Growth Hormone Receptor Ubiquitination Coincides with Recruitment to Clathrin-coated Membrane Domains*

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Endocytosis of the growth hormone receptor (GHR) depends on a functional ubiquitin conjugation system. A 10-amino acid residue motif within the GHR cytosolic tail (the ubiquitin-dependent endocytosis motif) is involved in both GHR ubiquitination and endocytosis. As shown previously, ubiquitination of the receptor itself is not required. In this paper ubiquitination of the GHR was used as a tool to address the question of at which stage the ubiquitin conjugation system acts in the process of GHR endocytosis. If potassium depletion was used to interfere with early stages of coated pit formation, both GHR endocytosis and ubiquitination were inhibited. Treatment of cells with methyl-β-cyclodextrin inhibited endocytosis at the stage of coated vesicle formation. Growth hormone addition to methyl-β-cyclodextrin-treated cells resulted in an accumulation of ubiquitinated GHR at the cell surface. Using immunoelectron microscopy, the GHR was localized in flattened clathrin-coated membranes. In addition, when clathrin-mediated endocytosis was inhibited in HeLa cells expressing a temperature-sensitive dynamin mutant, ubiquitinated GHR accumulated at the cell surface. Together, these data show that the GHR is ubiquitinated at the plasma membrane, before endocytosis occurs, and indicate that the resident time of the GHR at the cell surface is regulated by the ubiquitin conjugation system together with the endocytic machinery.

Clathrin-mediated endocytosis involves the formation of clathrin-coated vesicles from coated pits at the plasma membrane. Recruitment of membrane proteins into clathrin-coated pits is mediated by specific amino acid sequences within their cytoplasmic domain (for review, see Refs. 1, 2). The best-defined coated pit localization signals are the tyrosine-based motifs NPXY as described for, e.g. the low-density lipoprotein receptor (3), and YXXΦ (where Φ is an amino acid with a bulky hydrophobic group) found in, e.g. the transferrin receptor (4). Alternatively, internalization of the insulin and β2-adrenergic receptor is mediated by a dileucine-containing motif (5, 6). Many receptors, including the low-density lipoprotein receptor and the transferrin receptor, are clustered in coated pits and internalized constitutively, independent of ligand occupancy.

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The heterotetrameric adaptor complex AP-2 binds directly to the tyrosine-based motif and nucleates assembly of clathrin triskelions onto the plasma membrane (for review, see Ref. 7). Invagination of the plasma membrane results in the formation of constricted coated pits, followed by the dynamin-dependent detachment of coated vesicles from the plasma membrane. More complex situations exist when plasma membrane proteins enter cells on stimuli such as hormone binding or specific signal transduction events. In this case the internalization signal is only recognized on ligand binding, or the stimulus induces the addition of a signal, which results in the subsequent recruitment of the receptor into the coated pit. The agonist-induced phosphorylation of the β2-adrenergic receptor resulting in the binding of β-arrestin, a specialized adaptor that binds to clathrin, is an example of a protein modification that regulates internalization (8). Recently, it was shown that the attachment of ubiquitin moieties is involved in the internalization of several plasma membrane proteins (for review, see Refs. 9, 10). In mammalian cells, the ubiquitin conjugation system regulates the endocytosis of the epithelial sodium channel (11) and the growth hormone receptor (GHR (12); Ref. 12).

The GHR was initially found to be ubiquitinated on amino acid sequences of the receptor from rabbit liver (13). The ubiquitin conjugation system is involved in GHR internalization (12, 14). In particular, a 10-amino acid motif within the GHR cytosolic tail (the ubiquitin-dependent endocytosis motif, DSWVEFIELD) is involved in both receptor ubiquitination and endocytosis (15). We have recently shown that the proteasome is also involved in GHR internalization (16). Ligand-induced internalization of the GHR is blocked in the presence of specific proteasomal inhibitors such as carbobenzoxyl-l-leucyl-l-leucinal and β-lactone, the more membrane-permeable analogue of lactacycin. Disruption of clathrin-mediated endocytosis by cellular potassium depletion (17), hypertonic medium treatment (18), or cellular cytosol acidification (19) inhibits internalization and ubiquitination of the GHR (14). Although ubiquitination of the GHR itself is not required for endocytosis (15), GHR internalization requires the activity of the ubiquitin conjugation system, which acts together with the endocytic machinery in targeting the receptor into the coated pit.

In this study the question was addressed of at which stage the ubiquitin conjugation system acts in the process of GHR endocytosis. GHR ubiquitination was taken as a biochemical marker for the location of the activity of the ubiquitin conjuga-

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† The abbreviations used are: GHR, growth hormone receptor; GH, growth hormone; MjCD, methyl-β-cyclodextrin; mAb, monoclonal antibody; HA, hemagglutinin; MEM, minimal essential medium; dyn15°, temperature-sensitive mutant of dynamin; wtdyn, wild-type dynamin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; EGF, epidermal growth factor.

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tion system. Conditions in which coated pit formation was prevented were compared with conditions that allowed coated pit formation but prevented allowed coated vesicle formation.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies—**Antibody mAb5 recognizing the luminal part of the GHR was from AGEN Inc. (Parcissany, NJ). Polyclonal antibodies against amino acid residues 271–318 of the cytosolic tail of the GHR (Anti-T; Ref. 16) and against human growth hormone (GH) were raised in rabbits. Antiserum specific for protein-ubiquitin conjugates was a generous gift from Dr. A. Ciechanover (Technion-Israel Institute of Technology, Haifa, Israel). Antibody 4G10 (anti-pY), recognizing phosphorysotyrosine residues, was obtained from Upstate Biotechnologies Inc. (Lake Placid, NY). Anti-biotin was from Rockland (Gilbertsville, PA); monoclonal anti-clathrin was from Transduction Laboratories (Lexington, KY); anti-mouse IgG was from Nordic Immunological Laboratories (Tilburg, The Netherlands); and monoclonal anti-hemagglutinin (HA) antibody 12CA5 was from Babco (Richmond, CA). Human GH was a kind gift from Lilly; methyl-β-cyclodextrin (MβCD) was from Sigma; LipofectAMINE was from Life Technologies, Inc.; and carbobenzoxy-L-leucyl-L-leucyl-L-leucinal was from Calbiochem.

**Plasmids, Cell Culture, and Transfection—**Wild-type rabbit GHR cDNA was cloned into the cytomegalovirus-Neo expression plasmid pcDNA3.1 (Invitrogen BV/Novex) and used for transient transfections. The internalization-deficient mutant GHR(F327A) was constructed by site-directed mutagenesis and cloned into pcDNA3.1 as described (20). The internalization-deficient mutant GHR(F327A) was constructed by site-directed mutagenesis and cloned into pcDNA3.1 as described (20).

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GHR Ubiquitination Occurs in Coated Pits

**Fig. 1. Effect of MβCD on GH internalization.** A, GHR-expressing ts20 cells were incubated for 30 min at 30 °C in the absence (Control) or presence of 10 mM MβCD and incubated with Cy3-GH for 30 min. Cells were fixed before (30 min, 30 °C) or after acid wash (Acid Wash). Cy3-GH was visualized by confocal microscopy. B, GHR-expressing ts20 cells were incubated for 30 min at 30 °C in the absence (control) or presence of 10 mM MβCD and put on ice for 2 h with 125I-GH. Unbound label was removed, and the cells were incubated at 30 °C in the absence or presence of MβCD as indicated. Background label was determined in the presence of excess unlabeled GH and subtracted. The amounts of 125I-GH internalized are plotted as a percentage of the cell-associated radioactivity at the start of the incubation. Each point represents the mean value of two experiments performed in duplicate ± S.D. ●, control; ○, MβCD.

pits, indicating that cholesterol is essential for clathrin coated vesicle formation (28). To monitor GH uptake, GHR-expressing ts20 cells were incubated for 30 min in the presence of Cy3-labeled GH, which resulted in the presence of Cy3-GH in endosomal and lysosomal compartments (Fig. 1A, Control). As expected, the Cy3-GH was protected against acid treatment, confirming that the label is in intracellular structures (Fig. 1A, Acid Wash). When cells were preincubated in the presence of MβCD, virtually no Cy3-GH entered the cells (Fig. 1A, MβCD), and the majority of label could be removed on acid treatment (Fig. 1A, Acid Wash). Neither uptake nor binding was observed when excess unlabeled ligand was added together with Cy3-GH or when untransfected cells were used (data not shown).

To confirm and quantify the effect of MβCD on endocytosis, uptake of 125I-GH was measured in a time course experiment. As seen in Fig. 1B, MβCD inhibited the internalization of GH efficiently. There was no effect of MβCD on the total binding of 125I-GH to the cells (data not shown). Two control experiments were performed to ascertain that MβCD treatment did not affect other relevant cellular processes. The effect of MβCD on GHR biosynthesis was measured using pulse-chase labeling with [35S]methionine (Fig. 2A). The receptor was synthesized as a 110-kDa glycoprotein precursor (double band; Fig. 2A, p) and on “complex glycosylation” in the Golgi complex converted to a 130-kDa mature species (Fig. 2A, m). Quantification of the radioactivity showed that the GHR signal in the MβCD cells is ~85–90% of the control cells, indicating a slight inhibition of protein synthesis. Conversion of precursor to mature receptor was detectable after 30 min of chase both in control and in MβCD-treated cells, indicating that transport to the Golgi compartment was not affected by the cholesterol depletion. To examine the effect of MβCD on GHR phosphorylation, a second control experiment was performed. Allevato and colleagues (29) showed that a mutated GHR, deficient in internalization, was capable of stimulating transcription of the serine protease inhibitor 2.1 promoter, and we showed that the GHR cytosolic tail is tyrosine-phosphorylated, while internalization is inhibited (14, 20). From these studies it was concluded that GHR phosphorylation is independent of GHR endocytosis. MβCD-pre-treated cells were incubated for various periods with GH. As seen in Fig. 2B, phosphorylation became detectable after 5 min of incubation, reaching a maximum after 15 min for both control and MβCD-treated cells. The blot was reprobed with anti-GHR (mAb5). p, precursor GHR (110 kDa); m, mature GHR (130 kDa).

**Fig. 2. Effect of MβCD on the biosynthesis and the tyrosine-phosphorylation of the GHR.** A, GHR-expressing ts20 cells were incubated for 30 min at 30 °C in methionine-free medium in the absence (Control) or presence of 10 mM MβCD. [35S]Methionine was added, and the incubation was continued for 10 min. Cells were chased in MEMα supplemented with 0.1% BSA, 100 μM methionine, and 8 nM GH in the absence or presence of MβCD for the periods indicated. Upper panel, GHR was immunoprecipitated using anti-T; p, precursor GHR (110 kDa); m, mature GHR (130 kDa). Lower panel, radioactivity was quantified and expressed as a percentage of the radioactivity incorporated in the precursor GHR. ●, precursor GHR; ○, mature GHR. B, GHR-expressing ts20 cells were incubated at 30 °C in the absence (control) or presence of 10 mM MβCD. All dishes were incubated for 60 min in total; GH was present during the last 5, 15, or 30 min. Upper panel, cells were lysed, and the GHR was immunoprecipitated with anti-T and immunoblotted using anti-pY. Lower panel, the same blot was reprobed using anti-GHR (mAb5). p, precursor GHR (110 kDa); m, mature GHR (130 kDa).
GHR Ubiquitination Occurs in Coated Pits

**MβCD Does Not Affect Accessibility of Clathrin-coated Pits for GHR**—Previously, it was shown that MβCD does not interfere with the association of clathrin to the plasma membrane but has an inhibitory effect on the invagination and fission of clathrin-coated pits (28). For the present study it was important to determine whether the GHR enters these clathrin-coated areas at the plasma membrane of MβCD-treated cells. Immunogold labeling of clathrin in GHR-expressing ts20 cells revealed that the majority of plasma membrane-associated clathrin was present on deeply invaginated pits and coated vesicles in close vicinity to the plasma membrane (Fig. 3A and Table I). GH was regularly found in the deeply invaginated clathrin-coated pits and vesicles (Fig. 3, C and D), as well as in later compartments of the endocytic pathway (data not shown). In MβCD-treated cells, the total number of clathrin-coated structures at the plasma membrane was increased twice compared with that in control cells. In contrast to control cells, >85% of the clathrin-containing structures were flattened coated pits (Fig. 3B). Deeply invaginated coated pits and coated vesicles were rarely seen (Table I). GH accumulated at the plasma membrane, where it regularly but not exclusively occurred in the flattened clathrin-coated pits (Fig. 3E). These findings suggest that the GH-GHR complex in MβCD-treated cells indeed enters the clathrin-coated areas of the plasma membrane.

**Effect of MβCD on GHR Ubiquitination**—To address the question of whether the GHR is ubiquitinated after MβCD treatment, cells were incubated with or without GH, and ubiquitinated proteins were immunoprecipitated and analyzed by Western blotting, as indicated in Fig. 4. Ubiquitinated GHR appeared as high molecular weight species in the top part of the gel (Fig. 4A). Control cells (lanes 3 and 4) showed increased GHR ubiquitination on ligand binding. Both in unstimulated and stimulated cells the level of GHR ubiquitination increased when MβCD was present (Fig. 4A, compare lane 3 with lane 9 and lane 4 with lane 10). The use of untransfected cells resulted, as expected, in the absence of signal for ubiquitinated GHR (ts20; Fig. 4A, lanes 1 and 2). When endocytosis was inhibited by potassium depletion, GHR ubiquitination was almost completely abolished (lanes 5 and 6). Ubiquitination was restored to control values by adding 10 mM KCl (lanes 7 and 8). Reprobing the blot from Fig. 4A with anti-ubiquitin showed that the amount of immunoprecipitated, ubiquitinated protein in each lane was comparable (Fig. 4B). These results show that the GHR is ubiquitinated at the cell surface before constriction of the coated pit occurs and suggest that assembly of the clathrin coat is a requirement for GHR ubiquitination.

**Effect of a Temperature-sensitive Dynamin Mutation on GHR Ubiquitination**—To examine the effect of inhibition of clathrin-mediated endocytosis on GHR ubiquitination by an independent method, we used a HeLa cell line expressing a temperature-
sensitive mutant of human dynamin, dyn\textsuperscript{TS}. Dyn\textsuperscript{TS} carries a point mutation corresponding to the Drosophila shibire\textsuperscript{ts1} allele (30). For this dynamin mutant it has been shown that transferrin internalization is inhibited at the nonpermissive temperature, that the phenotype is rapid and reversible, and that invaginated but not constricted coated pits accumulate on the cytoplasmic surface of the plasma membrane. HeLa cells were transiently transfected with wild-type GHR cDNA and incubated in the presence of GH; afterward the GH-GHR complex was immunoprecipitated with anti-GH. Using this approach, only mature GHR species from the cell surface were immunoprecipitated. At the permissive temperature, ubiquitinated protein was detected in both the wild-type and mutant dynamin cells (Fig. 5A, anti-Ubi). After shifting the cells to the nonpermissive temperature, an increase in the amount of ubiquitinated GHR was detected in dyn\textsuperscript{TS} and wild-type dynamin cells (Fig. 5A, anti-Ubi). Ubiquitination of proteins is dynamic with rapid addition and removal of ubiquitin (31). The increase in ubiquitination in the wild-type dynamin cells might reflect with rapid addition and removal of ubiquitin (31). The increase in ubiquitination in the wild-type dynamin cells might reflect with rapid addition and removal of ubiquitin (31). The increase in ubiquitination in the wild-type dynamin cells might reflect with rapid addition and removal of ubiquitin (31). The increase in ubiquitination in the wild-type dynamin cells might reflect with rapid addition and removal of ubiquitin (31). The increase in ubiquitination in the wild-type dynamin cells might reflect with rapid addition and removal of ubiquitin (31). The increase in ubiquitination in the wild-type dynamin cells might reflect with rapid addition and removal of ubiquitin (31). The increase in ubiquitination in the wild-type dynamin cells might reflect with rapid addition and removal of ubiquitin (31). The increase in ubiquitination in the wild-type dynamin cells might reflect with rapid addition and removal of ubiquitin (31). The increase in ubiquitination in the wild-type dynamin cells might reflect with rapid addition and removal of ubiquitin (31). The increase in ubiquitination in the wild-type dynamin cells might reflect with rapid addition and removal of ubiquitin (31). The increase in ubiquitination in the wild-type dynamin cells might reflect with rapid addition and removal of ubiquitin (31). The increase in ubiquitination in the wild-type dynamin cells might reflect with rapid addition and removal of ubiquitin (31). The increase in ubiquitination in the wild-type dynamin cells might reflect with rapid addition and removal of ubiquitin (31).
cultured in the presence of tetracycline showed no detectable HA signal (results not shown). To ascertain that indeed GH uptake was inhibited, we measured internalization of 125I-GH. At the permissive temperature the percentage of GH uptake was comparable in the two cell lines, whereas at the nonpermissive temperature GH internalization was strongly inhibited in dynTS cells compared with wild-type dynamin cells (Fig. 5B). The results demonstrate that overexpression of a dominant negative mutant of dynamin-1 inhibits the clathrin-mediated endocytosis of the GHR. Because we analyzed a complex of proteins immunoprecipitated with anti-GH to monitor ubiquitination of the GHR, the possibility exists that other ubiquitinated proteins coimmunoprecipitate in this complex. To show that the ubiquitination of this complex depends on the GHR, we transfected the internalization-deficient GHR (F327A) mutant in dynTS cells. Previously, we have shown that this mutant is not ubiquitinated because of a defective ubiquitin-dependent endocytosis motif (15). After immunoprecipitation of the GH-GHR (F327A) complex, almost no ubiquitinated protein was isolated either at the permissive or the nonpermissive temperature (Fig. 5C, left panel). This result shows that the ubiquitination of the GH-GHR complex is dependent on an intact ubiquitin-dependent endocytosis motif and that most likely the receptor itself is ubiquitinated, perhaps in complex with other ubiquitinated proteins. Control experiments with mock-transfected cells and incubations without GH showed no signal on the ubiquitin blots (results not shown). As seen in the Fig. 5C, right panel, mature GHR and GHR (F327A) were detected in the complex, and virtually no GH signal is present at the top of the lanes, indicating that only a small percentage of total GHR is ubiquitinated. Most likely, the GHR is ubiquitinated during a very short period, presumably the resident time in the coated pit.

DISCUSSION

In this study two independent methods were used to inhibit clathrin-mediated endocytosis at the level of clathrin-coated vesicle formation. Both methods inhibited the endocytosis of the GHR, resulting in an accumulation of ubiquitinated receptors at the cell surface. A morphological approach detected the GHR in deeply invaginated coated pits in control cells and in flattened clathrin-coated pits in MjCD-treated cells. Disruption of clathrin-mediated endocytosis by hypertonic medium treatment, cytosol acidification, or potassium depletion resulted in the accumulation of nonubiquitinated receptors at the cell surface (Ref. 14 and Fig. 4A). Why is the GHR not ubiquitinated under these conditions? The ubiquitination state of a protein is the result of a dynamic process of ubiquitination and deubiquitination. Changing the intracellular milieu by depleting potassium or modifying the pH could alter the balance between those two processes. Do the used methods interfere with the ubiquitination machinery itself and cause a complete inhibition of cellular ubiquitination? Analysis of cell lysates from potassium-depleted or hypertonic medium-treated cells showed no reduction in total cellular ubiquitin conjugates but rather an increase in high molecular mass ubiquitinated proteins (Ref. 14 and Fig. 4B). The amount of free ubiquitin under these circumstances as measured with Western blotting was reduced (data not shown). However, cellular cytosol acidification showed increased free and less conjugated ubiquitin (data not shown). Cytosol acidification causes the same precipitation of small clathrin microages as seen after hypertonicity and potassium depletion (32). Recently, it was shown that after hypertonic treatment or cytoplasmic acidification, free clathrin triskelions within the cytosol are depleted, and all of the clathrin becomes associated with membranes (26). Because the presence of free clathrin triskelions is required for the stabilization of AP-2 coated pit nucleation sites, depletion of clathrin interferes with coated pit formation. Because the methods used have a varying effect on the ratio of free versus conjugated ubiquitin, it is most likely that the inhibition of GHR ubiquitination is the result of the interference with the coated pit formation rather than with ubiquitin conjugation itself.

The observation that GHR ubiquitination coincides with the recruitment of the GHR to clathrin-coated membrane areas suggests that the ubiquitin conjugation system and the endocytosis machinery act together in the endocytosis of the GHR. The earlier observation that ubiquitination of the receptor itself is not important for endocytosis suggests that ancillary proteins might be ubiquitinated or that factors of the ubiquitin conjugation system itself might act as adaptors for the endocytosis machinery. Ubiquitin-protein ligases have been implicated in endocytosis. For the epithelial sodium channel, it was shown that the ubiquitin-protein ligase Nedd4 mediates the down-regulation of the Na+ channel activity by ubiquitinating the channel, which leads to its endocytosis and degradation (33). The yeast homologue of Nedd4, Npl1/Rsp5, participates via its C2 domain in the endocytosis of Gap1 permease. A truncated Npl1 protein lacking the C2 domain can still promote ubiquitination but not the endocytosis of Gap1 permease, which is consistent with direct participation of Npl1 in endocytosis of the permease (34). Whether an E2/E3 ubiquitin ligase directly serves as an endocytic adaptor for GHR, analogous to the role of arrestin for the β2-adrenergic receptor (8) or binding of the ubiquitin conjugation system, results in the interaction with an endocytic adaptor (e.g. AP-2), remains to be established. Recently, GH-dependent association of AP-2 with the chicken GHR was reported (35). Also, a possible role for the ubiquitin polypeptide itself, conjugated to a GHR-associated protein, cannot be excluded, as has been described for Ste2p (36) and Ste3p (37). Ligand-induced ubiquitination was shown for Eps15, a clathrin-coated pit associated protein that is ubiquitinated on epidermal growth factor (EGF) receptor activation (38). Eps15 is required for clathrin-mediated endocytosis, and perturbation of Eps15 function inhibits receptor-mediated endocytosis of transferrin (39). The biological significance of Eps15 monoubiquitination is not known. Recently, it was shown that polyubiquitination of the EGF receptor occurs at the plasma membrane on ligand-induced activation (40). Inhibition of endocytosis caused by overexpression of mutant dynamin resulted in a transient polyubiquitination of the EGF receptor. The mechanisms for GHR and EGF receptor ubiquitination are probably different. Ubiquitination of the EGF receptor is mediated by Cbl adaptor proteins, and both EGF receptor and Cbl must undergo phosphorylation on specific sites for productive ubiquitination (41), whereas GHR ubiquitination occurs in the absence of GHR tyrosine phosphorylation and is dependent on the ubiquitin-dependent endocytosis motif (20).

The amount of ubiquitinated GHR is very low compared with the total amount of cell surface GHR. The fact that only a small percentage of total GHR is ubiquitinated indicates a coated pit restricted function of the ubiquitin conjugation system. Whether the ubiquitinated GHR is (partially) degraded soon after endocytosis or perhaps rapidly deubiquitinated is not clear at present. A role for deubiquitinating enzymes in regulating endocytosis cannot be excluded, because deubiquitination has been recognized as an important regulatory step (31, 42). Recently, genetic data were presented that support a model whereby the Drosophila fat facets deubiquitinating enzyme removes ubiquitin from the product of the liquid facets gene. The liquid facets locus encodes epsin, a protein involved in clathrin-mediated endocytosis (43). Thus the ubiquitin system may add another layer of complexity to the membranesorting machinery at the plasma membrane and regulate, to-
together with the classical endocytosis machinery, the time span of the GHR at the cell surface.

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REFERENCES
1. Mellman, I. (1996) Annu. Rev. Cell Dev. Biol. 12, 575–625
2. Schmid, S. L. (1997) Annu. Rev. Biochem. 66, 511–548
3. Chen, W.-J., Goldstein, J. L., and Brown, M. S. (1990) J. Biol. Chem. 265, 3116–3123
4. Collawn, J. F., Stangel, M., Kuhn, L. A., Essekogwu, V., Jing, S. Q., Trowbridge, I. S., and Tainer, J. A. (1990) Cell 63, 1061–1072
5. Haft, C. R., Klauser, R. D., and Taylor, S. L. (1994) J. Biol. Chem. 269, 26286–26294
6. Gabilondo, A. M., Hegler, J., Krasel, C., Bevinjahns, V., Hein, L., and Lohse, M. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12285–12290
7. Kirchhausen, T. (1999) Annu. Rev. Cell Dev. Biol. 15, 705–732
8. Goodman, O. B., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagon, A. W., Keen, J., and Benovic, J. L. (1996) Nature 383, 447–450
9. Strous, G. J., and Govers, R. (1999) J. Cell Sci. 112, 1417–1423
10. Hicke, L. (1999) Trends Cell Biol. 9, 107–112
11. Staub, O., Gausche, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., and Rotin, D. (1997) EMBO J. 16, 6325–6336
12. Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A., and Schwartz, A. L. (1996) EMBO J. 15, 3806–3812
13. Lueh, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. J., and Wood, W. I. (1987) Nature 330, 537–544
14. Govers, R., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1997) EMBO J. 16, 4851–4858
15. Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1999) EMBO J. 18, 26–36
16. van Kerkhof, P., Govers, R., Alves Dos Santos, C. M., and Strous, G. J. (2000) J. Biol. Chem. 275, 1575–1580
17. Larkin, J. M., Brown, M. S., Goldstein, J. L., and Anderson, R. G. W. (1983) Cell 33, 273–285
18. Hansen, S. H., Sandvig, K., and VanDeurs, B. (1993) J. Cell Biol. 121, 61–72
19. Sandvig, K., Olsen, S., Petersen, O. W., and van Deurs, B. (1987) J. Cell Biol. 105, 679–689
20. Strous, G. J., van Kerkhof, P., Govers, R., Botwin, P., and Schwartz, A. L. (1997) J. Biol. Chem. 272, 40–43
21. Bentham, J., Aplin, R., and Norman, M. R. (1994) J. Histochem. Cytochem. 42, 103–107
22. Slot, J. W., Geuze, H. J., Gislen, S., Lienhard, G. E., and James, D. E. (1991) J. Cell Biol. 113, 123–135
23. Liu, W., Geuze, H. J., and Slot, J. W. (1996) Histochem. Cell Biol. 106, 41–58
24. Ilondo, M. M., Petro, P. J., Geiger, D., Carpenter, J., Rousseau, G. G., and de Meyts, P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6440–6446
25. Ilondo, M. M., Smil, J., DeMeyts, P., and Curtis, P. J. (1991) Endocrinology 128, 1597–1602
26. Brown, C. M., and Petersen, N. O. (1999) Biochem. Cell Biol. 77, 439–448
27. Subtil, A., Gaidarov, I., Kebdrak, Z., Lampson, M. A., Keen, J. H., and Mcgraw, T. E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6775–6780
28. Rodal, S. K., Skretting, G., Garred, O., Vilhardt, F., Vanderbe, S., and Sandvig, K. (1999) Mol. Biol. Cell 10, 961–974
29. Allevato, G., Bililstrup, N., Goujon, L., Galagaard, E. D., Norstedt, G., and Nielsen, J. H. (1995) J. Biol. Chem. 270, 17210–17214
30. Damke, H., Bahn, T., van der Blik, A. M., and Schmid, S. L. (1995) J. Cell Biol. 131, 69–80
31. Hochstrasser, M. (1996) Annu. Rev. Genet. 30, 405–439
32. Heuser, J. (1989) J. Cell Biol. 108, 401–411
33. Harvey, K. F., Dinudom, A., Komwatanana, P., Jolliffe, C. N., Day, M. L., Paravishvam, G., Cook, D. I., and Kumar, S. (1999) J. Biol. Chem. 274, 12525–12530
34. Springael, J. Y., Decraene, J. O., and Andre, B. (1999) Biochem. Biophys. Res. Commun. 257, 561–566
35. Vleirick, L., Pezet, A., Kuhn, E. R., Decuyt, E., and Edery, M. (1999) Mol. Endocrinol. 13, 1823–1831
36. Shih, S. C., Sliper-Muul, K. E., and Hicke, L. (2000) EMBO J. 19, 187–198
37. Roth, A. F., and Davis, N. G. (2000) J. Biol. Chem. 275, 8143–8153
38. van Delft, S., Govers, R., Strous, G. J., Verkleij, A. J., and van Bergen en Hengouwen, P. M. (1997) J. Biol. Chem. 272, 14013–14016
39. Benmerah, A., Bayrou, M., Cerf-Benzisus, N., and Dautry-Varsat, A. (1999) J. Cell Sci. 112, 1303–1311
40. Stang, E., Johannsen, L. E., Knaardal, S. L., and Madshus, I. H. (2000) J. Biol. Chem. 275, 13840–13847
41. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Avoy, L., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Mol. Cell 4, 1029–1040
42. Chung, C. H., and Baek, S. H. (1999) Biochem. Biophys. Res. Commun. 266, 633–640
43. Cadavid, A. L., Ginzel, A., and Fischer, J. A. (2000) Development 127, 1727–1736
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