Phosphorylation of the human asialoglycoprotein receptor

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The human asialoglycoprotein receptor was isolated via immune precipitation from hepatoma Hep G2 cells following incubation with [^{32}P]P_i. Analysis on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed incorporation of ^{32}P into both the 46000 Da mature form of the receptor as well as the 40000 Da precursor. The incorporated ^{32}P was associated with phosphoserine. The degree of ^{32}P incorporation was not substantially altered in cells endocytosing asialoglycoprotein ligand at maximal rates nor in cells in which receptor recycling was abolished by incubation with primaquine. That endocytosis and phosphorylation can be dissociated is supported by the observation that ^{32}P is incorporated from [γ - ^{32}P]ATP into the asialoglycoprotein receptor in isolated plasma membranes of Hep G2 cells.

Once the terminal sialic acid is removed, circulating plasma glycoproteins are rapidly and specifically cleared from the circulation by a receptor-mediated process (Ashwell & Harford, 1982; Schwartz, 1984). This receptor, the asialoglycoprotein receptor (ASGP-R), localized in the hepatic parenchymal cell, is specific for galactose-terminal carbohydrates of glycoproteins. We have identified a human hepatocyte-derived cell line, Hep G2, which expresses abundant ASGP-R (Schwartz *et al.*, 1981). Using this cell line as a model, we have defined the kinetics of receptor-mediated endocytosis of asialoglycoprotein ligands and ASGP-R recycling (Schwartz *et al.*, 1982). Our studies indicate that a single ASGP-R can recycle from the cell surface into endosomal sorting compartments (i.e. compartment of uncoupling receptor and ligand; CURL) within the cell and back to the cell surface within 8 min (Schwartz *et al.*, 1982; Ciechanover *et al.*, 1983; Geuze *et al.*, 1983). ASGP-R molecules constitutively (i.e. without ligand) recycle from the cell surface, although this rate is considerably slower than that found in the presence of ligand (Schwartz *et al.*, 1984).

We have recently examined the biosynthesis and intracellular processing of the human ASGP-R in Hep G2 cells (Schwartz & Rup, 1983). The receptor is initially synthesized as a 40000 Da

precursor which is converted to the mature 46000 Da species with a $t_{1/2}$ of approx. 45 min. This conversion is coincident with the loss of sensitivity to endo- β -*N*-acetylglucosaminidase H, a marker of Golgi oligosaccharide processing. From the Golgi the newly synthesized ASGP-R rapidly reaches the cell surface where only the mature 46000 Da species is present.

Thus, the ASGP-R provides a well-characterized system for the examination of the mechanisms involved in receptor-mediated endocytosis. This process involves the specific binding of macromolecular ligands (e.g. virus, toxin, transferrin, low-density lipoprotein, asialoglycoprotein) to specialized cell-surface receptors and their internalization via a coated pit/coated vesicle pathway (Anderson *et al.*, 1977; Besterman & Low, 1983). Many ligands, including the asialoglycoproteins, dissociate from their receptors in a pre-lysosomal compartment and allow the receptor to recycle back to the cell surface (Brown *et al.*, 1983; Harford *et al.*, 1983).

Recent studies have shown that epidermal growth factor, platelet-derived growth factor, and insulin, all of which enter cells via receptor-mediated endocytosis, induce the rapid phosphorylation of certain membrane proteins, as well as of their respective receptors (Ushiro & Cohen, 1980; Kasuga *et al.*, 1982a; Shia & Pilch, 1983; Ek *et al.*, 1982). In order to examine the generality of this process to receptor-mediated endocytosis, as well as to examine this potential post-translational modification of the ASGP-R, the phosphorylation

Abbreviations used: ASGP-R, asialoglycoprotein receptor; SDS, sodium dodecyl sulphate; Ig, immunoglobulin.

of the human ASGP-R was evaluated both in intact Hep G2 cells as well as in a broken-cell preparation.

Experimental

Cells

The Hep G2 cell line (clone a16) was grown in monolayer culture as described earlier (Schwartz *et al.*, 1981; Schwartz & Rup, 1983).

Plasma membranes

Purified plasma membranes were prepared from 20 15-cm diameter (Lux) tissue culture dishes of 90% confluent Hep G2 cells according to Ray (1970). Briefly, cells were rinsed in Dulbecco's phosphate-buffered saline at 4°C, scraped from the dishes and homogenized in 1mM-NaHCO₃/0.5mM-CaCl₂ (pH 7.5) with a Dounce homogenizer. The post-nuclear supernatant was prepared and purified plasma membranes were isolated by equilibrium density centrifugation in sucrose. The purified membranes at the 35–41% (w/w) interface were washed with the 10mM-Tris (pH 7.4)/150mM-NaCl, pelleted free from the sucrose and stored at –80°C until use.

Anti-(human ASGP-R) antibodies

Affinity-purified monospecific polyclonal rabbit antibodies to the isolated human ASGP-R were prepared and characterized as described previously (Schwartz & Rup, 1983).

Radioactive labelling of Hep G2 cells

For labelling of the ASGP-R, cells (in general, 1×10^6) were rinsed in phosphate-free Eagle's minimum essential medium without foetal bovine serum and preincubated for 15 min at 37°C in phosphate-free minimum essential medium. Thereafter, [³²P]P_i (carrier-free; Amersham International) was added at 1mCi/ml and the incubation continued at 37°C. After the appropriate time, the cells were washed twice at 4°C with phosphate-buffered saline and thereafter solubilized in phosphate-buffered saline containing 1% Triton-X-100 and 1mM-phenylmethanesulphonyl fluoride. The solution was clarified by centrifugation and prepared for immunoprecipitation with staphylococcal protein A as described previously (Schwartz & Rup, 1983). The immune precipitates were isolated following centrifugation and washing at 4°C and the antigen and antibody were released from the protein A by boiling in SDS/polyacrylamide gel electrophoresis sample buffer (Laemmli, 1970). The supernatant containing radioactive antigen was separated on a SDS/10% polyacrylamide-gel electrophoresis, dried, and exposed to pre-fogged Kodak SB-5 film

with an enhancing screen (Lightning Plus, DuPont) at –80°C. ¹⁴C-labelled *M_r* markers (Amersham International) were included on all gels. These markers denote molecular masses of 200, 94, 68, 46 and 30kDa.

Phosphorylation of purified Hep G2 plasma membranes

Purified plasma membranes (50μg of protein) were preincubated for 60 min at 4°C in 100mM-NaCl/50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid] (pH 7.6)/10mM-CaCl₂ in the absence or presence of 4mM-MgCl₂ or MnCl₂ without or with 100μg of galactose-cytochrome *c*/ml. [³²P]ATP (50μCi; sp. radioactivity 5000Ci/mmol; Amersham International) was added to a final concn. of 3μM and the incubation was continued for 20 min at 4°C. Thereafter the reaction was stopped by addition of 1/7 volume of 200mM-NaF/20mM-EDTA/1.4% Triton X-100/40mM-Na₂HPO₄/40mM-Na₄P₂O₇/40mM-ATP/40mM-Hepes (pH 7.6). After 20 min at 4°C, samples were clarified by centrifugation. Aliquots of the supernatant were either prepared for SDS/polyacrylamide-gel electrophoresis directly or were immunoprecipitated with anti-ASGP-R antibody prior to analysis on SDS/polyacrylamide-gel electrophoresis as described above.

Identification of phospho-amino acids

The immunoprecipitated ASGP-R was hydrolysed for 2 h at 110°C in 6M-HCl. In addition, following localization by autoradiography, the 46000 Da band was excised and electroeluted from the gel prior to hydrolysis. The hydrolysate was freeze-dried and applied to a 250μm cellulose t.l.c. plate. Electrophoresis conditions were exactly as described by Hunter & Sefton (1980). Standards of phosphoserine, phosphothreonine and phosphotyrosine were co-chromatographed with the radioactive sample and visualized by staining with ninhydrin. The t.l.c. plate was then exposed to X-ray film as described above.

Results

Incubation of Hep G2 cells at 37°C with [³²P]P_i for 6 h followed by solubilization in non-ionic detergent and immunoprecipitation revealed ³²P incorporation into the human ASGP-R (Fig. 1a). Both the mature 46000Da form of the receptor as well as the 40000Da biosynthetic precursor species were labelled. Under these conditions, there was approx. 10–20-fold more radioactivity in the 46000Da species. Characterization of the ³²P-containing amino acids following immunoprecipitation, hydrolysis and two-dimensional

electrophoresis demonstrated that essentially all radioactivity comigrated with phosphoserine (Fig. 1b). The minor spot between the phosphotyrosine and the origin is most probably incompletely digested phosphoprotein.

In order to determine whether the degree of phosphorylation of the human ASGP-R was correlated with the extent of receptor-mediated endocytosis, Hep G2 cells were exposed to 200 μ g of asialofetuin/ml prior to and throughout the 6 h [32 P]P_i labelling period. These conditions ensure a maximal rate of receptor-mediated uptake of asialoglycoproteins in Hep G2 cells (Schwartz *et al.*, 1982). As seen in Fig. 2, there was no significant alteration in the phosphorylation of either the 46000 Da species or the 40000 Da precursor species under these conditions (Fig. 2, lane c).

Primaquine, a weak base, completely abolishes ASGP-R recycling and receptor-mediated uptake of ligand in Hep G2 cells (Schwartz *et al.*, 1984). Incubation of Hep G2 cells with 0.2 mM-primaquine during both preincubation and the 6 h incubation with [32 P]P_i reduced both the total incorporation of 32 P into the cellular proteins as well as into the ASGP-R. Nonetheless, labelling was still

clearly apparent in the 46000 Da mature receptor species (Fig. 2, lane e). No labelling was identified in the 40000 Da precursor. This may have resulted simply from the reduced 32 P incorporation alone or from a diminished rate of protein synthesis in these cells during the 6 h incubation with primaquine. Similarly, in the presence of 100 μ g of cycloheximide/ml, a concentration that completely abolishes protein synthesis in these cells (Schwartz *et al.*, 1982), only the 46000 Da mature receptor species was phosphorylated (Fig. 2, lane d).

The incorporation of 32 P from [γ - 32 P]ATP into the human ASGP-R in isolated plasma membranes of Hep G2 cells was next examined. As seen in Fig. 3, phosphorylation *in vitro* of membrane proteins was supported by the presence of either Mg²⁺ or Mn²⁺. Mn²⁺ appeared more effective. The presence of Ca²⁺ as the sole divalent cation did not support phosphorylation. Analysis of immunoprecipitates of the human ASGP-R from the plasma membranes phosphorylated *in vitro* revealed substantial incorporation of 32 P into the receptor in the presence of Mn²⁺. Little incorporation was seen in the presence of Mg²⁺, and no incorporation was found in the presence of Ca²⁺ alone (Fig. 3, lanes f-j). In order to determine the

Fig. 1. Incorporation of 32 P into ASGP-R in human hepatoma Hep G2 cells

(a) Approx. 5×10^6 Hep G2 cells were washed and incubated for 6 h in P_i-free minimum essential medium containing 10 mCi of [32 P]P_i. Following washing, the cells were solubilized and immune precipitated with either control IgG or anti-ASGP-R. The immune precipitates were analysed via SDS/polyacrylamide-gel electrophoresis and fluorography as described in the text. Large arrow, 46000 Da mature ASGP-R; small arrow, 40000 Da precursor ASGP-R. (b) Two-dimensional thin-layer electrophoresis of phosphoaminoacids was performed on the immunoprecipitated phosphorylated ASGP-R following hydrolysis for 2 h at 110°C in 6M-HCl as described in the text. The dotted lines represent the positions of the ninhydrin-stained standards superimposed on the autoradiograph.

Fig. 2. Effect of ligand, cycloheximide or primaquine on ^{32}P incorporation into ASGP-R in Hep G2 cells. 10^6 Hep G2 cells were washed and incubated for 6 h in P_i -free minimum essential medium containing 1 mCi of $[\text{}^{32}\text{P}]\text{P}_i$. Thereafter cells were solubilized, immunoprecipitated with either control IgG (a) or anti-ASGP-R (b-e) and analysed via SDS/polyacrylamide-gel electrophoresis and fluorography as described in the text. The cells in sample (c) were incubated in the presence of 200 μg of asialofetuin ligand/ml during the 6 h incubation. The cells in sample (d) were incubated with 100 μg of cycloheximide/ml both 30 min prior to and during the 6 h incubation. The cells in sample (e) were incubated with 200 μM primaquine both 30 min prior to and during the 6 h incubation. Large arrow, 46000Da mature ASGP-R; small arrow, 40000Da precursor ASGP-R.

effect of ligand occupation on ^{32}P incorporation into ASGP-R in isolated plasma membranes, membranes were incubated with a saturating concentration of a high-affinity (K_d 10^{-8}M) synthetic ligand for the ASGP-R, galactose-cytochrome c (Simmons & Schwartz, 1984). This neoglycoprotein ligand was selected because of its size (13000Da), so as to not obscure the 40000–55000Da area on gel-electrophoresis, an area in which asialofetuin and asialo-orosomucoid migrate. As seen in Fig. 3 (lane e) there is no major alteration in the pattern of phosphorylation of whole plasma membranes in the presence of saturating concentration of ligand. In addition, the incorporation of ^{32}P into the ASGP-R was not substantially altered by the presence or absence of saturating ligand (Fig. 3, lane j).

Discussion

The human ASGP-R in Hep G2 cells is a phosphoprotein. The mature 46000Da receptor

species is phosphorylated upon incubation of Hep G2 cells with $[\text{}^{32}\text{P}]\text{P}_i$. In addition, the major intracellular precursor form (40000Da) is also phosphorylated. However, the phosphorylation of the receptor does not require protein synthesis. Thus, at least a portion of the phosphorylation occurs prior to the late Golgi. This is consistent with the recent observation of Rees-Jones *et al.* (1983), who demonstrated phosphorylation *in vitro* of the 190000Da insulin receptor precursor. This precursor species was isolated from the mature insulin receptor by lectin-chromatography of total rough and smooth microsomes.

The extent of phosphorylation is not markedly altered in Hep G2 cells endocytosing asialoglycoprotein ligand at maximal rates. This is of interest in that the lifetime of the human ASGP-R in Hep G2 cells is also not appreciably altered at maximal rates of ligand endocytosis (Schwartz & Rup, 1983). However, since this receptor recycles constitutively, i.e. in the absence of added ligand, and since the rate of constitutive receptor recycling may be approx. 30–50% of the rate seen in the

Fig. 3. Incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into ASGP-R in Hep G2 plasma membranes

Purified plasma membranes from Hep G2 cells were preincubated at 4°C in 100mM-NaCl/50mM-Hepes (pH 7.6)/10mM- CaCl_2 in the absence or presence of 4mM- MgCl_2 or MnCl_2 without or with 100 μg of galactose-cytochrome *c*/ml. After the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at 3 μM the incubation was continued for 20 min at 4°C . The reaction was terminated as described in the text and the membranes were solubilized. A portion of the solubilized membranes was directly analysed via SDS/polyacrylamide-gel electrophoresis (a–e) and a second portion (100-fold greater) was immunoprecipitated with anti-ASGP-R (f–j) as described in the text.

presence of ligand (Schwartz *et al.*, 1984), the relationship of receptor phosphorylation and the dynamics of the receptor endocytosis and recycling remain unclear.

The human ASGP-R is phosphorylated on a serine residue. This finding is similar to the phosphorylation of serine in the *N*-acetylglucosamine-specific glycoprotein receptor of chicken liver, as demonstrated by Drickamer & Mahon (1982). The phosphorylation of other receptors involved in receptor-mediated endocytosis has been recently documented as well. The receptors for the growth factors, epidermal growth factor, platelet-derived growth factor and insulin are all autophosphorylated at tyrosine residues in the presence of their respective ligands. In addition, in the absence of ligand, phosphorylation of the insulin (Kasuga *et al.*, 1982b) and epidermal growth factor (Carlin *et al.*, 1983) receptors occurs at serine residues as well. In a broken-cell system, phosphorylation of the insulin receptor at phos-

phoserine residue(s) was demonstrated upon incubation of liver plasma membranes and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and required the presence of Mn^{2+} or Mg^{2+} (White *et al.*, 1984). The human ASGP-R in Hep G2 plasma membranes displays a similar requirement. These requirements for Mg^{2+} or Mn^{2+} , but not Ca^{2+} , and the site of phosphorylation being serine are most consistent with kinase activity similar to that found with the cyclic AMP-stimulated kinases (Krebs & Beavo, 1979; Rangel-Aldao & Rosen, 1976) or with the casein kinases (Zick *et al.*, 1983).

The human transferrin receptor in both foetal liver and a T-cell lymphoblastic leukaemic cell line is phosphorylated at (a) phosphoserine residue(s) (Schneider *et al.*, 1982). The phosphorylation site appears to be on the cytoplasmic domain of this transmembrane molecule. Phosphorylation has also been found on the cytoplasmic portion of the transmembrane IgE-receptor in RBL cells (Fewtrell *et al.*, 1982). While the degree of phosphoryl-

ation of the IgE receptor was not significantly altered in RBL cells exposed to IgE (i.e. to ligand) (Fewtrell *et al.*, 1982), similar to the present results with the human ASGP-R, analogous studies have not been reported for the transferrin receptor. As phosphorylation is a cytoplasmic event (Krebs & Beavo, 1979), the labelling of the human ASGP-R by ^{32}P provides indirect evidence for the transmembrane orientation of this integral membrane protein. This finding is consistent with the data of Harford & Ashwell (1981), which suggested a transmembrane orientation of the rat ASGP-R from studies which examined antibody-binding sites. Thus, the human ASGP-R is a single 46000Da subunit which is oriented as a transmembrane protein with an extracytoplasmic domain containing both the ligand binding site and two *N*-linked complex-type oligosaccharide chains. The cytoplasmic domain contains (a) phosphorylation site(s) on (a) serine residue(s).

Nonetheless, significant questions remain as to the nature of the phosphorylation mechanism of the human ASGP-R and its potential role in intracellular targetting, receptor-mediated endocytosis and receptor turnover. Direct examination of many of these issues will make use of the purified receptor species.

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