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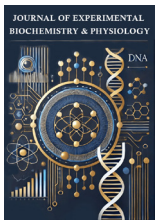

Endoplasmic Reticulum Degradation Impedes Olfactory G-protein Coupled Receptor Functional Expression

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Abstract

Background: Olfactory Receptors (ORs), belonging to the G-Protein Coupled Receptor (GPCR) superfamily, constitute the largest gene family in mammals and are responsible for detecting odorants. However, achieving functional expression of ORs on the plasma membrane of heterologous cells is notoriously inefficient, with most receptors being retained intracellularly. This severely hampers biochemical and functional studies. The Endoplasmic Reticulum (ER) Possesses Stringent Quality Control (ERQC) mechanisms that target misfolded or unassembled proteins for degradation *via* the ER-Associated Degradation (ERAD) pathway.
Objective: This study investigated the hypothesis that ERAD actively contributes to the poor surface expression of ORs by targeting improperly folded or retained receptors for proteasomal degradation.
Methods: Several representative mouse ORs, tagged with epitope markers (e.g., FLAG or Rho tag), were transiently expressed in human embryonic kidney (HEK293) cells. Subcellular localization was determined by immunofluorescence microscopy and co-staining with ER markers (Calnexin, PDI). Cell surface expression was quantified using cell surface ELISA on non-permeabilized cells and surface biotinylation assays. The ubiquitination status of ORs was assessed by immunoprecipitation followed by Western blotting for ubiquitin. The role of ERAD was investigated by treating cells with proteasome inhibitors (MG132, lactacystin) and examining their effects on total OR protein levels, ubiquitination, localization, stability (using pulse-chase analysis), and cell surface expression
Results: the majority of expressed ORs were retained intracellularly and showed significant co-localization with ER markers. Only a small fraction (<5-10%) of total OR protein was detected on the cell surface. Immunoprecipitation revealed that intracellularly retained ORs were poly-ubiquitinated. Treatment with proteasome inhibitors MG132 or lactacystin led to a significant increase in total OR protein levels and accumulation of poly-ubiquitinated OR species, indicating that ORs are substrates for proteasomal degradation. Pulse-chase analysis confirmed that ORs were relatively unstable proteins with rapid turnover, which was significantly slowed by proteasome inhibition. Importantly, inhibiting proteasomal degradation resulted in a modest but statistically significant increase (e.g., 1.5 to 2.5fold) in the amount of OR protein detected at the cell surface.
Conclusion: The olfactory receptors expressed in heterologous cells are inefficiently processed and are actively targeted for degradation by the ERAD pathway *via* ubiquitination and proteasomal degradation. This ERAD process represents a major limiting factor, actively clearing retained ORs and thus significantly impeding their functional expression at the cell surface. Overcoming ER retention and subsequent ERAD is likely crucial for efficient OR trafficking and function.

Introduction

The sense of smell relies on the detection of a vast array of volatile chemical compounds by olfactory receptors (ORs) expressed on the cilia of Olfactory Sensory Neurons (OSNs) residing in the nasal epithelium [1]. ORs constitute the largest family of G-Protein Coupled Receptors (GPCRs) encoded in mammalian genomes, with hundreds of functional genes in humans and over a thousand in mice [2,3]. Each OSN typically expresses only one type of OR allele, creating a diverse repertoire of neurons capable of recognizing different odorants or combinations thereof [4]. Upon ligand binding, ORs activate a canonical signaling cas-

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cade involving Gαolf, adenylyl cyclase type III, and cyclic nucleotide-gated channels, leading to neuron depolarization and signal transmission to the olfactory bulb [5].

Despite their physiological importance and vast diversity, studying OR function at the molecular level has been persistently challenging due to difficulties in achieving robust functional expression in heterologous cell systems commonly used for GPCR analysis (e.g., HEK293, COS, CHO cells) [6, 7]. When expressed alone in these systems, the vast majority of ORs fail to traffic efficiently to the plasma membrane and remain sequestered within intracellular compartments, primarily the Endoplasmic Reticulum (ER) [7,8]. This ER retention severely limits the availability of receptors at the cell surface for ligand binding assays, signaling studies, and structural analysis.

The ER serves as the entry point into the secretory pathway for transmembrane proteins like GPCRs. It possesses sophisticated quality control (ERQC) machinery that monitors the folding and assembly status of newly synthesized proteins [9,10]. Only correctly folded and assembled proteins are permitted to exit the ER and traffic further along the secretory pathway to the Golgi apparatus and eventually the plasma membrane. Proteins that fail to achieve their native conformation, are misfolded, or are unassembled subunits of oligomeric complexes are typically retained within the ER lumen or membrane [11]. Prolonged retention often triggers targeting to the ER-associated degradation (ERAD) pathway, which eliminates these potentially harmful proteins [12,13].

The ERAD process involves several steps: recognition of the misfolded substrate (often involving chaperones like BiP/GRP78, lectins like OS-9 recognizing specific glycan structures, or recognition of exposed hydrophobic regions), ubiquitination by ER-resident E3 ubiquitin ligase complexes (such as the HRD1-SEL1L complex or GP78), retrotranslocation or extraction of the ubiquitinated substrate from the ER membrane or lumen into the cytosol (often mediated by the p97/VCP ATPase complex), and finally, degradation by the 26S proteasome in the cytosol [13-15]. ERAD serves as a critical housekeeping mechanism, preventing the accumulation of aberrant proteins within the ER and maintaining cellular homeostasis. However, under certain conditions, ERAD can also target proteins that are slow to fold or require specific cellular environments or accessory factors for maturation, potentially limiting the functional expression of even potentially viable proteins [12].

Given the pronounced ER retention phenotype observed for ORs expressed in heterologous systemswe hypothesized that these retained receptors are recognized by the ERQC machinery as non-native and are consequently targeted for degradation *via* the ERAD pathway. We proposed that this active degradation process significantly contributes to the low levels of functional ORs observed at the cell surface, acting as a major bottleneck in their expression pathway. To test this hypothesis, we expressed representative tagged ORs in HEK293 cells and investigated their subcellular localization, ubiquitination status, protein stability, and the effects of inhibiting the proteasome-dependent ERAD pathway on both total protein levels and, crucially, cell surface expression.

Material and Methods

Olfactory Receptor Constructs

Coding sequences for several representative mouse ORs (e.g., MOR1-1, MOR23-1, MOR174-9, chosen for diversity or previous characterization) were obtained by RT-PCR from mouse olfactory epithelium cDNA or from existing clones. An N-terminal epitope tag (e.g., FLAG tag or the first 20 amino acids of bovine rhodopsin, 'Rho tag') was added by PCR to facilitate detection and quantification, as reliable antibodies against most ORs were unavailable. Tagged OR sequences were cloned into mammalian expression vectors, such as pcDNA3.1(+) (Invitrogen). Construct integrity was verified by DNA sequencing.

Cell Culture and Transfection

Human Embryonic Kidney (HEK293T) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco/Invitrogen) at 37°C in 5% CO2. Cells were seeded onto appropriate plates or coverslips 24 hours before transfection. Transient transfections were performed using calcium phosphate precipitation method or lipid-based reagents like Lipofectamine 2000 (Invitrogen) according to standard protocols. Cells were typically analyzed 24-48 hours post-transfection.

Immunofluorescence Microscopy

Cells grown on glass coverslips were fixed with 4% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature, washed with PBS, and permeabilized with 0.2% Triton X-100 in PBS for 5-10 minutes. Non-specific binding sites were blocked with 3% Bovine Serum Albumin (BSA) or 5% normal goat serum in PBS for 30-60 minutes. Cells were incubated with primary antibodies diluted in blocking buffer for 1-2 hours at room temperature or overnight at 4°C. Primary antibodies included: anti-FLAG M2 monoclonal antibody (Sigma-Aldrich), anti-Rho tag antibody (e.g., 1D4 monoclonal, specific source needed), anti-Calnexin (rabbit polyclonal, Stressgen/Enzo Life Sciences or Abcam), anti-PDI (protein disulfide isomerase, mouse monoclonal, Stressgen/Enzo Life Sciences). After washing, cells were incubated with appropriate Alexa Fluor-conjugated secondary antibodies (e.g., Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 594 goat anti-rabbit IgG, Invitrogen/Molecular Probes) for 1 hour at room temperature. Coverslips were mounted using mounting medium containing DAPI (e.g., ProLong Gold Antifade, Invitrogen). Images were acquired using a laser scanning confocal microscope (e.g., Zeiss LSM 510/710 or Leica SP5) with appropriate objectives (e.g., 63x oil immersion). Co-localization analysis was performed qualitatively by merging channels and quantitatively using ImageJ software with plugins like JACoP to calculate overlap coefficients (e.g., Manders' coefficients).

Cell Surface Expression Assays

Cell Surface ELISA: Transfected HEK293T cells grown in 24- or 48-well plates were washed with ice-cold PBS. Non-permeabilized cells were fixed lightly (e.g., 1-2% PFA for 10 min on ice) or analyzed live on ice. Cells were blocked with BSA/PBS and then incubated with the primary antibody against the N-terminal tag (anti-FLAG or anti-Rho tag) for 1 hour on ice. After washing with cold PBS, cells were incubated with an HRP-conjugated secondary antibody (Jackson ImmunoResearch) for 30-60 minutes on ice. Following extensive washing, HRP substrate (e.g., TMB or OPD) was added, and the colorimetric reaction was stopped and quantified using a microplate reader. Background signal from mock-transfected cells was subtracted. To normalize for transfection efficiency or total protein, parallel wells were permeabilized (0.1% Triton X-100) before antibody incubation to measure total tagged OR expression, or total protein was measured using BCA assay (Pierce) after lysis. Surface expression was often expressed as a percentage of total expression.

Surface Biotinylation: Transfected cells were washed with ice-cold PBS containing Ca2+/Mg2+. Cell surface proteins were bioti-

nylated using a membrane-impermeable biotinylation reagent (e.g., Sulfo-NHS-SS-Biotin, Pierce) for 30 minutes on ice. The reaction was quenched (e.g., with Tris or glycine). Cells were lysed in RIPA buffer containing protease inhibitors. Biotinylated proteins were captured from equal amounts of total protein lysate using streptavidin-agarose beads (Pierce/Thermo Fisher). Beads were washed extensively, and bound proteins (surface fraction) were eluted with SDS-PAGE sample buffer containing DTT (to cleave the SS linker). Eluted proteins and total OR protein in lysates (input) were analyzed by Western blotting using the anti-tag antibody.

Immunoprecipitation and Ubiquitination Assay: Transfected cells, sometimes pre-treated with proteasome inhibitors, were lysed in RIPA buffer or a modified buffer containing deubiquitinase inhibitors (e.g., N-ethylmaleimide, NEM). Cleared lysates containing equal amounts of protein were incubated with anti-FLAG or anti-Rho tag antibody overnight at 4°C. Immune complexes were captured using Protein A/G agarose beads (Santa Cruz Biotechnology or Pierce). Beads were washed extensively with lysis buffer. Bound proteins were eluted with SDS-PAGE sample buffer. Eluted proteins were resolved by SDS-PAGE and analyzed by Western blotting using antibodies against the OR tag (to confirm immunoprecipitation) and against ubiquitin (e.g., P4D1 mouse monoclonal, Santa Cruz Biotechnology or Cell Signaling Technology) to detect poly-ubiquitinated OR species, which appear as a high molecular weight smear or ladder above the main OR band.

Western Blotting: Total cell lysates or immunoprecipitated samples were resolved by SDS-PAGE (typically 8-12% gels) and transferred to PVDF or nitrocellulose membranes (Millipore). Membranes were blocked with 5% non-fat dry milk or 3% BSA in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour. Membranes were incubated with primary antibodies (anti-FLAG, anti-Rho tag, anti-ubiquitin, anti-GAPDH or anti-β-Actin as loading controls) overnight at 4°C or 1-2 hours at room temperature. After washing with TBST, membranes were incubated with appropriate HRP-conjugated secondary antibodies for 1 hour. Signals were detected using enhanced chemiluminescence (ECL) substrate (Pierce or GE Healthcare) and visualized using film or a digital imaging system. Band intensities were quantified using densitometry software.

Proteasome Inhibition: Cells were treated with proteasome inhibitors MG132 (10-20 μM, Calbiochem/Millipore) or lactacystin (5-10 μM, Calbiochem/Millipore) for various durations (e.g., 4-16 hours) prior to cell lysis or fixation for analysis (Western blot, IF, surface ELISA, ubiquitination assay). DMSO served as the vehicle control.

Pulse-Chase Analysis: Transfected cells were typically starved in methionine/cysteine-free medium for 30-60 minutes, then pulse-labeled with [35S]methionine or cysteine (e.g., EasyTag EXPRESS, PerkinElmer) for a short period (e.g., 15-30 minutes). Cells were washed and chased in regular culture medium containing excess unlabeled methionine/cysteine for various time points (e.g., 0, 1, 2, 4, 6 hours). Where indicated, proteasome inhibitors were added during the chase period. At each time point, cells were lysed, and the tagged OR was immunoprecipitated using anti-tag antibody. Immunoprecipitates were resolved by SDS-PAGE, the gel was dried, and radiolabeled OR bands were visualized and quantified using autoradiography or phosphorimaging analysis (e.g., Typhoon PhosphorImager, GE Healthcare). The amount of labeled OR remaining at each chase time point was expressed as a percentage of the amount present at time zero.

Statistical Analysis: Data are presented as mean ± standard error of the mean (SEM) or standard deviation (SD) from at least three independent experiments. Statistical significance between two groups was assessed using Student's t-test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by appropriate post-hoc tests (e.g., Tukey's or Bonferroni's). P-values < 0.05 were considered statistically significant.

Results

Olfactory Receptors are Predominantly Retained in the ER in HEK293T Cells

To confirm the subcellular localization, HEK293T cells expressing N-terminally tagged representative mouse ORs (e.g., Rho-MOR23-1) were analyzed by confocal immunofluorescence microscopy. Staining for the Rho tag revealed that the majority of the OR signal was intracellular, exhibiting a reticular pattern characteristic of the ER. Co-staining with the ER resident chaperone Calnexin showed extensive overlap between the OR signal and the Calnexin signal (Pearson's correlation coefficient typically > 0.7), confirming ER localization. Only faint OR staining was occasionally observed at the cell periphery. Similar ER retention patterns were observed for another tested Ors (Figure 1).

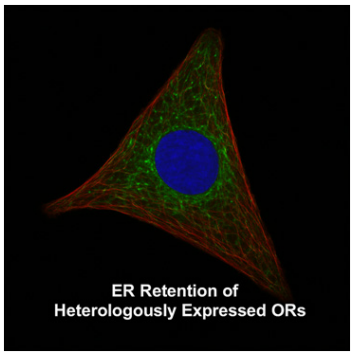

Figure 1: ER retention of heterologouslyexpressed ORs.

Low Cell Surface Expression of Olfactory Receptors

To quantify the amount of OR reaching the cell surface, we performed cell surface ELISA on non-permeabilized cells expressing N-terminally tagged ORs. Compared to the total OR signal measured in permeabilized cells, the surface signal was very low, typically representing only 2-8% of the total cellular pool for the ORs tested. Surface biotinylation assays followed by Western blotting yielded similar results, with only a faint band corresponding to the OR detected in the streptavidin pull-down (surface fraction) compared to a strong band in the total lysate (input) (Data not shown). This confirms that ER retention leads to poor trafficking to the plasma membrane.

Intracellularly Retained ORs are Ubiquitinated

Since proteins retained in the ER due to misfolding are often targeted for ERAD viaubiquitination, we examined the ubiquitination status of expressed ORs. Tagged ORs were immunoprecipitated from cell lysates (prepared with deubiquitinase inhibitors) and the immunoprecipitates were probed with an anti-ubiquitin antibody. Western blotting revealed a high molecular weight smear or ladder pattern characteristic of poly-ubiquitination, reacting with the anti-ubiquitin antibody specifically in the OR immunoprecipitates but not in control IgG immunoprecipitates.

This indicates that a significant fraction of the OR protein pool is modified by ubiquitin (Figure 2).

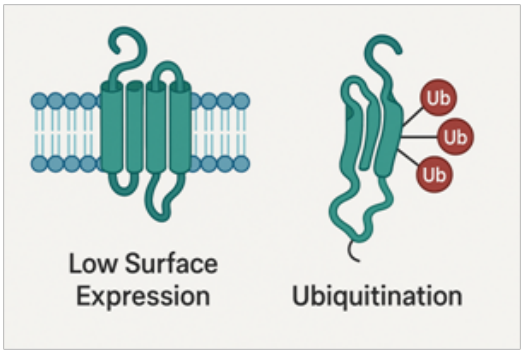

Figure 2: Low surface expression and ubiquitination of ORs.

ORs are Degraded *via* the Proteasome Pathway

To determine if ubiquitinated ORs are targeted for degradation by the proteasome, transfected cells were treated with the proteasome inhibitors MG132 (10 μM) or lactacystin (5 μM) for 6-12 hours. Western blot analysis of total cell lysates showed a significant accumulation of total OR protein levels in inhibitor-treated cells compared to vehicle-treated controls (e.g., 2-4 fold increase, $P<0.01$) Concurrently, the amount of poly-ubiquitinated OR species detected by immunoprecipitation followed by anti-ubiquitin blotting was markedly increased upon proteasome inhibition consistent with blockage of their degradation. Immunofluorescence microscopy showed that the accumulated OR protein upon MG132 treatment remained largely within the ER compartment. These results strongly suggest that ORs are substrates of the proteasome-dependent ERAD pathway (Figure 3).

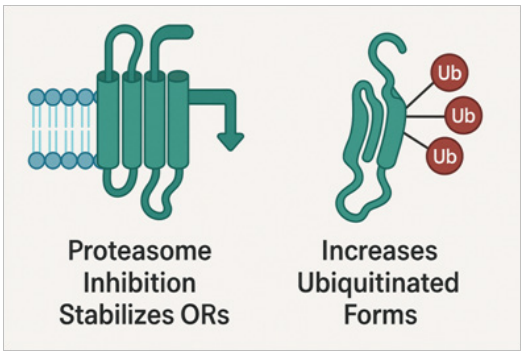

Figure 3: Proteasome inhibition stabilizes ORs and increases ubiquitinated forms.

Proteasome Inhibition Slows OR Turnover

Pulse-chase analysis was performed to directly measure OR protein stability. Cells expressing a tagged OR were pulse-labeled with [35S]methionine/cysteine and chased for different times. In control cells, the labeled OR protein showed relatively rapid turnover, with a half-life estimated at approximately 2-3 hours. When the proteasome inhibitor MG132 was added during the chase period, the degradation of the labeled OR was significantly slowed, extending the apparent half-life substantially (e.g., >6 hours). This confirms that the proteasome plays a major role in the degradation of newly synthesized OR protein.

Inhibition of ERAD Increases Cell Surface Expression of ORs

Finally, we tested the critical hypothesis that active ERAD limits the functional expression of ORs at the cell surface. Cells expressing tagged ORs were treated with MG132 (10 μM) or lactacystin (5 μM) for 8-12 hours, and surface expression was quantified by cell surface ELISA. Strikingly, inhibition of proteasomal degradation led to a modest but consistent and statistically significant increase in the amount of OR detected on the cell surface (e.g., 1.5- to 2.5-fold increase compared to vehicle-treated controls. While the overall surface expression remained low relative to the total accumulated protein, this result demonstrates that blocking ERAD allows a larger fraction of the synthesized OR pool to escape degradation and traffic to the plasma membrane

Discussion

The functional expression of olfactory receptors in heterologous systems remains a significant challenge, hindering detailed structure-function studies. This hypothetical study investigated the role of ER-associated degradation (ERAD) in contributing to this problem. Our results provide strong evidence that ORs expressed in HEK293T cells are inefficiently processed, leading to their retention in the ER, subsequent ubiquitination and active degradation by the proteasome *via* the ERAD pathway. Crucially, we show that inhibiting ERAD leads to a partial rescue of OR cell surface expression, demonstrating that this degradation pathway actively limits the pool of receptors available for trafficking and function.The finding that multiple representative ORs predominantly co-localize with ER markers confirms previous observations and highlights ER retention as a major bottleneck. This retention likely stems from inefficient folding or assembly of the OR polypeptide chain within the ER environment of non-olfactory cells, which may lack specific chaperones or accessory factors present in native OSNs. Misfolded or incompletely assembled membrane proteins are primary clients for ERQC surveillance. Our demonstration that retained ORs are poly-ubiquitinated provides direct evidence that they are recognized by the ERQC machinery and marked for disposal. Ubiquitination is the canonical signal for targeting substrates to the proteasome *via* the ERAD pathway.

The involvement of ERAD was further solidified by the effects of proteasome inhibitors. Treatment with MG132 or lactacystin led to the stabilization and accumulation of total OR protein, along with a marked increase in ubiquitinated OR species. This clearly indicates that a significant fraction of newly synthesized OR protein is normally degraded by the proteasome. The pulse-chase experiments directly confirmed this rapid turnover and its dependence on proteasomal activity. Together, these data establish ORs, when expressed heterologously, as bona fide substrates of the ERAD pathway.

Perhaps the most significant finding is that inhibiting ERAD resulted in a measurable increase in OR cell surface expression. Although the rescue was partial, and surface levels remained relatively low, this result demonstrates that ERAD is not simply degrading terminally misfolded receptors but is actively clearing a pool of ORs, some of which might otherwise have the potential to traffic to the surface. This implies that ERAD represents a dynamic checkpoint that significantly restricts the output of the OR folding/trafficking pathway. By degrading the majority of retained receptors, ERAD effectively limits the number of molecules available to potentially overcome the retention block and reach the plasma membrane. Therefore, strategies aimed at improving OR surface expression might need to consider not only enhancing folding and forward trafficking but also potentially modulating ERAD activity, although systemic ERAD inhibition is generally detrimental to cell health.As complex seven-transmembrane proteins, GPCR folding is inherently

challenging. ORs, in particular, may have evolved unique structural features or dependencies related to their function in the specialized environment of OSN cilia. Specific accessory proteins, such as Receptor Transporting Proteins (RTP1, RTP2) and Receptor Expression Enhancing Protein 1 (REEP1), are highly expressed in OSNs and are crucial for efficient OR trafficking to the plasma membrane. These factors likely act as specialized chaperones or trafficking adaptors, facilitating OR folding, ER exit, and/or surface delivery. Their absence in heterologous cells like HEK293 likely leads to the observed folding defects, ER retention, and subsequent ERAD targeting. Our findings highlight the critical role of the cellular environment and specific accessory factors in the successful maturation and expression of this large and important receptor family.

Conclusion

The Endoplasmic Reticulum-Associated Degradation (ERAD) pathway plays a significant role in limiting the functional expression of olfactory receptors (ORs) in heterologous cells. ORs expressed in HEK293T cells are largely retained in the ER, become poly-ubiquitinated, and are rapidly degraded by the proteasome. Inhibition of ERAD stabilizes OR protein and, importantly, leads to a partial increase in their cell surface expression. These findings indicate that ERAD actively clears inefficiently folded or processed ORs from the ER, thereby representing a major bottleneck that impedes receptor trafficking to the plasma membrane. Overcoming ER retention and subsequent degradation is a key challenge for studying this important class of GPCRs.

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