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A Specific Heat Shock Protein Enhances the Expression of Mammalian Olfactory Receptor Proteins

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Abstract

Multiple trials failed to express significant amounts of olfactory receptors in heterologous cells as they are typically retained in the endoplasmic reticulum (ER). Evidence is accumulating that cell-type–specific accessory proteins regulate the folding of olfactory receptors, their exit from the ER, and the trafficking to the plasma membrane of the olfactory cilia where the receptors gain access to odorants. We found Hsc70t, a testis-enriched variant of the Hsp70 family of heat shock proteins which is specifically expressed in post-meiotic germ cells, in the olfactory epithelium of mouse and human. Cotransfected HEK293 cells with Hsc70t and different green fluorescent protein–tagged odorant receptors (ORs) from mouse and man showed a significantly enhanced OR expression. Hsc70t expression also changed the amount of cells functionally expressing olfactory receptors at the cell surface as the number of cells responding to odorants in Ca^{2+}-imaging experiments significantly increased. Our results show that Hsc70t helps expression of ORs in heterologous cell systems and helped the characterization of an "orphan" human olfactory receptor.

Key words: heat shock protein, heterologous expression, olfactory receptor

Introduction

The sense of smell detects and discriminates among many thousands of volatile substances with a remarkably large subfamily of G-protein–coupled receptors (GPCRs). Humans have around 350 so-called odorant receptor (OR) genes with complete open reading frames, and mice have nearly 1000 OR proteins (Zozulya *et al.*, 2001; Zhang and Firestein, 2002). These receptors are expressed in the neurons of the olfactory epithelium, where they are concentrated at the plasma membrane of the cilia and at axon terminals (Barnea *et al.*, 2004; Feinstein *et al.*, 2004; Strotmann *et al.*, 2004). ORs also reside in tissues other than the olfactory epithelium as, for example, spermatogenic cells (Vanderhaeghen *et al.*, 1997), where at least some of the receptor proteins are localized to the sperm flagellar midpiece (Vanderhaeghen *et al.*, 1993) and function in sperm chemotaxis (Spehr *et al.*, 2003). Proper transport of ORs to the cell surface is critical for the receptors to recognize their ligands, and the transport machinery required for targeting receptors to the plasma membrane should therefore be present in all cell types that functionally express ORs.

In nonchemosensory cells used so far for heterologous expression of ORs, the proteins are typically retained in the endoplasmic reticulum (ER) despite multiple trials to improve expression (McClintock *et al.*, 1997; McClintock and Sammeta, 2003). Even expression of ORs in neuronal cell lines and primary neuronal cultures failed to result in plasma membrane localization of the OR protein (Gimelbrant and McClintock, 1997; McClintock *et al.*, 1997). Modifications of ORs have been used to improve trafficking to the plasma membrane of heterologous cells. N-termini of ORs were extended by addition of an N-terminal fragment of rhodopsin (Krautwurst *et al.*, 1998; Kajiya *et al.*, 2001) or by addition of a cleavable signal sequence (Wellerdieck *et al.*, 1997; Wetzel *et al.*, 1999; Gaillard *et al.*, 2002), and shuttle expression vectors were described that use the TM II–VII of an receptor of interest cloned between the N- and C-terminal regions of a relatively well-expressed OR (Spehr *et al.*, 2003). Although being successful in some cases, these methods still have the disadvantage of investigating not the native, but more or less modified versions of the proteins. Another attempt to circumvent the trafficking problem and obtain protein for functional investigations was the introduction of ORs into olfactory receptor neurons (ORNs). Using an adenoviral vector for expression allowed the identification of agonists for the mouse ORs I7 and MOR23 (Zhao *et al.*, 1998; Touhara *et al.*, 1999; Araneda *et al.*, 2004), and

imaging odor responses in ORNs from genetically engineered mice identified M71 agonists (Bozza *et al.*, 2002). These approaches were successful in ligand identification but are time consuming and can therefore not be used for large-scale investigations of ORs. Taken together, it seems that the folding and membrane trafficking requirements of olfactory receptors appear to be available only in mature olfactory sensory neurons and other specialized cell types where ORs are functionally expressed on the membrane, for example, mature spermatozoa.

Most GPCRs can be functionally expressed in heterologous cells, although there are exceptions as, for example, the calcitonin receptor–like receptor, which is only trafficking to the plasma membrane if a member of the receptor activity modifying protein family of proteins is present (McLatchie *et al.*, 1998). Other GPCRs traffic normally in heterologous cells but are known to interact with unrelated accessory proteins that modulate their trafficking. Adrenergic receptor trafficking is affected by interaction with EIF2Ba (Klein *et al.*, 1997) and opsin trafficking by prolyl *cis–trans* isomerases and related Ran-binding proteins (Ferreira *et al.*, 1996). These findings suggest that olfactory neurons and other chemosensory cells may also have a selective molecular machinery that promotes proper targeting of OR proteins to the cell surface. Some components of this machinery have recently been identified in *Caenorhabditis elegans* (Dwyer *et al.*, 1998, 2001) and in mice (Saito *et al.*, 2004). The transmembrane proteins RTP1, RTP2, and REEP1, which were found to be expressed specifically in olfactory neurons, promote functional cell-surface expression of ORs expressed in HEK293T cells and enhance the OR responses to odorants (Saito *et al.*, 2004).

In principle, membrane proteins in heterologous cells may be retained in the ER because of a lack of recognition as a cargo for ER export or because of misfolding (Ellgaard and Helenius, 2003). The sixth and seventh transmembrane domains of OR proteins seem to be important to prevent plasma membrane expression of the proteins (Gimelbrant *et al.*, 1999), indicating that intramolecular interactions are important for OR trafficking. Failures in the correct assembly of intramolecular interactions in heterologous cells are indicative of misfolding of the ORs, which could be caused by a requirement for special chaperone proteins. The cytosol and the ER contain molecular chaperones, which among other functions assist the folding process. One class of molecular chaperones are heat shock proteins (HSPs), which are ubiquitous proteins initially described to be upregulated in response to a change in the ambient temperature and in other conditions causing physiological stress. In mammals, the HSP70 family consists of HSP70 (inducible) and HSC70 (constitutive expressed) proteins, which are thought to bind to unfolded or incorrectly folded proteins assisting in their correct folding (Young *et al.*, 2004). An inducible member of the HSP70 family was found to characterize the response of olfactory tissue to heat shock and toxic substances within the nasal cavity of the rat (Simpson *et al.*, 2004, 2005). Hsc70t is a testis-enriched variant and is specifically expressed in the cytoplasm of spermatids during the last steps of spermatogenesis. The Hsc70t gene is located in the so-called major histocompatibility complex (MHC) region of chromosome 17 in mice and chromosome 6 in man, respectively (Ito *et al.*, 1998).

We found expression of this testis-enriched protein (Hsc70t) in the olfactory epithelium by a proteomics approach. Due to the fact that Hsc70t is specifically expressed in mature sperm and in olfactory epithelium, two cell types where OR proteins are present, we assumed that it might be involved in the folding or trafficking of these receptors. We investigated the role of this protein for the expression and folding of ORs and found that coexpression of Hsc70t with ORs leads to increased expression levels and functionality of the heterologous expression system.

Materials and methods

Protein analysis

For MudPIT protein expression analysis, mouse olfactory epithelium was homogenized with a tight-fitting pestle (60–70 strokes) in standard Ringer's solution with protease inhibitors (Roche complete protease inhibitor mixture) followed by sonification two times for 10 s at 40 W (Sonifier B12, Branson Sonic Power Co., Danbury, CT). Expression analysis via MudPIT, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and in-gel digestion was performed as described (Spehr *et al.*, 2004).

Reverse transcription–polymerase chain reaction

The RNAs of mouse and human testis tissue and of mouse and human olfactory epithelium were isolated with Trizol reagent (Invitrogen, Carlsbad, CA) and DNaseI digested before isolation of the polyA + mRNA by binding to oligo-dT–coated paramagnetic particles (Dynal, Oslo, Norway). cDNA was constructed by using the Moloney murine leukemia virus reverse transcriptase (Invitrogen) and oligo(dT12–18) primer. Amplifications were performed with about 1 ng cDNA and specific primer pairs for human and mouse Hsc70t, respectively.

DNA constructs and plasmids

Human (NM_005527) and mouse (NM_013558) Hsc70t were amplified from cDNA obtained from testis tissue by reverse transcription–polymerase chain reaction (RT–PCR) using specific primers which amplify the complete open reading frame and contain restriction sites for further subcloning (HS-Hsc70t-fw gcgaattcaccatggctactgccaagggaatcg, HS-Hsc70t-rw gctctagattaatctacttcttcaattgtggggcctgtgg, MM-Hsc70t-fw gcgaattcatggctgctaataaaggaatggcg, MM-Hsc70t-rw gctctagactaatctacttcctcgatggtagggcctgtgg). The PCR products were cloned in pcDNA3 and verified by sequencing.

Olfactory receptors were amplified from genomic DNA, which was isolated from blood using a commercial kit (Qiagen, Hilden, Germany). PCRs were done with 100 ng genomic DNA and specific primers for the human olfactory receptors 1F2, 1D2, 3A1, 52E2, 51I1, 2AG1, 52B4, 4C15 and mouse olfactory receptor I7. OR–green fluorescent protein (GFP) fusion constructs were generated by cloning the PCR products into a linearized GFP-containing plasmid with restriction sites for the generation of N-terminal fusion constructs (Neuhaus *et al.*, 2005).

Cell culture, transfection, and microscopy

HEK293 cells were maintained under standard conditions in a minimum essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and streptomycin, and 2 mM L-glutamine. Transfections were performed using a standard calcium phosphate precipitation technique. Cells were transfected with a 1:2 ratio of the different olfactory receptor–GFP constructs and Hsc70t or Gal4/empty plasmids (control). Two days after transfection, growth media were removed and replaced with standard Ringer solution. Cells were investigated for GFP expression with a Zeiss fluorescence microscope (Axiovert, Zeiss, Göttingen, Germany). Images were imported into Adobe Photoshop and adjusted for contrast.

Western blotting

Sample aliquots of fractionated HEK293 cells were mixed with Laemmli buffer (30% glycerol, 3% SDS, 125 mM Tris/Cl, pH 6.8), resolved by 10% SDS-PAGE, and transferred to nitrocellulose membrane (Protran; Schleicher & Schuell, Dassel, Germany). The nitrocellulose membranes were stained with Ponceau S (Sigma, Munich, Germany), blocked with TBST (150 mM NaCl, 50 mM Tris-Cl, Tween 20, pH 7.4) containing 5% nonfat dried milk (Biorad, Munich, Germany), and incubated with primary anti-GFP antibody (Abcam, Cambridge, UK) diluted in 3% dry milk in TBST. After washing and incubation with horseradish peroxidase–coupled secondary antibodies, detection was performed with ECL plus or ECL Advance (Amersham, Braunshweig, Germany) on Hyperfilm ECL (Amersham).

Single-cell Ca^{2+} imaging

Transfected HEK293 cells were incubated for 45 min in loading buffer (pH 7.4) containing Ringer solution, 7.5 μM Fura-2-AM (Molecular Probes, Leipzig, Germany), and 0.1% Pluronic F-127 (Sigma). After removal of extracellular Fura-2 by washing, ratiofluometric Ca^{2+} imaging was performed as described (Spehr *et al.*, 2003) using a Zeiss inverted microscope equipped for ratiometric imaging. Images were acquired in randomly selected fields of view, and integrated fluorescence ratios ($f340/f380$ ratio) were measured. Exposure to odorants (provided by Dr T. Gerke, Henkel KGaA, Düsseldorf, Germany) was accomplished using a specialized microcapillary application system. All odorants assayed for activation of OR2AG1 were tested in at least three different series of transfection series. Compounds regarded as ligands led to clear Ca^{2+} responses in several different experiments, whereas they did not elicit any Ca^{2+} signals in untransfected cells. The physiological state of the cells and their ability to produce phospholipase C–mediated Ca^{2+} signals was monitored by application of adenosine triphosphate (ATP) (200 μM) at the end of each experiment (Wetzel *et al.*, 1999).

Results

A testis-enriched HSP is expressed in the olfactory epithelium

We found expression of the testis-enriched protein Hsc70t in the mouse olfactory epithelium using a proteomics approach. Due to the fact that Hsc70t is known to be specifically expressed in mature mouse spermatozoa (Tsunekawa *et al.*, 1999), a cell type where also ORs are shown to be expressed (Fukuda *et al.*, 2004), we checked expression of Hsc70t in the olfactory epithelium by RT–PCR. Specific bands of ~1900 kD could be detected in the cDNAs from mouse and human olfactory epithelium under the same conditions that allowed amplification of the cDNAs from testis tissue (Figure 1).

Effect of Hsc70t on OR expression

Due to the fact that we found a spermatid-specific HSP in olfactory epithelium, we speculated that it might be involved in the proper folding or trafficking of OR proteins. We investigated the role of this protein for the expression ORs by coexpression of Hsc70t together with randomly selected GFP-tagged ORs in HEK293 cells. The coexpression of Hsc70t with ORs leads to an increased number of GFP-expressing cells compared to the control conditions (Figure 2A). The effect varied quantitatively for the different tested ORs, but for all tested receptors from mouse and human, we found an increased number of GFP-expressing HEK293

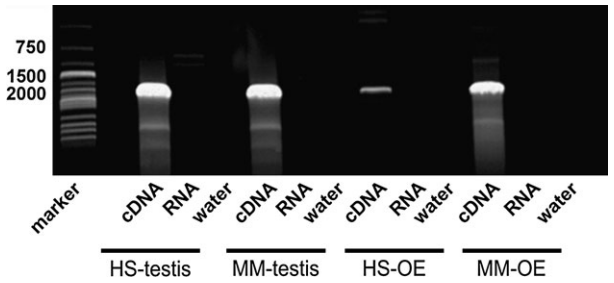
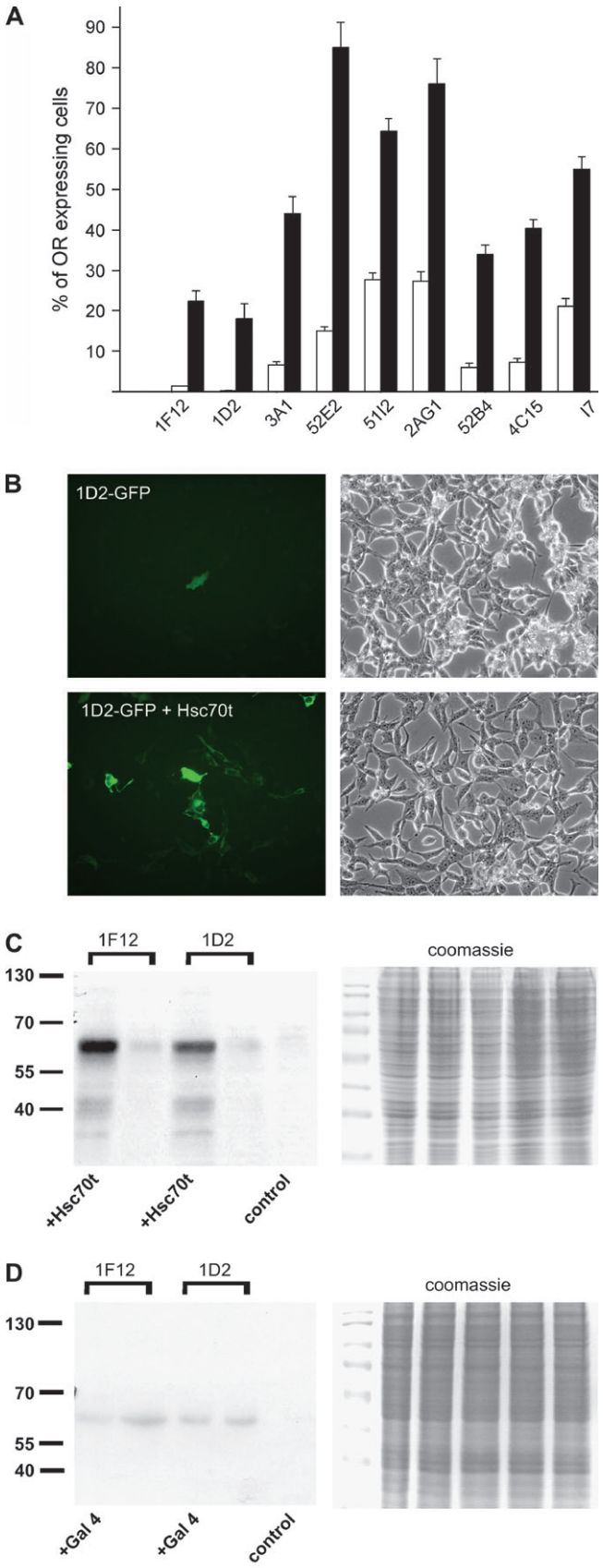


Figure 1 Hsc70t expression in the olfactory epithelium. RT–PCR from human and mouse testis and human and mouse olfactory epithelium. Shown is the PCR with synthesized cDNAs and with RNA without cDNA synthesis and water as control. Hsc70t can be detected in the testis of both, human and mouse, as expected. In addition, the cDNAs can be detected in the olfactory epithelium.



cells. The effect was remarkably strong for ORs which are known to be expressed in spermatozoa (OR1D2 = hOR17-4) (Figure 2B). To further confirm the increased expression level of the OR–GFP constructs, we performed western blot analysis. Coexpression of Hsc70t caused an increase in the amount of OR protein in the case of the olfactory receptors OR1F12 and OR1D2 (Figure 2C).

To control whether the increase in the number of OR-expressing cells upon cotransfection of Hsc70t is simply caused by an alteration of the transfection efficiency, we performed cotransfection with Gal4. Gal4 is also a purely cytosolic protein with a similar size as Hsc70t (2500 compared to 1900 nt), and it is well expressed in HEK293 cells, ruling out that a lack of effect is due to poor expression levels of Gal4. Moreover, it is cloned in the same expression plasmid as Hsc70t and the ORs. Western blot analysis showed that the expression levels of the OR1F12 and OR1D2 are not enhanced upon cotransfection of Gal4 (Figure 2D).

Enhanced OR function by Hsc70t

Poor odorant-evoked signaling activity in heterologous cell culture systems expressing ORs has been attributed to the poor cell-surface expression of ORs. To investigate whether Hsc70t alters the amount of functional ORs at the cell surface, we employed a Ca^{2+}-imaging assay to address this issue. We tested OR1D2-mediated Ca^{2+} responses when the receptor was coexpressed with Hsc70t protein in HEK293 cells and was activated by its ligand bourgeonal (Figure 3). We observed Ca^{2+} responses upon bourgeonal application in fewer than 1% of the cells using the unmodified receptor protein. The number of reponding cells increased to 5–10% of the cells when Hsc70t is present, indicating that Hsc70t increases the number of cells expressing the receptor in the heterologous expression system. We did not observe changes in the maximal amplitude and the time until decay of the signal to the basal level after stimulus onset upon Hsc70t coexpression (approximately 20 s).

Deorphanization of OR2AG1

Finally, we attempted to demonstrate the use of Hsc70t for the improvement of the heterologous OR expression and

Figure 2 Coexpression of Hsc70t improves the expression of ORs. **(A)** Increased expression of ORs in HEK293 cells upon coexpression of Hsc70t, shown as percentage of OR–GFP expressing cells. Bars represent SEM from at least three independent experminents. **(B)** Fluorescence and phase-contrast pictures of HEK293 cells, which were transfected with GFP-tagged OR1D2. Coexpression of Hsc70t increases the amount of OR1D2-GFP–expressing cells. **(C)** HEK293 cells were transfected with GFP-tagged ORs, with or without coexpression of Hsc70t and the amount of expressed OR protein is detected by western blotting using α-GFP antibodies. Coexpression of Hsc70t increases the amount of OR1F12 and OR1D2 expressed in HEK293 cells. **(D)** HEK293 cells were transfected with GFP-tagged ORs, with or without the coexpression of Gal4 as control, OR protein is detected by western blotting using α-GFP antibodies. Coexpression of Gal4 did not change the amount of expressed OR1F12 and OR1D2 in HEK293 cells. Coomassie-stained gels are shown as loading control.

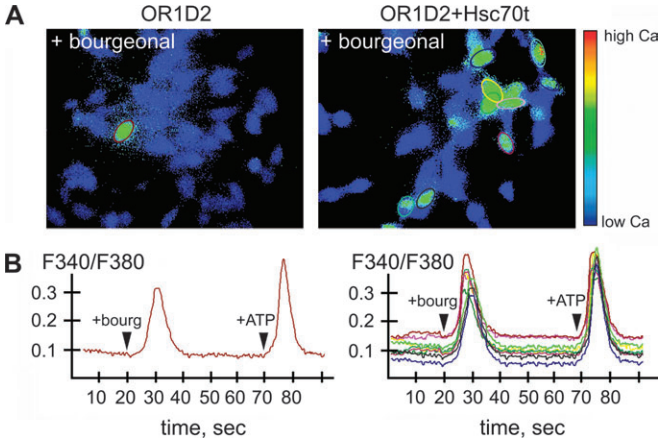


Figure 3 Expression of Hsc70t enhances OR activation. Ca^{2+}-imaging experiment with OR1D2 expressed in HEK293 cells in the presence and absence of Hsc70t. Bourgeonal application results in increased numbers of cells responding upon coexpression of Hsc70t. Representative ratiofluorometric recordings are shown. **(A)** Randomly selected field of view of fura-2–loaded cells and some responding cells are encircled in different colours. **(B)** Cytosolic Ca^{2+} levels of the encircled cells over time as integrated fluorescence ratio (*f*340/*f*380); traces are shown in the respective color. ATP (200 μM) or bourgeonal (500 μM) were applied for 5 s.

tested an additional human orphan class II OR, OR2AG1, against a panel of odorants to identify potential ligands. Therefore, a previously described (Wetzel *et al.*, 1999; Spehr *et al.*, 2003) complex mixture of 100 compounds (Henkel 100), including aromatic and short-chain aliphatic hydrocarbons, was used for initial ligand screening. Henkel 100, diluted 1:1000 in Ringer's solution, induced transient Ca^{2+} responses in 5–10% of all OR2AG1 and Hsc70t-expressing HEK293 cells tested. This rate is much higher as typically observed for transient OR transfection (Wetzel *et al.*, 1999; Spehr *et al.*, 2003). By subdivision into smaller fractions, we identified amylbutyrate as the best ligand in the Henkel 100 mixture (Figure 4). To determine the importance of the ester group and to rule out the possibility of the receptor being a broadly tuned, low-affinity receptor, we tested heptanal, an aliphatic odorant known to stimulate the mouse I7 receptor, but found it to be inactive. Also aliphatic alcohols, ketones, and ketals were inactive. In HEK293 cells expressing only Hsc70t, no Ca^{2+} signals were observed after application of any of the tested mixtures or single substances at the same concentrations (data not shown). OR2AG1, when expressed in the absence of Hsc70t, did also respond

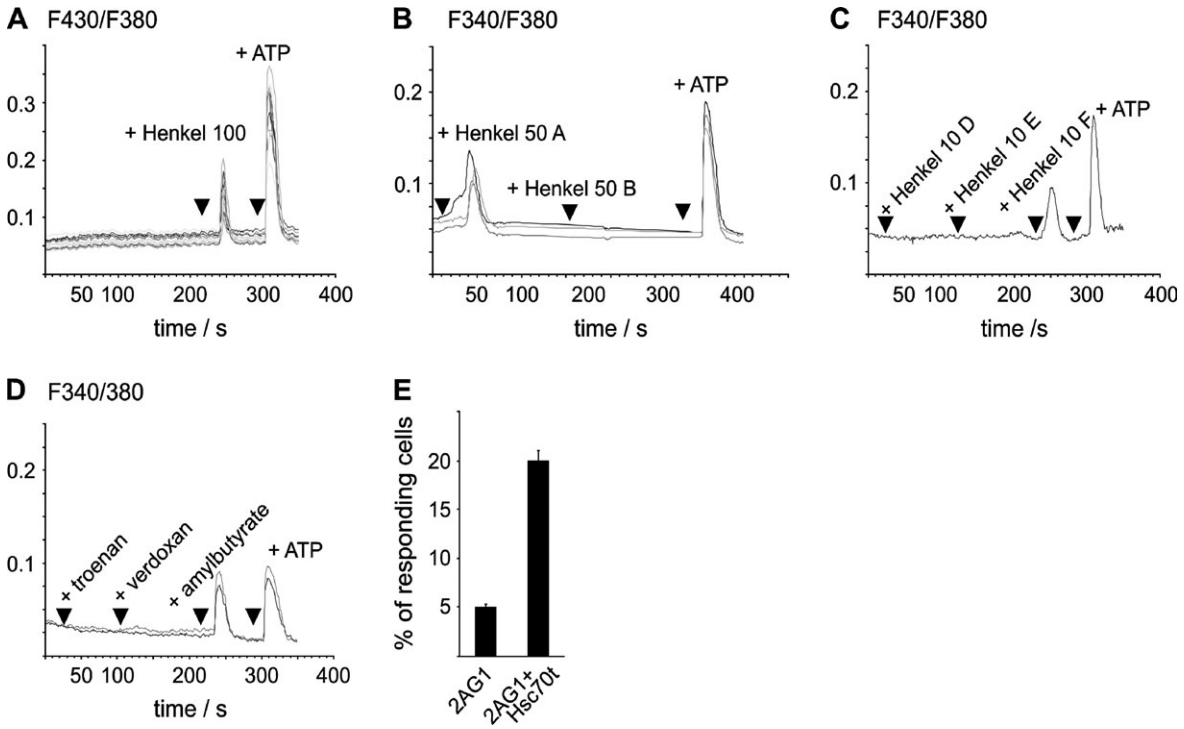


Figure 4 The molecular receptive field of OR2AG1. **(A)** In a randomly selected field of view, the complex odorant mixture Henkel 100 (1:1000) induced transient Ca^{2+} signals in OR2AG1-transfected HEK293 cells. **(B, C)** To identify the effective components in the Henkel 100 solution, the odorant mixture was subdivided into smaller fractions of 50 and then 10 substances each (1:1000) and tested for activity. **(D)** The active mixture was then further subdivided, yielding in the identification of amylbutyrate as ligand for OR2AG1 (single substances were tested at 500 μM). ATP (200 μM) served as a control. The integrated fluorescence ratio (*f*340/*f*380) for various fura-2–loaded cells is shown as a function of time; traces from individual cells are shown in different colors. All tested odorant mixtures, single compounds, and ATP were applied for 5 s. **(E)** Percentage of responding cells that express either OR2AG1 alone or together with Hsc70t. Amylbutyrate was applied at 500 μM; bars represent SEM.

to the Henkel 100 mixture or to amylbutyrate alone, indicating that Hsc70t is not changing or modifying the ligand specificity of the OR. Similar as in the case of OR1D2 (Figure 3), a smaller number of cells responded to the stimulus when only the receptor is expressed (Figure 4E). Hsc70t coexpression can therefore be helpful in deorphaning ORs by increasing the number of responding cells in ligand screening experiments with heterologous expressed receptors.

Discussion

Continued progress in the understanding of olfactory receptor function has been significantly hampered by the difficulty in expressing ORs in heterologous cells. We found the testis-enriched HSP Hsc70t in a proteomic analysis of mouse olfactory epithelium and confirmed the expression in human and mouse olfactory epithelium by RT–PCR. Due to the fact that olfactory receptors are expressed in the human (Spehr *et al.*, 2003) and mouse (Fukuda *et al.*, 2004) sperm, we speculated that a testis and olfactory epithelium chaperone might contribute to folding or cell-surface targeting of OR proteins. We were able to demonstrate that Hsc70t plays a role in functional expression of ORs and can thereby help to identify ligands for orphan ORs.

The identified protein belongs to the highly conserved and ubiquitous Hsp70 family of HSPs, which is composed of Hsp70 (inducible) and Hsc70 (constitutive expressed) proteins. Hsp70s assist folding processes, assembly of newly synthesized proteins, refolding of misfolded and aggregated proteins, membrane translocation of proteins, and control of the activity of regulatory proteins (Young *et al.*, 2004; Mayer and Bukau, 2005). Hsp70s have thus housekeeping functions in the cell in folding and signal transduction pathways and quality control functions in which they proofread and repair misfolded proteins. Hsp70, the dominant stress-inducible member of the Hsp70 family, was found to be predominantly localized to the sustentacular cells, basal cells, and Bowman's glands of the olfactory epithelium (Simpson *et al.*, 2004). Odorant exposure, heat shock, or toxic chemicals lead to a transient induction of Hsp70, Hsc70, Hsp25, and ubiquitin immunoreactivities in supporting cells and subepithelial Bowman's gland acinar cells, but not in ORNs (Carr *et al.*, 2001; Simpson *et al.*, 2004, 2005). Hsp70 has also been localized in human ORNs (Getchell *et al.*, 1995) and in a subpopulation of rat ORNs (Carr *et al.*, 1994) but not in mice, gerbils, guinea pigs, or hamster ORNs (Carr *et al.*, 1999).

Hsc70t, which belongs to the *Hsp70* family of genes, is expressed exclusively after meiosis in mouse spermatogenesis (Matsumoto and Fujimoto, 1990; Matsumoto *et al.*, 1993). The mouse *Hsc70t* gene and orthologous genes of the rat and human (Milner and Campbell, 1990; Walter *et al.*, 1994; Ito *et al.*, 1998) were mapped to the MHC class III region with linkage to the stress-inducible *Hsp70* genes. The specialized function of spermatozoa is acquired during the final steps of spermatogenesis and correlates with the accumulation of a large number of proteins that are not found in any other cell type. Appearance of the *Hsc70t* gene products during spermatogenesis demonstrates that it is synthesized in elongated spermatids which are transcriptionally inactive and is distributed in the cytoplasm of late spermatids. Mature spermatozoa contain Hsc70t in the midpiece region (Tsunekawa *et al.*, 1999). The Hsc70t protein in the cytoplasm of late spermatids is likely to be involved in chaperoning functions in the final steps of spermatogenesis, a time during which structures such as the acrosome, a special form of mitochondria, and the flagellum are formed.

Hsc70t promotes the heterologous expression of OR proteins, but the exact mode as well as time and place of action during the synthesis and transport of the OR protein are still largely unclear. Due to the broad spectrum of known functions of Hsp70 proteins not only in conventional protein folding but also in adjusting protein conformational switches in a multitude of cellular processes, elucidation of the exact function of Hsc70t needs further experimentation. Expression and targeting of GPCRs is a complex process that includes protein folding, posttranslational modifications, and transport through different cellular compartments. In the proper conformation, the hydrophobic domains in the polypeptide chain of GPCRs are buried inside the molecules, but hydrophobic segments are exposed on the protein surface during their synthesis. Constitutively expressed cytosolic heat shock cognate proteins (Hsc70s) are known to interact with hydrophobic peptide segments of proteins in an ATP-controlled fashion thereby helping in their cotranslational folding (Young *et al.*, 2004; Mayer and Bukau, 2005). Hsp70s in addition prevent proteins that are destined for posttranslational insertion into membranes from aggregation and assist in the transport to the translocation pore (Young *et al.*, 2003). Cytosolic Hsc70 is known to be important for the posttranslational translocation of proteins across intracellular membranes, most probably involving the prevention of precursor protein aggregation (Deshaies *et al.*, 1988a,b; Chirico, 1992; Walton *et al.*, 1994).

On the other hand, overexpression of Hsc70t may have a cytoprotective function in improving the cells unfolded protein response upon OR expression. Heterologously expressed ORs are typically retained in the ER (McClintock *et al.*, 1997; McClintock and Sammeta, 2003), likely in part due to inefficient folding (Lu *et al.*, 2003). In general, the presence of misfolded proteins elicits rapid and complex signal transduction cascades that are responsible for a number of cytoprotective mechanisms against the toxic buildup of misfolded proteins. This unfolded protein response includes the transcriptional upregulation of ER chaperones and folding enzymes that prevent polypeptide aggregation, a transient attenuation in the rate of protein synthesis accompanied by an arrest of cells in the G_1 phase of cell cycle, as well as retrotranslocation and degradation of ER-localized proteins (Harding *et al.*, 2002; Dobson, 2004). If

the misfolded proteins accumulate in excessive amounts, apoptotic pathways are activated to destroy the damaged cell (Paschen, 2003; Schroder and Kaufman, 2005). The unfolded protein response in mammals has therefore both cytotoxic (proapoptotic) functions as well as cytoprotective ones to enable the cell to tolerate and survive conditions which compromise protein folding in the ER. The fact that coexpression of Hsc70t increases the amount of cells heterologously expressing ORs might be explained by the fact that the increase in the amount of this chaperone protein helps the cells to cope with the otherwise deleterious effects of misfolded OR proteins in the ER and find a better balance between the synthesis of new proteins and the ER's ability to process the newly synthesized proteins. By increasing the capacity of the ER machinery for folding and degradation due to overexpression of Hsc70t, the ability of the ER to cope with the unusual load of OR protein could be enhanced. Minimizing the accumulation and aggregation of misfolded proteins, increased survival of cells expressing ORs could result in the observed increase in OR-expressing cells upon cotransfection of Hsc70t.

Receptor transporting proteins and receptor expression enhancing proteins-1 (REEP1) have also been shown to promote functional cell-surface expression of ORs expressed in HEK293 cells. These proteins were proposed to either act as chaperones to promote folding, to facilitate the transport of ORs, or to act as coreceptors for ORs, which mask otherwise exposed ER retention signals (Saito *et al.*, 2004). Due to the lack of any amino acid sequence similarity or specific sequence motif, Hsc70t might have a different or even complementary role, acting at other, maybe earlier stages of OR biosynthesis.

However, the data presented in this study provide that the chaperone environment of the ER plays an important role in the biogenesis of ORs. Coexpression of Hsc70t may moreover present a substantial improvement in the process of ligand screening of OR, a class of GPCRs, where the knowledge about their function is still far behind other classes of GPCRs.

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