A Novel Adenylylation Process in Liver Plasma Membrane-bound Proteins*

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Rat liver plasma membrane contains five distinct polypeptides of apparent molecular mass of 130, 120, 110, 100, and 86 kDa which are labeled upon incubation with [α-32P]ATP as well as with [γ-32P]ATP. Covalently bound adenosine 5’-monophosphate to some of the polypeptides was identified using nonhydrolyzable analogues of ATP. Chase experiments of α-32P-nucleotide-labeled polypeptides with different nonradioabeled phosphocompounds and sensitivity to different inhibitors demonstrate that the 86-kDa polypeptide is a phosphoesterase, forming a catalytic intermediate. On the other hand, the comparative slow rate of turnover of the polypeptides of higher molecular mass (130, 120, 110, and 100 kDa) suggests that the bound AMP could play a regulatory rather than a catalytic role. Using the nonhydrolyzable ATP analogue [α,β-methylene]ATP and dilution experiments with Triton X-100-solubilized membranes, it has been possible to identify the 130-kDa adenylylated polypeptide as a possible target of an adenylylating system. These polypeptides, except the 86-kDa phosphoesterase, are affected in their electrophoretic mobility in the absence of β-mercaptoethanol. An intercatenary disulfide bond(s) appears to link the polypeptide(s) of 120 kDa and/or 110 kDa in a dimeric structure of apparent molecular mass of 240 kDa. All five polypeptides labeled with [α-32P]ATP are glycoproteins bound to the cell plasma membrane.

The reversible covalent modification of enzymes is a common mechanism in eukaryotic and prokaryotic organisms directed toward the regulation of enzymatic activities (Holzer and Duntze, 1971; Krebs and Beavo, 1979; Springer et al., 1979). In addition, proteins without enzymatic activities could also be subject to such covalent modifications.

Protein adenylylation (i.e. the covalent link of the AMP moiety of ATP to an amino acid residue of a protein with the concomitant release of pyrophosphate) is a process that could take place as a reversible covalent modification of a protein to achieve a regulatory function. Alternatively, adenylylation could represent the formation of a catalytic intermediate of some enzyme during its mode of operation.

In prokaryotic cells it has been described that the glutamine synthetase (Shapiro et al., 1967; Shapiro and Stadtman, 1968; Kustu et al., 1984, 1985), the lysine-sensitive aspartykinase (Niles and Westhead, 1973), and the RNA polymerase (Chelata et al., 1971) exist in two interconvertible adenylylated and unadenylated forms. The adenylylation process results in all cases in a decrease in their respective enzymatic activities (Shapiro et al., 1967; Kustu et al., 1984, 1985; Niles and Westhead, 1973; Chelata et al., 1971).

Several enzymes involved in nucleic acid synthesis, processing, or degradation are also adenylylated. Simian virus 40 large tumor antigen is a DNA-binding phosphoprotein that is reversibly adenylylated, and it has been suggested that this reaction is necessary for the initiation of viral DNA synthesis acting as a primer (Bradley et al., 1984). Similarly, the analogous protein P3 from the bacteriophage ϕ29 initiates DNA replication as a protein-DAMP complex (Penalva and Salas, 1982). Moreover, adenylylation has been also described as an essential catalytic intermediate in both the RNA ligase from the bacteriophage T4 (Cranston et al., 1974; Thegersen et al., 1985; Heaphy et al., 1987) and the RNA 3’-terminal phosphate cyclase from HeLa cells (Filipowicz et al., 1985). Furthermore, the existence of an adenylylated catalytic intermediate of the enzyme 5’-nucleotidase phosphodiesterase from bovine intestine has been also demonstrated (Landt and Butler, 1978).

Moreover, several bacteria genera (Benveniste et al., 1970; Shimizu et al., 1981; Yagi et al., 1988) and the fungus Streptomyces (Marshall et al., 1986) contain enzymes able to adenylylate, and therefore to inactivate, different types of antibiotic molecules. These observations suggest that adenylylation is a process that goes beyond the modulation of protein functions.

In previous work by our group on phosphorylation of plasma membrane-bound proteins from normal and neoplastic liver, we detected the presence of several polypeptides that bind nucleotides (Church et al., 1988). Nevertheless, we did not study in detail the nature of these proteins. In this report, however, we describe several plasma membrane-bound polypeptides that are reversibly adenylylated as well as phosphorylated. Adenylylation was identified upon labeling with [α-32P]ATP and [2,5,8-3H]ATP and phosphorylation upon labeling with [γ-32P]ATP. In addition, we have detected the presence of putative adenylylating and deadenylylating systems as well as target adenylylated polypeptides.

MATERIALS AND METHODS

Chemicals—The radiolabeled compounds [γ-32P]ATP (triethylammonium salt), (3000–5000 Ci mmol−1) (1 Ci = 37 GBq), [α-32P] ATP (triethylammonium salt) (3000 Ci mmol−1), and [2,5,8-3H]ATP (ammonium salt) (62 Ci mmol−1) were purchased from Amersham Corp., and X-Omat AR x-ray blue-sensitive films were purchased from Eastman. 5'-Phosphodiesterases (RC 3.1.4.1) from bovine in-
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testine mucosa and from \textit{Crotalus adamanteus} venom (type II), 3'-phosphodiesterase (EC 3.1.1.6.1) from bovine spleen (type I-S), neuraminidase (EC 3.2.1.18) from \textit{Clostridium perfringens} (type VII), \(\alpha\beta\text{-methylene}\)ATP (lithium salt), \([\gamma\text{-methylene}]\text{ATP (sodium salt)}\), ATP (disodium salt), cyclic AMP (sodium salt) (type II), phenylmethylsulfonyl fluoride, pNP-3'-T' (ammonium salt), and AMP (sodium salt) and using bovine acid, pNP-3'-T (sodium salt) was obtained from Sigma and Calbiochem, and calmodulin was from Calbiochem. Hepes was obtained from Merck, and molecular weight standards for electrophoresis were purchased from Bio-Rad. All other chemicals used in this work are of analytical grade.

Preparation of Liver Plasma Membrane Fractions—Liver plasma membrane fractions from medium sized Sprague-Dawley albino rats (250–300 g) were prepared following the method of Brown et al. (1976) as modified by Church et al. (1988) except that the homogenization was increased up to 20 strokes in a glass-Teflon homogenizer, and the initial 15-s homogenization with the Polytron was omitted. When required, the membrane fractions were prepared in the presence of 1 mM EGTA to deplete the membranes of the calcium-dependent and bound calmodulin pool. Some preparations were also carried out in the presence of 1 mM phenylmethylsulfonyl fluoride to prevent proteolysis.

The crude membrane fraction resulting from the first sucrose gradient was enriched in plasma membrane enzymatic markers 5'-nucleotidase an average of 15-fold (15 preparations) and alkaline phosphatase an average of 17-fold (6 preparations) with respect to the crude homogenate. The further purified membranes (light fraction) resulting from the second sucrose gradient were enriched, however, with respect to the crude homogenate, an average of 43-fold, both in 5'-nucleotidase (10 preparations) and alkaline phosphatase (6 preparations).

The five polypeptides labeled by \([\alpha\text{-}^{32}\text{P}]\text{ATP under study were identified in both crude and further purified plasma membrane fractions. Therefore, both fractions were used to perform adenylylation experiments. However, the further purified plasma membrane fraction was generally used to perform phosphorylation experiments to avoid interferences by a larger number of phosphorylated polypeptides observed in the crude plasma membrane fractions. The fractions employed for each experiment are indicated in the legends to the figures.}

Phosphorylation Experiments—Phosphorylation experiments, unless indicated otherwise, were performed at 37 °C for 1 min in a total volume of 100 µl of medium containing 15 mM Hepes-Na (pH 7.4), 6 mM MgCl₂, 1 mM EGTA, 10 µM \([\gamma\text{-}^{32}\text{P}]\text{ATP (2–5 µCi)}\), and appropriate amounts of membrane protein. The reaction was initiated upon addition of the radiolabeled ATP and stopped upon addition of ice-cold 10% (w/v) trichloroacetic acid. The supernatant was discarded after centrifugation in a microcentrifuge (14,900 \times g, 15 min), and the pellet was processed for electrophoresis.

Adenylylation Experiments—Adenylylation experiments, unless indicated otherwise, were performed at 37 °C for 5 min in a total volume of 100 µl of medium containing 15 mM Hepes-Na (pH 7.4), 6 mM MgCl₂, 100 µM CaCl₂, 10 µM \([\alpha\text{-}^{32}\text{P}]\text{ATP (1.5–5 µCi)}\), and appropriate amounts of membrane protein. Some experiments were performed with 1.6 mM \([2,5',8'-\text{H}]\text{ATP (9.9 µCi)}\). The reaction was initiated and stopped as indicated above, and the pellet was processed for electrophoresis.

Analytical Procedures—Slab gel electrophoresis (10–30 µg of protein per track) was performed according to Laemmlli (1970) at 12 mA overnight in a linear gradient 5–20% (w/v) polyacrylamide gel in the presence of 0.1% (w/v) sodium dodecyl sulfate at pH 8.3. The gels were stained with Coomassie Brilliant Blue R-250, and, after drying the gels under vacuum at 70 °C on top of Whatman 3MM Chr filter paper, a blue-sensitive x-ray film was exposed in the dark at –20 °C for appropriate periods of time. The intensity of the labeled bands on the autoradiographs was read in a scanning photodensitometer.

Protein concentrations were determined by the method of Lowry et al. (1951) after precipitating the proteins with 10% (w/v) trichloroacetic acid. The further purified plasma membrane fractions containing 15 mM Hepes-Na (pH 7.4), 10 mM MgCl₂, 1 mM EGTA, and 10 µM \([\gamma\text{-}^{32}\text{P}]\text{ATP Tracks 2–5, crude plasma membranes (60 µg of protein) were incubated for 1 min in 100 µl of a medium containing 15 mM Hepes-Na (pH 7.4), 6 mM MgCl₂, 100 µM CaCl₂, and 10 µM \([\alpha\text{-}^{32}\text{P}]\text{ATP. The reaction was arrested upon addition of 10% (w/v) trichloroacetic acid and processed for electrophoresis as described under "Materials and Methods." In track 3, the gel was incubated for 20 min in 5% (w/v) trichloroacetic acid at 90 °C. In track 4, the gel was incubated for 3 h in 0.3 N HCl at 37 °C. In track 5, 10% (w/v) trichloroacetic acid was present during the assay. Heating the membranes at 100 °C for 5 min before the assay gave the same results as the one presented in track 5. Track 6, purified plasma membranes (100 µg of protein) were incubated for 1 min at 37 °C in 100 µl of a medium containing 15 mM Hepes-Na (pH 7.4, 7 mM MgCl₂, 100 µM CaCl₂, and 1.6 mM \([2,5',8'-\text{H}]\text{ATP. The reaction was arrested upon addition of 10% (w/v) ice-cold trichloroacetic acid and processed for electrophoresis as described under "Materials and Methods." In track 6, the gel was incubated for 20 min at 37 °C and subsequently processed for electrophoresis as described above. The autoradiographs were obtained from the dry gels as described under "Materials and Methods."}

RESULTS

Enzymatically Catalyzed Covalent Binding of \(\alpha\text{-}^{32}\text{P-nucleotide to Liver Plasma Membrane Glycoproteins—Membrane-bound protein kinase(s) autophosphorylate(s), and/or phosphorylate(s) served as target polypeptides in isolated rat liver plasma membrane fractions upon incubation with \(\gamma\text{-}^{32}\text{P}\text{ATP (Fig. 1A, track 1). In addition, incubation of these membranes with \([\alpha\text{-}^{32}\text{P}]\text{ATP results in the labeling of five distinct polypeptides of apparent molecular mass of 130, 120, 110, 100, and 86 kDa (Fig. 1A, track 2). Moreover, the incubation of the membranes with \(2,5',8'-\text{H}\text{ATP results in the labeling of the 130-, 120-, 110-, and 100-kDa polypeptides, although the 86-kDa polypeptide was not labeled (Fig. 1A, track 6). The five \(\alpha\text{-}^{32}\text{P-nucleotide-labeled polypeptides coincide in their electrophoretic mobilities with five polypeptides of identical apparent molecular mass which are also phosphorylated (compare tracks 1 and 2 of Fig. 1A).}}

To ascertain the covalent nature of the bond between the

FIG. 1. Covalent binding of \(\alpha\text{-}^{32}\text{P-nucleotide to plasma membrane-bound phosphoproteins (A) and adenylylated catalytic intermediates of 5'-phosphodiesterases (B). A: track 1, purified plasma membranes (52 µg of protein) were incubated for 1 min at 37 °C in 100 µl of a medium containing 15 mM Hepes-Na (pH 7.4), 10 mM MgCl₂, 1 mM EGTA, and 10 µM \([\gamma\text{-}^{32}\text{P}]\text{ATP Tracks 2–5, crude plasma membranes (60 µg of protein) were incubated for 1 min in 100 µl of a medium containing 15 mM Hepes-Na (pH 7.4), 6 mM MgCl₂, 100 µM CaCl₂, and 10 µM \([\alpha\text{-}^{32}\text{P}]\text{ATP. The reaction was arrested upon addition of 10% (w/v) ice-cold trichloroacetic acid and processed for electrophoresis as described under "Materials and Methods." In track 3, the gel was incubated for 20 min in 5% (w/v) trichloroacetic acid at 90 °C. In track 4, the gel was incubated for 3 h in 0.3 N HCl at 37 °C. In track 5, 10% (w/v) trichloroacetic acid was present during the assay. Heating the membranes at 100 °C for 5 min before the assay gave the same results as the one presented in track 5. Track 6, purified plasma membranes (100 µg of protein) were incubated for 1 min at 37 °C in 100 µl of a medium containing 15 mM Hepes-Na (pH 7.4, 7 mM MgCl₂, 100 µM CaCl₂, and 1.6 mM \([2,5',8'-\text{H}]\text{ATP. The reaction was arrested upon addition of 10% (w/v) ice-cold trichloroacetic acid and processed for electrophoresis as described under "Materials and Methods." In track 6, the gel was incubated for 20 min at 37 °C and subsequently processed for electrophoresis as described above. The autoradiographs were obtained from the dry gels as described under "Materials and Methods."}

1The abbreviations used are: pNP-3'-T, thymidine 3'-monophosphate; pNP-5'-T, thymidine 5'-monophosphate; pNP-phosphotyrosyl ester; pNP-5'-T', thymidine 5'-monophosphate p-nitrophenyl ester; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid.
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$\alpha$-32P-nucleotide and the polypeptides were subjected to different drastic treatments after its electrophoretic separation in polyacrylamide slab gels. The $\alpha$-32P-nucleotide-labeled polypeptides were resistant to incubation with 5% (w/v) trichloroacetic acid at 90 °C for 20 min (Fig. 1A, track 3) or 0.3 N HCl at 37 °C for 3 h (Fig. 1A, track 4). On the other hand, treatment of the membranes with trichloroacetic acid or heating at 100 °C for 5 min, before the addition of $[\alpha$-32P]ATP, prevents the binding of the $\alpha$-32P-nucleotide to the polypeptides (Fig. 1A, track 5).

Some of the $\alpha$-32P-nucleotide-labeled polypeptides could be adenylylated catalytic intermediates of phosphodiesterases (Landt and Buttler, 1978) associated to the liver plasma membrane. Therefore, we assayed different commercially available phosphodiesterases with $[\alpha$-32P]ATP in the same conditions as the plasma membranes to ascertain that their adenylylated catalytic intermediates were detected. Fig. 1B presents autoradiographs of the $[\alpha$-32P]ATP-bound catalytic intermediates of 5'-phosphodiesterases from bovine intestine mucosa (65 kDa with a minor labeled band of 125 kDa) (track 1) and from snake venom (102 kDa) (track 2).

As presented in Fig. 2, treatment of the membranes with neuraminidase prior to the incubation with $[\alpha$-32P]ATP results in an increased electrophoretic mobility of the five $\alpha$-32P-nucleotide-labeled polypeptides (compare tracks 1 and 2 of Fig. 2). The apparent molecular masses of the neuraminidase-treated polypeptides were 120, 110, 100, 90, and 75 kDa. The maintenance of the same relative pattern and intensity of labeling of the five polypeptides before and after treatment with neuraminidase suggests that all five polypeptides equally decrease their apparent molecular mass by approximately 10 kDa. These experiments demonstrate that at least from 8 to 13% by weight of these polypeptides are sialic acid-containing glycosylated, and the 120-kDa and/or 110-kDa polypeptide(s) forms(s) a disulfide bond-linked dimer. Tracks 1 and 2, crude plasma membranes (prepared in the presence of EGTA) (52 µg of protein) were preincubated for 15 min at 37 °C in the absence (track 1) or presence (track 2) of neuraminidase (6 µg of protein) in 100 µl of the medium containing 15 mM HEPES-Na (pH 7.4). Thereafter, 10 µM $[\alpha$-32P]ATP was added, and assay was made for 5 min at 37 °C. The protein was processed for electrophoresis and autoradiographs as described under “Materials and Methods.” Tracks 3 to 5, crude plasma membranes (prepared in the presence of EGTA) (52 µg of protein) were assayed for 1 min (track 3) or 5 min (tracks 4 and 5) at 37 °C in 100 µl of a medium containing 15 mM HEPES-Na (pH 7.4) and 10 µM $[\alpha$-32P]ATP (track 3) or 15 mM HEPES-Na (pH 7.4), 6 mM MgCl2, and 10 µM $[\alpha$-32P]ATP (tracks 4 and 5). After the assay, the trichloroacetic acid-precipitated proteins were processed for electrophoresis in the presence (track 4) or absence (tracks 3 and 5) of β-mercaptoethanol. Arrowheads show the bands of 240 and 140 kDa in the autoradiographs.

Fig. 2. The five $\alpha$-32P-nucleotide-labeled polypeptides are glycosylated, and the 120-kDa and/or 110-kDa polypeptide(s) forms(s) a disulfide bond-linked dimer. Tracks 1 and 2, crude plasma membranes (prepared in the presence of EGTA) (52 µg of protein) were preincubated for 15 min at 37 °C in the absence (track 1) or presence (track 2) of neuraminidase (6 µg of protein) in 100 µl of the medium containing 15 mM HEPES-Na (pH 7.4). Thereafter, 10 µM $[\alpha$-32P]ATP was added, and assay was made for 5 min at 37 °C. The protein was processed for electrophoresis and autoradiographs as described under “Materials and Methods.” Tracks 3 to 5, crude plasma membranes (prepared in the presence of EGTA) (52 µg of protein) were assayed for 1 min (track 3) or 5 min (tracks 4 and 5) at 37 °C in 100 µl of a medium containing 15 mM HEPES-Na (pH 7.4) and 10 µM $[\alpha$-32P]ATP (track 3) or 15 mM HEPES-Na (pH 7.4), 6 mM MgCl2, and 10 µM $[\alpha$-32P]ATP (tracks 4 and 5). After the assay, the trichloroacetic acid-precipitated proteins were processed for electrophoresis in the presence (track 4) or absence (tracks 3 and 5) of β-mercaptoethanol. Arrowheads show the bands of 240 and 140 kDa in the autoradiographs.
the bond of ATP must be hydrolyzed for the binding of AMP to these polypeptides, suggesting that the nonhydrolyzable ATP analogue \([\alpha,\beta\text{-methylene}]\text{ATP}\) does not induce such inhibition. Therefore, these experiments demonstrate that adenylylation of the 120- and 110-kDa polypeptides takes place.

Most significantly, the labeling of the 130- and 86-kDa polypeptides was not inhibited by \([\alpha,\gamma\text{-methylene}]\text{ATP}\) at this concentration (7.5 mM). However, a higher concentration of \([\alpha,\beta\text{-methylene}]\text{ATP}\) (100 \(\mu\text{M}\)) also produces a strong inhibition of the binding of the nucleotide to the 130 kDa polypeptide (results not shown).

The 240-kDa \(\alpha\text{-32P}-\text{nucleotide-labeled} \) band observed in electrophoresis in the absence of \(\beta\)-mercaptoethanol could be formed by two subunits of the 120-kDa polypeptide or a 120/110-kDa heterodimer since \([\alpha,\beta\text{-methylene}]\text{ATP}\) strongly inhibits the labeling of this band (results not shown).

**Nature of the \(\alpha\text{-32P}-\text{nucleotide-labeled Polypeptides}\)**

As demonstrated in Fig. 1B, different purified 5'-phosphodiesterases form adenylylated catalytic intermediates using \([\alpha,\beta\text{-methylene}]\text{ATP}\) as substrate and assayed under the same conditions utilized for the labeling of liver plasma membrane proteins. Therefore, it was suspected that some of the \([\alpha,\beta\text{-methylene}]\text{ATP}\) labeled polypeptides could represent adenylylated catalytic intermediates of plasma membrane-bound phosphodiesterases. In fact, the isolated liver plasma membrane fractions have an average \(\pm\) S.E. 5'-phosphodiesterase activity, of 3.9 \(\pm\) 0.7 \(\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}\) (three preparations) using pNP-5'-T as substrate. In contrast, 3'-phosphodiesterase activity was not detected in these membrane fractions using pNP-3'-T as substrate and assayed under the same conditions than the 5'-phosphodiesterase.

It is reasonable to postulate that if the \(\alpha\text{-32P}-\text{nucleotide-labeled polypeptides}\) are adenylylated catalytic intermediates of enzymes, they should present a high rate of turnover. Therefore, the nature of the adenylylated polypeptides was investigated by chase experiments of the \(\alpha\text{-32P}-\text{nucleotide-labeled polypeptides}\) with different nonradiolabeled phospho-compounds.

In Fig. 7, A and B, we present typical chase experiments using 5 \(\mu\text{M}\) nonradiolabeled ATP and 5 \(\mu\text{M}\) nonradiolabeled pNP-5'-T, respectively. In both cases it becomes clear that the \(\alpha\text{-32P}-\text{nucleotide-labeled} \) 86-kDa polypeptide loses its labeling very fast (less than 30 s) upon addition of nonradiolabeled ATP or pNP-5'-T, as expected for an adenylylated catalytic intermediate of a phosphodiesterase. However, non-labeling of this polypeptide was observed with \([\gamma\text{-32P}]\text{ATP}\) (see Fig. 1A, track 6).

In contrast, the other \(\alpha\text{-32P}-\text{nucleotide-labeled} \) polypeptides of apparent molecular mass 130, 120, 110, and 100 \(\text{kDa}\) were chased by nonradiolabeled ATP or pNP-5'-T at a very slow rate (Fig. 7, A and B). In fact, one-third of the total \(\alpha\text{-32P}-\text{nucleotide-labeled} \) polypeptides (results not shown).

This atypical slow rate of chase suggests that the bound \(\alpha\text{-32P}-\text{nucleotide-labeled} \) high molecular mass polypeptides are exchanged to the medium and/or transferred to other proteins at a very slow rate of turnover. This observation is not consistent with the idea that these high molecular mass \(\alpha\text{-32P}-\text{nucleotide-binding} \) polypeptides are catalytic intermediates of phosphodiesterases or other enzymes of similar nature.

Moreover, similar differential behaviors for the polypeptide of 86 \(\text{kDa}\) and the other four polypeptides of higher molecular mass (130, 120, 110, and 100 \(\text{kDa}\)) were observed in chase experiments with nonradiolabeled \([\alpha,\beta\text{-methylene}]\text{ATP}\), pNP-3'-T, ADP, and AMP (results not shown). In contrast, 5 \(\mu\text{M}\) cyclic AMP induces a slow rate of chase in all five \(\alpha\text{-32P}-\text{nucleotide-labeled} \) polypeptides (results not shown).

Vanadate partially inhibits the pNP-5'-T phosphodiesterase activity present in the membranes with an apparent \(K\text{f} \) of 30–40 \(\mu\text{M}\) (Fig. 8, panel A). The labeling of the 86-kDa polypeptide using \([\alpha\text{-32P}]\text{ATP}\) as substrate was inhibited by somewhat lower concentrations of vanadate with an apparent \(K\text{f} \) of 3–4 \(\mu\text{M}\) (Fig. 8, panel B). However, the higher molecular mass polypeptides were inhibited very slightly by 250 \(\mu\text{M}\) vanadate (see inset in Fig. 8B) although they were inhibited extensively at much higher concentrations of the inhibitor (results not shown). Moreover, AMP, another inhibitor of the pNP-5'-T phosphodiesterase activity, also inhibits the labeling of the 86-kDa polypeptide but does not affect the labeling of the 130-, 120-, 110-, and 100-kDa polypeptides (results not shown).

It is expected that a protein that is able to autoadenylylate...
membranes as a previous and necessary step to uncover their physiological functions. We have strong evidence suggesting that the five $\alpha_{-32P}$-nucleotide-labeled polypeptides under study are distinct polypeptides and not proteolytic products of a single protein. We have obtained identical electrophoretic patterns of $\alpha_{-32P}$-nucleotide-labeled polypeptides in the presence or absence of proteinase inhibitors. We tested 1 mM phenylmethylsulfonyl fluoride during the preparation of the membranes and 200 $\mu$M leupeptin during the preincubation and assay of the membranes. Furthermore, preparation and assay of membranes in the presence of 1 mM EGTA to prevent the action of calcium-dependent proteases (Pontremoli and Melloni, 1986) gave also the same electrophoretic pattern of $\alpha_{-32P}$-nucleotide-labeled polypeptides.

We have observed that the five $\alpha_{-32P}$-nucleotide-labeled polypeptides from rat liver membranes coincide in their electrophoretic mobilities with five polypeptides that are also phosphorylated. It is difficult to establish whether or not these plasma membrane polypeptides that bind nucleotide(s) and phosphate(s) and coincide in electrophoretic mobilities in gels of polyacrylamide are identical polypeptides. Nevertheless, we demonstrated that treatment of the membranes with neuraminidase before labeling with either $[\alpha_{-32P}]$ATP or $[\gamma_{-32P}]$ATP results in an identical increase in electrophoretic mobilities of the 130-, 120-, 110-, 100-, and 86-kDa adenylated or phosphorylated polypeptides. Furthermore, electrophoresis in the absence of $\beta$-mercaptoethanol of membranes subjected to adenylolation or phosphorylation with $[\alpha_{-32P}]$ATP or $[\gamma_{-32P}]$ATP, respectively, gave identical electrophoretic patterns of the polypeptides under consideration. Therefore, we propose that adenylolation and phosphorylation likely occur in the same polypeptides. We do not have a ready explanation for the occurrence of both adenylolation and phosphorylation processes in the same polypeptides. However, preliminary experiments suggest that blocking the adenylolation of some of the high molecular mass polypeptides affects their levels of phosphorylation as well.

The most probable subcellular location of these adenylolated glycoproteins is the plasma membrane. This is supported by the finding that they are detected not only in crude plasma membrane preparations (13-17-fold purified) but in highly purified plasma membrane preparations (43-fold purified) where there is a strong increase in the labeling of the five polypeptides.

The absence of the reducing agent $\beta$-mercaptoethanol during the treatment of the $[\alpha_{-32P}]$ATP- or $[\gamma_{-32P}]$ATP-labeled polypeptides, before the electrophoresis, clearly shows that the polypeptide of 86 kDa does not appear to form oligomeric structures linked by intercatenary disulfide bond(s). However, the electrophoretic mobilities of the polypeptides of higher molecular mass are strongly affected by the absence of this reducing agent.

The detection of a 240-kDa adenylolated band in the absence of $\beta$-mercaptoethanol strongly points to the existence of a dimer of the 120-kDa polypeptide(s) or a heterodimer of the 120-kDa plus the 110-kDa polypeptides. Using the nonhydrolyzable ATP analogue $[\alpha_{-32P}]$ATP, we observed a strong inhibition of the nucleotide-binding process to the 240-kDa band as occurs with the 120-kDa polypeptide. However, it could not be excluded that a heterooligomer formed by monomers of lower molecular mass could be responsible for the formation of the 240-kDa adenylolated protein observed in nonreducing conditions.

In addition, in the absence of $\beta$-mercaptoethanol, another $\alpha_{-32P}$-nucleotide-labeled 140-kDa band was also observed. This 140-kDa band could represent binding by disulfide bonds.
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Fig. 5. Time courses of $\alpha$-$\beta$-P-nucleotide labeling (A and B) and $\gamma$-$\beta$-P phosphorylation (C). A and B, crude plasma membranes (66 $\mu$g of protein) were incubated at 37°C for 4 min in 100 $\mu$L of a medium containing 15 mM Hepes-Na (pH 7.4), 6 mM MgCl$_2$, 50 mM CaCl$_2$, and 10 $\mu$M [\$\alpha$-$\beta$-P]ATP. At the indicated times, 100-$\mu$L aliquots were taken, and the reaction was stopped with 10% (w/v) ice-cold trichloroacetic acid. C, purified plasma membranes (28 $\mu$g of protein) were incubated at 37°C in 0.9 mL of a medium containing 15 mM Hepes-Na (pH 7.4), 6 mM MgCl$_2$, 1 mM EGTA, and 10 $\mu$M [$\gamma$-$\beta$-P]ATP. At the indicated times, 100-$\mu$L aliquots were taken, and the reaction was stopped with 10% (w/v) ice-cold trichloroacetic acid. The trichloroacetic acid-precipitated proteins were processed for electrophoresis and autoradiographs as described under "Materials and Methods." A plot of the intensities of the labeled bands in the autoradiographs versus time is presented. The different nucleotide-labeled (nb) or phosphorylated (pp) polypeptides are indicated by a number corresponding to their apparent molecular mass $\times 10^3$.

Fig. 6. The nonhydrolysable ATP analogue [\$\alpha$-$\beta$-methylene]ATP inhibits the nucleotide-binding process in the 120- and 110-kDa polypeptides. Crude plasma membranes (86 $\mu$g of protein) were incubated at 37°C for 4 min in 100 $\mu$L of a medium containing 15 mM Hepes-Na (pH 7.4), 6 mM MgCl$_2$, 100 mM CaCl$_2$, and 10 $\mu$M [\$\alpha$-$\beta$-P]ATP (track 1), 7.5 $\mu$M [\$\alpha$-$\beta$-P]ATP plus 7.5 $\mu$M [\$\alpha$-$\beta$-methylene]ATP (track 2), or 7.5 $\mu$M [\$\alpha$-$\beta$-P]ATP plus 7.5 $\mu$M [\$\beta$-$\gamma$-methylene]ATP (track 3).

of a small nonlabeled polypeptide to one of the adenylated polypeptides of higher molecular mass. Alternatively, the presence of intracatenary disulfide bonds in a nucleotide-binding polypeptide could alter their electrophoretic mobility because of the absence of complete unfolding. We propose that the most likely candidate for the formation of this 140-kDa band is the 130-kDa polypeptide observed in the presence of $\beta$-mercaptoethanol. This is supported by two observations. First, the 140-kDa band is labeled in the presence of [\$\alpha$-$\beta$-methylene]ATP to the same extent as that of the 130-kDa band; and second, both bands have similar intensity of labeling.

Adenylylation could result from the phosphodiester bond of AMP to a hydroxyl group of an amino acid residue of serine (Landt and Butler, 1978; Bradley et al., 1988), threonine (Landt and Butler, 1978), or tyrosine (Shapiro and Stadtman, 1968), or the phosphoramide link of AMP to an amino group of an amino acid residue such as lysine (Thøgersen et al., 1985; Heaphy et al., 1987).

To investigate the nature of the bond of the $\alpha$-$\beta$-P-nucleotide to these polypeptides we have treated $\alpha$-$\beta$-P-nucleotide-labeled polypeptides that were electrotransferred from the polyacrylamide gels to nitrocellulose paper with 5'-phosphodiesterases from snake venom or bovine intestine mucosa, and with a 3'-phosphodiesterase from bovine spleen. A significant decrease in labeling of any band was not observed with those treatments (results not shown). This could suggest that the bound nucleotide could be linked to these polypeptides by a phosphoramide bond. However, the high resistance of this bond to acid conditions appears to exclude this possibility (Landt and Butler, 1978). One probability is that the bound nucleotide could be linked to the polypeptides by a phosphodiester bond not accessible to the exogenously added phosphodiesterases.

The rate of chase by different phosphocompounds of the $\alpha$-$\beta$-P-nucleotide-labeled polypeptides also gave some evidences on the nature of the adenylated proteins. We have demonstrated that the label of the 86-kDa polypeptide is chased very rapidly by nonradio-labeled ATP, ADP, AMP, pNP-5'-T, pNP-3'-T, and [$\alpha$-$\beta$-methylene]ATP. In contrast, cyclic AMP induces a slow rate of chase of this labeled polypeptide. The rapid rate of chase of the label of the 86-kDa polypeptide suggests that this polypeptide could be an adenylated intermediate of a phosphoesterase with low specificity for its substrate and/or position of the hydrolyzed bonds. However, the absence of labeling by [5',5',8'-3H]ATP appears to suggest a different kind of intermediate. As demonstrated previously by us with some of the polypeptides (Church et al., 1988), we show now that the 130-, 120-, 110-, and 100-kDa polypeptides are labeled by [5',5',8'-H]ATP (see Fig. 1A, track 6), demonstrating that the adenosine moiety of the nucleotide is indeed bound to the polypeptides. However, the absence of labeling of the 86-kDa polypeptide by [5',5',8'-H]ATP could readily be explained by the simultaneous or sequential hydrolysis of the $\alpha$, $\beta$-bond of ATP and the 5'-phosphate bond from the resulting AMP as expected for an unspecific phosphoesterase. In agreement with this suggestion, we observed that nonradio-
be of 190-180 kDa (Ross and Gilman, 1980). Furthermore, if the presence of mercaptoethanol, since it has been described to
with the reported values for the catalytic subunit of the
polypeptides under study has a coincidental molecular mass
that could form a putative adenylylated intermediate during
the formation of such intermediate has been proposed for this
enzyme (Walsh, 1979). Moreover, none of the adenylylated
polypeptides detected. However, the extremely slow rate of chase of the 86-kDa polypeptide upon addition of ATP or pNP-5'-T. The intensities of the different bands were normalized considering 100% the maximum value obtained for each one.

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labeled AMP was able to chase effectively the label of the 86-
kDa polypeptide as expected if this polypeptide were able to
hydrolyze AMP to adenosine plus phosphate.

The experiments with vanadate further support the idea that the 86-kDa-labeled polypeptide is a phosphoesterase. However, the extremely slow rate of chase of the \( \alpha^{32}\text{P}-\)nucleotide bound to the polypeptides of higher molecular mass suggests that the nature of these polypeptides is different.

Adenylylate cyclase is a plasma membrane-bound enzyme that could form a putative adenylylated intermediate during its catalytic cycle. However, a mechanism of catalysis without the formation of such intermediate has been proposed for this enzyme (Walsh, 1979). Moreover, none of the adenylylated polypeptides under study has a coincidental molecular mass with the reported values for the catalytic subunit of the adenylylate cyclase from rat liver, either in the absence or presence of \( \beta\)-mercaptoethanol, since it has been described to be of 190–180 kDa (Ross and Gilman, 1980). Furthermore, if the adenylylate cyclase were one of the high molecular mass nucleotide-binding polypeptides observed in the liver plasma membrane preparations, it could be expected that the bound \( \alpha^{32}\text{P}-\)nucleotide was rapidly chased by 5 mM nonradiolabeled ATP. However, this exchange is very slow. Therefore, we exclude the adenylylate cyclase as one of the adenylylated polypeptides detected.

Especially significant is the fact that the 120- and 110-kDa polypeptides are strongly inhibited by low concentrations of the nonhydrolyzable ATP analogue \( \alpha,\beta\text{-methylene}\)ATP, suggesting that these polypeptides could themselves hydrolyze ATP between the phosphates in positions \( \alpha \) and \( \beta \). The 86-kDa polypeptide was not expected to be inhibited by \( \alpha,\beta\text{-methylene}\)ATP since this protein appears to be able to hydrolyze many different types of phosphoester bonds, and therefore it could hydrolyze the \( \alpha,\beta\text{-methylene}\)ATP at alternative positions from the \( \alpha,\beta\)-bond.

As mentioned above, the prominent 130-kDa \( \alpha^{32}\text{P}-\)nucleotide-binding polypeptide has a slow rate of chase by different phosphoanions, and also it is not inhibited by low concentrations of \( \alpha,\beta\text{-methylene}\)ATP. This suggests (but does not exclude) that this polypeptide does not hydrolyze ATP between the positions \( \alpha \) and \( \beta \). However, it appears to exclude that this polypeptide is a catalytic intermediate of an enzyme with a high turnover. We excluded ADP as the bound \( \alpha^{32}\text{P}-\)nucleotide since \( \beta,\gamma\text{-methylene}\)ATP does not inhibit the labeling of this polypeptide. Furthermore, the inhibition observed at high concentrations of \( \alpha,\beta\text{-methylene}\)ATP suggests that the bound nucleotide is indeed AMP. Therefore, we suggest that this 130-kDa polypeptide could receive the nucleotide from an adenylytransferase system. Nevertheless, it cannot be excluded that the 130-kDa polypeptide could adenylylate itself.
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Fig. 9. Effect of protein dilution on the \( \alpha^{32}P\)-nucleotide-labeling process in detergent-solubilized membranes. Crude plasma membranes (from 10.4 to 625 \( \mu \)g of protein) were solubilized in 0.5 ml of a medium containing 15 mM Hepes-Na (pH 7.4), 6 mM MgCl\(_2\), 100 \( \mu \)M CaCl\(_2\), and 4% (w/v) Triton X-100. Thereafter, 10 \( \mu \)M [\( \alpha^{32}P\)]ATP was added, and assay was done for 5 min. The reaction was stopped upon addition of 10% (w/v) ice-cold trichloroacetic acid, and the samples were processed for electrophoresis and autoradiographs as described under "Materials and Methods." A plot is presented on the binding of \( \alpha^{32}P\)-nucleotide per \( \mu \)g of protein (specific activity of binding) versus the fold-dilution of proteins in the assay system. The different nucleotide-labeled (nb) polypeptides are indicated by a number corresponding to their apparent molecular mass \( \times 10^3\).

Dilution experiments in which Triton X-100-solubilized membrane proteins are labeled with [\( \alpha^{32}P\)]ATP at decreasing concentrations of protein in the assay system show that the 130-kDa \( \alpha^{32}P\)-nucleotide-binding polypeptide dramatically increases its labeling when it is progressively diluted in the medium containing the detergent. This suggests that a putative deadenylylating system decreases its interaction with its target 130-kDa polypeptide. Moreover, these results also suggest that the adenylylating system could be closely associated with its 120-kDa target polypeptide. A specific adenylylating system is expected to have high affinity for recognizable features of its target protein, and therefore to remain in close association even at low concentrations of protein in the assay system.

The complex oscillatory time courses of \( \alpha^{32}P\)-nucleotide binding to the polypeptides of higher molecular mass (130, 120, 110, and 100 kDa) also suggest superimposition in time of two phenomena, one of adenylylation and the other of deadenylylation.

These polypeptides have high affinities for ATP with apparent \( K_{s(MATP)} \) of 5-7 \( \mu \)M, during nucleotide binding or phosphorylation. This indicates that the binding of the nucleotide to the polypeptides could take place very efficiently at low concentrations of ATP and suggests that it could be a physiological process. Moreover, the existence in isolated plasma membranes of a high ATPase activity (i.e., average \( \pm S.E. \) of 808 \( \pm 74 \) nmol min\(^{-1} \)mg protein\(^{-1}\), in four different preparations) also points out that the actual \( K_{s(MATP)} \) during adenylylation and phosphorylation could be even lower than estimated, since a significant amount of ATP is expected to be hydrolyzed during the time of assay.

The effect of calcium ion on the binding of AMP to these proteins is also a complex process. The existence of two systems for adenylylation and deadenylylation working simultaneously also makes it difficult to ascertain which system is affected by calcium ion. Nevertheless, it becomes clear from our experiments that in the presence of magnesium ion, the 120- and 110-kDa polypeptides increase their capacity to bind \( \alpha^{32}P\)-nucleotide. However, in the absence of magnesium ion, the 120-kDa polypeptide is significantly inhibited by calcium ion in its capacity to bind \( \alpha^{32}P\)-nucleotide. This suggests the existence of competition between both divalent cations.

Plasma membranes contain two pools of bound calmodulin, one easily removable by EGTA and the second one more tightly bound to the membranes (Cloor and Cazzotti, 1986). Therefore, it was not expected that a clear-cut answer could be given to the question on the possible stimulation by exogenous calmodulin of some of the \( \alpha^{32}P\)-nucleotide-binding polypeptides using plasma membrane prepared in the presence of EGTA. Nevertheless, we found in EGTA-prepared membranes a small (approximately 20-30%) increase in \( \alpha^{32}P\)-nucleotide binding to the 130-kDa polypeptide in the presence of the exogenously added Ca\(^{2+}\)-calmodulin complex.

Further experiments with solubilized and purified \( \alpha^{32}P\)-nucleotide-binding polypeptides should be performed to clarify the effect of calmodulin.

We have initiated a search on the presence of adenylylated proteins in normal and neoplastic cells from different origin. In membrane preparations from mouse liver we detected the presence of 120- and 86-kDa \( \alpha^{32}P\)-nucleotide-labeled polypeptides. In previous studies (Church et al., 1988) we also detected \( \alpha^{32}P\)-nucleotide-labeled polypeptides of 130 and 120 kDa in the AS-30D rat ascites hepatoma. However, membrane fractions from the mouse Ehrlich ascites tumor do not present similar bands of \( \alpha^{32}P\)-nucleotide-labeled polypeptides (results not shown). We have also detected several bands (the most prominently labeled of 54 kDa) in whole permeabilized Swiss 3T3 fibroblasts grown in culture and two 75- and 62-kDa \( \alpha^{32}P\)-nucleotide-labeled polypeptides in plasma membranes isolated from the yeast Saccharomyces cerevisiae (results not shown). These results demonstrate that adenylylation is a common process in plasma membrane-bound proteins.

The physiological role of these novel adenylylated proteins is not yet known. However, the detection of an adenylylating/deadenylylating system in plasma membranes of eukaryotic cells points out the existence of regulatory mechanisms for their target protein(s) mediated by adenosine 5'-triphosphate.

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