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Endocytosis by the asialoglycoprotein receptor is independent of cytoplasmic serine residues

(galactose lectin/serine phosphorylation/internalization signal)

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ABSTRACT **The human asialoglycoprotein (ASGP) receptor, like most other plasma membrane receptors, has previously been shown to be phosphorylated at serine residues within the cytoplasmic domain. Phorbol esters, which activate protein kinase C, cause hyperphosphorylation and down-regulation of the ASGP receptor in HepG2 cells. To test the importance of serine residues for receptor traffic and function, we have mutated all the cytoplasmic serines of the two receptor subunits H1 (at positions 16 and 37) and H2 (at positions 12, 13, and 55) to alanines or glycines. Stable transfected fibroblast cell lines expressing either mutant H1 alone or both mutant subunits together were created and compared to cell lines expressing the respective wild-type proteins. Mutant and wild-type subunits were found to have very similar distributions between the cell surface and intracellular compartments. Constitutive internalization of H1 alone and ligand uptake and degradation by cells expressing both receptor subunits were not affected by the mutations. Cytoplasmic serines and serine phosphorylation are thus not essential for receptor function and intracellular traffic. Analysis of individual serine mutations identified serine-12 of subunit H2 as the major site of phosphorylation in the ASGP receptor.**

Receptor-mediated endocytosis is the mechanism by which proteins and other macromolecules of the extracellular medium (e.g., nutrients, hormones, substances to be cleared from the circulation) are taken up into the cell. Surface receptors involved in this process are clustered in specialized, clathrin-coated regions of the plasma membrane that invaginate and pinch off as coated vesicles. After removal of the clathrin coat, they fuse to acidic endosomal compartments where ligand–receptor complexes dissociate. In many cases the ligands are transported to lysosomes for degradation, whereas the receptors recycle to the plasma membrane for reuse (1).

Within such a cycle, receptors are sorted at least at two points: in the plasma membrane they are concentrated in coated pits for internalization, and in endosomes they are collected into tubular extensions for recycling. At least the first of these sorting processes appears to be mediated by a signal contained within the receptor structure. Most endocytic receptors are single-spanning membrane proteins, and for several of them, the low density lipoprotein (LDL), transferrin, polymeric immunoglobulin, and mannose-6-phosphate receptors, it has been shown that the cytoplasmic domain is necessary for internalization (2–5). Upon its deletion, these receptors became resident plasma membrane proteins largely excluded from coated pits. Yet the cytoplasmic receptor domains are very diverse in length, orientation, and primary structure and do not contain any obvious consensus sequences. Individual tyrosine residues have been shown to be necessary for rapid internalization of the LDL, the mannose-6-phosphate, the transferrin, and the asialoglycoprotein (ASGP) receptor (5–8). In addition, other residues near the tyrosine also appear to be important—e.g., the sequence Asn-Pro-Xaa-Tyr in the LDL receptor (9).

Another feature common to most endocytic receptors is phosphorylation of cytoplasmic serine or threonine residues (10). This modification has been proposed to play an important role in regulating intracellular receptor traffic. This proposal is based on the observation that phorbol esters, which cause hyperphosphorylation of cell surface receptors by activating protein kinase C, have a marked effect on the kinetics of endocytosis of the epidermal growth factor receptor and the β-adrenergic receptor (10) and on the intracellular distribution of the transferrin, the ASGP, and the mannose-6-phosphate receptor (11–14).

In this study, we have analyzed the importance of constitutive serine phosphorylation (as opposed to phorbol ester-induced hyperphosphorylation) and of the cytoplasmic serines in general for the intracellular traffic of the human ASGP receptor. This receptor (reviewed in ref. 15) is a constituent of the plasma membrane of hepatocytes and is responsible for the clearance of desialylated (galactosyl-terminal) glycoproteins from the circulation. Serine residues of the rat and the human ASGP receptor have been shown to be phosphorylated at steady state (16, 17). After addition of phorbol esters to HepG2 hepatoma cells, surface ASGP receptors were rapidly hyperphosphorylated; coordinately the number of surface receptors was reduced by 40–50% with a half-time of 20 min, and the majority of phosphorylated receptors accumulated intracellularly (13, 18, 19). The rate of internalization and the total number of binding sites were found not to be affected, but the phosphorylated receptor population appeared not to be recycled. These findings suggested that phosphorylation/dephosphorylation might control receptor traffic, in particular recycling to the cell surface.

To test this hypothesis, we have mutated the cytoplasmic serine residues of the two subunits, H1 and H2, that constitute the ASGP receptor. After expression of the mutant subunits in COS-7 cells, we identified the sites of phosphorylation. Mutant subunits lacking any cytoplasmic serines were stably expressed in fibroblasts and analyzed with respect to their intracellular transport and to receptor function.

MATERIALS AND METHODS

DNA Constructs. Site-directed mutagenesis was performed according to Kramer *et al.* (20) or using the kit from Amersham. H1 cDNA (from plasmid pSA1; refs. 21 and 22) subcloned into phage M13mp9 was used as the template and the antisense oligonucleotides GTGGTCTCCCTCCTCAT

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Abbreviations: ASGP, asialoglycoprotein; ASOR, asialoorosomucoid; PBt_2, phorbol 12,13-dibutyrate; LDL, low density lipoprotein; BSA, bovine serum albumin.

and AGGTCCGGCGCAGAGAC as mismatched primers to mutate serine-16 to glycine and serine-37 to alanine, respectively (the mismatched nucleotides are underlined). H2 cDNAs were cloned in two differently spliced versions, H2a and H2b, differing in the presence or absence of five codons following serine-86 (23). H2b has been shown to account for ≈90% of H2 mRNA in HepG2 cells (24). A full-length H2b cDNA was prepared by ligating the complete 5′ portion up to the *Nco* I site of the full-length H2a cDNA clone pA34 to the 3′ portion of the H2b cDNA of clone pA33. The resulting cDNA was subcloned into M13mp19 for mutagenesis. The oligonucleotide GTCTCTGCGCCATGGTCTG was used to mutate serine-55 to alanine and the degenerate oligonucleotide GCAGCTG(A/G)GC(T/G)CGGAGGA to mutate serine-12 to glycine and/or serine-13 to alanine. Successfully mutated M13 clones were identified by DNA sequencing.

Cell Culture and Transfection. Cell culture reagents were purchased from GIBCO. HepG2 and COS-7 cells were grown in Eagle's minimal essential medium (MEM) with 10% fetal bovine serum, and fibroblasts in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine calf serum (Inotech, Wohlen, Switzerland). The media were supplemented with L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml). The cell lines 1-7 and 1-7-1 were derived from mouse NIH 3T3 fibroblasts as described by Shia and Lodish (25). To produce F1(16/37) cells, the mutant cDNA of H1 was subcloned into the retroviral shuttle vector pLJ (26) and transfected into NIH 3T3 cells according to Chaney *et al.* (27). After 2 days, selective medium with G418 sulfate (1 mg/ml) was added, and after 10–14 days resistant colonies were cloned and screened for expression by immunoblot analysis. F1(16/37) cells were further transfected with the mutant H2b cDNA (with all three serine codons 12, 13, and 55 altered) subcloned into the expression plasmid pBamHis (a derivative of pLJ with the neomycin-resistance gene replaced by the *his* gene of *Salmonella*; from R. Mulligan, Whitehead Institute). Resistant cell lines were selected in medium containing histidinol (2 mg/ml) and were screened for expression of mutant H2 by immunoprecipitation of cells labeled with [^{35}S]methionine and [^{35}S]cysteine. The resulting cell line producing both mutant subunits H1 and H2 with all five cytoplasmic serines mutated was called F1-2(S5). For transient expression, wild-type and mutant cDNAs were subcloned into pECE (28) and transfected into COS-7 cells according to Cullen (29). The cells were processed after 2–3 days.

Receptor Phosphorylation. For labeling with [^{32}P]phosphate, cells were incubated at 37°C for 30 min in phosphate-free medium and then for 60 min in the same medium supplemented with [^{32}P]phosphate (Amersham) at 0.5 mCi/ml (1 Ci = 37 GBq). For phorbol ester treatment, 200 nM phorbol 12,13-dibutyrate (PBt_2; Sigma) was added for a further 10-min incubation. The cells were then rinsed with phosphate-buffered saline (PBS) at 4°C and permeabilized with 0.1% saponin in MEM for 30 min prior to antibody addition. Subunit-specific rabbit antisera directed against synthetic oligopeptides corresponding to the carboxyl-terminal sequence of either the H1 or the H2 subunit were added at a 1:50 dilution in PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% bovine serum albumin (BSA) for 3 hr at 4°C. The cells were then lysed in PBS containing 1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 2 mM PMSF, 50 mM β-glycerophosphate, and 100 μM sodium orthovanadate (Sigma). Immune complexes were precipitated with protein A-Sepharose, dissociated in SDS sample buffer by boiling for 2 min, and analyzed by SDS/PAGE and autoradiography.

Receptor Distribution. The distribution of the receptor was determined either by immunoblot analysis of proteinase K-shaved and untreated cells (30) or by immunoprecipitation of total and cell surface receptors following metabolic labeling. For this, the cells were incubated for 30 min in medium free of methionine and cysteine, then for 30 min with [^{35}S]methionine (150 μCi/ml) and [^{35}S]cysteine (150 μCi/ml), and then for 4 hr with complete medium to allow the labeled receptor to complete the biosynthetic pathway. The cells either were left intact or were permeabilized with 0.1% saponin in DMEM for 30 min at 4°C before incubation with subunit-specific antisera in PBS containing 1% BSA for 2 hr at 4°C. The cells were then washed three times with PBS containing 1% BSA and solubilized in lysis buffer. The immune complexes were precipitated with protein A-Sepharose and analyzed by SDS/PAGE and fluorography. The complex glycosylated ≈46-kDa forms of H1 and H2 were quantitated by densitometric scanning of the fluorographs.

Assays for Receptor Internalization and for Ligand Uptake and Degradation. Constitutive internalization of wild-type and mutant H1 in 1-7 and F1(16/37) cells was analyzed by the surface iodination and protease protection assay (30). Asialoorosomucoid (ASOR) was prepared and iodinated and ligand binding was performed as described by Shia and Lodish (25), and ligand uptake and degradation were assessed according to Goldstein and Brown (31).

RESULTS

Distribution and Internalization of Subunit H1 Lacking Cytoplasmic Serine Residues. The ASGP receptor consists of two homologous subunits, H1 and H2, with 58% sequence identity (21, 23). They form a heterooligomeric complex that is necessary for the efficient transport of both subunits to the cell surface and for the formation of high-affinity ligand binding sites (25). In hepatocytes and HepG2 cells, H1 is 4–5 times more abundant than H2. Recent experiments employing fluorescence photobleaching recovery suggest a ratio of three H1 subunits to one or two H2 subunits in the receptor complex (32).

To assess the role of serine phosphorylation, we mutated the serine residues in the cytoplasmic portions of the two receptor subunits (Fig. 1). Since the major subunit H1 alone possesses all the signals necessary for constitutive endocytosis and recycling (30), we first analyzed the behavior of a mutant subunit H1 in which the serines at positions 16 and 37 were changed to glycine and alanine, respectively, in the absence of H2. Upon site-directed mutation of the corresponding codons, the mutant H1 cDNA was transfected into mouse 3T3 fibroblasts, and F1(16/37), a cell line stably expressing the mutant H1, was isolated. A defect in internalization or recycling would be expected to cause the receptor proteins to accumulate either on the cell surface or intracellularly. The distribution of the receptor protein was determined by immunoblot analysis of untreated cells and of

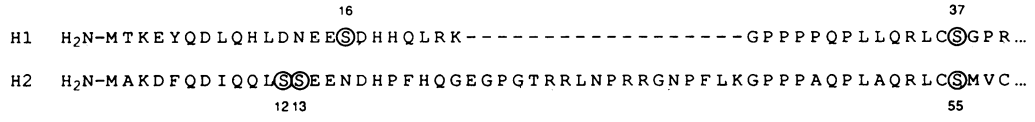

FIG. 1. Amino acid sequences of the cytoplasmic domains of the human ASGP receptor subunits H1 and H2. To align homologous segments, a gap of 18 residues (indicated by dashes) has been inserted in the H1 sequence. Serine residues, which are potential phosphorylation sites, are circled.

cells shaved with proteinase K at 4°C. Approximately 45% of complex glycosylated mutant H1 was accessible to the protease. Similarly, in the fibroblast line 1-7, which expresses wild-type H1, ≈50% was found to be on the cell surface (25, 30). The distribution of subunit H1 was thus not significantly affected by mutation of the two cytoplasmic serines.

The rate of constitutive internalization of mutant $H1^{16/37}$ was directly determined by the endocytosis assay of Geffen *et al.* (30). The surface of F1(16/37) and 1-7 cells was first labeled at 4°C with the [^{125}I]iodinated impermeant reagent sulfosuccinimidyl 3-(4-hydroxyphenyl)propionate. The cells were incubated at 37°C for various times, and the amount of internalized receptor was determined by protease digestion of surface proteins and immunoprecipitation. The kinetics by which wild-type and mutant H1 acquired resistance to exogenous protease were very similar (Fig. 2). Both the initial rate of internalization and the equilibrium distribution of surface-labeled H1 were essentially the same in both cell lines. This indicates that the recycling rate is not significantly altered in the mutant that is unable to be phosphorylated on serine residues. Clearly, the cytoplasmic serine residues are not important for the intracellular transport of subunit H1 alone.

Ligand Uptake and Degradation by Mutant H1 and H2 Lacking Cytoplasmic Serines. To analyze the importance of serine residues on the trafficking of the functional hetero-oligomeric receptor, F1(16/37) cells were transfected with the cDNA of H2 in which the codons for the serines at positions 12, 13, and 55 were mutated to codons for glycine or alanine. A stable cell line expressing both subunits H1 and H2 with all five cytoplasmic serines mutated, F1-2(S5), was isolated. Its properties were compared with those of 1-7-1 cells, a fibroblast line characterized previously (25, 30) that expresses both wild-type receptor subunits. The distribution of H1 and H2 between the surface and intracellular compartments was essentially the same in 1-7-1 and F1-2(S5) cells: 49% of wild-type and 44% of mutant H1 and 55% of wild-type and 47% of mutant H2 were detectable on the cell surface (average of three determinations). The mutant subunits assembled to form high-affinity ligand binding sites: 0.33 pmol of ASOR binding sites per mg of cellular protein was detected on the surface of F1-2(S5) cells, and 0.45 pmol/mg on 1-7-1 cells.

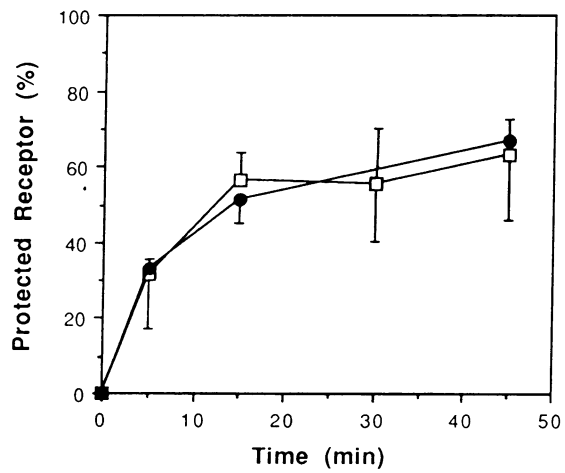

FIG. 2. Time course of internalization of the mutant H1(16/37) and wild-type H1. The surface of 1-7 and F1(16/37) cells was labeled with ^{125}I-sulfosuccinimidyl 3-(4-hydroxyphenyl)propionate at 4°C. The cells were incubated at 37°C for increasing chase periods (as indicated in minutes), after which the cells were chilled and surface proteins were digested with proteinase K at 4°C. Protease-resistant, labeled receptor protein was immunoprecipitated and quantitated by gel electrophoresis and autoradiography. The mean and standard deviation of triplicate determinations are shown.

To assess receptor function (i.e., continuous endocytosis of ligand and its delivery to lysosomes for degradation) the fibroblast lines expressing wild-type or mutant subunits were incubated at 37°C with ^{125}I-labeled ASOR. Cell-associated ^{125}I-ASOR (Fig. 3, ○) and radioactive degradation products released into the medium (△) were quantitated. The sum of these two values (□) corresponds to the cumulative uptake of ligand into the cells. Mutant and wild-type receptors displayed identical characteristics. Uptake of ^{125}I-ASOR continued linearly during the 6 hr of the experiment with a rate of 7.4 ligand molecules per surface binding site per hour. Based on the distribution of the receptor proteins in the two cell lines, there are approximately twice as many total cellular binding sites as there are surface binding sites. Accordingly, each receptor complex internalized ≈3.7 ligand molecules per hour, independently of whether it was composed of wild-type or mutant subunits. This value is similar to what has previously been reported for 1-7-1 and HepG2 cells (25, 33). These findings unambiguously show that the serine residues in the cytoplasmic portions of the receptor subunits are not essential for receptor-mediated endocytosis of ASGP.

Receptor Phosphorylation in Transfected Fibroblasts. To characterize the state of phosphorylation of the receptor subunits in transfected fibroblasts (cell lines 1-7 and 1-7-1) and (for comparison) HepG2 cells were labeled with [^{32}P]phosphate for 1 hr at 37°C, incubated for 10 min with or without PBt_2, and analyzed by immunoprecipitation, gel electrophoresis, and autoradiography (Fig. 4). The receptor proteins in 1-7 and 1-7-1 cells, like those in HepG2 cells, were labeled and susceptible to phorbol ester treatment. Both the high-mannose glycosylated precursors of 40 kDa and the complex glycosylated mature forms of 46 kDa were phosphorylated, yet PBt_2 treatment affected mainly the mature forms. This is consistent with the known mode of action of phorbol esters to activate protein kinase C and to induce its association with the plasma membrane (34) and with the previously observed hyperphosphorylation of the surface receptor pool in HepG2 cells (19). An important finding is that both in HepG2 and in 1-7-1 cells, subunit H2 incorporated significantly more [^{32}P]phosphate than subunit H1, for which phosphorylation could be detected only after PBt_2 treatment (Fig. 4, compare lanes 1–4 with lanes 5–8). Since subunit H2 is present in approximately equal amounts to H1 in 1-7-1 cells (25) and is only about one-third as abundant as H1 in HepG2 cells, the specific phosphorylation of subunit H2 is dramatically higher.

Identification of the Phosphorylated Serines. To identify the sites of receptor phosphorylation, we transfected the cDNAs of wild-type and mutant subunits into COS-7 cells for transient expression using the plasmid vector pECE (28). Maximal rates of synthesis and accumulation of the proteins were found at the second day after transfection. The individually transfected subunits were synthesized as high-mannose glycosylated precursors in COS-7 cells; while H1 readily matured and accumulated as the complex glycosylated 46-kDa form, H2 was mostly retained in its precursor form (data not shown), consistent with previous observations in transfected fibroblasts (25, 35).

Incorporation of [^{32}P]phosphate into receptor subunits expressed in COS-7 cells after PBt_2 treatment is shown in Fig. 5. Wild-type subunit H1 and two mutants in which either serine-16 was changed to glycine or serine-37 was changed to alanine were all labeled (Fig. 5, lanes 1–3), indicating that neither mutation completely abolished phosphorylation. Therefore, both serine-16 and serine-37 are targets for phosphorylation. However, the labeling was not detectable without phorbol ester stimulation (data not shown). Longer labeling times of up to 5 hr did not significantly enhance the signal (data not shown), indicating that the low level of [^{32}P]phosphate incorporation was not due to a long-lived

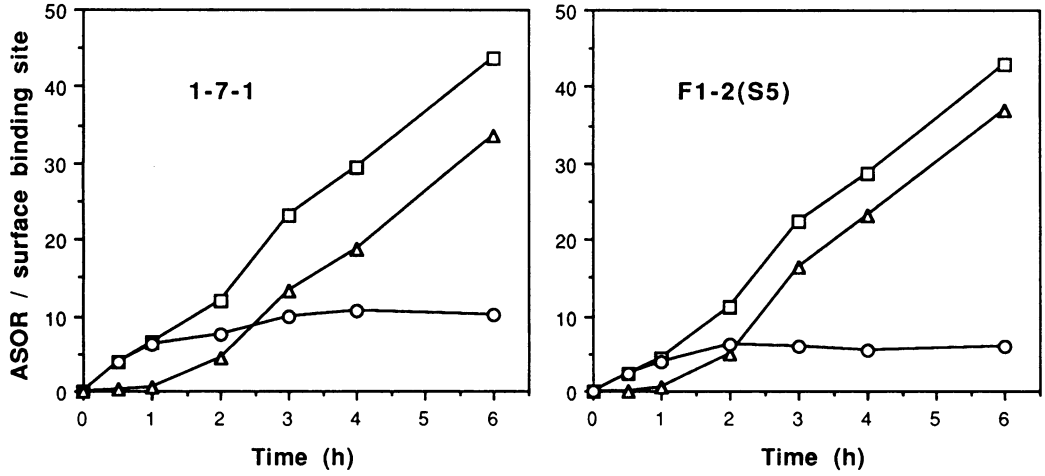

FIG. 3. Ligand uptake and degradation by 1-7-1 and F1-2(S5) cells. The cells were incubated at 37°C with ^{125}I-ASOR (2 μg/ml) for up to 6 hr. Cell-associated radioactive material (○) and degradation products released into the medium (△) were quantitated and added up to yield the cumulative ligand uptake (□). The values are expressed as ASOR molecules per surface binding site.

saturating phosphorylation of H1. As in fibroblasts and HepG2 cells (Fig. 4), labeling of H1 in COS-7 cells was considerably weaker than labeling of H2.

All possible combinations of serine mutations in subunit H2 were analyzed whereby serine-12 was changed to glycine, and the serines at positions 13 and 55 to alanines. None of the mutants lacking serine-12 was labeled with [^{32}P]phosphate, while all proteins retaining serine-12 were strongly labeled (Fig. 5, lanes 4–11). The same result was obtained without PBt_2 treatment (data not shown), indicating that under both induced and control conditions the same residue was modified. Clearly, serine-12 is the only site of phosphorylation in subunit H2.

DISCUSSION

Endocytosis and recycling of plasma membrane receptors require specific sorting mediated by signals within their structure. For several endocytic receptors, phosphorylation of their cytoplasmic domain has been proposed to play such a role. Many different receptors are phosphorylated at cytoplasmic serine or threonine residues by protein kinase C, which can be activated by phorbol esters. Receptor hyperphosphorylation induced by phorbol esters is often temporally correlated with changes in receptor distribution (10), suggesting that phosphorylation might be involved in regulating receptor traffic.

In several cases this has indeed been demonstrated. By site-specific mutagenesis, it has been shown that phosphorylation of threonine-654 of the epidermal growth factor receptor is necessary for ligand-independent internalization triggered by phorbol esters (36). Similarly, phorbol ester-induced internalization of the T-cell surface antigen CD4 depends directly on the three serines that are phosphorylated by protein kinase C (37), and for the polymeric immunoglobulin receptor, serine-664, the single site of phosphorylation, is required for efficient transcytosis (38). In contrast, the only phosphorylated residue of the transferrin receptor, serine-24, is not necessary for receptor cycling (3). In addition, mutation of this residue to threonine or alanine did not affect the phorbol ester-induced receptor redistribution (39, 40), suggesting that there is no causal relationship between transferrin receptor phosphorylation and redistribution.

In this study we have analyzed another recycling transport receptor, the human ASGP receptor, which consists of two homologous subunits assembled in a heterooligomeric complex. After transfection into mouse 3T3 fibroblasts, the subunits were phosphorylated as in HepG2 cells. The major subunit H1 was labeled only very weakly with [^{32}P]phosphate unless the cells were treated with phorbol ester. This did not rule out the possibility that a very short-lived phosphorylation—e.g., at a distinct step of the endocytic cycle—might occur. Labeling experiments with transfected COS-7 cells expressing mutant H1 subunits with individual serines changed to glycine or alanine revealed that both serine-16 and serine-37 are potential targets for phosphorylation by protein kinase C. However, an H1 mutant protein with both cytoplasmic serines mutated retained an unchanged distribution and rate of constitutive internalization when the mutant cDNA was stably expressed in a fibroblast cell line. This indicated that serine residues and their phosphorylation are

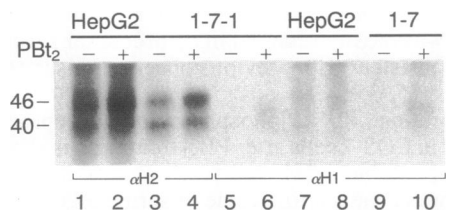

FIG. 4. Steady-state and PBt_2-induced phosphorylation. HepG2, 1-7-1, and 1-7 cells were labeled with [^{32}P]phosphate for 1 hr and, after addition of either 200 nM PBt_2 (+) or the inactive phorbol ester 4α-phorbol (−), for another 10 min. Receptor proteins were immunoprecipitated with antiserum specific for subunit H1 (αH1) or H2 (αH2) and analyzed by SDS/PAGE and autoradiography. The positions of the 40-kDa precursor and the 46-kDa mature forms are indicated.

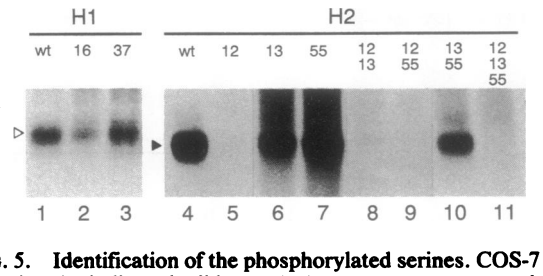

FIG. 5. Identification of the phosphorylated serines. COS-7 cells expressing the indicated wild-type (wt) or mutant receptor subunits were labeled with [^{32}P]phosphate for 1 hr and, after addition of 200 nM PBt_2, for another 10 min. Receptor proteins were immunoprecipitated and analyzed by SDS/PAGE and autoradiography. Similar amounts of wild-type and mutant subunits were synthesized, as judged by metabolic labeling and immunoprecipitation from parallel samples. Filled and open arrowheads indicate the positions of the mature form of H1 and the precursor form of H2, respectively.

not necessary for the constitutive cycling of H1 in the absence of the second subunit.

Subunit H2 was found to be much more highly labeled with [^{32}P]phosphate than H1. The major site of phosphorylation in the human ASGP receptor was identified to be serine-12 of H2. It has been reported (17) that the rat ASGP receptor is predominantly phosphorylated in its minor subunits 2 and 3 (two differently glycosylated forms of the same gene product). While the subunit specificity of phosphorylation appears to be conserved between the two species, the target residue is not. In the homologous position 12 of the rat sequence 2/3, an aspartic acid is found. At least one of the serines at positions 13, 28, 46, and 55 of the rat sequence 2/3 must be phosphorylated. Interestingly, the sequence context of serine-12 of H2 does not resemble the typical recognition sequences for protein kinase C, which are usually preceded by basic residues (41).

We have generated a fibroblast cell line stably expressing mutant forms of both receptor subunits H1 and H2 in which all five cytoplasmic serines were changed. The relative levels of expression of the two subunits and their intracellular distribution were very similar to those in 1-7-1 cells, which express the wild-type proteins. The mutant subunits formed high-affinity ligand binding sites and supported ligand internalization at a rate indistinguishable from that of the wild-type receptor. This result showed unambiguously that cytoplasmic serines are not essential for receptor function and that the phosphorylation of serine residues is not involved in the mechanism of intracellular receptor transport.

The ASGP receptor thus appears to behave similarly to the transferrin receptor, which has also been shown to cycle independently of serine phosphorylation (3, 39, 40). The ubiquity and specificity of phosphorylation of endocytic receptors suggest a physiological role for this modification, which is not clear at present. The human ASGP receptor is also phosphorylated at tyrosine (42), a modification that has not been reported for other transport receptors. Tyrosine-5 of subunit H1 is the only cytoplasmic tyrosine of the ASGP receptor. The significance of its modification for receptor function remains to be investigated.

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