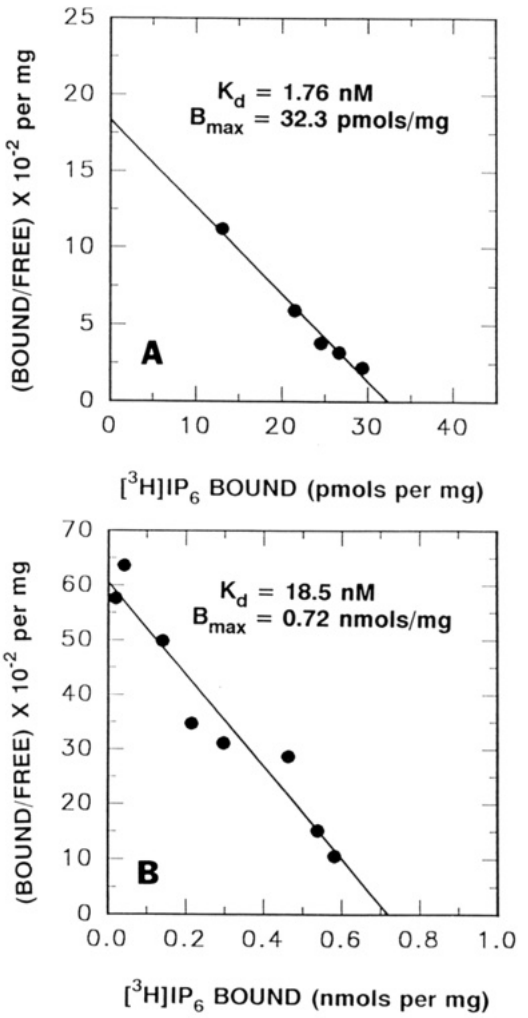

FIGURE 8: Scatchard analysis of IP_6 binding to fractions obtained from rat liver cytosol. Specific binding was determined as described in Materials and Methods. The data shown are for one preparation, which is typical of two different preparations. (A) Binding characteristics of the 40% ammonium sulfate fraction; (B) Binding characteristics of purified IP_6BP.

when these preparations were probed using mAb3A5, a monoclonal antibody that specifically recognizes the βCOP component of coatomer, only coatomer showed a positive band at about 105 kDa (data not shown). In addition, Western blot analysis of the gel illustrated in Figure 7 was carried out using an affinity-purified polyclonal anti-AP-2, which reacts with both the 111- and 110-kDa subunits of AP-2 (Timmerman et al., 1992). This antibody reacted positively with AP-2, but not with coatomer or with either preparation of IP_6BP (data not shown). These observations, summarized qualitatively in Table 3, support the conclusion that IP_6BP is immunologically distinct from IP_6 binding proteins known to be coat proteins of either clathrin-coated or non-clathrin-coated transport vesicles.

IP_6BP from rat liver was investigated for its ability to metabolize $[^3H]IP_6$ or the pyrophosphate derivative of this compound, $[^3H]IP_7$. For these assays, 0.4 μg of purified IP_6-BP in 0.2 mL of Hepes (pH 7.2) contained 100 mM KCl, 6 mM $MgSO_4$, 100 μg bovine serum albumin, 1 mM DTT, 5 mM ATP plus 8 units of phosphocreatine kinase, and either 22 000 dpm of $[^3H]IP_6$ (~5 nM) or 3000 dpm of $[^3H]IP_7$ (mass unknown). After 15 min at 37 °C, the assays were quenched with perchloric acid and neutralized with Freon–octylamine, and inositol polyphosphates were analyzed by HPLC (Menniti et al., 1993). No metabolism of IP_6 or IP_7

Table 3: Immunological Comparison of IP_6BP with Purified Coat Proteins[a]

antibody	antigen detected (kDa)	bovine brain IP_6BP	rat liver IP_6BP	bovine brain AP-2	bovine liver coatomer
anti-IP_6BP (bovine brain)	60, 45, and 43	+	+	–	–
anti-AP-2	111 and 110 (α- and β-adaptins)	–	–	+	–
anti-βCOP	110 (βCOP)	–	–	–	+
anti-AP-1	110 (γ-adaptin)	nt	–	nt	–

[a] Analyses were carried out using Western blots. Anti-IP_6BP is a rabbit antiserum prepared using purified IP_6BP isolated from bovine brain cytosol as antigen and was used at 1/500 dilution. Anti-AP-2 is an affinity-purified polyclonal antibody made using purified AP-2 from bovine brain as antigen (Timerman et al., 1992b) and was used at 1/20 dilution. Anti-βCOP is monoclonal antibody M3A5, which specifically recognizes the βCOP component of Golgi coatomer (Allen & Kreis, 1986) and was used at 1/3000 dilution. Anti-AP-1 is monoclonal antibody M100/3, which specifically recognizes the γ-adaptin component of AP-1 (Ahle et al., 1988) and was used at 1/1000 dilution. Methodological details and sources of the antibodies used are given in Materials and Methods. nt, not tested.

was detected (the limit of detection was about 5% of the inositol polyphosphate present; data from Stephen B. Shears, personal communication).

Analyses of IP_6 binding of the ammonium sulfate fraction and the purified IP_6BP from rat liver by Scatchard plots is shown in Figure 8. The ammonium sulfate fraction had a single class of high-affinity binding sites with a somewhat lower K_d than that found for bovine brain (1.8 versus 6.0 nM). The purified fraction, however, bound IP_6 with a significantly lower affinity (K_d about 19 nM). It is possible that IP_6 binding is modulated by the association of IP_6BP with the 97-kDa protein.

DISCUSSION

We have purified a soluble protein complex from bovine brain cytosol that specifically binds IP_6 with high affinity. The apparent enrichment achieved over the cytosol is approximately 500-fold. Estimation of the exact enrichment of IP_6BP is complicated by the presence of an endogenous inhibitor in the cytosol, probably IP_6 itself (Hogson & Shears, 1990). The estimate of purification is based on the 6-fold-enhanced activity of the fraction obtained by precipitation with 40% saturated ammonium sulfate as compared with the cytosol, which removes the endogenous inhibitor. From SDS–PAGE analysis of the purified fractions, we estimate the purity of the preparation to be about 50%. Using these calculations, the abundance of the protein in the cytosol can be estimated to be about 0.1% of the total protein.

Under the assay conditions used, the purified complex binds IP_6 with an apparent K_d of 6.0 nM and a B_{max} of 1.8 nmol of IP_6 bound/mg of protein. The size of the complex from gel exclusion data is 266 ± 14 kDa, consisting of eight polypeptides. The molar ratio of binding is about 0.48 mole of IP_6 bound/mol of complex. This also could be indicative of the purity of the preparation, although some loss of binding activity could also contribute to a less than 1/1 molar binding ratio. Inclusion of protease inhibitors in the homogenizing medium did not alter the electrophoretic pattern of the purified protein, nor did it improve the binding capacity.

The purified binding protein shows a reproducible pattern after SDS–PAGE analysis. Eight bands are present with calculated M_r values of 60 000, 51 000, 45 000, 43 000, 38 000, 33 000, 24 000, and 23 000. If we assume that the complex contains a single molecule of each protein, the calculated molecular weight of the complex would be 317 000. This is somewhat larger than the measured value of 266 000 ± 14 000, obtained using gel exclusion chromatography. We cannot exclude the possibility that one (or more) of the protein bands could result from proteolysis. If so, the altered protein remains associated with the complex during the isolation procedure.

Application of the purification procedure to rat liver supernatant yielded a similar, but not identical, protein complex. It has a larger size (512 kDa) and an additional protein band (M_r = 97 000). The association of this protein to IP_6BP may be responsible for the significantly higher K_d observed for IP_6 binding in this preparation, compared to the rat liver cytosol fraction obtained after precipitation with 40% saturated ammonium sulfate (19 versus 1.8 nM). If so, it suggests that the extra protein is not associated with IP_6BP in the fraction precipitated with ammonium sulfate. Additional studies to define this interaction are necessary.

The IP_6BP described here significantly differs from the inositol polyphosphate binding proteins described previously. It differs from both IP_3 receptors isolated from brain (Supattapone et al., 1988) or smooth muscle (Chadwick et al., 1990), in that the IP_3BP has a more complex protein profile, is present primarily in the cytosolic fraction, and binds IP_3 poorly (in the micromolar rather than the nanomolar range). It also differs from the inositol polyphosphate receptor isolated from cerebellum (Chadwick et al., 1992) and subsequently shown to be similar to clathrin assembly protein AP-2 (Timerman et al., 1992a; Voglmaier et al., 1992). Its protein profile (Figure 7), immunological reactivity (Table 3), and binding properties are all distinct. IP_6BP binds IP_6 much better than IP_4 (Table 2), whereas AP-2 binds both with about equal affinity. The presence of salt has little or no effect on the binding of IP_6 by IP_6BP, whereas the presence of even isotonic salt decreases the affinity of AP-2 for IP_6 by about 5-fold.

At least two important cytosolic proteins are known to bind IP_6 in a physiologically significant manner. Hemoglobin binds both IP_6 and IP_5 with K_d values of about 1 and 12 μM, respectively, and the reaction significantly decreases its oxygen binding affinity by 2–4-fold, depending on the isoform of hemoglobin used (Isaacks et al., 1976). The binding is physiologically relevant in avian erythrocytes, where high levels of IP_5 appear during the maturation of fetal red blood cells (Isaacks & Harkness, 1980). Arrestin is a cytosolic 48-kDa protein present in bovine rod outer segments that specifically binds phosphorylated rhodopsin in its light-activated form, thereby blocking its ability to activate the G-protein transducin (Wilden et al., 1986). IP_6 inhibits the arrestin–rhodopsin interaction by binding to free arrestin (K_d = 5.5 μM; Palczewski et al., 1991). β-Arrestin, a 46-kDa homologue of arrestin, is expressed in bovine brain (Lohse et al., 1990). It is believed to function by inhibiting the GTPase activity of phosphorylated β_2-adrenergic receptors bound to G_s (Lohse et al, 1990). Its binding to inositol polyphosphates has not been investigated as yet. Both arrestin and β-arrestin are easily purified by sequential ion-exchange chromatography followed by Superose 12 gel exclusion chromatography, in which they migrate as monomers. Thus, it is unlikely that these proteins are present

in our IP_6BP preparations, which behave as high molecular weight multimers under similar conditions.

IP_6BP also appears to differ from several inositol polyphosphate metabolizing enzymes that have been isolated from brain cytosol and shown to bind IP_6. Inositol 1,3,4,5-tetrakisphosphate 3-phosphatase from rat liver has a high affinity for IP_6 (K_m = 0.3 nM) (Nogimori et al., 1991). The enzyme displays a single band of M_r = 66 000 on SDS–PAGE. In contrast, IP_6BP is a heterooligomeric complex and has no detectable phosphatase activity. Interestingly, the 3-phosphatase appears to be compartmentalized inside the endoplasmic reticulum (Ali et al., 1993) and thus is not likely to metabolize IP_6 in vivo, since IP_6 is generally believed to be almost entirely in the cytosol.

The function of IP_6BP remains obscure. It does not appear to be involved in IP_6 formation or breakdown. Since the function of IP_6 itself is not understood, it is difficult to speculate as to the function of a protein that specifically binds it. From the studies presented here, we can conclude that IP_6BP, or proteins with similar binding properties, appears to be widely distributed in eukaryotes. Cell supernatants from organisms as diverse as *S. cerevisiae*, spinach leaves, and various mammalian sources exhibit high-affinity (nanomolar) binding for IP_6. The apparent widespread distribution of this IP_6BP suggests that it is involved in a fundamental process in eukaryotic cells.

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