Analysis of 100–180-kDa Phosphoproteins in Clathrin-coated Vesicles from Bovine Brain*

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Protein kinases which co-purify with clathrin-coated vesicles are known to phosphorylate in vitro the 50-kDa subunit of the HA-II adaptor complex and upon inclusion of polylysine the β-light chain of clathrin and polypeptides above 100 kDa. Here we relate the high molecular mass phosphoproteins to the known subunits of the adaptor protein complexes and to other clathrin-associated proteins by means of immunoprecipitation with monoclonal antibodies, two-dimensional electrophoresis, or electrophoresis in urea-sodium dodecyl sulfate-polyacrylamide gels. Our results show that some of the labeling of the 100–120-kDa region is accounted for by the β- and γ-subunits of the HA-I adaptor complex, the αc, and, to a lesser extent, by the β-subunits of the HA-II adaptor complex. In addition, we found the assembly protein AP 180 and a hitherto undescribed 110-kDa coat polypeptide to be heavily phosphorylated upon release of these proteins from the coated vesicle membrane. In all cases, labeling was confined to serine residues.

The coat of clathrin-coated vesicles from bovine brain is constructed from clathrin (heavy and light chains) in association with several other proteins (1). Three of these have been specified and partially characterized in structural and functional terms. They are referred to as AP 180 (2, 3) and the HA-I and HA-II adaptor protein complexes (4). The former, a monomeric neuron-specific protein, supports in vitro the assembly of clathrin triskelia into cage-like structures and was therefore termed assembly protein (AP). The ubiquitous HA-I and HA-II adaptor proteins are heterotetramers that were shown to interact with clathrin and certain receptor proteins (5–7). The ubiquitous HA-I and HA-II adaptor protein complexes and to other clathrin-associated proteins by means of immunoprecipitation with monoclonal antibodies, two-dimensional electrophoresis, or electrophoresis in urea-sodium dodecyl sulfate-polyacrylamide gels. Our results show that some of the labeling of the 100–120-kDa region is accounted for by the β- and γ-subunits of the HA-I adaptors, the αc, and γ-subunits of the HA-II adaptor complex. In addition, we found the assembly protein AP 180 and a hitherto undescribed 110-kDa coat polypeptide to be heavily phosphorylated upon release of these proteins from the coated vesicle membrane. In all cases, labeling was confined to serine residues.

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EXPERIMENTAL PROCEDURES

Materials

Fresh bovine brains were obtained from a local abattoir and processed within 1 h of slaughter. Adenosine 5’-[γ-32P]triphosphate, triethylammonium salt ([γ-32P]ATP), ~110 TBq/mmol was from Amerham Buchler, Braunschweig (Federal Republic of Germany). EGTA and HEPES were from Serva, Heidelberg (FRG). Reagents for SDS-PAGE were from LKB Instruments GmbH, Gräfelfing (FRG). MES, poly-DL-lysine hydrobromide (Mf = 30,000–70,000), and phenylmethyldifluorofluoride (PMSF) were from Sigma Chemie GmbH, Deisenhofen (FRG). Sepharose CL-4B and CNBr-activated

The abbreviations used are: [γ-32P]ATP, adenosine 5’-[γ-32P]triphosphate triethylammonium salt; EGTA, [ethylene bis(oxyethylenemine)]tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MES, 2-(N-morpholinio)ethanesulfonic acid; DTT, dithiothreitol; mAb, monoclonal antibody.
Seapharose 4B were from Deutsche Pharmacia, Freiburg (FRG). Bio-Gel HPHT hydroxypatite column was from Bio-Rad Laboratories GmbH, Munich (FRG). Immobilon transfer membranes were from Millipore, Eschborn (FRG). All other chemicals and reagents were of analytical grade.

Methods

Preparation of Coated Vesicles—Coated vesicles were prepared from bovine brain according to the method of Campbell et al. (17).

Tris Extraction of Coat Proteins and Preparation of the Assembly Protein Fraction—Coated vesicles were extracted at pH 7.0 with 0.5 M Tris, containing 0.1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, and 1 mM DTT according to the procedure of Keen et al. (18). The extract was clarified by ultracentrifugation at 100,000 × g for 45 min. For the preparation of the assembly protein fraction, consisting of HA-I and HA-II adaptors, AP 180, and other yet uncharacterized proteins, the extract was subjected to gel filtration on a Sepharose CL-4B column (2.6 × 95 cm) (18). The column was equilibrated in 0.5 M Tris-HCl, pH 7.0, 1 mM DTT, 0.02% N3, and eluted at a flow rate of 30 ml/h.

Hydroxyapatite Chromatography—For further purification of HA-I and HA-II adaptors, the assembly protein fraction was chromatographed on a Bio-Gel HPHT hydroxypatite column (100 × 7.8 mm) connected to a fast protein liquid chromatography system. The sample was applied at a flow rate of 0.5 ml/min. The column was subsequently washed with 5 ml of starting buffer and then eluted with a 30-ml gradient ranging from 1 mM NaHPO4, 50 mM Tris-HCl, 1 mM DTT, pH 8.0, to 500 mM NaHPO4, 50 mM Tris-HCl, 1 mM DTT, pH 8.0. Fractions of 0.5 ml were collected. HA-I adaptor eluted between 130 and 190 mM NaHPO4, and the HA-II adaptor at 290–360 mM NaHPO4 (4).

Phosphorylation Conditions—Samples were dialyzed into 0.15 M KCl, 50 mM HEPES, 5 mM MgCl₂, pH 7.2 (phosphorylation buffer). Polylysine was added to 100 μg/ml and [γ-32P]ATP (500 mCi/mmol) to 100 μM. Phosphorylation was carried out for 30 min at room temperature, after which NaF was added to a concentration of 0.1 M to inhibit phosphatases.

Immunoprecipitations—Immunoprecipitations of AP 180 and the HA-I adaptor were carried out using the monoclonal antibodies mAb AP 180-1 (2) and mAb 100/3 (7), respectively, that were coupled to Sepharose 4B. Unbound protein was removed by spinning the Sepharose beads through 10% sucrose in 0.5 M Tris, pH 7.0, precipitated protein was washed for SDS-PAGE by boiling the Sepharose beads in an equal volume of 2-fold sample buffer.

Gel Electrophoresis—SDS-PAGE was performed either according to Laemmli (19) or to the urea-SDS system described by Bretscher and Weber (20). To resolve the 104-112-kDa polypeptides of the HA-II adaptor, these systems were combined in a two-dimensional electrophoresis method (21). Radiolabeled bands were detected by autoradiography of dried gels using Fuji RX x-ray film.

Quantitation of Labeled Protein—The relative specific activities of the various labeled peptides were determined by relating radioactivity to the staining intensity with Coomassie blue R-250. The bands were excised from the gel and counted directly by Cerenkov counting. The Coomassie stain was then eluted with 25% pyridine and quantified spectrophotometrically (21).

Phosphoamino Acid Analysis—32P-Labeled polypeptides were electrophoretically transferred onto Immobilon membranes, where they were hydrolyzed and further analyzed as described by Kamps and Sefton (22).

RESULTS

Phosphorylated Subunits of the HA-II Adaptor Protein Complex—Coat proteins were first dissociated from coated vesicles by incubation in 0.5 M Tris to yield “Tris-extracted” coat proteins. These were dialyzed against phosphorylation buffer and then incubated in the presence of polylysine with γ-labeled ATP. For analysis of the phosphorylated HA-II subunits, the labeled coat proteins were immediately applied to a hydroxypatite column which allows rapid purification of this adaptor in a single chromatographic step (4). HA-II adaptor protein, which eluted with approximately 0.3 M phosphate, was concentrated by acid precipitation and then analyzed by two-dimensional electrophoresis using a urea-SDS system in the first dimension and standard SDS-PAGE in the second. This gel system has previously been shown to separate the three major 104–112-kDa subunits of the HA-II adaptor complex present in neuronal tissues (7). The resolved spots in this molecular weight range correspond to β, αδ, and αε subunits (Fig. 1a). The autoradiograph of the gel showed that besides the heavily phosphorylated 50-kDa subunit, the αε- and, to a lesser extent, also the β-subunit are phosphorylated (Fig. 1b). The specific activity of the 50-kDa subunit was ~0.2 mol of phosphate/mmol of protein, exceeding that of the αδ (7 × 10⁻³ mol of phosphate/mmol of protein) by a factor of 30, while phosphorylation of β was approximately one-third of that of αδ (2 × 10⁻³ mol of phosphate/mmol of protein). Phosphoamino acid analysis of the β-, αδ-, and αε-subunits showed that only serine residues were labeled. The 16-kDa subunit of HA-II adaptor, which was resolved on one-dimensional gradient gels, was not labeled under our phosphorylation conditions (data not shown).

Overnight storage at 4°C of the hydroxyapatite-purified HA-II adaptor resulted in a dramatic reduction of specific activity of all phosphorylated polypeptides indicating the presence of co-purifying soluble phosphatases. The presence of phosphatase activities associated with coated vesicles has been previously reported (23, 24). However, these activities were found to be associated with the vesicle membrane and to be absent from membrane-free extracts of coat proteins (23, 24). Why the soluble phosphatase activity found here was not seen in these earlier reports is unknown, but may be explained by a fortuitous enrichment of a phosphatase in the HA-II adaptor fractions after hydroxyapatite chromatography.

Phosphorylation of the HA-I Adaptor Complex—Phosphorylation of the HA-I adaptor was carried out under three different conditions. For the first experiment, the phosphorylation reaction was initiated in the unfractionated Tris extract, for the second in the assembly protein fraction which was obtained by gel filtration of the Tris-extracted coat proteins, and for the third after further purification of the HA-I adaptor by hydroxyapatite chromatography. In each case, the HA-I adaptor was rapidly purified by immunoprecipitation with the monoclonal antibody mAb 100/3, which is directed to the γ-subunit. The immunoprecipitate was then analyzed by standard SDS-PAGE. When the HA-I adaptor was incubated with ATP in the unfractionated Tris extract of coated vesicles, only the γ-subunit was phosphorylated (Fig. 2b). But, when the phosphorylation reaction was carried out in the assembly protein fraction, both the β' and γ-subunits were phosphorylated with a 1:3 ratio in specific activity (Fig. 2c). A similar result, with the β'-subunit incorporating about 5 × 10⁻³ mol mol⁻¹ protein⁻¹.
Phosphorylation of HA-I adaptor subunits. HA-I adaptor was phosphorylated at different stages of purification and then immunoprecipitated with the monoclonal antibody mAb 105/3. All lanes in panel a were stained for protein with Coomassie Blue. Lane 1, Tris-extracted coat proteins used for phosphorylation; lane 2, supernatant of the immunoprecipitate; lane 3, immunoprecipitate. Panel a, autoradiograph of lane 3; panel b, autoradiograph of immunoprecipitated HA-I adaptor after phosphorylation of the assembly protein fraction; panel d, autoradiograph of immunoprecipitated HA-I adaptor after phosphorylation of hydroxyapatite-purified HA-I adaptor. Note the differences in the ratio between the labeled γ- and β-subunit in panels b and c and d.

Phosphorylation of AP 180. An assembly protein fraction was labeled as described under "Methods," and protein AP 180 was immunoprecipitated with the monoclonal antibody mAb AP 180-1. Panel a shows the Coomassie-stained purified AP 180. Panel b shows the Coomassie-stained immunoprecipitate. Panel c shows the corresponding autoradiogram panel b.

DISCUSSION

The adaptor proteins in clathrin-coated vesicles are believed to function as specific linkers between the cargo of coated vesicles and the clathrin coat. The HA-I adaptor serves this function only in the trans-Golgi network, while the HA-II adaptor is confined in its action to the plasma membrane (7). The highly related β-class subunits of the two adaptors are assumed to contain clathrin binding sites (7, 10), while the other subunits are likely to be involved in binding the adaptor-specific cargo proteins and in the recognition of the correct intracellular membrane compartment (the Golgi membrane for the HA-I adaptor and plasma membrane of the HA-II adaptor). Our findings that the β-class subunits as well as the α- and γ-subunits can be phosphorylated under certain in vitro conditions by coated vesicle-associated kinases suggests that this type of protein modification may be used to modulate the interactions of adaptors with clathrin and the membrane. While the HA-I and HA-II adaptors show analogy in their overall structural organization, a difference is seen here in their phosphorylation characteristics, in that the 50-kDa component of the HA-II adaptor undergoes strong autophosphorylation, whereas the 47-kDa component of the HA-I adaptor displays no such activity, nor is it a substrate for other coated vesicle-associated kinases.

The cellular function of the neuronal specific protein AP 180 is not yet known. Its difference in primary structure and overall structural organization to the HA-I and HA-II adaptors makes it unlikely to function as an additional adaptor. In vitro AP 180 has been shown to possess a strong clathrin assembly promoting activity (2). Here we show that AP 180 is a major substrate for coated vesicle-associated kinases. This strengthens the proposed relationship between AP 180 and 155-kDa phosphoprotein isolated from rat sympathetic neurons (25) and bovine brain-coated vesicles, where it is referred to as AP 3 (26).

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Phosphoproteins in Clathrin-coated Vesicles

Phosphorylation of AP 180—Phosphorylation of protein AP 180 was investigated in the unfractionated Tris extract, in the assembly protein fraction, and after hydroxyapatite chromatography using the very same fractions which also contained the HA-I adaptor. In all cases, the phosphorylation reaction was terminated with the rapid removal of AP 180 by immunoprecipitation with the monoclonal antibody mAb AP 180-1 (2). Analysis of the immunoprecipitate by standard SDS-PAGE and autoradiography showed that AP 180 was phosphorylated when the reaction took place either in the assembly protein fraction (Fig. 3) or after further purification on hydroxyapatite chromatography (Fig. 4). This result also confirms an earlier study, which had shown that a kinase activity co-elutes with the HA-I adaptor from hydroxyapatite (9). Phosphorylation of the 47- and 20-kDa subunits was not detected. Phosphoamino acid analysis showed that β'- and γ-subunits were labeled only at serine residues.

A New High Molecular Mass Phosphoprotein in Coated Vesicles—The hydroxyapatite fraction, which was used to phosphorylate the subunits of the HA-I adaptor and protein...
of the HA-I adaptor when the reaction was carried out in the assembly protein fraction or after further purification of the proteins by hydroxyapatite chromatography. But neither was phosphorylated in the unfractionated Tris extract. This finding is most readily explained by the removal of soluble phosphatases upon gel filtration.

The fact that, in the course of this study, we came unintentionally across a new highly phosphorylated coat-associated protein, here referred to as p110, highlights the need for a systematic study designed to identify all clathrin-associated proteins present in the Tris extract of coated vesicles. The reason why p110 might have escaped previous detection is surely related to the fact that it migrates like the β'-subunit in standard Laemmli-type SDS-PAGE.

The description of phosphoproteins in coated vesicles is a necessary first step in understanding the functional significance of this modification. Apart from pinpointing the modified amino acid residues within the polypeptide chains, it remains now to be shown experimentally which of the known interactions of the adaptors or AP 180 are affected by their phosphorylation states.

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