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INOSITOL HEXAKISPHOSPHATE RECEPTOR IDENTIFIED AS THE CLATHRIN ASSEMBLY PROTEIN AP-2

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To clarify the function of the receptor binding protein for inositol hexakisphosphate (IP_6), we obtained a partial amino acid sequence from the purified protein and a partial nucleotide sequence from a cDNA clone of the gene. The sequences are essentially identical to those of the α-subunit of the clathrin assembly protein AP-2. The IP_6 receptor protein analyzed by SDS-PAGE contains a series of subunits which are the same as those of AP-2. Antibodies to AP-2 react with the IP_6 receptor protein in immunoblot analysis. © 1992 Academic Press, Inc.

A number of inositol polyphosphates serve important messenger functions. Insight into functions of inositol phosphates has been obtained by study of their receptor proteins, with the inositol 1,4,5-trisphosphate (IP_3) receptor having been best characterized as a ligand regulated calcium channel (1). To clarify functions of inositol 1,3,4,5-tetrakisphosphate (IP_4) and inositol hexakisphosphate (IP_6), we identified and isolated receptor binding proteins for these substances (2). Utilizing photoaffinity labels we identified the subunits that possess recognition sites for the inositol phosphates (3).

Abbreviations: IP_6, inositol hexakisphosphate; IP_6R, inositol hexakisphosphate receptor protein; IP_4, inositol 1,3,4,5-tetrakisphosphate; TFA, trifluoroacetic acid; ACN, acetonotrile; PCR, polymerase chain reaction; CHAPS, 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate.

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We have now obtained partial amino acid sequence for the IP_6 receptor protein (IP_6R) binding subunit and conducted polymerase chain reaction (PCR) analysis using degenerate primers based on this amino acid sequence. We demonstrate that the amino acid and nucleotide sequences for IP_6R are essentially identical to those of the clathrin assembly protein AP-2 and that antibodies to AP-2 react with IP_6R.

Materials and Methods

Materials.

IP_4 affigel was synthesized as described by Estevez and Prestwich (4). Heparin-agarose, CHAPS, and protease inhibitors were obtained from Sigma. Concanavalin A was purchased from Pharmacia. Clathrin-sepharose was synthesized as described (5). D-myo-inositol phosphates were obtained from Calbiochem. Other reagents were from Sigma. Rabbit polyclonal antibodies were generated to peptides corresponding to sequences in the mouse brain α_A (residues 720-736) and rat brain β (residues 511-536) subunits of AP-2. Peptides with an N-terminal cysteinyl residue were coupled to maleimide-activated keyhole limpet hemocyanin (Pierce, Rockford, IL) and injected following a previously described immunization protocol (6).

Isolation of IP_6R.

IP_6R was purified as described previously (2). Briefly, cerebellar membranes were isolated and solubilized with 1% CHAPS detergent and the crude CHAPS extract was chromatographed on Heparin agarose. The heparin eluate was collected and chromatographed on Concanavalin A-sepharose to remove the IP_3 receptor. The proteins which did not interact with Con-A were incubated with IP_4-affigel 10 and the various inositol phosphate binding proteins were eluted from the IP_4 resin with increasing ionic strength buffer (NaCl). IP_6R was eluted from the IP_4 column with 200-500 mM NaCl. Proteins were analyzed by SDS-PAGE on 7.5% Laemmli gels and stained with Coomassie blue.

Isolation of AP-2 Protein.

The clathrin assembly protein AP-2 was purified from bovine brain coated vesicles as described (5,7). Briefly, brain coated vesicles were obtained by density gradient centrifugation and purified AP-2 was prepared either by clathrin-sepharose affinity chromatography or by hydroxylapatite fractionation.

Generation and Purification of Peptides from IP_6R.

Affinity purified IP_6R (approximately 1 mg) was separated by SDS-PAGE on a 7.5% Laemmli gel. After separation, the proteins were transferred to nitrocellulose (0.45 μm Schleicher and Schuell) and visualized with Ponceau S (Sigma). The 105-115 kD protein band was cut from the blot, reduced and alkylated. The nitrocellulose was cut into 2 mm squares and placed in a microfuge tube with 1 ml of 1 mg/ml trypsin (Boehringer) for 18 hr at 25°C in 50 mM NH_4CO_3. The nitrocellulose was removed by centrifugation at 11,000 x g for 10 min and the supernatant was lyophilized, resuspended in 0.1% trifluoroacetic acid (TFA) and applied to an HPLC (Hewlett Packard, HP109) C-8 column, (RP-300 μM 220 x 21 mM, Aquapore) that had been equilibrated in 0.1% TFA. A gradient of 0 - 70% acetonitrile (ACN) was run over 20 ml (1 ml/min) and the peptide peaks were monitored at 214 nm and collected by hand. Peptides were repurified on a diphenyl column (208TP52, 2.1 x 250 mm, Vydac) using a gradient of 0-70% ACN. Peptides were sequenced using a pulsed-liquid phase sequencer and compared to amino acid sequences in the GenBank sequence database.

PCR.

Fully degenerate oligonucleotide primers were designed against 6-8 amino acids at the N and C termini of the two peptides isolated from digestion of the IP_6R. All

primers include nine base pair sequences at the 5' end which allowed subcloning into EcoR1 and BamH1 sites and had the following sequences:

V5': CCCGAATTCGTNGGNGGNTA(C/T)AT(A/C/T)(C/T)T
V3': CCCGGATCCGG(G/A)TCNCCNGC(T/G/A)ATNA(G/A)(G/A)TT
15': CCCGAATTCAT(A/C/T)AT(A/C/T)GGNTT(C/T)GGN(T/A)(C/G)NGC
13': CCCGGATCCAT(T/G/A)ATNCCNGCNCCNAC(A/G)AA(A/G)TT

First strand cDNA derived from total rat cerebellar RNA was subjected to 35 cycles of PCR at 42°C annealing temperature to amplify the short intervening sequences. A single prominent band of the expected size was excised from a 1% agarose gel, subcloned into a Bluescript vector (Stratagene) and sequenced by the dideoxy chain termination method using a USB Sequenase kit.

Results

We generated several tryptic peptides of the 105-115 kD IP_6 binding subunits of IP_6R and determined the amino acid sequence of two of the peptides (Table 1). The sequences of the 17 and 25 amino acid peptides are identical to amino acid numbers 498-514 and 869-893 of the predicted amino acid sequence of the α_A and α_C subunits, respectively, of the mouse AP-2 protein (8).

Fully degenerate oligonucleotide primers were designed to the N- and C-termini of the two tryptic peptides. PCR analysis enabled us to determine the intervening nucleotide sequences. These rat sequences are nearly identical to the corresponding sequences of the mouse α_A and α_C subunits of the AP-2 protein (Table 2).

The AP-2 protein comprises multiple subunits, a doublet at 114 and 116 kD, a doublet at 104 and 106 kD and single bands at 50 and 17 kD on SDS-PAGE (7). Our previous purification of IP_6R had revealed doublets at about 115 kD and 105 kD as well as a single band at 50 kD (2). Reexamination of our SDS-PAGE preparations now reveals a relatively faint band at 17 kD. To further assess the relationship between IP_6R and AP-2, we have utilized two anti-peptide antibodies generated to sequences in the α_A and β subunits. In immunoblots of IP_6R preparations (Fig. 1), the α_A antibody reacts with a closely spaced doublet at ≈115 kD corresponding to the dye-stained α_A bands in AP-2 preparations (5). The β antibody reveals a strong band

Table 1
Sequence Comparison Between Peptides of the IP_6R 105-115 kD Subunits and the Predicted Amino Acid Sequence of Mouse α-Adaptin

Mouse α_A Adaptin	495 NMVKVGGYILGEFGNLIAGDPRSSP
	::::::::::::::::::
Rat brain IP_6R (peptide #55)	VGGYILGEFGNLIAGDP
Mouse α_C Adaptin	865 TKAKIIGFGSALLEEVDPNPANFVGAGIIHTKT
	::::::::::::::::::::::::::
Rat brain IP_6R (peptide #72)	IIGFGSALLEEVDPNPANFVGAGII

Table 2
Comparison of Nucleotide Sequence from Rat Brain IP_6R and Mouse α-Adaptin cDNAs

Mouse α_C Adaptin	2620	TCTGCGCTCCTGGAAGAAGTTGACCCGAATCCTGCAAATTT
		:::::::::::::::::: :: :: ::::::
Rat brain IP_6R		GCTCCTGGAAGAAGTTGATCCCAACCCTGCA
Mouse α_A Adaptin	2737	TCTGCTCTGGACAATGTGGATCCCAACCCTGAGAACTTTGT
		::::::::::::::::::::::::::::::::
Rat brain IP_6R		TCTTCTGGACAATGTGGATCCCAACCCTGAG
Mouse α_C Adaptin	1504	ATCCTGGGGGAGTTTGGAAACTT
		::: :::::::::
Rat brain IP_6R		GGGCGAGTTTGGA

consistent with the major β subunit at ≈105 kD. The band at ≈114 kD is likely to be the quantiatively more minor β* subunit identified in AP-2 preparations (7) and coded for by the AP105a clone described earlier (9) as its sequence is identical to β in the 26-mer peptide used for immunization (except for a C-terminal alanine instead of threonine).

Beck and Keen (10) photolabeled AP-2 with an azido-ATP derivative which labeled selectively α_A and α_C subunits of AP-2 which are about 116 and 104 kD respectively. Careful analysis of our photoaffinity labeling of IP_6R with an [^{125}I]labeled azidosalicylamide derivative of IP_4 (3) also reveals that the photoaffinity label modifies the smaller of the 115 and 105 kD doublets (Table 3). The selective labeling of the lower bands which is fully displaced by 1000-fold excess IP_6, is also evident when using chiral D-myo-IP_4 as the competitor and the optically pure [^{125}I]ASA-aminopropyl-IP_4 photolabel (11). Thus, the IP_6 binding site is associated with the subunit whose peptide sequence we have shown to be identical to AP-2.

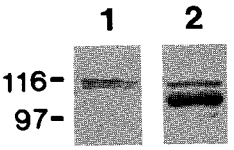

Figure 1. Antibodies to the α and β subunits of AP-2 react with IP_6R preparations. Immunoblot of IP_6R preparations challenged with anti-α_A peptide antiserum (lane 1) and anti-β peptide antiserum (lane 2). Molecular weight markers (x 10^{-3}) are indicated at left.

Table 3
Inositol Hexakisphosphate Recognition Sites in AP-2

AP-2 Subunit Designation	Apparent Size (kD)	IP_6 Binding
β*	116	No
α_A	114	Yes
β	106	No
α_C	104	Yes
AP-50	50	No
AP-17	17	No

Discussion

The principal finding of this study is that IP_6R is identical to the clathrin assembly protein AP-2. While this work was in progress Timerman et al. (12) in an abstract reported that a 20 amino acid peptide isolated from the 50 kD subunit of a cerebellar protein that binds IP_4, IP_3 and IP_6 is identical to the corresponding sequence in AP-2. What might be the role of IP_6 or lipid-linked inositol phosphates in regulating AP-2? The clathrin assembly proteins, also referred to as adaptors or associated (AP) proteins, promote the assembly of clathrin into coated vesicles (13). AP-1 is localized to the Golgi apparatus of cells and is implicated in the regulation of intracellular membrane trafficking. AP-2 occurs at the plasma membrane and is proposed to be involved in recycling receptors from the plasma membrane surface (14-16). The enrichment of AP-2 in the brain suggests that it may be associated with recycling of synaptic vesicles and/or regulation of neurotransmitter receptors. Inositol phosphates and phosphatidyl inositol phosphates inhibit self-assembly of AP-2 and clathrin coat formation (10). The Kd of IP_6 in binding to IP_6R is about 12 nM, substantially lower than endogenous concentrations of IP_6 in most tissues (17,18).

Several lines of evidence indicate that the effects of polyphosphoinositides and/or inositol phosphates on AP-2 assembly activity is likely to be mediated by the core portion of the α subunit of AP-2. This domain is specifically photoaffinity-labeled by 8-azido-ATP in a reaction that is totally blocked by inositol phosphates (10), and has also been shown to bind to clathrin in a polyphosphate-inhibitable manner (19,20).

The exact nature of the physiologically relevant effector that binds to this site is unclear. Though IP_6 has the greatest affinity under the experimental conditions used, and is present at substantially higher concentrations in most tissues, AP-2 also binds some isomers of IP_4 with high affinity (12). Furthermore, AP-2 does not seem to discriminate between polyphosphoinositides and free inositol phosphates, at least for PIP_2 and 1,4,5-IP_3 (10). The localization of AP-2 at the plasma membrane (8) raises the possibility that PIP_2, which is thought to be present

at the plasma membrane at relatively high local concentrations, or other, more highly phosphorylated plasma membrane phosphoinositides such as PIP_3, may modulate clathrin assembly activity.

Acknowledgments

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