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Minireview

Dimerization, ubiquitylation and endocytosis go together in growth hormone receptor function

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Abstract Internalization of membrane proteins has been studied for more than three decades without solving all the underlying mechanisms. Our knowledge of the clathrin-coated endocytosis is sufficient to understand the basic principles. However, more detailed insight is required to recognize why different proteins enter clathrin-coated pits with different rates and affinities. In addition to clathrin coat components, several adapter systems and even more accessory proteins have been described to preselect membrane proteins before they can enter cells. Recent experimental data have identified the ubiquitin–proteasome system as a regulatory system both in endocytic and lysosomal membrane traffic. This system is well-known for its basic regulatory function in protein degradation, and controls a magnitude of key events. In this review, we will discuss the complexity and implications of this mechanism for membrane trafficking with emphasis on the growth hormone receptor.

Key words: Growth hormone receptor; Dimerization; Ubiquitin; Endocytosis; Endoplasmic reticulum

1. The ubiquitin–proteasome system

Initially, the ATP-dependent conjugation of the 76 amino-acid-long ubiquitin to abnormal or misfolded cytosolic proteins was found to mediate their degradation (review in [1]). At present, however, ubiquitylation is considered to be involved in a wide variety of cellular processes that do not only involve degradation, but play a role in regulation of cell cycle, differentiation and development, stress response, DNA repair, gene transcription, immune response, degradation of misfolded proteins in the endoplasmic reticulum (ER) (ER-associated degradation or ERAD), modulation of cell surface receptors and ion channels, muscle wasting, and apoptosis. Considering the involvement in so many processes it is not surprising that aberrations in the ubiquitylation pathway are implicated in many pathological disorders.

Ubiquitin conjugation to substrate proteins requires the successive action of several enzymes [1]. First, in an ATP-dependent step, the ubiquitin-activating enzyme (E1) forms a high-energy thiol-ester between the active site cysteine residue and the C-terminal glycine residue of ubiquitin. Subsequently, a ubiquitin-conjugating enzyme (E2) accepts the activated ubiquitin from the E1 to form an E2-S∼ubiquitin intermediate involving the E2 active-site cysteine. The final conjugation of the ubiquitin molecule to the substrate can be mediated directly by E2, but frequently requires an ubiquitin protein ligase (E3), in addition. The ubiquitin moiety is conjugated via its C-terminal glycine to the ε-amino-group of a lysine residue or in some cases the α-amino-group of the N-terminal residue of the substrate [2]. A substrate can be modified with a single ubiquitin moiety (monoubiquitylation) but since ubiquitin itself contains seven lysine residues, repetition of the conjugation reaction can also result in the formation of polyubiquitin chains. The fate of a polyubiquitylated protein depends on the length and linkage type of the ubiquitin chain [3]. In general, substrates with chains of four or more ubiquitin molecules linked via lysine 29 or 48 are efficiently targeted for degradation by a large multimeric cytosolic protease, the 26S proteasome. Unlike the substrate, the ubiquitin molecules are not degraded but recycled for new conjugation reactions. Rather than proteasome-targeting signals, lysine 63-linked polyubiquitin chains might be involved in regulation of endocytosis of membrane channel proteins [4], DNA repair [5], and kinase activation [6]. Analogous to protein phosphatases, deubiquitylating enzymes (DUBs) play an important role in the timing and intensity of ubiquitylation-controlled events [7–9] and are required for ubiquitin homeostasis [10].

In every instance, class and variety of the E3 ligase mainly determines the specificity of the ubiquitin system (Fig. 1). The E3 enzymes are divided into three categories: HECT domain, RING finger and U-box E3s. *HECT domain E3s* contain a conserved cysteine residue required for thiol-ester linkage of ubiquitin within a 350 amino-acid region with *h*omology to the *E6*-AP *C-t*erminus [11,12]. Most HECT domain E3s contain WW domains that interact with proline-rich, phosphorylated tyrosine (PY) motifs of the substrate and a N-terminal C2 domain that mediates translocation to the plasma membrane upon intracellular Ca^{2+} increase [4]. A well-studied example of HECT domain E3s is Nedd4, which mediates the ubiquitylation and endocytosis of the amiloride-sensitive sodium channel (ENaC). ENaC is apically located and expressed primarily in the polarized epithelia of the distal nephron, lung, distal colon, and other organs. It is composed of three related subunits, α, β, and γ, arranged in a stoichiometry of 2α:1β:1γ. Mutation of the PY motifs of ENaC, as seen in Liddle's syndrome patients, prevents ENaC ubiquitylation

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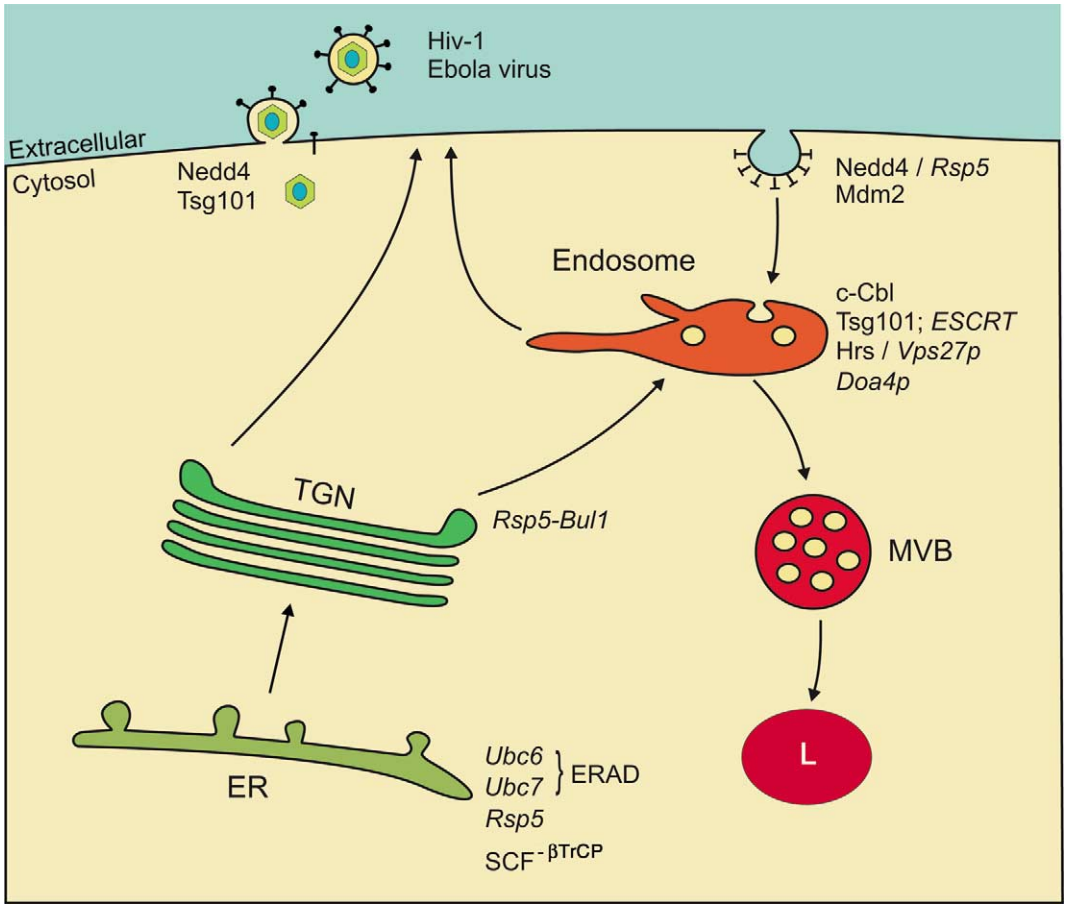

Fig. 1. A schematic view of the regulatory functions of the ubiquitin system in membrane protein transport. Proteasome-mediated ERAD is involved in protein quality control of newly synthesized proteins via Ubc6 and -7 degradation. Rsp5 and $SCF^{\beta-TrCP}$ are involved in (partial) degradation of ER membrane proteins. At the Golgi complex the combination of Rsp5–Bul1 can direct Gap1 directly into the degradation route. Endocytosis of ENaC via Nedd4 and GHR is regulated by the ubiquitin system, while the combination Nedd4/Tsg101 is involved in virus budding from the cell surface. A crucial step in downregulation of growth factor receptors and other regulatory membrane proteins is at the endosome, where the ubiquitin interacting motif containing protein Hrs/Vps27p, the deubiquitinase Doa4p and the ESCRTs co-operate to direct them to the multivesicular bodies (MVB) and lysosomes (L) for degradation. The specificity of the selection process is probably provided by E3 ligases, as is reported for the EGFR and c-Cbl.

and causes hypertension due to the prolonged activation of the sodium channels [13,14]. The yeast homolog of Nedd4, Npi1/Rsp5, is involved in regulating internalization of several transporters and plasma membrane receptors [4]. In addition, Rsp5 induces the release of two transcription factors, Spt23 and Mga2, by facilitating the ubiquitin- and proteasome-dependent cleavage of their membrane-anchored ER precursor proteins (Fig. 1) [15]. Under certain nutritional conditions, Rsp5 (together with Bul1) was found to regulate the sorting of the general amino acid permease, Gap1, from the *trans*-Golgi network (TGN) to the vacuole [16]. Nedd4 is also involved in the budding of some viruses. Both the VP40 protein of Ebola virus and Gag and M proteins of specific retroviruses and rhabdoviruses, respectively, possess a Nedd4 interacting PY motif [17–19]. Together, Nedd4/Rsp5 is an exquisite example of a single E3 that regulates the activity of different proteins at distinct cellular locations.

The second group of E3 ligases comprises the *RING* (really interesting new gene) E3s. These ligases contain a structural element, the RING finger, which is defined by a pattern of conserved cysteine and histidine residues that bind two zinc ions [20]. In complex RING E3s, the RING domain is required for the recruitment of E2 enzymes, while single-subunit RING E3s contain both the E2 and substrate binding sites on the same molecule. Examples of the latter are c-Cbl, involved in ubiquitylation of cell surface growth factor receptors [21], Mdm2 that ubiquitylates the tumor suppressor protein p53 [22], and the inhibitors of apoptosis (IAPs)[23]. The multisubunit E3 complexes comprise the anaphase promoting complex or cyclosome involved in the degradation of cell cycle regulators [24], the Skp1/cullin1/F-box (SCF) complex that mediates the degradation of many signal- and cell cycle-induced phosphorylated proteins [25], and the von Hippel-Lindau/elongin C/elongin B/cullin2 complex, a regulator of hypoxia-inducible transcription factor 1α [26]. The $SCF^{\beta-TrCP}$ E3 interacts via the WD40 motif with Hiv-1 Vpu resulting in CD4 degradation in the ER [27].

The third class of E3 enzymes are the *U-box* proteins, which contain a RING domain lacking the canonical cysteines for Zn^{2+} coordination [28]. Nevertheless, U-box proteins can still bind E2 enzymes as exemplified by the recruitment of Ubc4/5 E2 enzymes to the Hsp90/Hsp70 co-chaperone CHIP [29]. In this way, misfolded proteins that are recognized by Hsp90/Hsp70 are proposed to be ubiquitylated and targeted for degradation by the proteasome as reported for the cystic fibrosis transmembrane conductance regulator (CFTR) [30].

Proteins with a lysine 48-linked polyubiquitin tag are efficiently recognized by the 26S proteasome and degraded. The 26S proteasome is a large (~2.5 MDa) multisubunit cytosolic protease composed of a 20S catalytic core particle, which is capped on both sides by a 19S regulatory multimeric complex (reviewed in [31,32]). Proteasome inhibitors bind to the catalytically active threonine residue of the β-subunits in the inner rings [33,34]. The 19S cap complex is structurally divided into an eight subunit 'lid' and a 'base', consisting of three non-ATPase subunits and six homologous ATPases, presumably involved in unfolding and translocating the substrate into the 20S catalytic chamber [35].

Mostly, the substrates are hydrolyzed into 8–11 amino-acid-long peptides, Occasionally, limited-processing yields new biological active polypeptides, e.g. the transcription factors Spt23 and Mga2 arise from their membrane-anchored precursor proteins by proteasome-dependent cleavage, probably via hairpin intermediates [15]. Another well-studied example is the generation of the p50 subunit of the nuclear factor (NF)-κB transcription factor from its p105 precursor [36]. In this case, a glycine-rich region prevents complete degradation of p105 [37]. Strikingly, a glycine–alanine repeat prevents proteasome degradation of the Epstein–Barr virus nuclear antigen 1 (EBNA1) [38]. Since this precludes the presentation of EBNA1 antigenic peptides by major histocompatibility complex (MHC) molecules, EBNA1 escapes the immune system.

2. Ubiquitylation in membrane protein traffic

As a major regulatory system of the cytosol and the nucleus, it is not unexpected that the ubiquitin system is involved in the trafficking of a variety of membrane proteins at several intracellular locations. An important functional parameter of membrane proteins is their residence time at the cell surface. The number of specific proteins at the cell surface is within certain limits regulated by their expression (gene expression, mRNA stability, translation rate). However, once a membrane protein, bound for the cell surface, has been synthesized and translocated into the lumen of the ER, its presence and abundance at the cell surface depends on a variety of factors, which differ for each individual protein: time and efficiency of multimerization and ER quality control, transport rate from ER to plasma membrane, rate of uptake via endocytosis, susceptibility to cell surface proteolysis, recycling efficiency in the endocytic route, and finally, sorting efficiency into lysosomes. The major control points are depicted in Fig. 1.

The first controlling organel in the life of a membrane protein is the ER. In general, misfolded proteins are recognized in the ER by a mechanism involving ER chaperones such as calnexin and Bip (Kar2p in yeast), translocated to the cytosol via the Sec61 translocon, polyubiquitylated and degraded by the 26S proteasome [39]. This quality control mechanism is referred to as ERAD and ensures that only correctly folded and/or assembled proteins reach their final destinations in the cell. Known examples of membrane proteins, whose number at the cell surface is controlled by ER-based sorting mechanisms, are CFTR [40] and the erythropoietin receptor (EpoR) [41].

At the TGN, ubiquitylation determines the routing of the yeast GAP1 and TAT2 tryptophan permease [42]. Depending on nutrient conditions, these permeases are polyubiquitylated and subsequently directed from the TGN to the vacuole (the yeast equivalent of the mammalian lysosome) without emerging at the plasma membrane.

Clathrin-mediated endocytosis is a major factor in cell surface residence time of membrane protein. In yeast, the α-factor (Ste2p) and a-factor (Ste3p) receptor, uracil (Fur4p), maltose (Mal61p) and general amino acid (Gap1p) permease, and the ABC-transporter Ste6p are ubiquitylated either constitutively or in response to ligand binding (reviewed in [43]). In mammalian cells, the GHR, epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), Met receptor and the epithelial sodium channel ENaC are also targets of the ubiquitin system. In most cases, these proteins are not degraded by the proteasome but, following ubiquitylation, internalized and transported to the vacuole/lysosome (reviewed in [66]).

Many of the yeast proteins are monoubiquitylated or tagged with a short lysine 63-linked polyubiquitin chain [43]. In fact, a single ubiquitin molecule appears to be sufficient to induce internalization, since in-frame fusion of ubiquitin to the stable plasma membrane protein Pma1 and Ste2p stimulates their endocytosis [44]. Despite the lack of classical dileucine or tyrosine-based internalization motifs that can mediate the recruitment to coated membranes, the three-dimensional structure of folded ubiquitin reveals an internalization signal composed of two surface patches surrounding the critical residues phenylalanine-4 and isoleucine-44 [45]. Thus, ubiquitin might bind adapter complexes directly. Recently, another indication for the involvement of ubiquitylation and the endocytosis machinery has been reported. Adapter proteins of the endocytosis machinery such as eps15(R), and epsins possess a ubiquitin-interacting motif (UIM), not only involved in ubiquitin recognition but also in monoubiquitylation of the proteins themselves [46,47]. Eps15 monoubiquitylation is also observed upon stimulation of mammalian cells with EGF and is important for the activity of the endocytosis machinery [48]. Because Eps15 can interact with clathrin adapter proteins, it is conceivable that endocytosis of a cell surface protein lacking a classical internalization motif can be induced by its monoubiquitylation. Whether polyubiquitylated proteins use a similar pathway remains to be determined. Internalization of the G protein-coupled β2-adrenergic receptor (β2AR) is also regulated via the ubiquitin system. Activation of β2AR results in the recruitment of β-arrestin, an adapter that can interact with clathrin. Although β2AR is ubiquitylated, it is the ubiquitylation of β-arrestin by the RING E3 Mdm2 that is essential for the internalization of the complex [49]. It is conceivable that the ubiquitin moieties on β-arrestin mediate the linkage to the endocytosis machinery [50].

Following internalization, membrane proteins are delivered to endosomes. Here, proteins, destined for degradation in the vacuole/lysosome, are incorporated into the inward budding vesicles (in later stages these structures are designated multivesicular bodies MVBs), whereas others recycle back to the plasma membrane via recycling endosomes [51]. The involvement of the ubiquitin system in the sorting into internal vesicles has been demonstrated in several ways. In response to EGF, the EGFR is ubiquitylated by the RING E3 c-Cbl, incorporated into internal vesicles of the MVB and degraded in lysosomes [52,53]. Oncogenic Cbl mutants that lack ubiquitin ligase activity, cause the recycling of the activated EGFR back to the plasma membrane, and prolong EGFR signalling

[54]. The yeast vacuolar carboxypeptidase S is monoubiquitylated on a single lysine residue and its mutation to arginine prevents sorting into MVB vesicles [55]. Recognition of the ubiquitylated proteins is mediated by ESCRT-1 (endosomal sorting complex required for transport), a 350-kDa protein complex consisting of Vps23p, Vps28p, and Vps37p. Vps23p contains a UBC-like domain that resembles the UBC domain of E2 enzymes but lacks the active site cysteine. This domain is essential for binding of ubiquitylated substrates and sorting into the internal vesicles [55]. The mammalian homolog of Vps23p is a protein encoded by the tumor susceptibility gene 101 (Tsg101) [56] and mutations in Tsg101 result in tumorigenesis presumably due to recycling of receptors, as seen for oncogenic Cbl mutants. Recently, mammalian hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) has been demonstrated to mediate sorting of ubiquitylated substrates to the lysosome [51,57]. Like eps15 and epsins, Hrs and its yeast homolog Vps27p contain a UIM that mediates the binding to ubiquitin [46]. Because Vps27p is not a component of the ESCRT-1 complex, it might function in recruiting ubiquitylated cargo into the bi-layered structures and MVB vesicles [51]. Another important factor in this sorting process is Vps4, an ATPase of the AAA protein family that appears to utilize the energy derived from ATP hydrolysis to disassemble endosome-associated Vps complexes and thereby allow multiple rounds of sorting [58]. Genetic studies indicate that removal of ubiquitin moieties is necessary for completion of the sorting at the MVBs [59]. The DUB Doa4p removes ubiquitin from cargo molecules and, in this way, rescues ubiquitin molecules from degradation and restores cellular ubiquitin homeostasis.

Recently, Tsg101 appeared to be essential for Hiv-1 and Ebola virus budding from the cell surface [60–62]. Short peptide motifs in the Hiv-1 Gag late domain and Ebola virus matrix proteins recruit Tsg101 to sites of particle assembly and a short, EbVp40-derived Tsg101-binding peptide sequence (PTAP) can functionally substitute for the Hiv-1 Gag late domain. This finding reveals functional homology between the process of inward vesicle budding as in MVBs and virus budding at the cell surface. Morphological studies have clearly illustrated that both Vps4 and Tsg101 are involved in the final step of virus release. To explain the nature of Tsg101 as a tumor suppressor, it would mean that a last step in MVB vesicle budding is still reversible, as a defective Tsg101 increases the number of growth factor receptors available for signalling [63].

Together, the ubiquitin system appears to regulate the transport/sorting of many membrane proteins both in the biosynthetic, endocytic pathway, and in virus budding. Monoubiquitylation and/or alternative linked polyubiquitin chains might be a common theme. More importantly, regulation of the trafficking of membrane proteins is highly specific due to the versatility of E3 enzymes involved.

3. Growth hormone receptor (GHR) function, controlled by the ubiquitin system

The number of GHRs per cell are regulated and this feature plays a major role in the growth hormone (GH) responsiveness of the body. The availability of membrane proteins at the cell surface can be regulated at different locations within the cell: (1) The amount of protein synthesized in the ER is largely controlled by gene transcription. In addition, the ER quality control system regulates the exiting of properly folded proteins from the ER. (2) In the TGN, proteins can either be diverted directly to the lysosomes or be transported to the cell surface. (3) At the plasma membrane, the endocytic machinery can select proteins for endocytosis via clathrin-coated pits or proteins may be subject to proteolysis, resulting in shedding of the extracellular domain (ECD). (4) In endosomes, internalized proteins either recycle back to the plasma membrane or are targeted to the lysosome for degradation. At each of these cellular locations the ubiquitin–proteasome pathway can specifically regulate protein levels via different mechanisms. In transfected Chinese hamster lung cells, GHR availability is determined by three factors: endocytosis (75%), shedding (10%), and other undetermined mechanisms (15%) [64]. Thus, the number of GHRs at the cell surface is mainly regulated by the ubiquitin–proteasome pathway.

Three features render the GHR unique compared to other receptors, signalling from the cell surface: (i) Endocytosis of the GHR is constitutive. This means that its residence time at the cell surface does not depend on the presence of ligand; upon arrival at the cell surface from the ER/Golgi complex, it is recruited into coated vesicles (like cargo receptors such as the low-density lipoprotein (LDL) receptor) and transported to lysosomes for degradation (unlike cargo receptors, which release their cargo in endosomes, and immediately return to the plasma membrane for the next round of endocytosis). (ii) Endocytosis of the GHR depends on an active ubiquitin system. We assume that this fact determines the average residence time at the cell surface. There is evidence from many clinical studies that in stress conditions the number of GHRs is decreased, due to rapid endocytosis [65].

Thus, the ubiquitin system is a key regulator of GHR internalization. Both genetic and molecular experiments show that GHR molecules accumulate at the plasma membrane if the ubiquitin system is inhibited [66]. Moreover, GHR ubiquitylation coincides with its recruitment into clathrin-coated pits [67]. Strikingly, ubiquitylation of the GHR itself is not required because replacement of all lysine residues by arginines in the GHR cytosolic tail does not inhibit internalization [68]. In our studies, we have identified the target of the ubiquitin system in the GHR cytosolic tail as a 10 amino-acid-long sequence, DSWVEFIELD, designated as the UbE motif for ubiquitin-dependent endocytosis [68]. This motif is also required for recruiting the GHR into the MVBs for lysosomal degradation [51,69]. Besides the ubiquitin-conjugating system, the 26S proteasome is involved in GHR downregulation. Proteasome inhibitors prevent both internalization of the GHR and endosome-to-lysosome transport [69,70]. The inhibitory effect is lost when the GHR is truncated beyond amino acid 369, suggesting that either the GHR or an associated protein must be degraded by the proteasome before internalization can occur [51,70]. Using a truncated receptor, it has been demonstrated that proteasome inhibitors force the GHR into recycling mode comparable to the trafficking route of cargo receptors. [51,69]. From these data, it is clear that the ubiquitin–proteasome system regulates the turnover of the GHR in two distinct events: internalization from the plasma membrane and transport from endosomes to lysosomes. There is a remarkable difference between the involvement of the ubiquitin system in GHR trafficking and of other membrane proteins: in most, if not all, systems reported, the activity of

the ubiquitin system is preceded by protein phosphorylation. For Ste2p and most permeases in yeast, phosphorylation of serine or threonine is required before the E3 Rsp5 can initiate their uptake [71,72]. In mammalian cells, uptake of ENaC requires serine phosphorylation before the WW domain of Nedd4 can bind to the tetrameric sodium channel (Fig. 1). Degradation via the RING motif $SCF^{\beta-TrCP}$ E3 requires previous phosphorylation as documented for NFκB, β-catenin, Hiv-1 protein vpu, cyclins, myo-D, while c-Cbl stimulates degradation of tyrosine kinase receptors only if their kinase domain is active. As the signal transduction pathway of GHR (via Jak2) is not involved in ubiquitin system-dependent endocytosis [73], and no other phosphorylation has ever been observed, it is likely that in this case the ubiquitin system acts directly on the GHR, raising the third unique point: (iii) The ubiquitin system acts as primary regulator in GHR trafficking. Until now only the interaction p53–Mdm2 seems to be a direct protein–protein interaction, independent of phosphorylation, implicating that the concentrations of these proteins in cytosol and/or nucleus is a major factor in controlling cellular life and death. Whether availability of GHRs at the cell surface is subjected to the same kind of mechanism remains to be elucidated.

4. GHR dimerization and ubiquitylation, a functional connection

To be functional, most proteins occur as complexes. Complex formation of membrane proteins can either be structural or part of functional regulation. In general, structural complex formation is part of the maturation process and takes place in the ER. Functional complex formation often occurs at the place of action; e.g. ligand binding might induce signalling receptors to dimerize. Based on structural studies, GH-induced dimerization of the GHR has long been the accepted model for activation [74]. According to this model, GH-induced dimerization of the GHR brings two Jak2 molecules in close proximity, thereby facilitating *trans*-phosphorylation of tyrosine residues in the kinase domain of the paired Jak2 [75]. Subsequently, these activated Jak2 molecules phosphorylate tyrosine residues of the GHR and signalling molecules. In general, PY residues serve as docking sites for proteins with Src homology 2 or phosphotyrosine binding domains. However, from a mechanistical point of view, there are several advantages in an alternative mechanism in which GH activates a preformed GHR dimer. It could considerably increase signalling rate and lower the signalling threshold of GH concentration in the circulation. Recently, we obtained evidence in favor of this alternative activation mechanism [76]. GHRs form dimers immediately after their synthesis in the ER, and these dimeric receptors continue to the cell surface. We used sucrose velocity gradient centrifugation combined with short pulse-labelling to show that indeed complex formation is ER-located. The ER probably offers the best environment due to the high concentration of chaperones and of GHR precursor polypeptides as multiple GHRs are inserted in close proximity into the ER membrane by GHR mRNA-programmed polyribosomes. Which ER chaperones play a role in dimerization remains to be determined. Previously, Ross and colleagues have shown that the number of binding sites for GH increases upon incubation with a monoclonal antibody directed against GHR subdomain 2, presumably by preventing dimer formation [77]. A similar increase was observed when the monovalent GH antagonist B2036 was used. They also suggested that the antagonist binds to a preformed dimer in the absence of the monoclonal antibody. Consistent with this finding, cross-linking of either B2036 or GH to the GHR revealed a similar-sized complex consisting of a single GH and two GHR molecules [64].

Ligand-independent oligomerization has been reported for various membrane proteins. In Table 1 the features of a selected number of membrane receptors are collected. Preformed dimers of the EpoR [78], the EGFR [79] and the MT1R and MT2R [80], are activated through a ligand-induced conformational change. Interestingly, in many cases the transmembrane domain (TMD) is sufficient for di- or oligomerization. Dimerization of glycophorin A (GpA) is currently one of the best-characterized examples. GpA is the most abundant sialoglycoprotein in human erythrocyte membranes and the formation of non-covalent SDS-stable dimers facilitated these studies considerably. In the GpA TMD a dimerization motif L75IXXGVXXGVXXT87 (L, leucine; I, isoleucine; X, any amino acid; G, glycine; V, valine; T, threonine) has been identified [81]. Nuclear magnetic resonance spectroscopy confirmed this by positioning the specific residues at the site of interaction [82]. The right-handed parallel TMD α-helices cross at an angle of −40° and form a supercoil with a closely packed interface, e.g. a ridges-into-grooves structure. The GXXXG motif is the core of the dimerization interface as the glycines stabilize the structure through Van der Waals interactions with residues on the opposite helix [83] and their mutation to alanine affects dimerization the most

Table 1
Ligand-independent oligomerization of selected membrane proteins

Protein	Function	Type of oligomerization	Interact. domain	Reference
GHR	metabol regulation	homo-dimer	TMD	[76]
EpoR	growth control	homo-dimer	TMD	[78]
LeptinR	metabol regulation	homo-dimer	n.d.	[93]
EGFR (ErbB1)	growth control	homo/hetero-dimer	TMD	[79]
ErbB2	growth control	homo/hetero-dimer	TMD	[94]
MHC class II	antigen presentation	hetero-dimer	TMD	[95]
TGFβ type I and II R	growth control	homo-di-/hetero-tetramer	n.d.	[96]
MT1R or MT2R	G-coupled receptor	homo-/hetero-dimer	n.d.	[80]
TCRα and CD3δ	immune response	hetero-oligomer	TMD	[97]
TNF p60 and p80 R	cytokine receptor	homo-trimer	ECD	[98]
GpA	struct. prot. erythrocyte	homo-dimer	TMD	[99]

EPO, erythropoietin; TGF, transforming growth factor; MT1R melatonin receptor 1; MT2R, melatonin receptor 2; TCR, T cell receptor; TNF, tumor necrosis factor; n.d., not determined.

[84]. Genetic and statistical data suggest that the GXXXG motif is a common framework for TMD helix–helix interactions [85]. The GpA TMD is a transferable dimerization unit that can dimerize and thereby activate the bacterial ToxR transcription activator [86] or induce transcytosis of the polymeric immunoglobulin receptor from basolateral to the apical membrane [87].

The ligand-independent dimerization of the GHR is not just an advantage for signal transduction, it appears also a condition for ubiquitin system-dependent endocytosis of this receptor [76]. We showed that monomeric GHRs were internalized rapidly and independent of an active ubiquitin conjugation system using the Chinese hamster lung cell line (CH-ts20) with a temperature-sensitive ubiquitin activating enzyme. The underlying mechanism for the different mode of internalization of monomeric and dimeric receptors is still unclear. Apparently, the ubiquitylation machinery only recognizes dimeric receptors. Alternatively, the ubiquitin–proteasome system may be required to degrade a protein that associates with and prevents the endocytosis of dimeric GHRs. To our knowledge, no other membrane protein has been described which is controlled by the ubiquitin system in a conformational-dependent fashion.

5. Mechanism of ligand-independent dimerization

How is dimerization achieved in the ER? For GHR, a role for the cytoplasmic domain is unlikely because elimination of 97% of the cytoplasmic domain does not affect the heterodimerization of the truncated GHR with full length GHR ([88] and J.G., unpublished results). An increasing number of membrane proteins has been reported to be mediated via their TMD) [89]. We investigated this possibility by mutating single and multiple amino acids in the TMD to alanine residues. In addition, the GHR TMD was replaced by heterologous TMDs known for their effect on protein dimerization. Because none of the mutations disrupted GHR dimerization, we conclude that the GHR TMD lacks specific interaction capacity. A role for amino acids in the membrane-proximal subdomain 2 of the ECD has been proposed based on crystallographic data [74]. Surprisingly, also mutations of these amino acids did not affect dimerization. Possibly, the single mutations are not sufficient to disturb the extensive interaction between the GHRs. In contrast, replacement of the complete GHR ECD with a part of the LDL receptor-related protein resulted in monomeric chimers [76]. Most likely, the ECD is only required for the initial contact between the GHRs, because protease-digestion of the ECD of cell surface-localized (already dimerized) GHRs does not disrupt dimerization of the membrane-bound remnant proteins. Once brought into close contact, dimerization is probably maintained via the weakly interacting TMDs.

The mechanism of GHR dimerization differs from that of the homologous EpoR. The EpoR TMD is sufficient to mediate ligand-independent dimer formation [90]. The EpoR TMDs interact strongly, in contrast to that of GHR and prolactin receptor (PrlR) [90]. Interestingly, there is only limited interaction between EpoR ECDs, whereas both GHR [74] and PrlR ECDs [91] appear to interact extensively. Therefore, EpoR dimerization requires a strong interaction between the TMDs, whereas GHR and probably PrlR use their ECD rather than TMD to induce dimerization.

6. Initiation of signal transduction

Although the GHR dimerizes in the absence of GH, signal transduction pathways are only activated upon GH binding. The activation upon GH binding most likely involves a conformational change of the GHR, which results in the activation of Jak2 tyrosine kinase at the cytoplasmic side. Mutations of amino acids of subdomain 2 prevent this GH-induced structural reorganization and yield signalling-deficient GHR mutants. Because GH binds the GHRs sequentially [92], the presence of preformed dimers offers an advantage for rapid signalling as no time is lost for recruiting a second GHR. This would be especially advantageous in cells with low GHR levels.

7. Concluding remarks

The number of functional GHRs at the cell surface can now be understood in terms of a balance between synthesis and degradation. Both processes add their special features: functionality is only achieved if the receptors appear as dimers at the cell surface, and only dimers obtain a special ticket to be endocytosed by the ubiquitin system. This procedure warrants that the number of receptors can be tightly and swiftly regulated upon changed in the metabolic status of the cell; it also excludes non-dimerized receptors from taking part in the signalling process. These features render the GHR unique, and the rationale is important to understand its role as a regulator of cellular metabolism. Although many aspects of the molecular mechanism of GHR downregulation have been established, the precise mechanisms by which the link with the ubiquitin–proteasome system is achieved remain to be resolved. Future work will need to establish the relative importance of the conformational changes inducing the Jak2 activation and signal transduction. It will also be crucial to obtain structural information on a range of different GHR constructs to see how flexible the domain structures are, as well as establishing the precise location and density of the interacting domains to enable dimerization. In addition, it will be necessary to establish which ER chaperones are involved.

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