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Partial Characterization of a New Nucleotide Binding Glycoprotein of Hepatocyte Plasma Membrane

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ABSTRACT. Hepatocyte plasma membranes contain a glycosylated 230-kDa Ca^{2+}-dependent, Mg^{2+}-stimulated ATPase (pgp230), which consists of two subunits, one of 120 kDa and the other of 110 kDa. pgp230 can be enriched by the use of affinity chromatography on Concanavalin A-Sepharose, wheat germ lectin-Sepharose, and 5′-AMP-Sepharose. It has a high-affinity Ca^{2+} binding site. In the presence of Ca^{2+}, it forms a phosphorylated intermediate by autocatalytic transfer of the terminal phosphate residue from ATP. Maximal Ca^{2+}-dependent autophosphorylation is observed at pH 5–6. Photoaffinity labeling using 8-azido-[α-^{32}P]ATP or [γ-^{32}P]ATP confirms the presence of ATP binding sites. Incubation with [α-^{32}P]ATP leads to a rapid but transient labeling of pgp230. Various nucleotides, nucleotide receptor agonists, or antagonists inhibit Ca^{2+}-dependent phosphorylation by [γ-^{32}P]ATP. The concentrations of half-maximal inhibition range from 10^{-7} M to 10^{-3} M. The rank order of inhibitory potency is: ATP > α,β-methylene-ATP > CTP = TTP > γ-4-aminophenyl-ATP = 2-methyl-thio-ATP > UTP = GTP > GDP = ADP = β,γ-methylene-ATP = β,γ-methylene-TTP = β,γ-methylene-GTP = adenosine-5′-O-2-thiodiphosphate = CMP = AMP > adenosine > cytidine > guanosine = suramin > Reactive blue 2 > iso-butyl-methyl-xanthine > thymidine > uridine. These data suggest a nucleotide binding capacity of this new hepatocyte membrane glycoprotein. Further investigations should be carried out to reveal its biological function. BIOCHEM PHARMACOL 51;10:1269–1276, 1996.

KEY WORDS. hepatocyte; purinoceptor; nucleotide binding; membrane glycoprotein; phosphorylation

Extracellular nucleosides or nucleotides regulate many important physiological processes, such as cardiac function, muscle contraction, neurotransmission, and platelet aggregation. In the liver, these substances influence glycogen metabolism, ion homeostasis, prostanoid and glucose efflux, and protein synthesis. The receptors involved in these processes are classified as P_1-purinoceptors with adenosine and P_2-purinoceptors with ATP as the typical agonist. Recently, P_3-purinoceptors activated by adenosine and ATP were classified. Biochemical, pharmacological, and receptor binding studies led to a subdivision of P_2-purinoceptors into P_{2x}-, P_{2y}-, P_{2u}-, P_{2t}-, P_{2z}, and P_{2d}-subtypes [1–5]. Mainly functional studies revealed that hepatocytes are endowed with P_{2y}-purinoceptors [6–15]. However, extracellular UTP exerts metabolic effects on these cells, as well [9, 12]. A 53 kDa protein of rat liver plasma membrane was labeled by photoaffinity radiolabeling with [^{32}P]3′-O-(4-benzoyl-ATP, leading to the conclusion that this protein is a hepatic P_{2y}-purinoceptor [16]. However, in the same study, further membrane proteins with a molecular weight of approximately 110 kDa and 120 kDa were also labeled. This suggests the existence of further nucleotide binding proteins in hepatocytes.

In a recent paper [17], we described a 230 kDa glycoprotein of rat liver plasma membrane that is composed of 120 kDa and 110 kDa subunits. It binds ATP, possesses Ca^{2+}-dependent, Mg^{2+}-stimulated ATPase activity, and forms a phosphorylated intermediate by incorporation of the terminal phosphate residue of ATP. Several characteristics suggest that this glycoprotein is an integral transmembraneous protein with a regulatory high-affinity Ca^{2+} binding site residing inside the cell.

In the present paper, we report on further enrichment of this glycoprotein and on experiments suggesting that it could be a hitherto unknown nucleotide-binding protein of the hepatocyte surface membrane.

MATERIALS AND METHODS

Chemicals and isotopes

Chemicals were of analytical grade and were supplied by E. Merck AG (Darmstadt, Germany), C. Roth OHG (Karlsruhe, Germany), and Serva (Heidelberg, Germany). Molecular weight calibration proteins were from Sigma (St Louis, MO, U.S.A.). [γ-^{32}P]ATP and [α-^{32}P]ATP (specific

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radioactivity of both substances: 3000 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, U.K.). 8-Azido-[α-^{32}P]ATP (specific radioactivity 7.42 Ci/mmol) was obtained from ICN (Costa Mesa, CA).

Protein Determination

Protein was determined according to Lowry *et al.* [18].

SDS-PAGE

Sodium dodecylsulfate polyacrylamide gelelectrophoresis was performed as described using Laemmle's procedure [19]. Usually, 7.5% slab gels were used. The gels were stained with Coomassie brillant blue and destained as described.

Autoradiography

SDS-polyacrylamide slab gels were dried under vacuum and exposed to Kodak X-OMat-AR™ films at −70°C.

Isolation of Rat Liver Plasma Membranes and Isolation of the Phosphoglycoprotein (pgp230)

Plasma membranes of rat liver were isolated and checked for purity, using marker enzymes as described [20]. From these membranes, a glycoprotein fraction was isolated [21]. Briefly, the membranes were solubilized in 10 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4, containing 1.5% (v/v) Triton X-100. Solubilized glycoproteins were bound to Concanavalin A-Sepharose. Unbound proteins were eluted with the same buffer containing 0.1% (v/v) Triton X-100, and bound glycoproteins were eluted with 0.1 M α-methyl-mannoside in the same buffer. The glycoproteins obtained were further separated on a wheat germ lectin-Sepharose column. The bound glycoproteins were eluted with 0.1 M N-acetyl-D-glucosamine in the same buffer. This glycoprotein fraction was dialyzed against H_2O. After lyophilization, the glycoproteins were dissolved in 10 mM Hepes, pH 6.0 and applied onto a 5′-AMP-Sepharose column. Unbound proteins were eluted using the same buffer. Bound glycoproteins were eluted by stepwise adding NaCl to this buffer. The fraction eluted with 500 mM NaCl in 10 Hepes, pH 6.0, contained the bulk of the phosphoprotein. After dialysis against H_2O, the glycoproteins of the latter fraction were lyophilized, then dissolved in buffer for phosphorylation experiments (see below).

Cell Culture

Rat hepatocytes were prepared and cultured as described [22]. Either freshly prepared cells or cells 2 days after seeding were used for the experiments. Hepatocytes were homogenized and a particulate fraction was prepared by centrifugation at 30,000 × *g* for 15 min. This particulate fraction was solubilized with Tris/NaCl/$CaCl_2$/$MgCl_2$ buffer containing Triton X-100 and fractionated using the two lectins as described for isolated plasma membranes.

Western Immunoblotting

Immunoblotting was carried out as described by Towbin *et al.* [23], using commercially available horseradish peroxidase-conjugated antimouse IgG as a second antibody. Immunoreactive proteins were visualized by the peroxidase reaction. The monoclonal antibody MAB 9.2 used in this study binds to gp110, the taurocholate transport protein of hepatocyte plasma membrane [24].

Phosphorylation

Unless otherwise indicated, the standard phosphorylation mixture contained in an end volume of 100 µL was: 40 µL glycoprotein fraction, 40 mM Hepes (pH 7.0), 80 mM KCl, 10 µL additions (ions, chelators), [γ-^{32}P]ATP (final concentration 0.27 µM). As a matter of routine, the reaction was carried out in the presence of 1 mM $CaCl_2$ and 2 mM CDTA. After incubations of between 5 sec and 15 min in the ice bath, the reactions were stopped by the addition of 100 mM Tris-HCl (pH 6.8), containing 4% (w/v) SDS and 5% (v/w) 2-mercaptoethanol (Tris/mercaptoethanol/SDS), and boiling for 5 min.

Binding of Nucleotides to pgp230

PHOTOAFFINITY LABELING USING 8-N_3-[α-^{32}]ATP. We employed exactly the same method as described in our previous paper. 40 µL of the solution containing pgp230 enriched by the three affinity chromatographic steps was incubated with 8-N_3-[α-^{32}P]ATP (end concentration 5.4 µM) for up to 30 min upon UV irradiation at 4°C in the presence of 1 mM $CaCl_2$ and 2 mM CDTA in the above-mentioned Hepes buffer. In competition experiments, we found that unlabeled ATP at a concentration of approximately 5–10 µM led to half-maximal inhibition of labeling. Incubation in the presence of 50 µM ATP completely suppressed incorporation of radioactivity. Radioactive proteins were identified by autoradiography after separation on SDS-PAGE.

DIRECT PHOTOAFFINITY LABELING WITH [γ-^{32}P]ATP. We used the methods described by Müller *et al.* [25] and Bo *et al.* [26]. The reaction mixture contained the standard solution for phosphorylation and 40 µL of the pgp230-containing fraction, as above. After the addition of the [γ-^{32}P]ATP solution (end concentration 0.27 µM), a further incubation was carried out for up to 15 min in the dark at 4°C. Thus, the time-dependent phosphorylation of pgp230 was monitored. In parallel, samples were incubated for the same times but were irradiated for the respective times with 254-nm wavelength UV light at a distance of 10 cm at 4°C. Under this condition, labeling of proteins corresponds to phosphorylation of pgp230 and also radiation-induced binding of [γ-^{32}P]ATP to this glycoprotein. For the

control, 10 μM unlabeled ATP was added prior to [γ-^{32}P]ATP. This suppressed labeling completely. Labeled proteins were identified by autoradiography after separation by SDS-PAGE. The autoradiographs were scanned, and the combined peak areas corresponding to pgp230 and its subunits measured. Peak areas were indicated as cm^2 (under standard scanning conditions).

BINDING OF [α-^{32}P]ATP TO PGP320. 40 μL of enriched pgp230 was incubated in the presence of 1 mM $CaCl_2$ and 2 mM CDTA with [α-^{32}P]ATP (end concentration 0.27 μM) at 4°C for times up to 5 min. The reaction was stopped by the addition of SDS and mercaptoethanol and by boiling. Labeled proteins were visualized by autoradiography after separation by SDS-PAGE. The peak areas (under standard scanning conditions) corresponding to pgp230 and its subunits were plotted against time. The addition of 10 μM ATP suppressed radioactive labeling completely.

RESULTS

To investigate whether or not the 230-kDa glycoprotein (pgp230) is a genuine hepatocyte glycoprotein, phosphorylation studies were performed using freshly prepared and cultured hepatocytes. A glycoprotein fraction obtained from a particulate fraction of these cells by serial affinity chromatography on Concanavalin A-Sepharose and wheat germ lectin-Sepharose was incubated with [γ-^{32}P]ATP in the presence of Ca^{2+} and CDTA. As shown in Fig. 1, lanes 1 and 2, the subunits of pgp230 (pgp120 and pgp110) could be detected in the glycoprotein fraction of hepatocytes. This shows that pgp230 is a glycoprotein of the hepatocyte and that it is expressed in freshly prepared cells, as well in cells 2 days after seeding.

Figure 2 shows the pH dependence of phosphoprotein formation in the glycoprotein fraction (from rat liver plasma membrane). At pH 5.0 or 6.0, radioactive labeling of pgp120 and pgp110 (the subunits of pgp230) was maximal. At pH 7.0, all the incorporated radioactivity was found in the 120 kDa and 110 kDa subunits. At pH 5.0 and 4.0, considerable radioactivity was incorporated into a 130 kDa phosphoglycoprotein and a 100 kDa phosphoglycoprotein. These phosphoproteins could not be detected under the standard conditions normally used in our studies on phosphorylation of plasma membrane glycoproteins.

Recently we showed that in addition to pgp120 and pgp110, the subunits from pgp230, two further proteins were found in the molecular weight range of 100 kDa to 120 kDa [17]: these were gp110, the taurocholate transport protein, and the ectoenzyme dipeptidyl peptidase IV (DPP IV), the main constituent of the glycoprotein fraction, both comigrating on SDS-PAGE with pgp110. As shown in Fig. 2 (at pH 4), at least one additional phosphoprotein was detectable in this glycoprotein fraction. Further purification of the 230 kDa glycoprotein was achieved by fractionation of the glycoprotein fraction on 5′-AMP-Sepharose. As shown in Fig. 3, using 10 mM Hepes buffer, pH 6.0

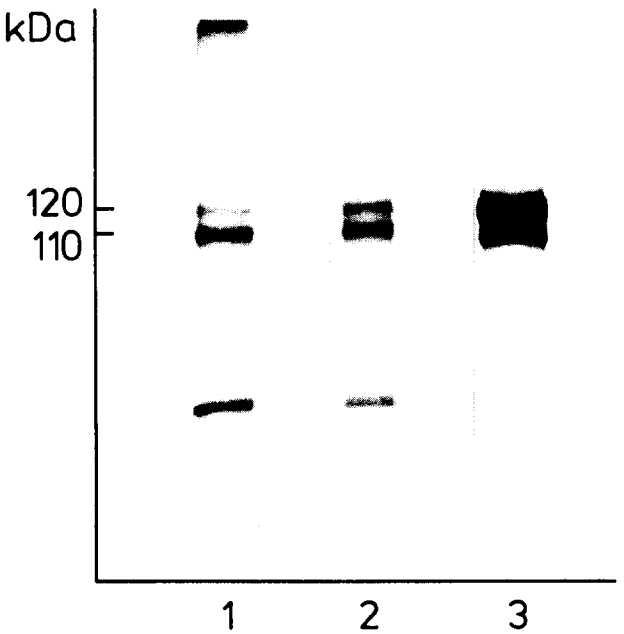


FIG. 1. Detection of pgp230 in freshly prepared and in cultured hepatocytes. Rat liver plasma membranes or a particulate fraction from freshly prepared or cultured hepatocytes were solubilized and a glycoprotein fraction was prepared as described. The glycoproteins were phosphorylated with [γ-^{32}P]-ATP in the presence of Ca^{2+} and CDTA. The proteins were separated on 7.5% SDS-PAGE. Labeled glycoproteins were visualized by autoradiography. Lane 1: Glycoprotein fraction from a particulate fraction of freshly prepared rat hepatocytes; Lane 2: Glycoprotein fraction from a particulate fraction of cultured rat hepatocytes 2 days after seeding; Lane 3: Glycoprotein fraction from rat liver plasma membranes.

pgp230 was bound to the affinity matrix but the taurocholate binding protein and dipeptidyl peptidase IV were not. Neither was the greatest part of the 130 kDa phosphoglycoprotein bound to the gel (lane 1). Adding increasing concentrations of NaCl to the elution buffer, the 130 kDa phosphoglycoprotein was eluted (lanes 2–4). At a concentration of 500 mM NaCl in 10 mM Hepes pH 6.0, enriched pgp230 could be obtained (lane 5). This glycoprotein fraction, which evidently contained only one phosphoprotein, namely pgp230, was used for further studies.

ATP binding of pgp230 was investigated by 3 different approaches:

1. Photoaffinity labeling using N_3-[α-^{32}P]ATP. As shown in Fig. 4a, lane 1, this affinity label for ATP binding proteins labeled only the 230 kDa glycoprotein and the 120 kDa and the 110 kDa subunits. In competition experiments, we found that ATP added in concentrations of approximately 5–10 μM led to half-maximal suppression of radioactive labeling. If 50 μM ATP was added, the radioactive labeling was virtually completely abolished.
2. Direct photoaffinity labeling using [γ-^{32}P]ATP. Fig. 4b shows a time-dependent increase of radioactive labeling of pgp230 upon incubation with [γ-^{32}P]ATP. This may

FIG. 2. pH Dependence of phosphoprotein formation. The glycoprotein fraction bound to Concanavalin A-Sepharose and to wheat germ lectin-Sepharose was incubated in Hepes buffer at varying pH (adjusted with HCl or NaOH) with [γ-^{32}P]ATP for 1 min at 4°C. After SDS-PAGE (7.5% polyacrylamide), phosphorylated proteins were visualized by autoradiography.

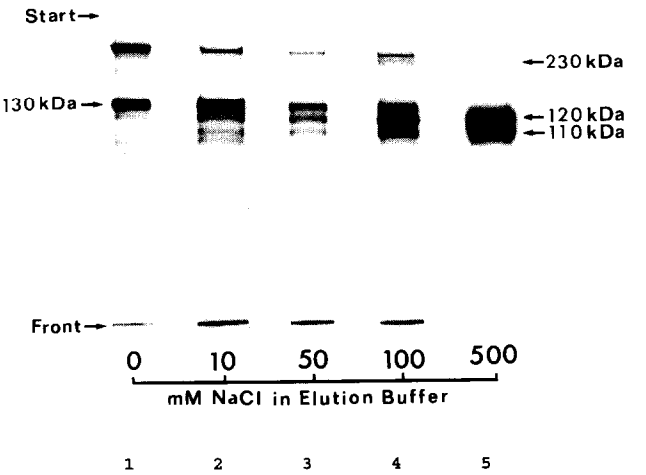


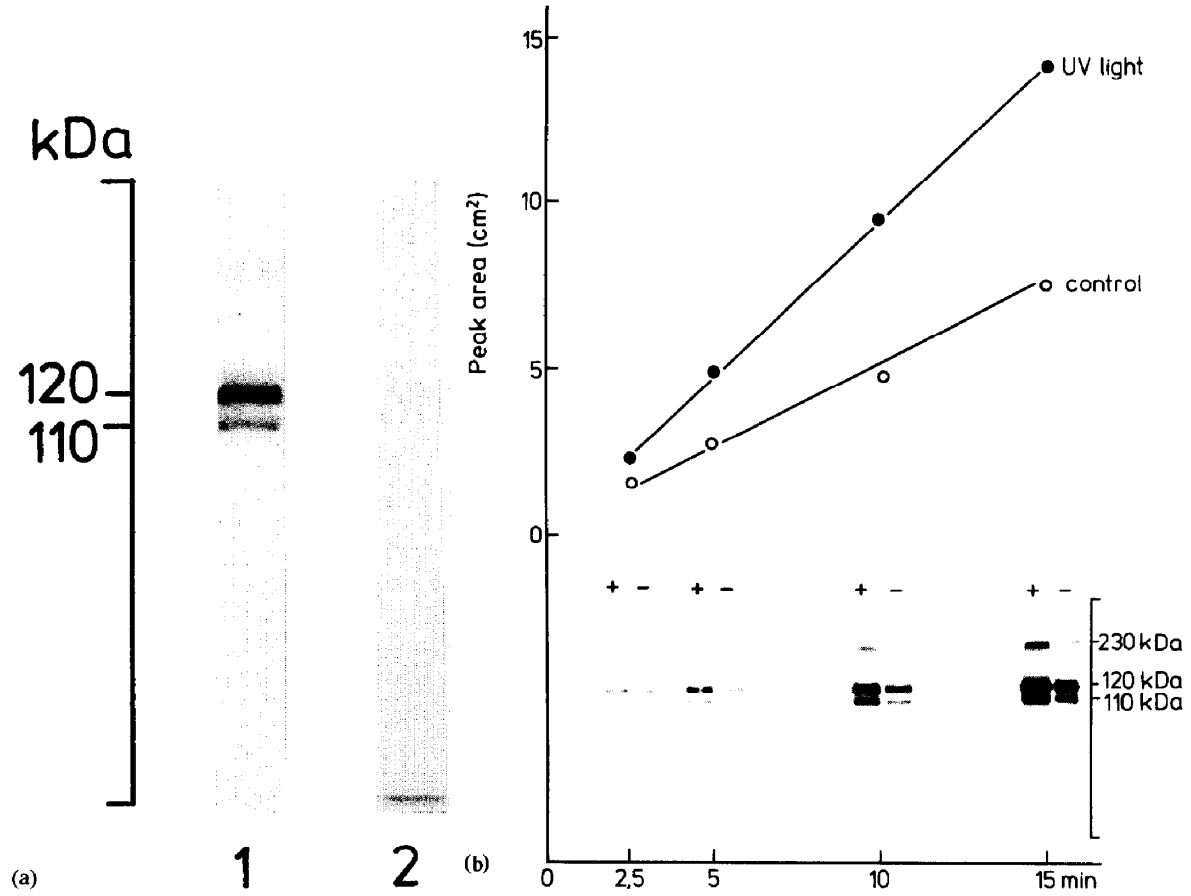
FIG. 3. Enrichment of the 230 kDa glycoprotein by affinity chromatography on 5′-AMP-Sepharose. The glycoprotein fraction obtained by sequential affinity chromatography on Concanavalin-A-Sepharose and wheat germ lectin-Sepharose was further fractionated on a 5′-AMP-Sepharose column as described in Methods. Aliquots of the fractions obtained by elution with increasing NaCl concentrations were dialyzed against H_2O, dissolved in phosphorylation buffer (see Methods) and incubated with [γ-^{32}P]ATP in the presence of Ca^{2+} and CDTA for 5 min at 4°C. After separation of 7.5% SDS-PAGE phosphorylated glycoproteins were visualized by autoradiography. Lane 1: Phosphorylated glycoproteins not bound to 5′-AMP-Sepharose; Lane 2: Phosphorylated glycoproteins bound to 5′-AMP-Sepharose and eluted with 10 mM Hepes buffer, pH 6.0, containing 10 mM NaCl; Lane 3: As Lane 2, containing 50 mM NaCl; or Lane 4: containing 100 mM NaCl; or Lane 5: containing 500 mM NaCl. The taurocholate transport protein gp110 can be detected by immunoblotting using MAB 9.2 only in the unbound fraction (not shown in this figure). Less than 5% of total dipeptidyl peptidase IV activity is bound to the gel and less than 1% of the activity is measured in the fraction eluted with 500 mM NaCl.

be interpreted as time-dependent autophosphorylation of this glycoprotein. If the glycoprotein was irradiated with UV-light during the incubation time, the radioactivity in pgp230 detected by autoradiography increased further. Under these conditions, radioactive labeling of pgp230 may, in part, result from phosphorylation and partly from direct photoaffinity labeling induced by UV light. Addition of 10 μM unlabeled ATP prevented radioactive labeling of the proteins completely (curve not shown in the figure).

3. Binding experiments using [α-^{32}P]ATP. Using this precursor, a marked labeling of pgp230 was achieved at a maximum at approximately 30 sec, followed by a rapid decline (Fig. 4c). Addition of 10 μM unlabeled ATP prevented incorporation of radioactivity. Because, in this type of experiment, the radioactivity in the protein was resistant to boiling in the presence of mercaptoethanol and SDS, a firm binding of pgp230 and the nucleotide must be assumed. It can be speculated that ATP formed a stable binding to the glycoprotein, whereas ADP or AMP arising from the action of the protein's inherent nucleotidase activity dissociated from the protein.

To study the influence of purine or pyrimidine nucleotides and known agonists or antagonists of purinoceptor

FIG. 4. ATP binding of pgp230 (a) Photoaffinity labeling of pgp230 with 8-azido-[α-^{32}P]ATP. The glycoproteins bound to 5′-AMP-Sepharose and eluted with 500 mM NaCl in 10 mM Hepes pH 6.0 were incubated with 8-azido-[α-^{32}P]ATP and irradiated with UV light for 30 min as described in Methods. Labeled proteins were visualized by autoradiography after separation on 7.5% SDS-PAGE. Lane 1: Glycoproteins + 8-azido-[α-^{32}P]ATP; Lane 2: As in Lane 1, +50 μM ATP. (b) Direct photoaffinity labeling of the glycoproteins using [γ-^{32}P]ATP. The glycoproteins were incubated with [γ-^{32}P]ATP without or with UV-irradiation for the times indicated. Details are given in Methods. Radioactivity incorporated into pgp230 was quantified by scanning autoradiographs after separation of the proteins by SDS-PAGE. The combined peak areas (expressed as cm^2) corresponding to pgp230 and its subunits are plotted against time. (Upper part) peak area corresponding to pgp230 and its subunits; Upper line, with UV light; Lower line, without UV light. Lower part of the figure: the respective autoradiographs. –, without UV light; +, with UV light. (c) Binding of [α-^{32}P]ATP to pgp230. The glycoproteins were incubated with [α-^{32}P]ATP in the presence of Ca^{2+} and CDTA as described in Methods for the times indicated at 4°C. After having stopped the reaction by addition of SDS and mercaptoethanol and boiling, the proteins were separated by SDS-PAGE. After autoradiography, the radioactivity corresponding to pgp230 and its subunits was quantified by scanning the gels. The combined peak areas (cm^2) were plotted against incubation time. Upper curve, time-dependent radioactive labeling of pgp230 by [α-^{32}P]ATP; Lower curve, same as upper, but experiment performed in the presence of 10 μM ATP. Radioactive labeling is completely suppressed. The insets show some of the autoradiographs.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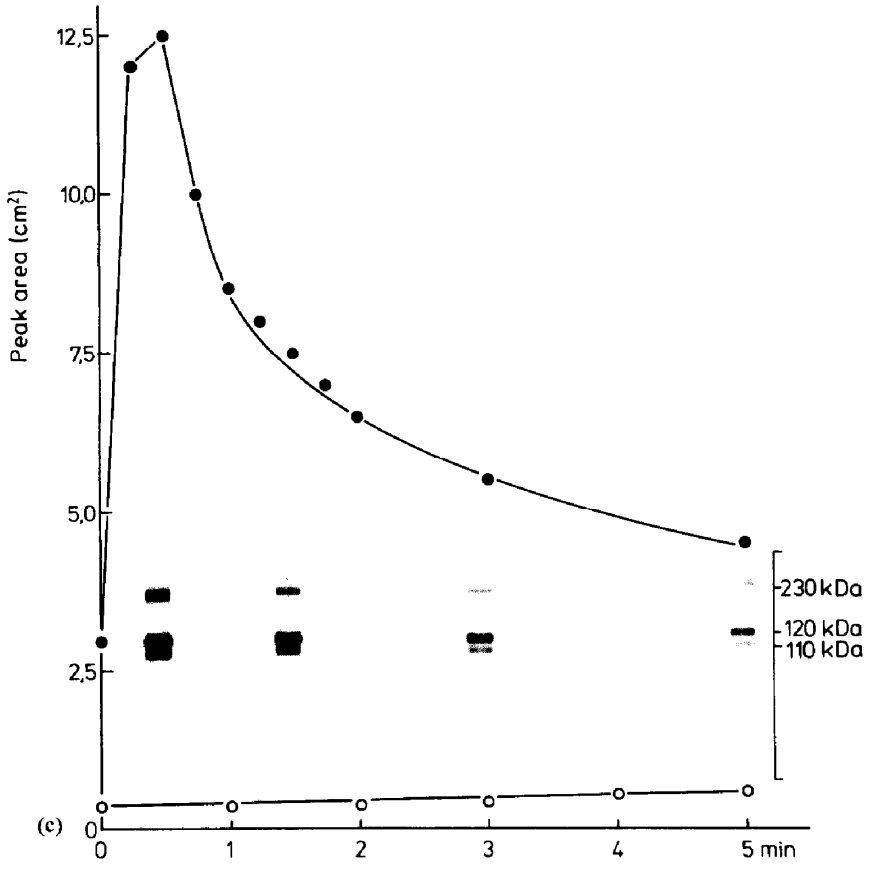
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function on the autophosphorylation of pgp230, the incorporation of radioactivity from [γ-^{32}P]ATP into pgp230 (in the presence of Ca^{2+} and CDTA) was measured after addition of increasing concentrations of the respective substances. Table 1 summarizes the concentrations causing half-maximal inhibition of phosphorylation of pgp230. As for ATP, this concentration amounted to 2×10^{-7} M. This might be explained by simple competition of labeled and unlabeled ATP. α,β-Methylene ATP, a P_{2x}-purinoceptor agonist, and 2-methyl-thio-ATP, a P_{2y}-purinoceptor agonist, inhibited autophosphorylation of pgp230 at micromolar or submicromolar concentrations. Even the pyrimidine triphosphates UTP and TTP interfered with pgp230 phosphorylation at micromolar or submicromolar concentrations. For GDP or ADP, the half-maximal inhibition concentration amounted to approximately one micromolar and for AMP or CMP to approximately 10 micromolar. Isobutyl-methyl-xanthin, an inhibitor of P_1-purinoceptor function, caused half-maximal inhibition only at concentrations above 5×10^{-3} M. Suramin and Reactive Blue 2, known to exhibit (among other functions) inhibition of P_2-purinoceptor activity, inhibited phosphorylation at approximately 4×10^{-4} M and 1×10^{-3} M, respectively (i.e. at concentrations used in pharmacological studies of purinoceptor function).

DISCUSSION

In a previous paper, we demonstrated that rat liver plasma membranes contain a hitherto undescribed 230 kDa glycoprotein (pgp230) composed of two subunits with relative molecular weights of 120 kDa and 110 kDa (pgp120 and pgp110), respectively [17]. pgp230 is a transmembranous protein with hybrid-type carbohydrate chains. It possesses a high-affinity Ca^{2+} binding site and autophosphorylation capacity; it binds ATP and has Ca^{2+}-dependent, Mg^{2+}-stimulated ATPase activity. In these studies, pgp230 was enriched from plasma membranes, which contain various contaminating intracellular hepatocyte membranes and membranes of cells different from hepatocytes. In the present paper, we demonstrate that pgp230 is a membrane protein of the hepatocyte and not a contaminant originating from other cells of the liver.

TABLE 1. Concentrations of various nucleotides, purinoceptor agonists, or antagonists causing half-maximal inhibition of pgp230 phosphorylation (μM)

ATP	0.2
α,β-Methylene-ATP	0.3
γ-4-Aminophenyl-ATP	3
2-Methyl-thio-ATP	3
β,γ-Methylene-ATP	10
Adenosine-5′-O-2-thiodiphosphate	10
ADP	10
AMP	10
Adenosine	100
TTP	0.5
β,γ-Methylene-TTP	10
Thymidine	10,000
CTP	0.5
CMP	10
Cytidine	200
UTP	5
Uridine	>10,000
GTP	5
β,γ-Methylene-GTP	10
GDP	10
Guanosine	400
Iso-butyl-methyl-xanthine	>5000
Suramin	400
Reactive blue 2	1000

By adding affinity chromatography on 5′AMP-Sepharose to our initial method, pgp230 can be further enriched. This method separates it from a 130 kDa phosphoglycoprotein, from the taurocholate carrier protein, and from dipeptidylpeptidase IV (the latter two proteins comigrate with pgp110 on SDS-PAGE). However, we could not obtain pgp230 as a homogenous band on SDS-PAGE or in sufficient quantities to obtain peptide sequence analyses for the identification of the nature of this glycoprotein. In preliminary attempts, after proteolytic digestion of the final glycoprotein fraction, we received sequences corresponding to the glucose transporting protein glut 2, dipeptidyl peptidase IV, a dicarboxylate carrier protein, and the Na^+/cholate cotransporter (details not described in this paper). Previously, we had excluded the identity of pgp230 with several hepatocyte membrane proteins. Another recently characterized ATPase of the hepatocyte cell surface, the multispecific organic anion transporter (MOAT), is a phosphorylatable glycoprotein and should be considered as a possible candidate for identity. In contrast to the protein described here, its phosphorylation is not inhibited by UTP even at high concentrations, and the molecular weight of the protein amounts to 90 kDa [27–29].

The present paper focuses on the nucleotide binding and inhibition of Ca^{2+}-dependent phosphorylation of pgp230. By photoaffinity labeling using 8-azido-[α-^{32}P]ATP, we had demonstrated binding sites for ATP [17]. Using this method, pgp230 and its two subunits were the only labeled proteins in the fraction obtained after affinity chromatography on 5′-AMP-Sepharose, largely excluding the presence of additional ATP-binding proteins. This suggests that the transfer of the terminal phosphate residue of ATP to this protein represents an autophosphorylation and not the action of a contaminating protein kinase. Further evidence for nucleotide binding to pgp230 came from direct photoaffinity labeling using [γ-^{32}P]ATP. A striking result was the rapid radioactive labeling of pgp230 by [α-^{32}P]ATP, which was resistant to boiling in the presence of mercaptoethanol and SDS, followed by the disappearance of the radioactivity. It may be assumed that ATP is firmly bound as a ligand followed by a dissociation from the protein after the action of the glycoprotein's inherent ATPase activity, which results in the formation of ADP.

Various nucleotides inhibit the phosphorylation of pgp230. The rank order of inhibitory potency is: ATP >

α,β-Methylene-ATP > CTP = TTP > y-4-aminophenyl-ATP = 2-methyl-thio-ATP > UTP = GTP > GDP = ADP = β,y-methylene-ATP = β,y-methylene-TTP = β,y-methylene-GTP = adenosine-5′-O-2-thiodiphosphate = CMP = AMP > adenosine. Suramin and Reactive Blue 2, known as P_2-purinoceptor antagonists, inhibit phosphorylation of the glycoprotein at concentrations currently used in pharmacological experiments. Iso-butyl-methyl-xanthine, an inhibitor of P_1-purinoceptors, inhibits phosphorylation at > 5 mM. This rank order of inhibition does not fit to a known purinoceptor subtype. However, the typing of purinoceptors is based on the influence of various nucleotides on a biological action (e.g. muscle contraction, Ca^{2+}-release, etc.) that is mediated by a specific receptor. No such biological or pharmacological function had been found for pgp230 until now and a phosphorylation has not been described for purinoceptors.

Concerning cells of the liver, the existence of purinoceptors was demonstrated for hepatocytes [6–15], Ito cells [30], and biliary epithelial cells [31] by functional studies. Purinoceptors of several cell types have been isolated, cloned, and expressed in different cells. Recently, a P_{2u}-receptor, which is activated by ATP and UTP, has been cloned and shown to be a 53-kDa protein [32, 33]. A [^{3}H]α,β-methylene-ATP binding protein of rat vas deferens, regarded as a P_{2x} purinoceptor, has a M_r of 62 kDa [26]. A P_{2y}-purinoceptor has been cloned from a chicken whole-brain cDNA library [34]. Rat and mouse P_{2y} purinoceptors were cloned by a different group [35] and were shown to have 97% amino acid identity; the rat P_{2y} purinoceptor sequence had 85.7% and 37.8% identity with chicken P_{2y} and rat P_{2u} purinoceptors, respectively. [^{32}P]3′-O-(4-benzoyl)benzoyl ATP was used as a radioactive photolabel to identify the presumed P_{2y} purinoceptor in turkey erythrocyte membranes [13]. This method, when applied to several other tissues (astrocytes, rat hepatocytes, rat brain, human astrocytoma cells, bovine pulmonary aortic endothelial cells), identified a 53 kDa protein as the supposed P_{2y} receptor. However, exclusively in hepatocytes, additional polypeptides were labeled using this procedure with an M_r between 100 kDa and 130 kDa, strongly resembling the phosphoprotein pattern obtained in our experiments.

It would be speculative to derive a purinoceptor function for pgp230 from our results. A phosphorylation has not been found for purinoceptors until now and the inhibition experiments using the various nucleotides can be interpreted as inhibition of ATP binding to a ATPase, which has autophosphorylating capacity. Recently, in aortic endothelial cells, another ATPase was described that also acts as an ATP receptor mediating NO release [36]. The data presented here broaden the spectrum of nucleotide binding membrane proteins found in the liver. In pharmacological studies on liver function, pgp230 has to be considered as a new candidate of a protein-mediating nucleotide action. The data presented here should stimulate further experimentation on the nature of this interesting new hepatocyte surface glycoprotein.

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