Ligand-induced endocytosis of the asialoglycoprotein receptor: evidence for heterogeneity in subunit oligomerization

Marc D. Bider, Martin Spiess*

Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

Received 22 July 1998; revised version received 27 July 1998

Abstract The hepatic asialoglycoprotein receptor, a non-covalent hetero-oligomer of two subunits, is a constitutively cycling endocytic receptor. However, the ligand asialoorosomucoid caused downregulation of up to 40% of surface binding sites and a twofold increase in internalization rate. This was not the result of receptor crosslinking, since monovalent ligands had the same effect. Ligand binding thus appears to transmit a signal to the cytosolic portion of the receptor not unlike in signaling receptors. The two subunits were endocytosed at different average rates lower than that of ligand, indicating heterogeneity in oligomer formation and potentially in ligand specificity.

Key words: Asialoglycoprotein receptor; Endocytosis; Hepatic lectin; Receptor downregulation

1. Introduction

Receptors internalized via clathrin-dependent endocytosis can be divided into two classes. Signal-transducing receptors, such as peptide hormone receptors, are internalized in a ligand-dependent manner. Since they transduce a signal to the cytoplasm, endocytosis serves to terminate the signal by dissociation and/or degradation of the ligand-receptor complex, and to desensitize the cell by reducing the number of available receptors on the cell surface. In contrast, transport receptors involved in the uptake of macromolecules, such as transferrin, low density lipoproteins (LDL), mannose-conjugated proteins, and asialoglycoproteins (ASGPs), cycle constitutively between the plasma membrane and endosomes both in the presence and in the absence of their ligands [1–4]. For both classes, clustering in clathrin-coated pits depends on cytosolic determinants, of which the tyrosine- and dileucine-containing motifs are the best characterized (reviewed in [5,6]). These motifs are recognized by clathrin-associated adaptor complexes (reviewed in [7]). The ligand-dependent activity of endocytosis motifs in signaling receptors may be regulated by a conformational change in the cytoplasmic domain [5], by receptor oligomerization that increases the valency of the internalization motif, and/or by a release of receptors from membrane domains incompetent for endocytosis (as in the case of the insulin receptor [8]). In contrast, the signals of constitutively cycling receptors are continuously exposed.

*Corresponding author. Fax: (41) (61) 267 2149.
E-mail: spiess@ubaclu.unibas.ch

Abbreviations: ASGP, asialoglycoprotein; ASOR, asialoorosomucoid; CRD, carbohydrate recognition domain; LDL, low density lipoprotein; MGP, monoglycosylated peptide; SSHPP, sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate

We have analyzed the distribution and internalization kinetics of the ASGP receptor, an endocytic transport receptor of hepatocytes which removes galactose-terminal (desialylated) glycoproteins with tri- or tetra-antennary *N*-linked glycans from the circulation [9,10]. The receptor consists of two homologous subunits (called H1 and H2 in the human system), which are both required to form high-affinity ASGP binding sites [11,12]. Despite numerous approaches, the exact stoichiometry of the subunits in the functional receptor complex has not been unambiguously determined. Based on the accumulated data, the minimal ASGP receptor complex must contain two H1 and one H2 [13]. Ligand uptake and receptor internalization are mediated by a tyrosine-containing endocytosis signal in the 40 amino acid cytosolic domain of subunit H1 [14,15]. Initial evidence for constitutive cycling of this receptor was obtained in studies on the effect of lysosomotropic agents on receptor traffic in hepatocytes and HepG2 cells [4,16–18]. These agents caused downregulation of surface receptors by inhibiting or blocking their recycling from endosomes back to the cell surface. In these experiments, it was observed that the disappearance of ASGP binding sites in chloroquine-treated cells was more rapid in the presence of ligand than in its absence [18], suggesting a stimulatory effect of ligand binding on endocytosis.

In this study, we analyzed the effect of ligand directly. Ligand caused downregulation of surface binding sites by increasing the internalization rate of the receptor. Surprisingly, the internalization rates of H1 and H2 with and without ligand were different from each other and from that of ligand alone, indicating heterogeneity in the composition of ASGP receptor subunit complexes.

2. Materials and methods

2.1. Cell culture

Cell culture reagents were purchased from Gibco. The human hepatoma cell line HepG2 was grown in Eagle's minimal essential medium with 10% fetal bovine serum supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.2. Ligand binding and internalization

Asialoorosomucoid (ASOR) was prepared and ^{125}I-iodinated as described [12]. Ligand binding was performed at 4°C for 2 h using 2 μg/ml [^{125}I]ASOR or [^{125}I]MGP in HEPES-buffered saline containing 1.7 mM $CaCl_2$ and 0.2 mg/ml cytochrome *c*. Specific (i.e. Ca^{2+}-dependent) binding was determined by stripping bound ligand with 5 mM EDTA at 4°C for 20 min. Non-specific binding was always less than 15% of total binding. After ligand binding and washing, the cells were incubated at 37°C to allow endocytosis. Surface ligand was stripped with 5 mM EDTA and internalized ligand was quantified by counting cell-associated radioactivity.

Downregulation of binding sites was assayed by incubating the cells at 37°C for 15 or 30 min with ASOR or a complex-type, triantennary, desialylated *N*-linked oligosaccharide (from Oxford Glycosystems,

PII: S0014-5793(98)00947-8

Oxford, UK). Surface ligand was stripped with 5 mM EDTA at 4°C and surface binding sites determined by [^{125}I]ASOR binding as described above.

2.3. Receptor internalization

Internalization of the ASGP receptor subunits H1 and H2 was analyzed by the surface iodination and protease protection assay described by Geffen et al. [20]. Briefly, the cell surface was labeled at 4°C using the impermeant, ^{125}I-iodinated reagent sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate (SSHPP; from Pierce) and, after incubation at 37°C for different times, digested at 4°C with proteinase K. Protease-resistant labeled receptor subunits were analyzed by immunoprecipitation, SDS-gel electrophoresis, and quantitative autoradiography using a phosphoimager. To study the effect of prebound ligand on internalization, cells were incubated for 2 h at 4°C with or without 10 μg/ml ASOR before surface labeling. To distinguish the two subunits, antibodies raised against synthetic peptides corresponding to residues 277–286 of H1 or 300–309 of H2 were used (anti-H1 and anti-H2, respectively). Initial endocytosis rates were calculated based on the earliest time point measured (after 1 or 2 min). To assess the effect of surface iodination on receptor functionality, binding and uptake of [^{125}I]ASOR were performed using cells treated with non-radioactive iodinated SSHPP. The stability of ligand binding was tested by first binding [^{125}I]ASOR to HepG2 cells, labeling the cells with non-radioactive iodinated SSHPP. Cell-associated [^{125}I]ASOR was measured before and after incubating the cells at 4°C for 2 h in the presence of 200 μg/ml cold ASOR. The surface iodination procedure had no significant effect on the number of ligand binding sites, on binding stability, and on the internalization rate (not shown).

2.4. Monoglycosylated peptide (MGP)

The peptide corresponding to residues 175–184 of asialofetuin was purified and ^{125}I-iodinated according to Bider et al. [19]. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a Vestec Benchtop II instrument in negative ion mode with 25 kV accelerating voltage and 337 nm laser wavelength. Aliquots of MGP (3 nmol in 1 μl 50 mM sodium citrate/phosphate buffer, pH 5, containing 25 mM zinc chloride) were incubated without or with neuraminidase (0.2 mU), or with neuraminidase and β-galactosidase (from bovine testes, 1.4 mU; both from Boehringer Mannheim) at 37°C for 23 h. Samples were mixed with an equal volume of saturated sinapic acid in water and dried onto the sample plate. For calibration, oxidized insulin chains A and B (Sigma) were used.

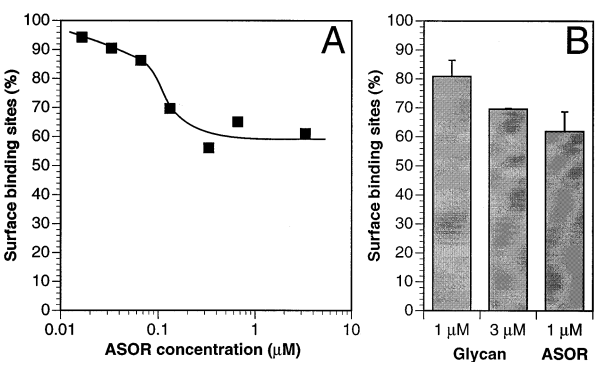

Fig. 1. Ligand-induced downregulation of surface ASGP-binding sites in HepG2 cells. Cells were incubated at 37°C for 15 min with the indicated concentration of ASOR or of a triantennary, galactose-terminal oligosaccharide (Glycan). At 4°C, any bound ligand was released with EDTA and the total surface binding sites were determined with [^{125}I]ASOR in the presence of $CaCl_2$ (as described in Section 2). The results are expressed in percent of binding sites on cells preincubated in the absence of ligand. The means of duplicate determinations are shown in panel A, and of triplicate determinations with standard deviations in panel B.

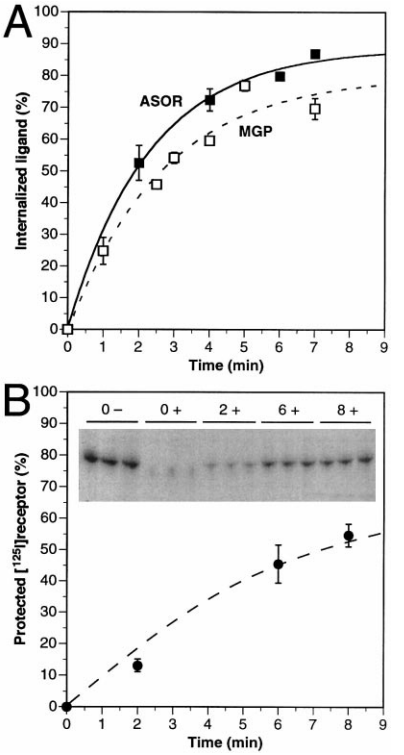

Fig. 2. Internalization kinetics of unoccupied ASGP receptor, ASOR, and MGP. A: Internalization of prebound [^{125}I]ASOR (■) and [^{125}I]MGP (□) was measured as described in Section 2. The means and range of 2–5 determinations are shown. B: The constitutive endocytosis of the ASGP receptor was determined using the surface iodination/protease protection assay with an antiserum against both receptor subunits. The means and standard deviations of triplicate determinations are shown. The fluorograph of the SDS gel is shown in the inset.

3. Results

3.1. Ligand-induced downregulation of surface binding sites

An effect of ligand on receptor cycling will be reflected in the number of ASGP receptors at the cell surface. To determine the number of surface binding sites upon ligand exposure, HepG2 cells were first incubated with different concentrations of ASOR at 37°C for 15 min (which is the average cycle time of the receptor in the presence of ligand [21]), washed at 4°C with EDTA to release bound unlabeled ASOR and incubated for 2 h with ^{125}I-labeled ASOR. The measured numbers of surface ASOR-binding sites were plotted as a percentage of cells preincubated without ligand (Fig. 1A). A concentration-dependent downregulation of up to 40% of the surface binding sites was observed with a half-maximal effect at approximately 0.1 μM ASOR.

To compare endocytosis of ligand-receptor complexes and free receptor protein, the internalization rates of [^{125}I]ASOR and of surface-labeled unoccupied receptor were measured. [^{125}I]ASOR was prebound at 4°C to receptors on the cell surface and then allowed to endocytose at 37°C for different times. After removal of surface ligand by washing with EDTA, internalized [^{125}I]ASOR was quantified and plotted as a percentage of initially bound ligand (Fig. 2A, filled squares). Bound ligand was internalized at an initial rate of ~26%/min. To measure the constitutive rate of receptor internalization, surface receptors were labeled with the amino

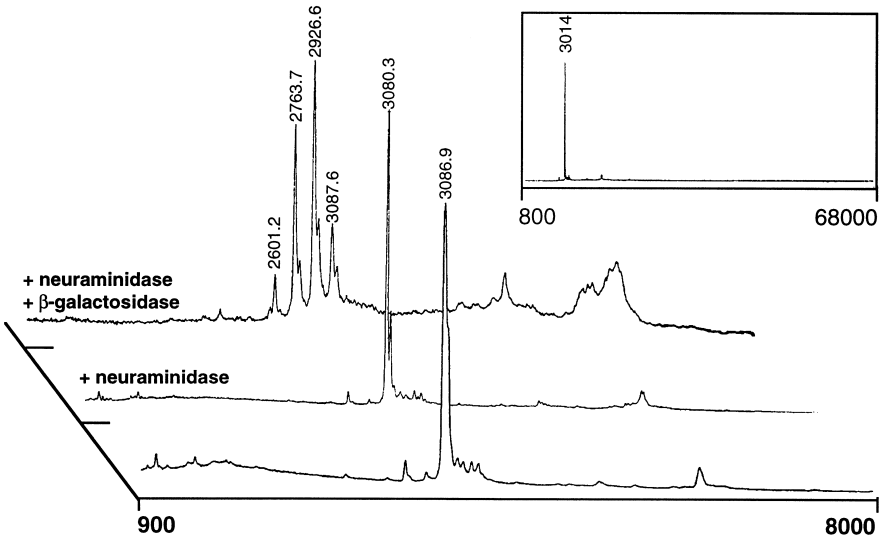

Fig. 3. Characterization of the purified MGP by mass spectrometry. MALDI-TOF mass spectrometry of the purified MGP without and with incubation with neuraminidase and β-galactosidase was performed as described in Section 2.

group-specific, membrane-impermeant reagent [^{125}I]SSHPP at 4°C and then incubated at 37°C for different times. Receptors at the cell surface were digested with proteinase K at 4°C, and the resistant, labeled receptors were immunoprecipitated with an antiserum recognizing both subunits, and quantified (Fig. 2B). Surface labeled receptor protein acquired protease resistance with an initial rate of ~7%/min. The total receptor protein in the absence of ligand was thus internalized at a rate of less than one third of that of ligand-receptor complexes, suggesting a dramatic induction of endocytosis by ligand.

The ligands generally used to measure ASGP binding and internalization, ASOR and asialofetuin, are glycoproteins with five and three *N*-linked glycans, respectively. A possible explanation for the ligand-induced increase in receptor internalization is the physical crosslinking of receptors by the multivalent ligands. To test this possibility, we determined the effect of a purified, desialylated, triantennary oligosaccharide of the *N*-linked type on ASGP receptor distribution. As shown in Fig. 1B, also the free glycan induced a significant downregulation of surface ASOR-binding sites. At a glycan concentration of 3 μM, the reduction was similar to that of ASOR at 1 μM (corresponding to 5 μM of bound *N*-linked oligosaccharides).

To directly measure the internalization rate of a monovalent ligand, we purified a MGP of asialofetuin [19]. The endoproteinase Glu-C fragment corresponding to residues 175–184 contains one of the *N*-glycans and a tyrosine that could be labeled with [^{125}I]iodine. MALDI-TOF mass spectrometry of this purified MGP recorded a single major species with a molecular mass of 3087, which is within the accuracy of the measurement of the calculated mass of 3098 (Fig. 3). Neuraminidase treatment did not alter the observed mass, whereas additional partial digestion with β-galactosidase revealed three steps of mass reduction of 162 each, corresponding to the mass of galactose. This confirmed that the glycan of MGP was triantennary and completely desialylated. Most importantly, contamination by products of incomplete proteolysis that might contain more than one glycan could not be detected in the entire mass range up to 68 000 (inset). Internalization of ^{125}I-iodinated, prebound MGP proceeded with an initial rate of ~25%/min (Fig. 2A, open squares), which is very similar to that of [^{125}I]ASOR. Hence, receptor crosslinking by ligand contributes little, if at all, to the rate of internalization.

3.2. Heterogeneity of ASGP receptor complexes

The direct comparison of the internalization rate of receptor protein without ligand with that of ligand-receptor complexes is only possible if the receptor proteins constitute a

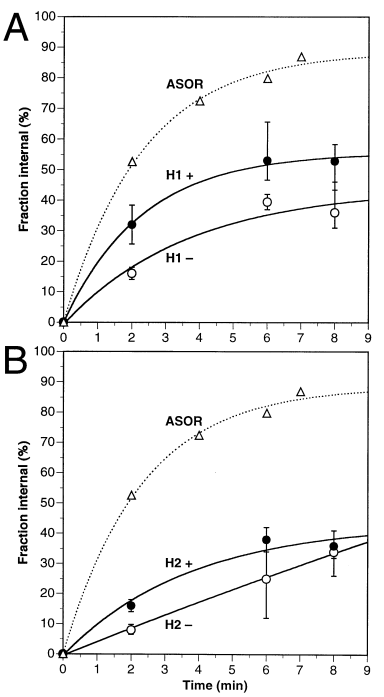

Fig. 4. Internalization kinetics of the receptor subunits H1 and H2 in the presence or absence of bound ASOR. The constitutive endocytosis of H1 (A) and H2 (B) in the absence (open symbols) or presence (filled symbols) of ligand was determined using the surface iodination/protease protection assay with subunit-specific antisera. The means and ranges of 2–3 determinations are shown.

homogeneous receptor population. Since the two subunits of the ASGP receptor have the potential to form homo-oligomers that are unable to bind ASOR [12,14,15], this condition is not necessarily fulfilled. We therefore separately analyzed the internalization rates of subunits H1 and H2 in HepG2 cells. Receptor subunits at the cell surface were preincubated with or without ASOR at 4°C, labeled with [^{125}I]SSHPP at 4°C followed by internalization at 37°C for different times, proteinase K digestion at 4°C, and subunit-specific immunoprecipitation of resistant H1 and H2 using antibodies raised against synthetic peptides corresponding to the C-terminal sequences of the subunits. In control experiments, we found that modifying the cell surface with non-radioactive iodinated SSHPP did not reduce the number of binding sites for [^{125}I]ASOR, the stability of its binding, or the rate of its internalization (not shown). In the absence of ligand (Fig. 4, open circles), subunit H1 was internalized twice as fast as H2 with initial rates of ~8%/min for H1 vs. ~4%/min for H2. This is not consistent with the notion that all subunits are assembled in a single type of receptor complex, but rather indicates heterogeneity in subunit oligomerization. Ligand binding increased the rates of both subunits approximately twofold (Fig. 4, filled circles) to ~16%/min for H1 and to ~8%/min for H2, confirming that both subunits are part of ligand binding receptor complexes. However, both subunits had a clearly lower average internalization rate than ligand itself, indicating that both subunits are also present in alternative subunit complexes not binding and not internalizing ASOR-binding sites.

4. Discussion

Transport receptors are characterized by constitutive internalization. However, only in a few cases have internalization rates in the presence and absence of ligand been quantified. For the transferrin receptor, there is clear evidence (despite an early publication to the contrary [22]) that ligand binding does not significantly alter receptor internalization [2,23] or coated pit localization [24]. Analysis of the LDL receptor did not suggest a ligand effect on the internalization rate per se [1], but experiments using monensin to inhibit recycling indicated a fraction of LDL receptors that do not cycle at all unless ligand is added [25]. The situation for the ASGP receptor is different, since monensin experiments showed that ASOR-binding sites constitute a single constitutively cycling pool [18]. Binding ASOR, however, induced the downregulation of up to 40% of these binding sites (Fig. 1) and approximately doubled the internalization rate of the receptor subunits (Fig. 4). This effect is not due to a simple crosslinking mechanism via ligand, since a monovalent glycopeptide was internalized with essentially the same kinetics as a multivalent ligand (Fig. 2).

That ligand binding strongly stimulates endocytosis of a constitutive receptor suggests that the distinction between transport and signaling receptors may not be as strict as generally assumed. Interestingly, Fallon et al. reported coimmunoprecipitation of a protein kinase activity with the ASGP receptor and with subunit H1 alone [26,27]. They proposed that an extrinsic kinase comparable to the intrinsic kinase in peptide hormone receptors might perform a regulatory function in trafficking of transport receptors. In this model, ligand binding might activate the associated kinase via a conformational change, thereby enhancing the association with clathrin coats. A ligand effect on the kinase activity remains to be demonstrated.

Previous studies revealed that tyrosine-5 of H1 in the cytoplasmic domain is necessary for constitutive as well as ligand internalization [14,15]. Ligand binds to the exoplasmic carbohydrate recognition domain (CRD) at the C-terminus. The subunits are oligomerized via a coiled-coil domain [28,29] that forms a stalk of approximately 12 nm on top of which the CRDs are presented. For high-affinity binding, at least three CRDs in a receptor complex interact each with a terminal galactose in the desialylated *N*-linked glycan. To induce increased internalization of the receptor complex, binding of ligand is likely to transmit a conformational change across the membrane to alter the exposure of the endocytosis signals or to influence putative associated proteins. The coiled-coil stalk domain and its transmembrane extension might transmit a ligand-induced change in the relative position of the CRDs across the membrane to the cytoplasmic portion of the subunits, possibly by a slight relative rotation of the α-helices in the coiled-coil.

Surprisingly, we found that the internalization rates of the two receptor subunits were different and, even with bound ASOR, lower than the rate of ASOR itself (Fig. 4). This suggests the existence of ASOR-binding and non-binding receptor complexes with different subunit compositions. In fluorescence photobleaching recovery experiments, immobilization of either H1 or H2 by antibody crosslinking led to an equal immobilization of the other subunit, indicating that most of H1 and H2 in HepG2 cells is assembled in stable hetero-oligomers [30]. In vitro analysis of the oligomerization specificity of the purified coiled-coil domains of H1 and H2 revealed the preferred formation of 2 H1:2 H2 as well as 1:3 hetero-tetramers [29]. Since it was shown by ligand-receptor crosslinking that two of the galactoses in a triantennary ligand specifically interact with the rat homolog of H1 and the third galactose with H2 [31], only the 2:2 oligomers are potential ligand binding complexes. 1:3 hetero-oligomers are thus candidates to account for the populations of H1 and H2 which are internalized but are not part of ASOR-binding receptor complexes. Endocytosis has been shown to be mediated by the tyrosine signal in H1, whereas H2 did not significantly contribute to internalization [15]. Thus, 2:2 complexes, which contain two copies of the H1 endocytosis signal, are expected to internalize more efficiently than 1:3 complexes with only one copy. Since H2 is more abundant in the latter oligomers than H1, this could explain that the average internalization rate of H2 is lower than that of H1.

The CRDs of H1 and H2 both recognize terminal galactose. Hetero-oligomerization is likely to be necessary for the precise asymmetric presentation of the individual CRDs' galactose-binding sites to fit the arrangement of the galactoses in the ligand [13,31]. The finding that the ASGP receptor subunits form different oligomeric complexes with distinct ASGP-binding capabilities raises the possibility that as yet unidentified ligands exist. Most studies to test natural and synthetic glycans for binding to the ASGP receptor were performed as competition experiments in which the inhibition of the binding of radioactive ASOR or asialofetuin (i.e. triantennary, desialylated *N*-linked glycans) was assessed. Interaction with distinct binding sites would thus have gone undetected. Our results raise the possibility that alternative oligosaccharide

ligands exist, specific for those ASGP receptor complexes that do not bind ASOR.

Acknowledgements: We are grateful to Nicole Beuret for technical assistance and to Dr. Paul Jenö and Thierry Mini for help with MGP purification. This work was supported by Grant 31-43483.95 from the Swiss National Science Foundation.

References

[1] Brown, M.S., Anderson, R.G.W. and Goldstein, J.L. (1983) Cell 32, 663–667.
[2] Watts, C. (1985) J. Cell Biol. 100, 633–637.
[3] Tietze, C., Schlesinger, P. and Stahl, P. (1982) J. Cell Biol. 92, 417–424.
[4] Tolleshaug, H. and Berg, T. (1979) Biochem. Pharmacol. 28, 2919–2922.
[5] Trowbridge, I.S., Collawn, J.F. and Hopkins, C.R. (1993) Annu. Rev. Cell Biol. 9, 129–161.
[6] Sandoval, I.V. and Bakke, O. (1994) Trends Cell Biol. 4, 292–297.
[7] Marks, M.S., Ohno, H., Kirchhausen, T. and Bonifacino, J.S. (1997) Trends Cell Biol. 7, 124–128.
[8] Carpentier, J.L. and McClain, D. (1995) J. Biol. Chem. 270, 5001–5006.
[9] Spiess, M. (1990) Biochemistry 29, 10009–10018.
[10] Weigel, P.H. (1994) BioEssays 16, 519–524.
[11] McPhaul, M. and Berg, P. (1986) Proc. Natl. Acad. Sci. USA 83, 8863–8867.
[12] Shia, M.A. and Lodish, H.F. (1989) Proc. Natl. Acad. Sci. USA 86, 1158–1162.
[13] Lodish, H.F. (1991) Trends Biochem. Sci. 16, 374–377.
[14] Fuhrer, C., Geffen, I. and Spiess, M. (1991) J. Cell Biol. 114, 423–432.
[15] Fuhrer, C., Geffen, I., Huggel, K. and Spiess, M. (1994) J. Biol. Chem. 269, 3277–3282.
[16] Berg, T., Blomhoff, R., Naess, L., Tolleshaug, H. and Drevon, C.A. (1983) Exp. Cell Res. 148, 319–330.
[17] Fiete, D., Brownell, M.D. and Baenziger, J.U. (1983) J. Biol. Chem. 258, 817–823.
[18] Schwartz, A.L., Bolognesi, A. and Fridovich, S.E. (1984) J. Cell Biol. 98, 732–738.
[19] Bider, M.D., Cescato, R., Jenö, P. and Spiess, M. (1995) Eur. J. Biochem. 230, 207–212.
[20] Geffen, I., Wessels, H.P., Roth, J., Shia, M.A. and Spiess, M. (1989) EMBO J. 8, 2855–2862.
[21] Schwartz, A.L., Fridovich, S.E. and Lodish, H.F. (1982) J. Biol. Chem. 257, 4230–4237.
[22] Klausner, R.D., Harford, J. and van Renswoude, J. (1984) Proc. Natl. Acad. Sci. USA 81, 3005–3009.
[23] Hanover, J.A., Beguinot, L., Willingham, M.C. and Pastan, I.H. (1985) J. Biol. Chem. 260, 15938–15945.
[24] Hansen, S.H., Sandvig, K. and van Deurs, B. (1992) Exp. Cell Res. 199, 19–28.
[25] Basu, S.K., Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1981) Cell 24, 493–502.
[26] Fallon, R.J., Danaher, M., Saylors, R.L. and Saxena, A. (1994) J. Biol. Chem. 269, 11011–11017.
[27] Fallon, R.J., Danaher, M. and Saxena, A. (1994) J. Biol. Chem. 269, 26626–26629.
[28] Beavil, A.J., Edmeades, R.L., Gould, H.J. and Sutton, B.J. (1992) Proc. Natl. Acad. Sci. USA 89, 753–757.
[29] Bider, M.D., Wahlberg, J.M., Kammerer, R.A. and Spiess, M. (1996) J. Biol. Chem. 271, 31996–32001.
[30] Henis, Y.I., Katzir, Z., Shia, M.A. and Lodish, H.F. (1990) J. Cell Biol. 111, 1409–1418.
[31] Rice, K.G., Weisz, O.A., Barthel, T., Lee, R.T. and Lee, Y.C. (1990) J. Biol. Chem. 265, 18429–18434.