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Phorbol ester-induced redistribution of the ASGP receptor is independent of receptor phosphorylation

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Like virtually all endocytic receptors, the human asialoglycoprotein (ASGP) receptor is phosphorylated by protein kinase C at serine residues within the cytoplasmic domains of its two subunits H1 and H2. Activation of protein kinase C by phorbol esters results in hyperphosphorylation and in a concomitant net redistribution of receptors to intracellular compartments (down-regulation) in HepG2 cells. To test whether there is a causal relationship between receptor hyperphosphorylation and redistribution, we examined the effect of phorbol ester treatment on the ASGP receptor composed of either wild-type subunits or of mutant subunits lacking any cytoplasmic serine residues in transfected NIH3T3 fibroblast and COS-7 cells. Although the wild-type subunits were hyperphosphorylated in fibroblast cells, the distribution of neither the wild-type nor the mutant receptors was affected. In contrast, phorbol ester treatment of transfected COS-7 cells induced down-regulation of both wild-type and mutant receptors. These findings indicate that redistribution of the receptor is independent of its cytoplasmic serines and is not caused by receptor phosphorylation.

Asialoglycoprotein receptor; Endocytosis; Phorbol ester; Protein kinase C; Serine phosphorylation

1. INTRODUCTION

Phorbol esters are tumor-promoting agents that activate protein kinase C. They mimick the action of the second messenger diacyl glycerol which, together with inositol phosphates, is produced from phosphoinositides by phospholipase C upon activation by hormone receptors at the cell surface. Phorbol esters are thus useful as pharmacological agents to analyze the effects of the diacyl glycerol branch of the signal pathway. Activated protein kinase C associates with the plasma membrane and phosphorylates a variety of proteins at cytoplasmic serine or threonine residues. This finally results in a complex and tissue-specific pattern of cellular responses (reviewed by Nishizuka [1]).

Among the targets of protein kinase C are virtually all endocytic receptors, both signal-transducing hormone receptors and transport receptors (reviewed by Backer and King [2]). Upon addition of phorbol esters to the cells, receptors are rapidly hyperphosphorylated at specific residues within their cytoplasmic portions. In some hormone receptors, this directly affects the affinity of ligand binding (epidermal growth factor [EGF] receptor [3]) or modulates signal transduction (insulin receptor [4]). In many receptor systems, hyperphosphorylation is also temporally correlated with changes in the kinetics of endocytosis and/or recycling and in the cellular receptor distribution (e.g. receptors for transferrin, asialoglycoproteins [ASGP], mannose-6-phosphate [5–8]). In most cases surface binding sites are 'down-regulated' upon phorbol ester treatment, i.e. the surface-to-intracellular distribution is shifted in favor of the intracellular pool. This phenomenon could suggest that receptor phosphorylation/dephosphorylation might be necessary for endocytosis and/or recyling also in unstimulated cells. Alternatively, receptor phosphorylation might play a role only in modulating receptor traffic.

The first suggestion has so far not been substantiated: site-specific mutagenesis of the phorbol ester-stimulated phosphorylation site in the EGF receptor (threonine-654) did not hinder constitutive or ligand-induced internalization [9]. Similarly, deletion of the carboxy-terminal portion of the insulin receptor, including the phosphorylation site threonine-1336, did not affect internalization in response to insulin [10]. Mutation of the unique phosphorylation site of the transferrin receptor, serine-24, did not alter ligand uptake and receptor cycling [11,12], nor was there any detectable functional difference between the wild-type ASGP receptor and a mutant receptor lacking all cytoplasmic serine residues, including its phosphorylation sites [13].

However, the phorbol ester effects have been shown to depend on receptor phosphorylation in at least two systems. It was found that phosphorylation of threonine-654 of the EGF receptor is necessary for ligand-independent internalization triggered by phorbol esters [9]. Similarly, phorbol ester-induced internalization of

Abbreviations: ASGP, asialoglycoprotein; ASOR, asialoorosomucoid; EGF, epidermal growth factor; PdB, phorbol dibutyrate.

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the T-cell surface antigen CD4 depends directly on the three serines that are phosphorylated by protein kinase C [14]. In contrast, it was reported for the transferrin receptor that its redistribution upon phorbol ester treatment was independent of phosphorylation at serine-24 [15,16].

In this study, we have analyzed the importance of phorbol ester-induced hyperphosphorylation for the intracellular distribution of the human ASGP receptor. This receptor (reviewed by Spiess [17]) is a constituent of the plasma membrane of hepatocytes and is responsible for the clearance of desialylated (galactosyl-terminal) glycoproteins from the circulation. The ASGP receptor consists of two homologous subunits, H1 and H2 [18], which form a hetero-oligomeric complex necessary for the formation of high-affinity ligand binding sites [19]. In hepatocytes and the human hepatoma cell line HepG2, H1 is approximately four times as abundant as H2. Mobility measurements suggest a ratio of three H1 subunits to one H2 subunit in the native receptor complex [20].

The ASGP receptor is exclusively phosphorylated at serine residues [21], predominantly at serine-12 of subunit H2, and weakly also at serines 16 and 37 of subunit H1, both at steady-state and upon phorbol ester treatment [13]. However, ligand uptake and degradation was not affected by mutation of all five cytoplasmic serine residues in the ASGP receptor subunits in untreated cells, demonstrating that serine phosphorylation is not essential for receptor cycling. Yet, addition of phorbol esters to HepG2 cells was shown to reduce the number of surface receptors by 40–50% coordinately with receptor hyperphosphorylation [7,22]. The rate of internalization remained unchanged, suggesting a phorbol ester effect on the rate of receptor recycling [23]. To test whether receptor down-regulation is caused by receptor phosphorylation, we have analyzed the phorbol ester effect on wild-type and mutant receptors in transfected cell lines. The results indicate that receptor redistribution is independent of cytoplasmic serine residues and is therefore not a consequence of receptor phosphorylation.

2. MATERIALS AND METHODS

2.1. *Cell culture and transfection*

Cell culture reagents were purchased from Gibco Laboratories. HepG2 and COS-7 cells were grown in minimal essential medium (MEM) with 10% fetal calf serum, and NIH3T3 fibroblast cells in Dulbecco's MEM containing 10% bovine calf serum (Inotech, Switzerland). The media were supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cell line 1-7-1 was derived from mouse NIH3T3 fibroblasts as described by Shia and Lodish [19]. The F1–2(S5) fibroblast cell line was described previously [13]. For transient expression in COS-7 cells, wild-type and mutant cDNAs were subcloned into the expression plasmid pECE [24] and transfected according to Cullen [25] in 10-cm dishes. To ensure uniform expression within each experiment, the cells were trypsinized, mixed, and seeded into 35 mm-dishes 12 h after transfection. The cells were used for experiments 24 h later (i.e., 36 h after transfection).

2.2. *Determination of surface ligand binding sites*

Preparation and iodination of asialoorosomucoid (ASOR) and ligand binding was performed as described previously [19]. Cells were incubated at 4°C for 2 h with 2 μg/ml ^{125}I-ASOR. Non-specific binding was determined in the presence of 200-fold excess of unlabeled ligand, or by selectively removing the bound ligand by 3 washes with PBS, pH 5, containing 10 mM EDTA. The effect of phorbol esters on surface receptors was determined after 1 h preincubation with 200 nM 4β-phorbol 12,13-dibutyrate (PdB) or with 200 nM 4α-phorbol (both from Sigma) at 37°C prior to ligand binding for 2 h at 4°C.

2.3. *Internalization assay*

Cells were incubated at 4°C for 2 h with 2 μg/ml ^{125}I-ASOR, washed 3 times with PBS, and incubated for various times at 37°C. Surface-bound ligand was removed by 3 washes with PBS, pH 5, containing 10 mM EDTA. Internalized ligand was quantified by SDS-gel electrophoresis of the lysed cells, autoradiography, and densitometric scanning of the ^{125}I-ASOR band.

2.4. *Determination of receptor distribution*

Cells were treated with or without 1 mg/ml proteinase K (Serva) at 4°C for 30 min, digestion was stopped by addition of 2 mM phenylmethylsulfonyl fluoride, and protease-resistant receptor was analyzed by immunoblotting as previously described [26]. Alternatively, cell surface receptors were quantitated using ^{125}I-labeled IgG purified from a rabbit antiserum directed against ASGP receptor subunit H1. Cells were incubated with saturating concentrations of ^{125}I-IgG in PBS containing 1 mg/ml bovine serum albumin at 4°C for 2 h and then washed 3 times with PBS. Cell-associated radioactivity was determined by gamma counting and by SDS-gel electrophoresis of the lysed cells, autoradiography, and densitometric scanning.

3. RESULTS AND DISCUSSION

3.1. *Phorbol ester-induced receptor redistribution is dependent on the cell type*

To examine the effect of phorbol esters on receptor distribution, stable cell lines expressing wild-type or mutant ASGP receptor subunits were preincubated with 200 nM 4β-phorbol 12,13-dibutyrate (PdB) or with the same concentration of the inactive compound 4α-phorbol for 1 h at 37°C. The cells were then chilled to 4°C and incubated with a saturating concentration of ^{125}I-iodinated ASOR for 2 h to determine the number of ligand binding sites on the cell surface. In agreement with previous reports [7, 23], PdB caused a 30–40% reduction of surface binding sites in HepG2 cells which naturally express the functional ASGP receptor (Fig. 1). This effect is the result of net internalization of receptors rather than a permanent change in affinity or total number of binding sites, since an equivalent reduction of H1 polypeptides on the surface was determined (Fig. 2 and [7]).

To compare the phorbol ester effect on wild-type and mutant receptors, stable transfected cell lines, which normally do not express the ASGP receptor, were examined. In the mouse NIH3T3 fibroblast cell line 1-7-1, both wild-type receptor subunits are expressed. It has previously been shown that they form high-affinity li-

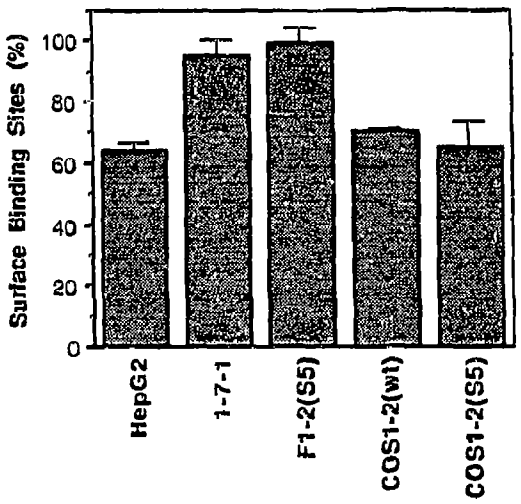

Fig. 1. Phorbol ester effect on surface ligand-binding sites. After pretreatment of the cells for 1 h at 37°C with either 200 nM PdB or the inactive compound 4α-phorbol, ^{125}I-ASOR was allowed to bind to the cell surface at 4°C. HepG2 cells, transfected fibroblasts (1-7-1(wt) and F1-2(S5)) and COS-7 cells (COS1-2(wt) and COS1-2(S5)) expressing wild-type (wt) or mutant (S5) receptor subunits were analyzed. Ligand binding to PdB-treated cells is shown as a percentage of that of control cells. The average and range of two experiments, each performed in triplicate, is shown.

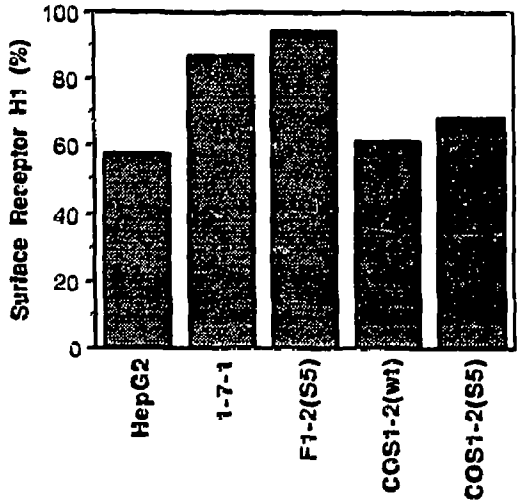

Fig. 2. Phorbol ester effect on surface receptor protein H1. The relative amount of the receptor subunit H1 was determined after treatment of the indicated cells with either 200 nM PdB or the inactive compound 4α-phorbol for 1 h at 37°C. Surface H1 was determined either by proteinase K digestion and immunoblot analysis of resistant H1 or by binding of ^{125}I-iodinated anti-H1 IgG to the cell surface (as described in section 2). Surface H1 of PdB-treated cells is shown as percentage of that of control cells. The average of duplicate or triplicate determinations is shown.

gand binding sites and mediate ligand uptake very similarly to the endogenous receptor in HepG2 cells [19], and that they are internalized and recycled also constitutively (i.e., in the absence of ligand [26]). As in HepG2 cells, predominantly subunit H2 was found to be phosphorylated in 1-7-1 cells and upon PdB treatment phosphorylation of both subunits was enhanced several-fold [13]. Yet, the receptor distribution was not significantly affected by phorbol ester treatment: both the number of surface binding sites and the amount of H1 protein on the cell surface remained unchanged after stimulation (Fig. 1 and 2). No change in receptor distrubution could be observed even at concentrations of up to 1 mM PdB and when another active compound, 4β-phorbol 12-myristate 13-acetate, was used (data not shown). Thus, while fibroblasts and HepG2 cells respond similarly to phorbol esters with respect to receptor phosphorylation, they differ in their response with respect to receptor redistribution.

Not surprisingly, a mutant ASGP receptor in which all cytoplasmic serines (at positions 16 and 37 of H1, and 12, 13, and 55 of H2) were exchanged by alanine or glycine also did not respond to PdB treatment in the stably expressing fibroblast cell line F1-2(S5) (Figs. 1 and 2).

3.2. *Transient expression of functional receptors in transfected COS-7 cells*

Transient expression in COS cells is a convenient method to rapidly produce proteins in sufficient amounts for structural and functional analysis. We have transfected wild-type and mutant receptor cDNAs into COS-7 cells using the plasmid vector pECE. Maximal rates of synthesis and accumulation of the receptor subunits were reached 48–72 h after transfection. In this period, approximately 5 times as many surface binding sites per milligram of protein could be measured as in HepG2 cells, while no specific binding was detectable in mock-transfected COS-7 cells. Moreover, during incubation at 37°C, bound ligand gradually acquired resistance to low pH/EDTA stripping, demonstrating ligand internalization, both in cells expressing wild-type or mutant receptor (not shown). However, the rates of endocytosis were very low, most likely because of substantial overexpression in the successfully transfected subpopulation of the cells resulting in saturation of the endocytic machinery.

If the cells were analyzed at earlier times after transfection (36 h), similar numbers of surface binding sites per mg of protein were expressed as in HepG2 cells. In addition, ligand internalization was found to be more efficient: close to 50% of ^{125}I-ASOR bound to the cells at 4°C acquired resistance to low pH/EDTA stripping within 20 min at 37°C. As in the fibroblast cell lines 1-7-1 and F1-2(S5) [13], ligand binding and uptake in transfected COS-7 cells does not depend on serine phosphorylation of the receptor subunits: wild-type and mutant subunits lacking all cytoplasmic serines yielded equally functional receptor complexes.

3.3. *Phorbol ester effects on transfected COS-7 cells*

The functional expression of ASGP receptors in

transfected COS-7 cells made it possible to examine the effect of phorbol esters on the distribution of either the wild-type receptor or the mutant lacking cytoplasmic serines in this cell type. Both ligand binding sites and receptor protein were found to be reduced on the cell surface upon PdB treatment by 30–40% in comparison to control cells (Figs. 1 and 2). Thus, COS cells do respond to PdB treatment by down-regulation of the ASGP receptor, similar to HepG2 cells but unlike fibroblasts. Importantly, redistribution occurred equally for the wild-type and the mutant receptor subunits. This result strongly argues against a causal relationship between serine phosphorylation of the receptor and its phorbol ester-induced redistribution.

The ASGP receptor thus appears to behave similarly to the transferrin receptor. The only phosphorylated residue of the transferrin receptor, serine-24, is not necessary for receptor cycling [11,12], and mutation of this residue to threonine or alanine did not affect the phorbol ester-induced receptor redistribution in Swiss 3T3 and CHO cells [15,16]. In these two cell lines, phorbol esters induce an increase in surface transferrin receptors. This is in contrast to K562 and HL60 leukemic cells in which the number of surface receptors are reduced due to a phorbol ester-induced increase in the rate of internalization [5,6,27,28]. It has been suggested that the two processes may involve different cellular mechanisms [2]; the dependence of transferrin receptor down-regulation on its phosphorylation has not been demonstrated directly.

Our study on the ASGP receptor in transfected COS-7 cells shows that PdB-induced down-regulation is independent of receptor phosphorylation and of cytoplasmic serine residues. Receptor redistribution is therefore more likely an indirect effect of protein kinase C activation acting more generally on membrane traffic, as has previously been suggested by Buys et al. [29] in studies on the phorbol ester effects on macrophages. Since in addition, serine phosphorylation of the ASGP receptor is not necessary for endocytosis and recycling [13], and mutation of the cytoplasmic serines of subunit H1 does not affect its correct transport to the basolateral surface in transfected Madin-Darby canine kidney cells (B. Leitinger, I. Geffen, and M. Spiess, unpublished results), no functional role is known for this modification in the ASGP receptor.

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