The 50 kDa protein subunit of assembly polypeptide (AP) AP-2 adaptor from clathrin-coated vesicles is phosphorylated on threonine-156 by AP-1 and a soluble AP50 kinase which co-purifies with the assembly polypeptides

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AP50 is a subunit of the assembly polypeptide (AP) subclass AP-2 from bovine brain coated vesicles. It can be phosphorylated *in vivo* and *in vitro* on a threonine residue by means of the AP50 kinase activity associated with AP. We have undertaken an analysis of the amino acid sequence around the AP50 phosphorylation site. After phosphorylation *in vitro* of AP50 followed by tryptic cleavage, only one radioactive peptide was isolated following Mono-Q ion-exchange f.p.l.c. and reverse-phase h.p.l.c. The amino acid sequence of this peptide: Glu¹⁴⁶-Glu-Gln-Ser-Gln-Ile-Thr-Ser-Gln-Val-Thr*-Gly-Gln-Ile-Gly-Trp-Arg¹⁶², displayed two threonine residues. Analysis of the yield and radioactivity of the product from automated Edman

degradation indicated that only Thr-156 was phosphorylated, reflecting the presence of a single phosphorylation site in AP50. AP phosphorylated the corresponding synthetic peptide on the same threonyl residue. We demonstrated that AP50 was a phosphorylation substrate unable to autophosphorylate. The enzyme involved in the AP50 phosphorylation was shown to be associated with AP-1 and with a soluble protein complex co-purified with APs but resolved from the latter by hydroxyapatite-column exclusion chromatography. This AP50 kinase activity corresponded to a 280 kDa protein complex according to gel-filtration data.

INTRODUCTION

The intracellular transport of macromolecules involves several organelles and organelle systems. It is mediated by vesicles which bud off from a donor membrane compartment, traverse the intervening cytoplasm and fuse with an acceptor membrane compartment, thus delivering their cargo. In eukaryotic cells, clathrin-coated pits and vesicles mediate the selective internalization of cell-surface receptors (Goldstein et al., 1985) and are associated with the *trans*-Golgi network in the targeting of newly synthesized proteins to lysosomes and secretory granules (Pfeffer and Rothman, 1987).

The major structural proteins of the coat are the heavy and light chains of clathrin (Pearse, 1975; Ungewickell and Branton, 1981). In addition to clathrin, coated vesicles contain clathrin-associated proteins called assembly polypeptides (APs), so called because of their apparent ability to promote the polymerization of clathrin triskelions into polyhedral coat structures under physiological conditions (Zaremba and Keen, 1983). Three types of AP (AP-1, -2 and -3) have so far been identified. AP-1 and AP-2, also called HA-1 and HA-2 because of their relative elution from hydroxyapatite (Pearse and Robinson, 1984), each consist of a 330 kDa protein complex (Pauloin and Jollés, 1984; Keen, 1987). Both have been shown to contain a heterodimer of proteins from the 100–120 kDa group named adaptins (Pearse, 1988) and two smaller associated proteins. Thus, AP-2 is composed of α and β adaptins associated with 50 kDa and 17 kDa subunits. Similarly, AP-1 is made up of β' and γ adaptins allied to 47 kDa and 20 kDa proteins (Ahle et al., 1988). AP-1 and AP-2 can be localized to distinct regions of the cell: one to the Golgi (AP-1) and the other to the plasma membrane (AP-2) (Robinson, 1987; Ahle et al., 1988). Pearse and co-workers have

demonstrated that these adaptor complexes selectively bind cytoplasmic receptor tails *in vitro* (Pearse, 1988; Glickman et al., 1989). AP-3 corresponds to three independently isolated clathrin-associated proteins, previously called 155 K protein (Murphy et al., 1991).

Previous observations from our laboratory describe the presence of a cyclic nucleotide- and Ca^{2+} /calmodulin-independent protein kinase activity in coated vesicles from bovine brain which specifically phosphorylates a constitutive 50 kDa protein (AP50 also called pp50) on a threonine residue (Pauloin et al., 1982, 1984). Both the AP50 kinase and its substrate belong to the AP family (Pauloin and Jollés, 1984). We also showed the antagonistic enzymic activity which dephosphorylates AP50 (Pauloin and Jollés, 1986; Pauloin et al., 1988). Although the functional role of AP50 phosphorylation has not yet been directly addressed, there are nevertheless indications that it may influence the association of adaptors with clathrin and/or membrane. Thus, the degree of phosphorylation of AP components differs between their soluble cytosolic and membrane-associated states (Bar-Zvi et al., 1988).

In this paper we have determined the sequence around the threonine residue phosphorylated *in vitro* in bovine brain AP50 by a combination of gas-phase and solid-phase amino acid sequencing. We have demonstrated that AP50 is a protein kinase substrate and that AP-2 is unable to phosphorylate its AP50 subunit. AP50 is mostly phosphorylated by means of a soluble AP50 kinase co-purified with AP.

EXPERIMENTAL

Materials

[γ -³²P]ATP PB 10168 and PB 132 were obtained from Amersham

Abbreviations used: AP, assembly polypeptide; PMSF, phenylmethanesulphonyl fluoride; TPCK, tosylphenylalanylchloromethane; IT₅₀, temperature required for 50% inactivation of enzyme activity; TFA, trifluoroacetic acid.

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(Aylesbury, Bucks., U.K.), $^2\text{H}_2\text{O}$ from Euriso-Top (CEA, CEN-Saclay, France), Sephacryl S200, S-400 and S-1000 from Pharmacia (Guyancourt, France), HA-Ultrogel from IBF (Villeneuve-la-Garenne, France) and all other chemicals from Sigma (St. Louis, MO, U.S.A.). Bovine brains were provided by the Institut National de Recherche Agronomique (Jouy-en-Josas, France).

Preparation of APs

All procedures were carried out at 4 °C. After the brains were cleansed of meninges, the grey matter was scraped off and homogenized for two 15-s bursts at high speed in a Waring blender in 1 vol. of Mes buffer: 0.1 M Mes, pH 6.5, 0.1 mM EGTA, 2 mM MgCl_2 , 1 mM 2-mercaptoethanol, 0.02 % NaN_3 , 1 mM phenylmethanesulphonyl fluoride (PMSF), and aprotinin, antipain, leupeptin, pepstatin A, and chymostatin at 0.5 mg/l each. The homogenate was centrifuged at 17000 *g* for 30 min in a Beckman JA-10 rotor. The resulting pellet was resuspended and then centrifuged a further two times under the same conditions. All the supernatants were pooled and clarified by centrifugation at 17000 *g* for 1 h, then centrifuged at 110000 *g* for 1 h in a Beckman 50.2Ti rotor. The pelleted material was resuspended in Mes buffer using a glass homogenizer and a motor-driven Teflon pestle and then treated with another round of low-speed/high-speed centrifugations. Crude coated vesicles were purified on an $^2\text{H}_2\text{O}$ -8 % (w/v) sucrose step gradient as described (Nandi et al., 1982). After centrifugation, the coated vesicle pellets were resuspended in Mes buffer and loaded on to a 1.6 cm $\times$ 92 cm Sephacryl S-1000 column pre-equilibrated in Mes buffer at 4 °C and connected to a chromatography Bio-Rad Econo-System (Hercules, U.S.A.). Fractions (4.5 ml) were eluted at 72 ml/h. The 280 nm absorbance profile of the eluate showed two peaks. The second peak, smaller than the first, contained pure coated vesicles (Bar-Zvi and Branton, 1986). The coated vesicle fractions were pooled, pelleted and then resuspended in 9 ml of 1 M Tris/HCl buffer, pH 7.5/1 mM 2-mercaptoethanol, homogenized using a Dounce glass homogenizer and left for 2 h at room temperature. The extract was clarified by two rounds of centrifugation at 150000 *g* for 1 h. The resulting supernatant was applied to a Sepharose CL-4B column (2.5 cm $\times$ 120 cm), equilibrated and eluted at room temperature with 0.5 M Tris/HCl buffer, pH 7.5/1 mM 2-mercaptoethanol. Fractions (5 ml) were collected at a flow rate of 72 ml/h. The third peak which contained the AP (Keen et al., 1979), was concentrated 16-fold by ultrafiltration on an XM 300 Amicon membrane at room temperature. This step was critical. Magnetic stirring was kept as low as possible to avoid aggregation. Then concentrated AP solution was dialysed overnight at 4 °C against 500 ml of 0.1 M Tris/HCl buffer, pH 6.5/2 mM MgCl_2 /1 mM 2-mercaptoethanol.

AP50 phosphorylation

Dialysed AP (about 14 ml; $A_{280} = 2.53$) was mixed with 300 μl of [γ - ^{32}P]ATP (specific radioactivity 40 Ci/mmol) and incubated in 300 μl aliquots for 15 min at 37 °C. At the end of this first incubation, 10 μl of buffered 40 mM unlabelled ATP was added to each AP aliquot (final ATP concentration: 1 mM) and then the 37 °C incubation was continued for an additional 15 min duration. The phosphorylation reaction was arrested by the addition of 50 μl of electrophoresis sample buffer (Laemmli, 1970).

Preparative SDS/PAGE of [^{32}P]AP50

Phosphorylated AP in the sample buffer containing SDS was

concentrated about 4-fold in a Speed-Vac concentrator (Savant Instrument, Farmingdale, U.S.A.) and then analysed on eight 0.15 cm $\times$ 17.5 cm SDS/10 % (w/v) polyacrylamide preparative slab gels (Laemmli, 1970). Proteins were visualized by potassium precipitation of dodecyl sulphate (Hager and Burgess, 1980). The white precipitated AP50 protein bands were excised as continuous gel slices, cut into 5-mm-long pieces and were electroeluted in a Biotrap apparatus (Schleicher and Schuell, Cera-Labs, France). The recovered eluate contained both the isolated AP50 and excess dodecyl sulphate. The latter was selectively precipitated by addition of KCl to a final concentration of 50 mM. Following a 10 min incubation on ice, the precipitate was removed by low-speed centrifugation. Protein concentration was determined by the modified Lowry method (Peterson, 1977), using BSA as standard. The pure AP50 was dialysed overnight at 4 °C against 500 ml of 100 mM Tris/HCl buffer, pH 7.5, and then digested with tosylphenylalanylchloromethane (TPCK)-treated trypsin at a substrate to enzyme weight ratio of 25:1 at 37 °C for 5 h.

Purification of phosphorylated peptides

The tryptic mixture was filtered on a 0.45 μm pore diameter SpinX (Costar, Cambridge, MA, U.S.A.) and was then directly applied to a Mono-Q f.p.l.c. ion-exchange column (Pharmacia) previously equilibrated with 20 mM Tris/HCl buffer, pH 7.3. A linear gradient of 0–300 mM NaCl was developed over 30 min at a flow rate of 1 ml/min and 0.5 ml fractions were collected. Absorbance values were measured at 214 nm, and the radioactivity was monitored in the fractions by Cerenkov counting in a Betamatic V liquid-scintillation spectrometer (Kontron Instrument, France). Five radioactive peaks (named 0, 1A, 1B, 2, 3) were pooled separately and concentrated under vacuum in siliconized tubes. Each pool was applied to a C_{18} -h.p.l.c. reverse-phase column Aquapore 300 (Brownlee, Santa Clara, U.S.A.) after the addition of trifluoroacetic acid (TFA) to a final concentration of 0.1 %. A linear gradient of 0–40 % acetonitrile in 0.1 % TFA was run over 60 min with a flow rate of 1 ml/min and 1 ml fractions were collected. The radioactive peaks were pooled and lyophilized in siliconized glass tubes.

Phosphoamino acid analysis

Radioactive peptide aliquots were hydrolysed under vacuum in 6 M HCl at 110 °C for 2 h. HCl was evaporated and the samples were dissolved in 25 μl of electrophoresis buffer, pH 3.5 (acetic acid/pyridine/water, 10:1:89, by vol.). Aliquots (5 μl) were mixed with 2 μl of a solution containing 5 mg/ml of phosphoserine, phosphothreonine and phosphotyrosine, spotted on Whatman 3MM chromatography paper and then electrophoresed for 90 min at 60 V/cm. Phosphoamino acids were detected with ninhydrin and radioactive spots by autoradiography.

Synthetic peptide preparation and phosphorylation

Synthetic peptide corresponding to the phosphorylated AP50 tryptic peptide was synthesized by the solid-phase method using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry and a model 430A peptide synthesizer (Applied Biosystems, U.S.A.). The peptide was purified by preparative reverse-phase h.p.l.c. (Delta-Prep 3000 Waters) on a Vydac C_{18} 50 mm $\times$ 300 mm column. Purity was estimated to be > 95 % by analytical h.p.l.c. and the authenticity of the 17-residue peptide was confirmed by amino acid sequence analysis. Phosphorylation assays were carried out at 37 °C in 1.5 ml capacity microfuge tubes in a final reaction volume of 140 μl containing (final concentration) 100 mM Tris/

HCl (pH 7.0), 2 mM MgCl_2 , 2 mM 2-mercaptoethanol, 5 μg of AP, 0.1 mM synthetic peptide and 5 μl of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific radioactivity 40 Ci/mmol). The reaction mixture was incubated for 30 min. The reaction was terminated by the addition of 140 μl of 60% (v/v) acetic acid. The acid-denatured proteins were removed by low-speed centrifugation. The phosphorylated substrate was separated from unreacted $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by anion-exchange chromatography using 2 ml of Dowex AG1-X8 resin in a disposable Pasteur pipette column as described previously (Kemp et al., 1976). The eluted ^{32}P -labelled peptide was lyophilized, redissolved in 250 μl of water/0.1% TFA, and purified by h.p.l.c. as described above.

Amino acid sequence analysis

Peptides selected for automated gas-phase amino acid sequencing were dissolved in 50% formic acid, spotted directly on to 'Biobrene'-coated filters and sequenced by automated Edman degradation using an Applied Biosystems model 470 A protein/peptide sequencer and the 03CPTH program supplied by the manufacturer. The resulting phenylthiohydantoin derivatives were identified with an on-line 120 A phenylthiohydantoin analyser.

Determination of phosphorylated residues was performed on a Beckman 890 C spinning-cup sequencer using a 0.1 M quadrol program. Polybrene (2 mg) and horse skeletal muscle apomyoglobin (60 nmol) were added to the sequencer cup to prevent washout of peptides and as a good running control. Phosphoric acid was added at 1.5 mM to the butyl chloride solvent (S4) used to transfer residues from the cup to the fraction collector. The inclusion of phosphoric acid gave some improvement in ^{32}P yields without resulting in a detectable decrease in the repetitive yield of the internal sequencing standard (Lees-Miller and Anderson, 1989).

Elastase digestion of AP

Aliquot fractions of dialysed AP (50 μg in 20 μl) were treated for various times with 4 ng of elastase. Ten minutes before the end of each proteolytic digestion time, 1 μl of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific radioactivity 40 Ci/mmol) was added. Phosphorylation was terminated by addition of 25 μl of electrophoresis sample buffer. Electrophoresis was performed in an SDS/10% polyacrylamide slab gel. The gel was then silver stained (Merril et al., 1981) and exposed to Kodak XAR-5 film for autoradiography.

Purification of AP-1 and AP-2

The AP-1/AP-2 preparation was performed according to a modification of the Manfredi and Bazari's method (Manfredi and Bazari, 1987) in order to avoid cross contamination between the two adaptor classes. The AP-containing fractions were pooled, brought to 50 mM K_2HPO_4 and then loaded (0.5 ml/min) directly on to an XK-16 column (Pharmacia) containing 30 ml of hydroxyapatite-Ultrogel equilibrated with the same buffer and connected to an f.p.l.c. system (Pharmacia). The column was washed with 500 ml of starting buffer [50 mM K_2HPO_4 , 0.1 M NaCl, 10% (w/v) glycerol, 2 mM 2-mercaptoethanol, 0.02% NaN_3 , 1 mM PMSF, and 0.5 mg/l each of aprotinin, antipain, leupeptin, pepstatin A, and chymostatin]. The flowthrough was saved and the column was eluted stepwise with 20 ml of 500 mM K_2HPO_4 in starting buffer. The column flow rate was 30 ml/h and 1 ml fractions were collected. The eluted material corresponded to AP-2. Its purity was greater than 95% and the yield was typically about 1.2 mg of protein per

100 g of grey matter (wet wt.). The flowthrough fractions were concentrated about 10-fold in a Centriprep-100 (Amicon, U.S.A.), dialysed overnight in the starting buffer and re-loaded on to the hydroxyapatite column. After washing with 5 column vol. of buffer as above, the column was eluted with a 54 ml 50–500 mM K_2HPO_4 gradient. Only one peak, corresponding to AP-1, was eluted.

Purification of the AP50 kinase

The flowthrough fractions from the hydroxyapatite-chromatography step were concentrated at 0 °C by means of a 150 ml Omegacell (Filtron, U.S.A.) set with a 100 kDa cut-off membrane and then applied to a Superose 12 f.p.l.c. gel-filtration column previously equilibrated with 100 mM Tris/HCl buffer (pH 7.0)/2 mM MgCl_2 /2 mM 2-mercaptoethanol. The Superose 12 column was calibrated using Blue Dextra 2000 (void volume), bovine thyroglobulin (669 kDa), horse spleen ferritin (440 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa) and BSA (67 kDa), each run in the same buffer.

AP50 kinase assay

Fractions (30 μl) to be tested were phosphorylated by means of 1 μl of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific radioactivity 40 Ci/mmol) followed by 10 min incubation at room temperature. Phosphorylation was terminated by the addition of 20 μl of electrophoresis sample buffer and the sample analysed by SDS/PAGE.

RESULTS

Isolation of the phosphopeptides

AP purified from three bovine brains was phosphorylated and the resulting ^{32}P -labelled AP50 was purified as described in the Experimental section. 1.2 mg of radioactive protein were obtained (specific radioactivity: 5000 c.p.m./mg). Following tryptic cleavage a total of 6×10^6 c.p.m. (based on Cerenkov radiation) was injected on to the Mono-Q f.p.l.c. column with an overall recovery of 95% (Figure 1).

Only 6.5% of the recovered radioactivity was eluted in the void volume (peak 0). The greater part (81%) was associated with peak 1 which eluted between 50 and 80 mM NaCl. As this

Figure 1 Initial fractionation of the tryptic digest of phosphorylated AP50 by ion-exchange Mono-Q f.p.l.c.

The ^{32}P -labelled tryptic mixture was directly applied to a Mono-Q f.p.l.c. ion-exchange column (see the Experimental section). A linear gradient of NaCl (...) was developed. Absorbance was monitored at 214 nm (—), and the radioactivity was measured in the fractions by Cerenkov counting (shaded areas). The position of each pooled peak (0, 1A, 1B, 2, 3) has been indicated.

Figure 2 Elution profile of the Mono-Q-fractionated 1A-peak analysed by reverse-phase h.p.l.c.

Reverse-phase h.p.l.c. was performed as described in the Experimental section. Peptides were eluted from the C_{18} reverse-phase column with a gradient of acetonitrile (...) in 0.1% TFA. Absorbance was monitored at 214 nm (—). Fractions were collected and radioactivity of each fraction was quantified by Cerenkov emission (shaded areas).

Figure 3 N-terminal sequence analysis of the ^{32}P -labelled AP50 phosphopeptide 1A

^{32}P -labelled peptide 1A from reverse-phase h.p.l.c. was submitted to the Edman degradation by means of an Applied Biosystems gas-phase sequanator (filled bar) and a Beckman spinning cup sequanator (opened bar).

peak showed a shoulder on its descending side, it was split into two pools labelled 1A and 1B. Two other peaks, containing more than 2% of the recovered Cerenkov radioactivity, were also collected: peak 2 (2.9%) and peak 3 (4.8%), they were eluted at 0.13 and 0.8 M NaCl respectively. Each of the five peaks was individually subjected to reverse-phase h.p.l.c. chromatography. In each case, except peak 3, a single radioactive peak was eluted. The major radioactive peptide was eluted at 22% acetonitrile (fraction 54 peak 1A) and contained 63.5% of the whole radioactivity recovered from the five h.p.l.c. experiments. Peak 1b (fraction 57) was eluted at 25% acetonitrile and contained 6.4% of total radioactivity (Figure 2).

Peak 0 (fraction 49) and peak 2 (fraction 55) were eluted at 20% and 25% acetonitrile respectively (not shown). Both of them represented around 2% of the h.p.l.c.-recovered radioactivity. As the ^{32}P -labelled peptides resulting from peak 3 (results not shown) represented less than 0.5% of recovered radioactivity, this material was ignored.

Figure 4 Action of elastase digestion of AP on AP50 phosphorylation

Dialysed fractions of crude AP (50 μg in 20 μl) were incubated for 20 min at 37 $^{\circ}\text{C}$ with the following additions: none (lane a), 0.16 $\mu\text{g}/\text{ml}$ (lane b), 0.8 $\mu\text{g}/\text{ml}$ elastase (lane c). Then 1 μl of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was added to each fraction. After 10 min incubation at 37 $^{\circ}\text{C}$, reaction was stopped with 25 μl of electrophoresis sample buffer. Lanes a–c are Coomassie Blue-stained gels and lanes a'–c' are the corresponding autoradiograms.

Aliquots of each h.p.l.c.-recovered radioactive peptide were subjected to phosphoamino acid analysis. Only ^{32}P phosphothreonine was observed (results not shown). This result confirmed our previous report on the exclusive phosphorylation of AP50 on its threonine residue (Pauloin et al., 1984).

Location of the phosphorylated threonine

The h.p.l.c.-recovered peptide which eluted at fraction 54 (peak 1A) was sequenced. The following sequence was obtained: Glu-Glu-Gln-Ser-Gln-Ile-Thr-Ser-Gln-Val-___-Gly-Gln-Ile-Gly-Trp-Arg. This sequence corresponded to residues 146–162 in the AP50 (Thurieau et al., 1988). The absence of phenylthiohydantoin-Thr at cycle 11 from gas-phase sequencing compared with the normal yield at cycle 12 (19% of theoretical) suggested not only that Thr-156 (cycle 11) was the phosphorylation site but also that the efficiency of phosphorus γ -transfer to this threonine was close to 100% (Figure 3). As all the applied radioactivity remained on the filter after automated gas-phase sequencing with little or no radioactivity in the fractions, a second Edman degradation of the ^{32}P -labelled peptide was carried out by using a Beckman 890C liquid-phase sequencer and the Cerenkov radiation associated with the phenylthiohydantoin was measured (Figure 3). Radiochemical sequence analysis of the labelled peptide gave a broad release of ^{32}P beginning with the eleventh residue. The sudden rise in released radioactivity was indicative of a phosphorylated residue at that position in the Edman degradation. This experiment confirmed that Thr-156 was indeed phosphorylated and that Thr-152 was not. Total radioactivity applied was 350000 c.p.m. The yield obtained in cycle 11 was 0.5% of the input radioactivity. This low recovery of ^{32}P and its extensive tailing into the following cycles were explained because the acid-catalysed cyclization reaction of a phenylisothiocyanate-treated peptide that had an N-terminal phosphothreonine caused the elimination of much of the phosphate during the cleavage rather than the formation of an

Figure 5 Hydroxyapatite-Ultrogel chromatography: comparison of the AP50 kinase activities contained in AP-1, AP-2 and flowthrough fractions (FF)

Samples were incubated in the presence of 1 μ l of [γ - ^{32} P]ATP, 2 mM $MgCl_2$ and 2 mM 2-mercaptoethanol for 10 min at 37 $^{\circ}C$. Total reaction volume was 61 μ l. The lane contents were as follows: 20 μ l of AP-1 alone (lane 1), 20 μ l of AP-2 alone (lane 3), 20 μ l of FF alone (lane 5), 20 μ l of AP-1 plus 20 μ l of FF (lane 2), 20 μ l of AP-2 plus 20 μ l of FF (lane 4), 20 μ l of FF plus 20 μ l of both AP-1 and AP-2 (lane 7), 20 μ l of AP-1 plus 20 μ l of AP-2 (lane 6). Panel A shows the Coomassie Blue-stained gel and panel B shows the corresponding autoradiogram.

anilinothiazolinone derivative. The phosphate released by elimination was not extracted from the spinning cup.

The relationship between the two AP50 ^{32}P -labelled phosphopeptides 1A and 1B was further investigated. Based on their amino acid sequence, they appeared to be identical. Trace amounts of secondary sequence not related to AP50 were detected associated to 1B. Gas-phase sequence analysis of peak 2 showed the following sequence: Ser-Gln-His-Gln-Thr-Lys-Glu-Glu-Gln... This peptide was an extended form of the main phosphorylated peptide obtained in peaks 1A and 1B. This apparent heterogeneity was caused by resistance to tryptic cleavage of the Lys-Glu bond. Gas-phase sequence analysis of peak 0 (Mono-Q flowthrough) revealed a complex mixture of peptides. No sequence related either to the phosphorylation site or to some other putative threonine site seemed to be present.

Phosphorylation of the synthetic peptide

The synthetic peptide analogue to the phosphorylated AP50 tryptic peptide was prepared by the solid-phase method. This peptide was readily phosphorylated by the AP50 kinase activity associated with AP. When subjected to partial acid hydrolysis and high-voltage paper electrophoresis, the ^{32}P radioactivity was associated with phosphothreonine. By amino acid sequencing, only the second threonine was phosphorylated (results not shown).

Action of elastase digestion of AP on AP50 phosphorylation

APs were treated with a low concentration of elastase and then phosphorylated with radioactive ATP. The time-course experiment showed that adaptins and other proteins were more or less hydrolysed when AP50 remained almost intact (Figure 4, lanes

Figure 6 Superose 12 chromatography of flowthrough fractions

The flowthrough fractions from the hydroxyapatite-Ultrogel chromatography were concentrated and chromatographed on a Superose 12 HR 10/30 f.p.l.c. column. Upper panel: AP50 kinase activity was assayed in each fraction using AP-2 fraction as substrate ($\bullet$). The column was calibrated using Blue Dextran 2000 (void volume), bovine thyroglobulin (669 kDa), horse spleen ferritin (440 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa) and BSA (67 kDa). Lower panel: SDS/PAGE fractions analysis and silver staining.

a-c). The corresponding autoradiography (lanes a'-c') showed that AP50 phosphorylation was not correlated with its integrity. Complete absence of AP50 phosphorylation could be observed even if this protein was still detectable. Similar results were obtained with trypsin instead of elastase (results not shown).

AP50 kinase activities of AP-1, AP-2 and flowthrough fractions

As previously described, two peaks were obtained after hydroxyapatite chromatography of crude AP (Pearse and Robinson, 1984; Manfredi and Bazari, 1987). The first peak eluted at 230 mM K_2HPO_4 and was termed AP-1 (or HA-1); the second peak, termed AP-2 (or HA-2), was eluted at 370 mM K_2HPO_4 . However, this method led to cross-contamination. Further purification of AP-1 and AP-2 was therefore undertaken in order to eliminate it. Our method allowed the separation of the two AP complexes and gave, for each of them, a purity greater than 95%. Incubation of AP-2 with [γ - ^{32} P]ATP gave a very low AP50 phosphorylation (Figure 5, lane 3). Mixing AP-1 or flowthrough fractions with AP-2 permitted the phosphorylation of AP50 (lanes 6 and 4). Simultaneous addition of flowthrough and AP-1 to AP-2 gave maximum signal, comparable with that of the starting material (lane 7). Taken together these results indicated that AP-2 was devoid of AP50 kinase activity, the latter being associated with flowthrough and AP-1.

The phosphorylation time-course of AP-2 in the presence of both AP-1 and flowthrough was not merely the result of the addition of the two AP50 kinase activities but showed a 2.6-fold synergism (results not shown).

Figure 7 Localization of AP50 kinase in clathrin-coated vesicle components

(a) Sephacryl S-400 column chromatography profiles. Tris extract of clathrin-coated vesicles was gel filtered on a Sephacryl S-400 column. Absorbance was monitored at 280 nm (—). Aliquots (5 μ l) of each fraction were diluted to 100 mM Tris, pH 7.0/2 mM MgCl_2 /2 mM 2-mercaptoethanol and then incubated in the presence of 1 μ l of [γ - ^{32} P]ATP with (○) or without (●) AP-2. Fractions were analysed by SDS/PAGE. The phosphorylated AP50 bands were cut out and the radioactivity was counted. (b) AP50 phosphorylation is not related to the amount of AP50. Phosphorylated clathrin-coated vesicles from Tris-extract fractions obtained after Sephacryl S-400 column chromatography were analysed by SDS/PAGE and autoradiography. Panel A shows the Coomassie Blue-stained gel and panel B shows the autoradiogram corresponding to the AP50 region.

Molecular mass determination of the AP50 kinase activity from the flowthrough fractions

The flowthrough fractions from the hydroxyapatite-Ultrogel chromatography were concentrated using ultrafiltration on a 100 kDa cut-off membrane. Ammonium sulphate precipitation (final concentration = 40%) was also effective for concentration (results not shown). Concentrated material was chromatographed on a Superose 12 HR 10/30 f.p.l.c. column. AP50 kinase activity was assayed in each fraction using AP-2 fraction as substrate. A major polypeptide of 160 kDa and a minor band of 60 kDa co-eluted with the AP50 kinase activity (Figure 6). Based on the elution volume of AP50 kinase activity relative to a standard series of markers during f.p.l.c. gel filtration with Superose 12, we estimated the native molecular mass of AP50 kinase to be 280 kDa.

Localization of the AP50 kinase in clathrin-coated vesicle components

Clathrin-coated vesicles were dissociated in 1 M Tris/HCl pH 7.5 and the Tris-extract was gel filtered on a Sephacryl S-400 column to separate coated vesicles components (Keen et al., 1979). Aliquot parts of each fraction were incubated in the presence of [γ - ^{32} P]ATP and with or without AP-2 as an exogenous specific

phosphorylation substrate, to check the presence of the AP50 kinase (Figure 7a).

Comparative study of intrinsic phosphorylation (without AP-2) and extrinsic phosphorylation (with AP-2) of AP50 clearly showed that the positions of maximum activity in both profiles were superimposable. The addition of exogenous AP-2 to the fractions indicated that AP50 kinase activity was associated only with AP. The shoulder on its descending side corresponded to the AP50 kinase activity associated with AP-1. Careful examination of each AP50 Coomassie Blue-stained band with respective autoradiography showed that phosphorylation was not proportional to the intensity of the staining (Figure 7b). For instance, AP50 phosphorylation was at least 50-fold higher in lane 2 compared with lane 7 with an equal amount of protein.

DISCUSSION

Identification of the phosphorylation site from a phosphoprotein is important for several reasons: (1) progress in the recognition of specific phosphorylation site motifs might lead to the possibility of identifying putative protein kinase substrate after scanning available protein sequences from databanks. The more phosphorylation site sequences we can determine, the more precise phosphorylation site consensus sequences will be; (2) structures termed pseudosubstrates, resembling protein kinase phosphorylation site sequences, also play an important role in the regulation of protein kinase (Soderling, 1990); (3) knowledge of the phosphorylation site is the first step before undertaking site-directed mutagenesis of the cloned protein kinase substrate cDNA. Such a genetic approach would shed some light on the biological significance of the phosphorylation/dephosphorylation of AP50.

We have shown that there is only one major tryptic phosphopeptide containing two threonine residues of which the second threonine (position 11) is phosphorylated. This latter residue, corresponding to Thr-156 in AP50, is situated at the third part from the N-terminus of the protein.

To complement studies that have demonstrated the prominent phosphorylation of AP50 *in vitro*, several laboratories have described phosphorylation of clathrin-coated membrane proteins in intact cells (Keen and Black, 1986; Cantournet et al., 1987; Bar-Zvi et al., 1988). Comparative one-dimensional peptide mappings from AP50 purified from reticulocytes phosphorylated either *in vivo* or *in vitro* showed no difference. This indicates that the phosphorylation site we observed *in vitro* may correspond to the amino acid phosphorylated *in vivo*. It was recently shown that *APM1* gene in *Saccharomyces cerevisiae* encodes a 54 kDa protein that is 56% and 36% identical with AP47 and AP50 respectively. It is noteworthy that the Thr residue in the 54 kDa yeast protein corresponding to Thr-156 in bovine brain AP50 is conserved. On the other hand, Thr-152 is replaced by a Pro residue in the yeast protein (Lemmon et al., 1991).

The synthetic 17-residue polypeptide analogous to the natural tryptic phosphopeptide is an effective substrate for the AP50 kinase associated with the AP. The enzyme phosphorylates the synthetic peptide at Thr-11, which corresponds to the Thr-156 in the parent protein. Significantly, this peptide contains most of the information necessary for its recognition by the AP50 kinase. We have compared its sequence with some other peptides containing serine and threonine kinase phosphorylation sites (Kemp and Pearson, 1990; Kennelly and Krebs, 1991). No obvious similarity appeared but one may note that the AP50 peptide contains a glutamine-rich motif QXQXTXQXT*XQ which resembles the T*QT*QXQ motif found in the 90 kDa heat

shock protein substrate of the double-stranded DNA-activated protein kinase (Lees-Miller and Anderson, 1989).

Cloning and sequencing of AP50 revealed the absence of an ATP-binding site in the molecule (Thurieu et al., 1988). This assumption was confirmed after [α - ^{32} P]8-azido-ATP failed to label AP-50 (Beck and Keen, 1991). This was a surprising result since AP50 was thought to undergo autophosphorylation according to an intramolecular process (Keen et al., 1987). The fact that AP50 phosphorylation is not directly proportional to the amount of AP50 constitutes another clue which is inconsistent with the autophosphorylation theory. This observation and the same conclusion have been reported also (Bar-Zvi and Branton, 1986). Finally, the fact that phosphorylation of AP50 was inhibited in spite of its resistance to proteolysis after trypsin or elastase treatment, strongly suggested that AP50 phosphorylation was strictly dependent on the integrity of some other proteins either associated with AP50 in AP or co-purified with them.

If AP50 is indeed devoid of AP50 kinase activity and AP-2 is really able to autophosphorylate, AP50 kinase activity must be associated either with adaptins or with the 17 kDa protein. We have explored the amino acid sequence of both α - and β -adaptin (Kirchhausen et al., 1989; Robinson, 1989). No protein kinase consensus sequence was found in the two classes of adaptin, but the nucleotide-binding site consensus sequence Gly⁷-Xaa-Gly-Xaa-Xaa-Gly-(19)-Lys³¹ was found in the N-terminal part of α -adaptin. The respective sequences are missing in β -adaptin. This finding has been confirmed experimentally by means of [α - ^{32} P]8-azido-ATP. The predominant photoproduct corresponded to the α -adaptins. No labelling was found associated with β -polypeptide (Beck and Keen, 1991). As the AP50 kinase-like adaptin is very sensitive to proteolysis and the N-terminal ends of α - and β -adaptin are supposed to interact with both AP50 and AP17, one may think that the complementarity between α -adaptin, which carries the nucleotide-binding site, and AP50 could allow its phosphorylation. To verify this hypothesis, we checked whether pure AP-2 is able to autophosphorylate. However, pure AP-2 is practically unable to do so (see Figure 5, lane 3). AP50 kinase activity appears to be associated in part with AP-1 but mostly with a soluble factor associated with the flowthrough fractions from the hydroxyapatite chromatography. This factor is a protein since it is elastase/trypsin sensitive, and heat inactivated ($IT_{50} = 43^\circ\text{C}$) (results not shown). It is noteworthy that AP-2 is heat inactivated at a significantly higher temperature ($IT_{50} = 52^\circ\text{C}$). SDS/PAGE analysis of flowthrough fractions followed by silver staining shows one major band of 160 kDa and one minor band of about 60 kDa. Addition of exogenous AP-2 as substrate to Tris-dissociated coated vesicle fractions shows that AP50 kinase activity is only detected in the AP fractions. This observation is in agreement with its 280 kDa molecular mass, estimated after gel filtration. Manfredi and Bazari described two AP50 kinase activities associated with AP-1 and AP-2. However, phosphorylation by AP-2 was not observed until 10–20 min (Manfredi and Bazari, 1987). As AP50 phosphorylation is a very rapid process (Pauloin et al., 1984, 1988), AP50 phosphorylation by AP-2 could be caused by some AP-1 contamination. We do not know yet whether the soluble AP50 kinase and the kinase associated with AP-1 are identical or whether they correspond to different proteins. However, the synergistic action observed when AP-1 and the flowthrough

fractions act together to phosphorylate AP50 may suggest that these two AP50 activities are different. It must be noted that AP50 kinase activity dropped dramatically after gel filtration or after any other purification method we have tested such as ATP-agarose chromatography, Cibacron Blue 36A-Sepharose chromatography or f.p.l.c. on a Mono-Q column. The reason for this decay is still unknown. Completion of the purification and complete characterization of these two AP50 kinases represent the future direction of this work.

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