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Identification of a Protein Kinase as an Intrinsic Component of Rat Liver Coated Vesicles†

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ABSTRACT: Purified rat liver coated vesicles phosphorylate two peptides, M_r 53 000 and M_r 51 000, in the presence of [γ-^{32}P]ATP. Incorporation of phosphate into these peptides is not stimulated by cAMP, Ca^{2+}, or Ca^{2+} plus calmodulin and occurs principally on a threonine residue. Mild conditions that result in removal of coat proteins from coated vesicles remove most of the protein kinase activity, suggesting the enzyme(s) is (are) not an integral membrane protein. Photolabeling of coated vesicles with 8-azido-[α-^{32}P]ATP results in specific labeling of only the M_r 53000 and M_r 51000 peptides. Preincubation with 10 mM *N*-ethylmaleimide inhibits kinase activity and concomitantly reduces photolabeling of the two peptides. Thus, the data are consistent with the hypothesis that protein kinase activity resides with these two coated vesicle proteins and that they are catalyzing an autophosphorylation reaction.

Coated vesicles are cagelike structures that function in eucaryotic cells to transfer membrane material to and from various subcellular structures (Goldstein et al., 1979; Pearse & Bretscher, 1981). Coated vesicles consist of membrane encapsulated by a protein coat. Coated vesicles are comprised principally (ca. 70%) of an M_r 180 000 protein called clathrin (Pearse, 1975) as well as several families of polypeptides whose molecular weights cluster respectively at 100 000, 50 000–55 000, and 34 000 (Blitz et al., 1977; Pearse, 1978; Woodward & Roth, 1978; Pfeffer & Kelly, 1981). Coated vesicles arise from coated regions of membranes usually referred to as coated pits, when they occur at the cell surface. The coated pits invaginate and assume an icosahedral surface to form coated vesicles. The coat proteins are rapidly lost at this point with the resultant formation of smooth vesicles. There is evidence that uncoating requires ATP and a specific uncoating enzyme (Patzer et al., 1982). There are minimal data on what factors govern, in vivo, formation of a coated pit, its evolution into a coated vesicle, and its movement within a cell. In vitro, assembly of coat structures occurs spontaneously from purified

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coat proteins and requires only clathrin and M_r 34000 proteins or light chains (Kirchhausen & Harrison, 1981; Ungewickell & Branton, 1981). The M_r 100000 protein has been proposed to mediate binding of triskelions (three clathrins plus three light chains) to vesicles (Unanue et al., 1981). It is possible that the M_r 50000–55000 proteins play a regulatory role in vivo in some phase of coated vesicle dynamics.

Coated vesicles from bovine brain have been demonstrated to possess protein kinase activity (Pauloin et al., 1982). An M_r 51000 protein was shown to be an endogenous kinase substrate whose phosphorylation was Ca^{2+} and cAMP[1] independent (Pauloin et al., 1982). This conclusion was very recently confirmed, and additionally a similar kinase activity was found associated with bovine liver coated vesicles (Pfeffer et al., 1983). In the present study, we show that purified rat liver coated vesicles, like bovine brain coated vesicles, possess protein kinase activity that is cAMP and Ca^{2+} independent. Moreover, we show that the kinase substrates, proteins of M_r 53000 and 51000, are themselves most probably protein kinases that self-phosphorylate almost exclusively on threonine residues in intact coated vesicles.

Materials and Methods

Coated vesicles were prepared from rat liver by a novel method of differential centrifugation. Livers (6–10) were excised from 150–250-g male Sprague-Dawley rats (Charles River Labs). The livers were minced and homogenized with six passes of a Potter–Elvehjem tissue grinder in a volume of ice-cold buffer equal to the weight of the liver. The buffer consisted of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES; Research Organics), 1 mM EGTA, 0.5 mM magnesium chloride, and 0.02% sodium azide adjusted to pH 6.5 with sodium hydroxide (MES buffer). The homogenate was centrifuged at 19000*g* in a Sorvall GSA head for 40 min. The supernatant was saved and centrifuged for 70 min at 43000*g* in a Sorvall SS34 head. The pellet containing the coated vesicles was resuspended in a small volume of MES buffer by using a loose-fitting Dounce homogenizer. The membrane suspension was diluted with an equal volume of 12.5% Ficoll and 12.5% sucrose (both in MES buffer) and spun at 43000*g* for 40 min. The supernatant was saved and diluted with 4–5 volumes of MES buffer, and coated vesicles were pelleted for 70 min at 33000 in a Beckman 35 rotor or a Sorvall 641 rotor. The pellet from this spin was resuspended in a small volume of MES buffer and spun for 12 min in an Eppendorf microfuge. The coated vesicles are found in the supernatant together with filamentous material. Additional spins in the microfuge will remove successively more of the filaments but will also reduce coated vesicle yield. Electron microscopy after two microfuge spins reveal the coated vesicles to be >75% pure with filaments as the major contaminant. Agarose gel electrophoresis (Rubenstein et al., 1981) reveals no smooth vesicle contaminants. A typical yield is 1.5 mg of coated vesicles per rat liver.

In some experiments we used the conventional two sucrose gradient procedure described previously (Pilch et al., 1983) to purify the coated vesicles from the microsomal pellet. In this case electron microscopy revealed the coated vesicles to be at least 85% pure with filaments again the major contaminant.

Phosphorylation of coated vesicle protein was accomplished under the following conditions: Coated vesicles at 4 °C were made 2.5 mM in $MgCl_2$ and warmed to 23 °C, and [γ-^{32}P]-ATP (New England Nuclear) was added to a final concentration of 30 μM (2–10 μCi). Additions, if any, were made to the coated vesicles prior to warm up. Phosphorylation was terminated by the addition of electrophoresis sample buffer (Laemmli, 1970) and boiling for 1 min prior to electrophoresis.

Incorporation of 8-azido-[α-^{32}P]ATP (ICN Corp.) into coated vesicles was accomplished essentially as previously described (Shia & Pilch, 1983). Two 100-μg aliquots of coated vesicles, in 10 mM $MgCl_2$ and 0.124 mmol of 8-azido[α-^{32}P]ATP (10 μCi) in the presence and absence of adenosine 5′-(β,γ-imidotriphosphate), 150 μL final volume, were incubated for 2 min at 4 °C in the dark and then directly illuminated for 45 s with a focused 200-W mercury lamp (Oriel Corp.) at a distance of 20 cm. An equal volume of electrophoresis sample buffer was then added, and the samples were boiled for 1 min and then subjected to electrophoresis by the procedure of Laemmli (Laemmli, 1970).

Two-dimensional gel electrophoresis was performed as described by O'Farrell (1975) as modified by Pfeffer et al. (1983). Agarose gel electrophoresis was performed as described by Rubenstein et al. (1981). Column purification of coated vesicles from rat liver was achieved by the procedure of Altsteil & Branton (1983) as modified below. Sucrose gradient purified coated vesicles were applied to a 1.5 × 100 cm Sephacryl S-1000 column (Pharmacia) equilibrated in MES buffer containing 0.25 M sucrose. The column was eluted at approximately 25 mL/h; 4-mL fractions were collected.

Two-dimensional thin-layer electrophoresis was accomplished by the procedure of Hunter & Sefton (1980a).

Protein content was evaluated by the method of Lowry et al. (1951).

Unless otherwise specified, all reagents were purchased from Sigma. Calmodulin (CAM) was a kind gift from Dr. James Head, Boston University School of Medicine. DEAE-purified bovine brain tubulin was the kind gift of Dr. George Bloom, Worcester Foundation for Experimental Biology.

Results

Protein kinases are very common enzymes that function in regulating a great variety of metabolic processes (Krebs & Beavo, 1979; Cohen, 1982). It is important therefore that, in assigning protein kinase activity specifically to coated vesicles, rigorous criteria be employed to assure that the kinase activity is not due to the presence of a trace contaminant. Agarose gel electrophoresis is a particularly useful method for separating coated vesicles from smooth vesicles and trace contaminants (Rubenstein et al., 1981). We subjected our coated vesicle preparation to agarose gel electrophoresis, assayed fractions for kinase activity, and analyzed the results by SDS–PAGE and autoradiography as shown in Figure 1. Figure 1A depicts the Coomassie blue staining pattern of the agarose gel fractions, and Figure 1B is an autoradiograph of the same gel. Fraction 6 contains >75% of the coated vesicles and >75% of the phosphopeptides. Electron microscopy on negative-stained preparations of material eluted from the agarose confirmed that fraction 6 contained essentially pure coated vesicles. The phosphopeptides in the autoradiograph (Figure 1B) correspond exactly to a protein stained faintly by Coomassie blue of M_r 53000 and to a more intensely stained band of M_r 51000. Thus, as was shown in bovine brain

[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; CAM, calmodulin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Ado-PPNHP, adenosine 5′-(β,γ-imidotriphosphate); NEM, *N*-ethylmaleimide; cAMP, adenosine cyclic 3′,5′-phosphate; CV, coated vesicle; TLC, thin-layer chromatography; kDa, kilodalton; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

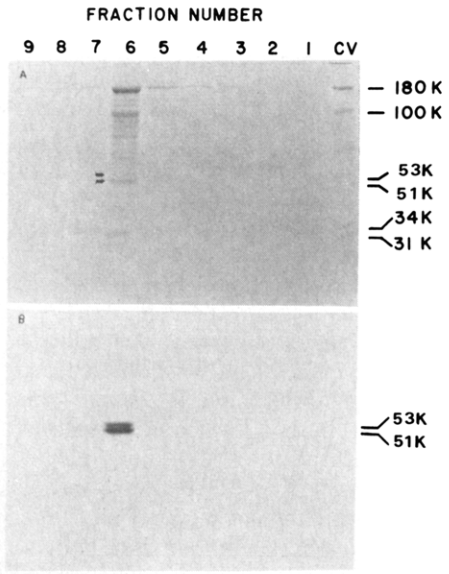

FIGURE 1: Protein kinase activity and its endogenous substrates are closely associated with agarose gel purified rat liver coated vesicles. A total of 75 μg of rat liver coated vesicles was subjected to agarose gel electrophoresis as described (Rubenstein et al., 1981). The coated vesicle containing lane was removed, and nine consecutive 3-mm slices from the origin toward the anode were excised and briefly homogenized. Fifty microliters of 5× concentrated MES buffer was added. [γ-^{32}P]ATP (30 μM final concentration) and enough 20 mM $MgCl_2$ to give a final 2.5 mM $MgCl_2$ concentration were added to each fraction after warming to 23 °C. After 5 min the reactions were terminated by the addition of electrophoresis buffer and boiling for 1 min; 100-μL aliquots of each fraction were placed onto a 10% acrylamide gel and subjected to electrophoresis as described (Laemmli, 1970). The gel was then stained for protein with Coomassie blue, dried, and autoradiographed for 2 h at room temperature. (A) The Coomassie blue staining pattern. The molecular weights of the major coated vesicle associated proteins are shown on the right. The lane marked CV contains 20 μg of rat liver coated vesicles. (B) The autoradiogram. The molecular weights of the phosphorylated proteins are shown on the right.

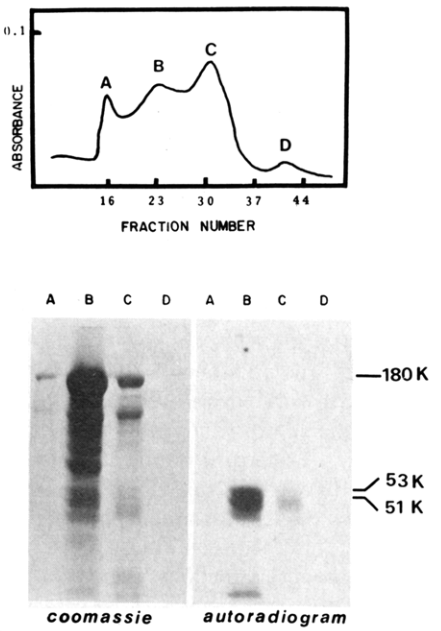

FIGURE 2: Protein kinase activity copurifies with liver-coated vesicles during gel filtration chromatography. Two milligrams of rat liver coated vesicles were chromatographed as described. Peaks were pooled as shown in the elution profile above. These four samples were centrifuged for 70 min at 33 000 rpm in a Beckman 35 rotor in order to pellet any coated vesicles present. The pellets were resuspended in 0.5 mL MES buffer. Aliquots of 100 μL of each sample were phosphorylated, placed onto a 9% acrylamide gel, and subjected to gel electrophoresis, stained, and autoradiographed as described in Figure 1. Molecular weights of major coated vesicle associated proteins are shown.

(Pfeffer et al., 1983), the phosphopeptides are tightly associated with the coated vesicle, implying that the protein kinase activity is an intrinsic one. In addition, we have confirmed that brain coated vesicles phosphorylate a single peptide (data not shown) of M_r 53 000 that likely corresponds to the previously described M_r 51 000 species (Pfeffer et al., 1983) whereas two phosphopeptides are always observed with rat liver coated vesicles.

We also used another recently described method to further purify coated vesicles to near homogeneity, Sephacryl S-1000 chromatography (Altsteil & Branton, 1983). Coated vesicles were passed through the column, and four peaks were obtained (Figure 2A). Peaks B and C contained coated vesicles of >95% purity as determined by electron microscopy. These two peaks also contained almost all the clathrin (Figure 2B) and kinase activity (Figure 2C). Peak I, which coincided with the void volume, contained large filament aggregates and membrane fragments. Peak IV contained very small particles and very little protein. These data further confirm that the kinase activity and the substrate for the reaction are integral coated vesicles components.

Intrinsic protein kinase activity could be associated with either the coat or the membrane portion of coated vesicles. The major protein constituents of coated vesicles can be easily removed from the membrane by incubation with 0.5 M Tris, pH 6.5 (Keen et al., 1979). We treated coated vesicles accordingly and incubated the various fractions with [γ-^{32}P]ATP. As shown in Figure 3, panel 1, it can be seen by the Coomassie blue staining pattern that most of the coated vesicle proteins

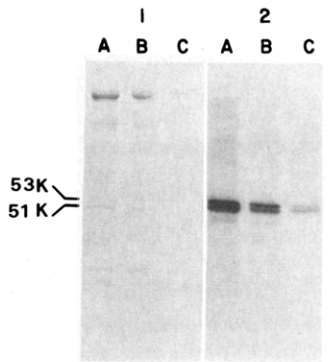

FIGURE 3: Most of the protein kinase activity can be solubilized by incubation in 0.5 M Tris-HCl. To 100 μg of rat liver coated vesicles in 0.1 mL of MES buffer was added 0.1 mL of 1 M Tris-HCl, pH 6.5. After a 30-min incubation at 23 °C, the suspension was centrifuged at 150000g for 20 min in a Beckman airfuge. The supernatant was removed, and the pellet was rinsed twice with 0.5 M Tris-HCl, pH 6.5, and then resuspended in the original volume (200 μL) of MES + 0.5 M Tris-HCl, pH 6.5. Aliquots of supernatant, pellet, and supernatant plus pellet were made 2.5 mM in $MgCl_2$. Protein kinase reactions, gel electrophoresis, protein staining, and autoradiography were performed as in Figure 1. Lanes marked 1 show the protein patterns obtained; lanes marked 2 show the corresponding autoradiograms. (A) 25 μL of supernatant + 25 μL of pellet; (B) 25 μL of supernatant + 25 μL of MES + 0.5 M Tris, pH 6.5; (C) 25 μL of pellet. The molecular weight of the major phosphorylated proteins are shown on the left.

including the M_r 53 000 and 51 000 species are rendered nonpelletable by Tris treatment (lane B, supernatant vs. lane C, membrane pellet). Similarly, panel 2 of Figure 3 depicts an autoradiograph showing that most of the kinase activity can be found extracted from the coated vesicle by Tris (lane B, soluble vs. lane C, membrane pellet). Lane A shows the supernatant plus the pellet, demonstrating the additive effect

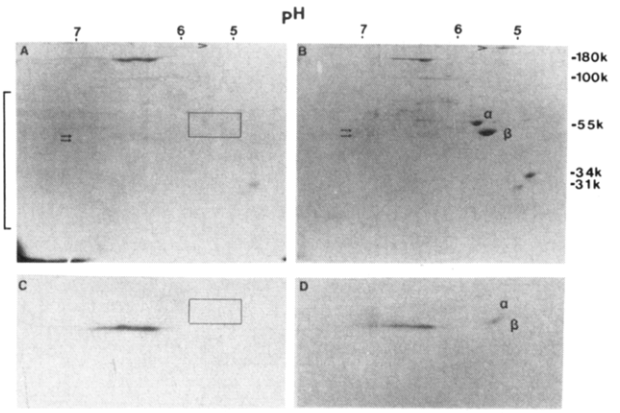

FIGURE 4: Two-dimensional gel electrophoresis of rat liver coated vesicles phosphorylated in the presence and absence of tubulin. Two 50-μg aliquots of rat liver coated vesicles, one containing 8 μg of bovine brain tubulin and one not, were incubated with [γ-^{32}P]ATP as described under Materials and Methods and then subjected to two-dimensional electrophoresis. The gels were then stained with Coomassie blue, dried, and autoradiographed for 16 h at −70 °C with Kodak X-OMAT AR film and Cronex lightening plus enhancing screen. (A) Coomassie blue stained gel of rat liver coated vesicles. (B) Coomassie blue stained gel of rat liver coated vesicles plus tubulin. (C) and (D) are autoradiograms of bracketed regions of (A) and (B), respectively. The positions of the M_r 53 000 and 51 000 coated vesicle polypeptides (→) and MAPs (⟩) are indicated. Molecular weights and pHs are as indicated.

of the two together. The addition of a final concentration of 0.5 M Tris-HCl, pH 6.5, to coated vesicles in MES buffer had no effect on the kinase reaction (data not shown). It appears therefore that the protein kinase activity of coated vesicles is not mediated by an integral membrane protein but rather resides in an easily solubilized, clathrin-associated fraction.

Very recently evidence has been obtained that α- and β-tubulin are components of highly enriched preparations of bovine brain and bovine and chicken liver coated vesicles (Pfeffer et al., 1983; Kelly et al., 1983). Both β-tubulin and, to a lesser extent, γ-tubulin were phosphorylated when coated vesicles from either source were incubated in the presence of [γ-^{32}P]ATP (Pfeffer et al., 1983). Since β-tubulin has the identical mobility on one-dimensional SDS gels as the M_r 53 000 phosphorylated peptide in our coated vesicle preparation, we subjected rat liver coated vesicles, phosphorylated in the presence and absence of exogenous DEAE-purified calf brain tubulin, to two-dimensional gel electrophoresis to determine whether or not β-tubulin is a major phosphorylated peptide.

Figure 4B shows the Coomassie blue stained pattern of a 2-D gel containing 50 μg of rat liver coated vesicles and 8 μg of tubulin after incubation with [γ-^{32}P]ATP. The pattern obtained was quite similar to that reported by Pfeffer et al. (1983). Two well-resolved spots corresponding to clathrin light chains are evident as are streaks corresponding to clathrin heavy chains and M_r 100 000 polypeptides. Two streaks corresponding to the M_r 53 000 and 51 000 components are denoted by arrows. α- and β-tubulins are also evident. A very high molecular weight peptide corresponding to MAP-2, a high molecular weight tubulin associated protein, is also seen (arrowhead). An essentially identical pattern is obtained when an identical aliquot of coated vesicles without added tubulin was incubated with [γ-^{32}P]ATP and subjected to two-dimensional gel electrophoresis, with two exceptions (Figure 4A). Very little material corresponding in mobility to either α-tubulin or β-tubulin is seen (rectangle); also a much reduced quantity of the high molecular weight polypeptide is seen (arrowhead). Figure 4D shows the autoradiogram of the bracketed region of the gel in Figure 4B. Two streaks are seen corresponding to the streaks seen in Figure 4B. Also, two spots corresponding to α- and β-tubulin, respectively, are seen.

Table I: Effects of Nucleotides and Divalent Cations on Coated Vesicle Protein Kinase Activity[a]

	% of control at final Ca^{2+} (mM) concn of		
	0	2	10
none	100	103	62
cAMP, 40 μM	93		
calmodulin, 10 μg		105	68
trifluoperazine, 10 μM		87	73
trifluoperazine, 50 μM		78	64
GTP, 20 μM	69		
GTP, 200 μM	61		

[a] To 25-μg aliquots of rat liver coated vesicles in MES buffer were added the various components listed above, and the final volumes were adjusted to 50 μL with MES containing a final concentration of 2.5 mM $MgCl_2$. [γ-^{32}P]ATP (30 μM final concentration) was added to each tube, and the reaction was carried out for 5 min at 23 °C. After termination by addition of electrophoresis buffer and boiling for 1 min, samples were placed on a 10% gel, and electrophoresis, staining, and autoradiography were carried out. The phosphorylated 53- and 51-kDa regions from each lane were excised and counted by Cerenkov radiation in a scintillation counter. Counts were normalized to the value obtained with no additions.

Figure 4C shows the autoradiogram of the bracketed region of Figure 4A exposed for the identical period. The two streaks are again seen corresponding to those in Figure 4A. No phosphorylation is seen in the region of the gel corresponding to tubulin. No phosphorylated bands in this area are seen when the gel is exposed for up to 4 days. When tubulin is incubated with [γ-^{32}P]ATP alone under conditions identical with those in Figure 4A, a similar amount of phosphorylation of α- and β-tubulin is found (data not shown). It is therefore likely that the phosphorylation of exogenous tubulin seen in Figure 4D is not the result of phosphorylation by the coated vesicle kinase but rather results from the action of the kinase activity associated with the high molecular weight MAP-2 (Sloboda, et al., 1976; Theurkauf and Vallee, 1982).

We do see a small amount of material in our coated vesicles corresponding to MAP-2. We think it is highly unlikely, however, that any associated kinase activity is responsible for phosphorylation of the M_r 53 000 and 51 000 polypeptides since the MAP-2 kinase is highly stimulated by cAMP (Theurkauf & Vallee, 1982) while our activity shows no stimulation (Table I).

The data shown in Figures 1–4 identify proteins of M_r 53 000 and M_r 51 000 as protein kinase substrates. To identify ATP binding sites possibly responsible for the protein kinase mediating these phosphorylations, we employed 8-azido-[α-^{32}P]ATP as a photoactive probe. This reagent will bind to ATP binding sites on proteins and become covalently attached upon photolysis (Haley & Hoffman, 1974). The use of this reagent to label protein kinases has been described (Shia & Pilch, 1983; Roth & Cassell, 1983). We incubated 8-azido-[α-^{32}P]ATP with coated vesicles in the presence and absence of excess adenosine 5′-(β,γ-imidotriphosphate) (Ado-PPNHP). The latter reagent is a nonhydrolyzable ATP analogue (Yount et al., 1971) useful in determining the specificity of photolabels such as 8-azido-[α-^{32}P]ATP (Shia & Pilch, 1983). As shown in Figure 4, the ATP photoprobe is specifically incorporated in substantial amounts only into the M_r 53 000 and M_r 51 000 proteins previously identified as protein kinase substrates. These bands are less well resolved in the experiment depicted in Figure 5 than in other experiments. In order to obtain further evidence concerning the

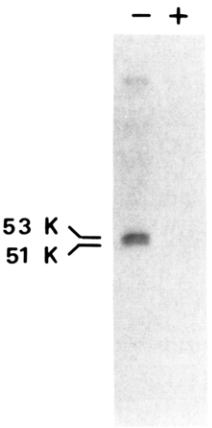

FIGURE 5: Photolabeling of the coated vesicle kinase(s) with 8-azido-[α-^{32}P]ATP. The reactions were carried out in the presence (+) and absence (-) of Ado-PPNHP as described under Materials and Methods. The reactions were terminated with electrophoresis buffer, boiled, and electrophoresed on a 10% acrylamide gel. Following staining and drying autoradiography was performed for 2 h at room temperature. 53 000 and 51 000 are the molecular weights of the polypeptides labeled only in the absence of Ado-PPNHP.

identity of the kinase(s), we preincubated coated vesicles with 10 mM *N*-ethylmaleimide (NEM), a sulfhydryl reagent which inhibits several classes of protein kinase (Krebs & Beavo, 1979). Figure 6 demonstrates that a preincubation with NEM does inhibit coated vesicle kinase activity in a time-dependent manner. As can be seen in the insert to Figure 6, preincubation with NEM also causes a time-dependent decrease in the amount of photolabeling of the 53–51-kDa polypeptides with 8-azido-[α-^{32}P]ATP. We also saw substantial photolabeling in other regions of the gel following NEM preincubation, especially at the top of the separatory gel. There was no time-dependent decrease in photolabeling in any region except the 53–51-kDa region, however, supporting the hypothesis that the coated vesicle kinase substrates are themselves the kinase(s).

The lifetime of a given coated pit-coated vesicle cycle is not known with any certainty. However, numerous studies of receptor-mediated endocytosis occurring via these structures suggests that it is on the order of minutes rather than seconds or hours (Goldstein et al., 1979; Pearse & Bretscher, 1981). Thus, the time course of the self-phosphorylation reaction of the coated vesicle kinase should be consistent with these limits if it is to be of regulatory significance. We have determined the time course of ^{32}P incorporation into the two substrate peptides using the methodology employed in Figure 1. It is quite rapid with half-maximal label incorporation occurring after 2–4 min at 23 °C (data not shown). Within reason, this time course is consistent with coated pit-coated vesicle dynamics.

Numerous types of protein kinases have been described [see Krebs & Beavo (1979) for review]. The best understood kinases play significant roles in the regulation of numerous metabolic processes and are either cAMP dependent or Ca^{2+} dependent, respectively (Krebs & Beavo, 1979; Cohen, 1983). We examined the properties of the coated vesicle kinase to determine what class of kinase it might be. As shown in Table I, the coated vesicle kinase is not stimulated by either cAMP or Ca^{2+}. High Ca^{2+} concentration is somewhat inhibitory, probably by competing for Mg^{2+} binding to ATP. GTP is only slightly inhibitory, suggesting this nucleotide may be utilized as a substrate for the coated vesicle kinase. The addition of 2-mercaptoethanol to the reaction had no effect (data not shown).

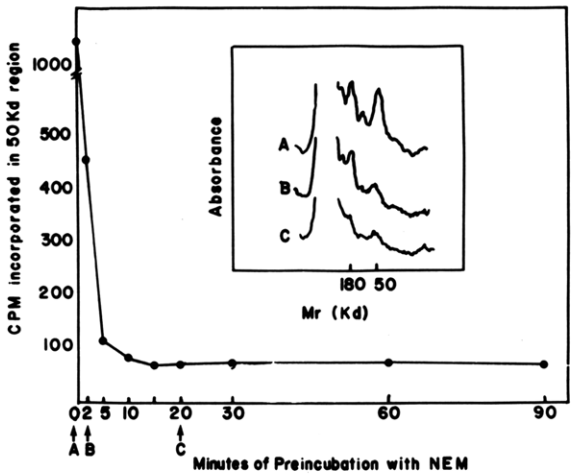

FIGURE 6: Preincubation of coated vesicles with NEM inhibits kinase activity and photolabeling of the 53–51-kDa polypeptides in a time-dependent manner. Nine 50-μg aliquots of rat liver coated vesicles were incubated with 10 mM NEM for the times indicated in the figure. The samples were assayed for kinase activity as described in Figure 1. The phosphorylated bands at 53 000 and 51 000 were cut from the dried gel and counted by Cerenkov radiation in a scintillation counter. (Insert) Fifty-microgram aliquots of coated vesicles were incubated above for 0, 2, or 20 min. The samples were then photolabeled with 10^6 cpm of 8-azido-[α-^{32}P]ATP as described under Materials and Methods. The samples were then electrophoresed, dried, and autoradiographed as in Figure 4. The autoradiogram was scanned by using an Isco UA5 absorbance monitor fitted with a Model 1316 gel scanner.

We further characterized the protein kinase activity of coated vesicles by determining what phospho-amino acid is formed in the M_r 53 000 and M_r 51 000 peptides following acid hydrolysis. Figure 7A shows an autoradiogram of a two-dimensional thin-layer electrophoresis plate. The positions of the standard phospho-amino acids are outlined, and it is evident that phosphothreonine is the predominant if not exclusive product of coated vesicle protein kinase activity.

We have recently carried out a similar experiment with coated vesicle proteins dissociated and separated from vesicles by incubation in 0.5 M Tris as described in Figure 3. As can be seen in Figure 7B, we now see serine as the predominant amino acid phosphorylated.

Discussion

We present evidence that rat liver coated vesicles contain a highly active protein kinase as a tightly associated constituent. This protein kinase exactly comigrates with coated vesicles on agarose gel electrophoresis (Figure 1) and Sephacryl S-1000 chromatography (Figure 2) and is readily dissociated from coated vesicles by mild conditions that remove clathrin and other major coated vesicle proteins (Figure 3). The coated vesicle protein kinase appears to catalyze autophosphorylation since the same proteins that are phosphorylated, M_r 53 000 and M_r 51 000, are the major species photolabeled by an ATP binding site probe (Figure 5). These polypeptides are the only species whose photolabeling is reduced by preincubation with NEM in a time-dependent manner correlated with the concomitant reduction in kinase activity. A number of different protein kinases are known to autophosphorylate (Shia & Pilch, 1983; Rangel-Aldao & Rosen, 1976; Cohen et al., 1980; Hunter & Sefton, 1980b), and we feel that this is highly likely in the present case as well, in view of the above; however, only purification of the kinase(s) to homogeneity will prove our hypothesis.

As mentioned in the introduction, other groups have reported only one major 50 K–55K polypeptide as the major

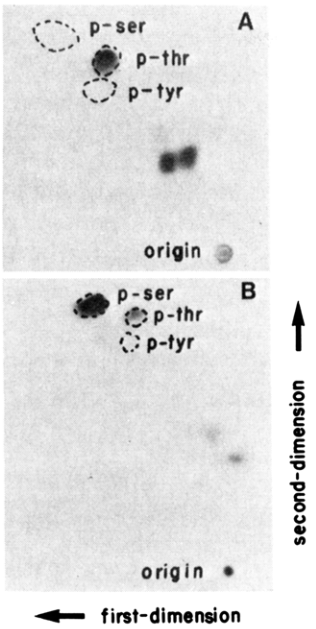

FIGURE 7: Phosphorylation of coated vesicle associated polypeptides occurs predominantly on threonine in intact coated vesicles but on serine in disrupted coat proteins. (A) A 75-μg sample of rat liver coated vesicles in MES buffer at 2.5 mM $MgCl_2$ was incubated with 30 μM [γ-^{32}P]ATP for 5 min at 23 °C. A 10-fold molar excess of unlabeled ATP was added to stop the reaction and the solution dialyzed against several changes of a 1000-fold excess of 10 mM ammonium bicarbonate. The solution was lyophilized and hydrolyzed for 2 h at 110 °C in 6 N HCl. The hydrolysate was freeze-dried, combined with phospho-amino acid standards, and applied to a 250 μM cellulose TLC plate. Electrophoresis buffers and conditions were exactly as described by Hunter & Sefton (1980a,b). The dotted lines represent the ninhydrin-stained standards superimposed on the autoradiogram. (B) A 100-μg sample of rat liver coated vesicles was disrupted by treatment with 0.5 M Tris-HCl, and the coat proteins were separated from the stripped vesicles as described in Figure 3. The conditions for phosphorylation, hydrolysis, and chromatography were identical with those in (A).

substrate for the bovine brain and liver coated vesicle protein kinase (Pauloin et al., 1982; Pfeffer et al., 1983), while we find two for rat liver. One obvious explanation for the difference is that the rat liver polypeptide is partially degraded during isolation. We have done the isolation in the presence of a battery of proteolytic inhibitors, and we still obtain two polypeptides. We do, however, see differences in the relative amounts of the two polypeptides from preparation to preparation. It is certainly possible that the two polypeptides we see are structurally very similar or identical and the different mobilities of the two species result from a posttranslational modification. We are investigating this possibility.

As mentioned under Results two groups have presented data suggesting that tubulin is a constituent of coated vesicles isolated from bovine and chicken liver as well as from bovine brain (Pfeffer et al., 1983; Kelly et al., 1983). Pfeffer et al. (1983) also report that the brain M_r 51 000 phosphopeptide can be specifically immunoprecipitated by a monospecific antibody against τ protein (a class of microtubule binding protein) isolated from brain. the M_r 51 000 protein is also immunoprecipitated with α- and β-tubulin by monospecific anti-tubulin. Pfeffer et al. (1983) suggest that phosphorylation of the M_r 51 000 polypeptide may play a role in the regulation of the coated vesicles interaction with microtubules.

The data presented in Figure 4 indicate that tubulin is present in very small amounts in purified rat liver coated vesicles. Also, there does not appear to be a significant amount of tubulin phosphorylation mediated by the coated vesicle kinase. It would therefore seem essential for a functional assay of microtubule–coated vesicle interactions to be developed for the role of microtubules and/or tubulin in liver coated vesicle dynamics to be evaluated.

Very recently it has been shown that a complex consisting of the brain M_r 51 000 polypeptide and polypeptides of M_r 100 000 and 110 000 is essential for the reassembly of clathrin triskelions into baskets under certain conditions (Zaremba & Keen, 1983). The function of the complex called the "assembly protein" may also be regulated by phosphorylation reactions. These workers also provided evidence that the kinase activity is associated with this complex (Keen & Zaremba, 1983).

The coated vesicle-associated protein kinase appears interesting in several respects. Its activity is not significantly influenced by cAMP and Ca^{2+}-CAM (Table I), and it appears to be relatively specific for threonine residues (Figure 7A) when the coated vesicles are intact; however, serine residues appear at the predominant sites of phosphorylation when disrupted coats are assayed (Figure 7B). Thus, it appears that there is no intrinsic preference for threonine groups in the kinase. It is rather that the constrained state of the protein in coated vesicles only permits threonine residues on the 53–51-kDa polypeptides to be accessible to the presumably autocatalytic kinase. In the dissociated state it is possible that one kinase molecule can phosphorylate another at now accessible serine residue(s) as well as autocatalytically at threonines. We are now testing this hypothesis.

Recently two reports have appeared on the purification of a CAM-dependent glycogen synthetase kinase (Amad et al., 1982; Payne, 1983). The protein consists of two polypeptides having molecular weights of 53 000 and 51 000, respectively, in a high molecular weight complex which autophosphorylates. Even though our kinase appears to be totally independent of Ca^{2+} and CAM for activity and not susceptible to inhibition by trifluoperazine (Table I) while the glycogen synthetase kinase is a totally Ca^{2+}-CAM-dependent enzyme completely inhibited by trifluoperazine, the relationship between the two kinases is worth investigating in view of the above-mentioned similarities in structure.

A large body of evidence implicates coated vesicles as intermediates in the endocytosis of numerous ligands as well as in the movement of vesicles within the cell (Goldstein et al., 1978; Pearse & Bretscher, 1981). There is, however, virtually no evidence as to what factors govern the formation of a coated pit (or coated intracellular membrane), its location on the membrane, and its subsequent conversion to a coated vesicle. Moreover, coated pits and coated vesicles have been shown to be selective with respect to which membrane substituents are included within or excluded by them (Goldstein et al., 1979). It seems very likely therefore that the formation of coated pits and coated vesicles is regulated in vivo, and this regulation may involve the content of coated vesicles as well. Numerous cellular processes are regulated by the phosphorylation and dephosphorylation of proteins (Krebs & Beavo, 1979; Cohen, 1981). The former reactions are mediated by protein kinases, many forms of which have been described (Krebs & Beavo, 1979). Thus, it is tempting to speculate that the protein kinase described herein has a regulatory role in some aspect of the coated pit-coated vesicle life cycle.

We do not know if the phosphorylation of the M_r 53 000 and M_r 51 000 proteins is a primary role of the coated vesicle protein kinase. The possibility exists that there are additional physiological substrates for this protein kinase, and we are therefore looking for such substrates in various fractions of rat liver. Recently, we have demonstrated that coated vesicles

are intermediates involved in the receptor-mediated encocytosis of insulin by rat liver (Pilch et al., 1983). We have developed methods to separate these insulin-containing endocytic vesicles from exocytic vesicles (Helmy et al., 1983), and we are in the process of determining if there are differences in kinase activity in the different types of coated vesicles. Thus, the discovery of the protein kinase(s) described herein is likely to open several new avenues of investigation into fundamental aspects of coated vesicle dynamics.

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