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Expression and intracellular localization of leptin receptor long isoform-GFP chimera

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Abstract

The leptin receptor (OBR) and its ligand leptin (OB) are key players in the regulation of body weight. The OBR is a member of the class I cytokine receptor family and is alternatively spliced into at least six different isoforms. The multiple forms are identical in their extracellular and transmembrane regions but differ in lengths. The two predominant isoforms include a long form (OBR_l) with an intracellular domain of 303 amino acids and a shorter form (OBR_s) with an intracellular domain of 34 amino acids. We have constructed a recombinant OBR_l chimera with the green fluorescent protein (GFP) by fusing GFP to the C-terminus of the OBR_l. The OBR_l-GFP chimera was transiently transfected and expressed in SHSY5Y and HEK293 cells. In a STAT-Luciferase assay we show that the GFP moiety in this chimera did not affect the signalling capacity of OBR_l-GFP. In both SHSY5Y and HEK293 cells transfected with OBR_l-GFP, a predominant intracellular green OBR_l-GFP fluorescence was detected in vesicles also positive for internalized fluorophore conjugated leptin. We also found that treatment with the lysosomotropic reagent monensin did not relocalize OBR_l-GFP together with the human transferrin receptor in recycling endosomes, indicating OBR_l-GFP not to participate in this pathway. In biotinylation-streptavidin pulse chase experiments, using antibodies raised against GFP and OBR, we observed that the rate of early appearance of OBR_s at the cell surface, upon leptin stimulation, was faster than that found for OBR_l-GFP. Taken together, our results provide novel data concerning the intracellular trafficking of the two different isoforms of the leptin receptor.

Keywords: Obesity; Leptin; Leptin receptor; Endocytosis; Green fluorescent protein

1. Introduction

The leptin receptor (OBR), a member of the cytokine receptor family, is a product from the diabetes (*db*) gene, which encodes several different spliced forms of various lengths [1,2]. The longest form of the receptor, OBR_l, has an extended cytoplasmic domain of 303 amino acids and is mainly expressed in the hypothalamus where it is thought to mediate reduction in food intake and increased energy consumption followed by weight loss [3–7]. The other forms are shorter in their cytoplasmic tails and are missing several motifs compared to the long form [3]. One of the shorter forms, here named OBR_s, is truncated at 34 amino acids in its cytoplasmic tail and is

Abbreviations: OB, leptin; OBR_s, the short form of the human OB receptor; OBR_l the long form of the human OB receptor; K278, polyclonal antibody recognizing both the long and the short form of the OB receptor; TfR, transferrin receptor

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the most broadly expressed form [2,8,9]. All isoforms contain a 'box 1' Janus kinase in the cytoplasmic tail. OBR_l also contains a 'box 2' motif and STAT binding sites. OBR_l contains a longer cytoplasmic carboxyl sequence with a STAT interaction motif and signals via the JAK-STAT pathway. It is therefore generally believed that the OBR_l is the main signalling form of the receptor [10–13]. However, it has been shown that also the shorter forms are competent in signalling [14]. Furthermore, divergent signalling capacities between the different short isoforms have been reported [15].

The major part of the cytokine receptor family is internalized in a ligand dependent manner, via clathrin-coated vesicles, into early endosomes. Intracellular transport of ligand-receptor complexes is dependent on an acidic environment and on specific targeting sequences in the cytoplasmic domains. Generally, after receptor-ligand internalization, the ligand is separated from the receptor and the receptor may either shuttle to lysosomes for degradation or efficiently recycle back to the cell surface for another turn of internalization. Receptor-mediated endocytosis and recycling of receptors contribute to desensitization and resensitization of receptors (for reviews see [17,18]). Thus active trafficking of receptors is important for regulation of cell signalling. In previous studies on isolated brain vessels, it has been proposed that leptin binds and endocytoses at the blood-brain barrier and that this receptor-mediated transport most likely is carried out by OBR_s since OBR_l is absent in the brain endothelium [19].

Studies of receptor trafficking with antibodies do not always allow for efficient observation of receptor translocation in intracellular compartments. The green fluorescent protein (GFP) from jellyfish is becoming widely used as a molecular reporter to monitor gene expression, localization and intracellular protein trafficking in living cells. Many GFP-tagged proteins retain their biological activity and have the same trafficking pattern as the native proteins [21,22]. Moreover, the proteins that are of interest for labelling with GFP for microscopy studies may be endogenously expressed at relatively low levels. Thus, the GFP labelling may make the detection of these proteins significantly easier.

In the present study, we have constructed a functional recombinant OBR_l chimera with GFP. In fluorescence microscopy, a predominant intracellular green OBR_l-GFP fluorescence was detected in vesicles positive for internalized fluorescent-conjugated leptin.

It has been discussed whether there are differences in the intracellular trafficking of OBR_s and OBR_l [8,15,20]. Uotani et al. [36] recently provided a characterization of the ability of OBR_s and OBR_l to mediate leptin uptake and degradation and found OBR_l to be the most efficiently degraded, whereas Barr et al. [37] were able to detect differences in the internalization of different OBR isoforms. To further address this question, we have chosen to study variations in cell surface appearance between OBR_s and OBR_l-GFP in HEK293 cells since this cell line has previously been reported to express significant levels of OBR_s endogenously [16]. In surface biotinylation experiments in cells expressing OBR_l-GFP, we show that OBR_s appeared faster at the cell surface as compared to OBR_l-GFP upon leptin stimulation.

2. Materials and methods

2.1. Reagents and antibodies

RPMI 1640, fetal calf serum (FCS), phosphate buffered saline (PBS) and sodium pyruvate were all purchased from Gibco (Paisley, UK). Protein-A Sepharose was from Pharmacia (Uppsala, Sweden). The following antibodies were used: the anti-human transferrin receptor (TfR) was obtained from Boehringer Mannheim (Germany). An OBR polyclonal antibody (K278) was produced by Neosystems (Strasbourg) and was raised against a peptide in the human leptin receptor corresponding to amino acids 478–498 present in both the short and the long form of the receptor. Texas red labelled donkey secondary antibodies were from Amersham, and Cy3 labelling kits were obtained from Molecular Probes (Oregon, USA). The pEGFP-N3 plasmid was from Clontech (USA). Plasmid mini- and maxiprep kits were from Qiagen (USA). Restriction enzymes and alkaline phosphatase were obtained from Boehringer Mannheim. Low melting preparative Grade Agarose gels were from Bio-Rad Laboratories. Ligation was performed according to Ready to go T4 DNA-ligase from Amersham Pharmacia Biotech (USA).

2.2. Cell culture, plasmids and transfection

SHSY5Y and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, UK) supplemented with 10% FCS and 5 mM glutamine (Gibco) in a 5% humidified CO_2 incubator. cDNA encoding the full-length sequence of the human OBR_l was cloned into the mammalian expression vector pCI-NEO (Promega, Madison, WI, USA) and the C-terminus was fused to the N-terminus of the gene for enhanced GFP (EGFP) from pEGFP-N3 (Clontech). In the construct the mature OBR_l was found at the N-terminus to the human IL3 signal peptide. The resulting construct, OBR_l-GFP, expressed the full-length OBR_l fused at the C-terminus with EGFP. Cells were transfected by lipofection using the TransFast Transfection Reagent (Promega) usually for 48 h before the experimental procedures.

2.3. Internalization of endocytic markers

Leptin was conjugated with Cy3 (Molecular Probes), according to the manufacturer's instructions. For internalization of fluorophore-conjugated leptin, cells were grown on 12 mm round coverslips and incubated in DMEM medium supplemented with 10% FCS, 4 mM glutamine in a 5% humidified CO_2 incubator for various times at 37°C before they were washed twice with ice cold PBS and fixed with 4% paraformaldehyde.

2.4. Immunofluorescence

For immunofluorescence experiments, cells were grown on 12 mm round glass coverslips, prior to fixation and quenching in 50 mM glycine. Cells were permeabilized in 0.2% saponin containing PBS and 1% BSA. Cells were then double labelled with antibodies against various proteins. As secondary antibodies, we used donkey anti-IgG antibodies coupled to FITC, Cy3 or marine blue (Molecular Probes). When indicated cells were also treated with 50 μM of the lysosomotropic reagent monensin for 30 min before fixation. Coverslips were mounted in Mowiol (Sigma). Double immunofluorescence analyses were examined with a Leica DMR-RXA microscope (Leica Microsystems, Heerbrugg, Switzerland) equipped with a three-chip charged-coupled device (CCD) camera (C5810, Hamamatsu Photonics, Hamamatsu City, Japan). The images were captured and quantified by densitometric analysis using a Bio-Rad Image analyser and processed with Adobe Photoshop (Adobe Systems, Mountain View, CA, USA).

2.5. STAT-Luciferase reporter assay

A reporter assay based on Stat3 signalling was used to analyse the OBR_l-GFP signalling capacities. SHSY5Y cells were transiently cotransfected with a plasmid containing luciferase as reporter gene coupled to a Stat3 responsive element together with either OBR_l or OBR_l-GFP. Non-transfected cells were used as control. After 48 h of transfection cells were treated with two different concentrations of leptin, 25 nM and 2.5 nM respectively. After 20 h of stimulation with leptin, the cells were lysed in Tris-EDTA buffer containing 1% Triton X-100. The luciferase activity was measured according to the Luciferase Assay Kit instructions (BioThema, Sweden).

2.6. Cell lysis and Western blotting

HEK293 cell lysates were prepared by solving cells in lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, 1% Triton X-100, 0.5% NP40 and 0.5% SDS, pH 7.4, and the lysates were cleared by centrifugation ($13\,000 \times g$, 30 min). They were then resolved on SDS-polyacrylamide gel electrophoresis through 12% gradient polyacrylamide gels from Novex and transferred to nitrocellulose membranes (Amersham) using Novex Transblot cells at 150 mA for 2 h. After transfer, the membranes were blocked in 5% BSA for 1 h in blotting buffer (PBS, 0.3% BSA and 0.05% Tween 20) at room temperature, before incubation with antibodies. Membranes were developed with the ECL kit (Amersham) and exposed on Kodak xormat film.

2.7. Surface biotinylation experiments of OBR_s and OBR_l-GFP

HEK293 cells were transfected with OBR_l-GFP and cultured in 10 cm petri dishes to 90% confluence. Non-transfected cells were used as control cells. The

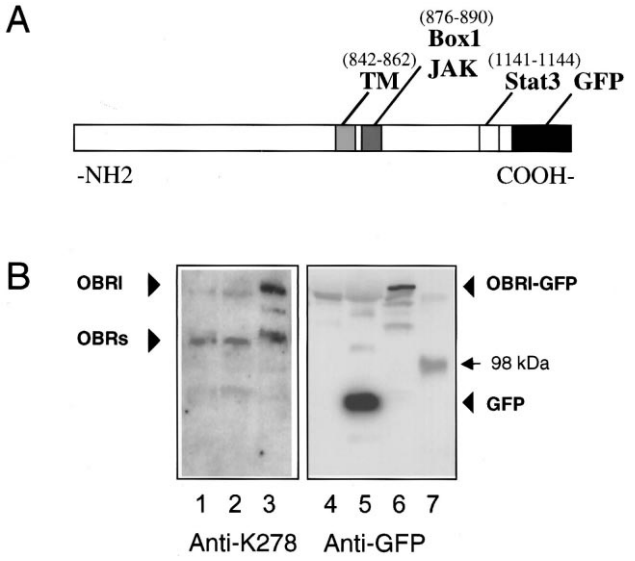

Fig. 1. Schematic presentation of the OBR_l-GFP chimera and immunoblot analysis of OBR_l-GFP expression in HEK293 cells. GFP was linked to the C-terminus of OBR_l following removal of the stop codon. Transmembrane regions (TM) and JAK and Stat binding motifs are indicated (A). (B) HEK293 cells were transiently transfected with empty vector (lanes 1 and 4), GFP plasmid (lanes 2 and 5) or OBR_l-GFP (lanes 3 and 6). Left panel shows Western blotting with cell lysates examined with antibodies against OBR (K278) (B, lanes 1–3). The right panel shows Western blotting with antibodies against GFP (B, lanes 4–7). In HEK293 cells, transfected with an empty plasmid, staining with K278 yielded an appropriate band around 150 kDa corresponding to OBR_s (B, lane 1). In lysates from cells transfected with GFP only (B, lanes 2 and 5), a strong band around 26 kDa was observed when staining with antibodies against GFP (B, lane 5). The same antibodies also recognized a strong band around 200 kDa in lysates from cells transfected with OBR_l-GFP (B, lane 6). This band was also visible when staining with the K278 antibody (B, lane 3). Lane 7 shows sea blue standard markers and arrow points on 98 kDa.

cells were serum starved for at least 4 h prior stimulation with 2 nM leptin at various times and then placed on ice, rinsed with cold PBS supplemented with 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$ (PBS++) and surface labelled for 30 min on ice (surface labelling) using 1 mg/ml EZ-linked NHS-LC-biotin (Pierce Chemical, Rockford, IL, USA) in PBS++. After biotinylation, cells were rinsed twice with ice cold PBS++ before harvested directly in lysis buffer for immunoprecipitation for 4 h at 4°C with antibodies against OBR_s (K278) or OBR_l-GFP (GFP). Immunoprecipitated biotinylated cell surface proteins were eluted with sample buffer and resolved by SDS-PAGE on 12% Novex gels and transferred to nitrocellulose membranes and probed with HRP-coupled streptavidin and developed by the ECL kit (Amersham).

3. Results

3.1. Expression of OBR_l-GFP in HEK293 cells

The human full-length OBR_l was fused to GFP as the schematic representation indicates in Fig. 1A. Cell lysates from HEK293 cells transfected with an empty plasmid (control cells), GFP only or the OBR_l-GFP chimera was analysed with antibodies against OBR (K278) and GFP (anti-GFP) in Western blotting (Fig. 1B). The K278 antibody detected significant levels of endogenous OBR_s with a molecular size of approx. 150 kDa in control cells (Fig. 1B, lane 1) as well as in GFP plasmid expressing cells (Fig. 1B, lane 2). However, in Western blot analysis of lysates from OBR_l-GFP transfected cells (Fig. 1B, lane 3) an additional band of about 200 kDa in molecular size was detected, corresponding to the predicted size of the recombinant OBR_l-GFP chimera. Western blotting with antibodies against GFP yielded no specific bands in control cells (Fig. 1B,

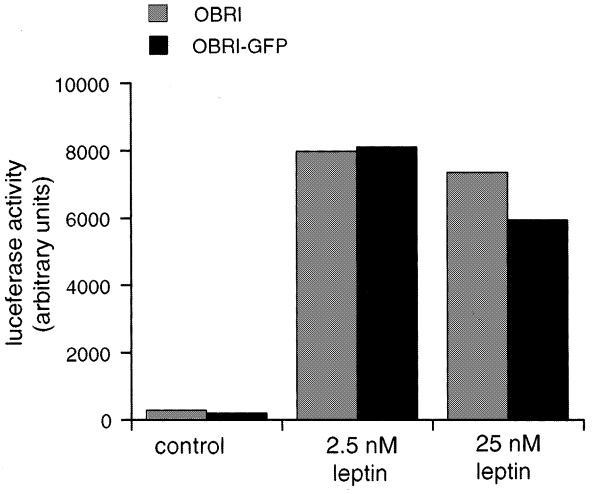

Fig. 2. Leptin-induced activation of STAT. A reporter assay based on Stat3 signalling was used to analyse the OBR_l-GFP signalling capacities. SHSY5Y cells were transfected with either OBR_l or OBR_l-GFP for 48 h prior to stimulation with leptin for 20 h. Cells were lysed and luciferase activity was measured. A representative of two experiments is shown.

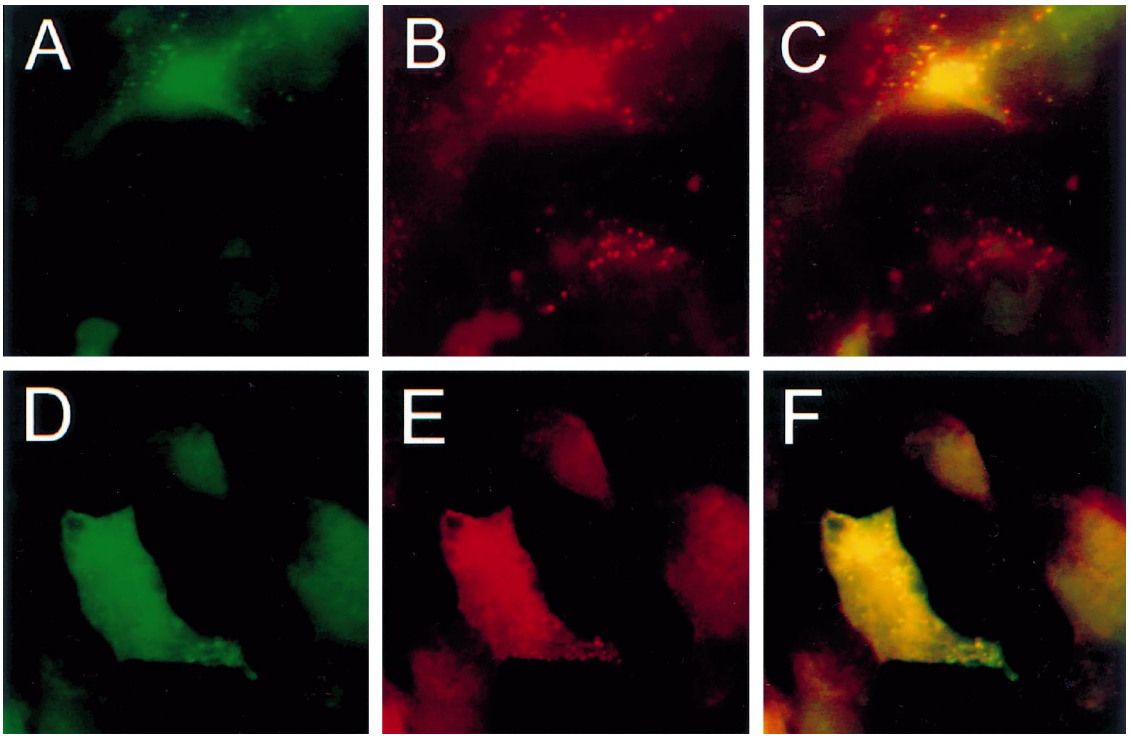

Fig. 3. Intracellular localization of GFP, OBR_l-GFP and fluorophore-coupled leptin. In HEK293 cells transfected with OBR_l-GFP a green fluorescence was visible in intracellular vesicles spread throughout the cell interior (A). The same cells were internalized with 5 μg/ml of Cy3-conjugated leptin for 60 min at 37°C prior to fixation. Vesicles containing OBR_l-GFP green fluorescence (A) and Cy3-conjugated leptin (B) strongly colocalized in superimposition (D). Note a non-transfected cell in the lower part of the same image that is not positive for OBR_l-GFP fluorescence but for Cy3-leptin. Cells transfected with OBR_l-GFP were also examined with polyclonal antibodies against GFP (D–F). The green OBR_l-GFP fluorescence (D) was clearly colocalized with the GFP containing vesicles detected with antibodies against GFP only (E). Superimposition of the same images is shown in F.

lane 4) but recognized a strong band of approx. 26 kDa (Fig. 1B, lane 5). Moreover, in agreement with the expected size of the recombinant OBR_l-GFP chimera, detection with GFP antibodies yielded a band of approx. 200 kDa (Fig. 1B, lane 6).

3.2. *Functional characterization of the OBR_l-GFP chimera in a STAT assay*

To assess whether the leptin induced signalling capacity is maintained in the OBR_l-GFP chimera, a luciferase reporter assay was used. The stat reporter contained a promoter specific for stat activation fused to the luciferase gene. SHSY5Y cells were co-transfected with either OBR_l or OBR_l-GFP and a stat luciferase reporter gene. After 48 h of transfection, cells were stimulated for 20 h with two different concentrations of leptin, 25 nM and 2.5 nM respectively. Results presented in Fig. 2 show that expression of OBR_l and OBR_l-GFP induced activation of the luciferase reporter gene. Thus, the level of luciferase induction observed with OBR_l and OBR_l-GFP truly resulted from the ability of the receptors to stimulate transcriptional activity of the stat luciferase gene.

3.3. *Expression and intracellular localization of OBR_l-GFP in HEK293 cells*

In HEK293 cells transfected with the OBR_l-GFP chimera we observed a distinct green fluorescent vesicular pattern spread throughout the cell interior (Fig. 3A). The same cells were also used to study subcellular localization of internalized fluorophore-coupled leptin. In cells incubated with 2 nM Cy3-conjugated leptin (Cy3-leptin) for 60 min at 37°C

(Fig. 3B), OBR_l-GFP fluorescence and Cy3-leptin displayed colocalized in a juxtapositional staining indicating a receptor-ligand association in these vesicles (Fig. 3C). In the same image but in the lower part, a non-transfected cell is seen that does not express OBR_l-GFP fluorescence but is positive for internalization with Cy3-leptin. This may confirm that also cells expressing only endogenous OBR_s are competent in internalizing leptin. The intracellular expression of the OBR_l-GFP chimera was also confirmed with polyclonal antibodies against GFP itself in permeabilized cells (Fig. 3D–F). OBR_l-GFP green fluorescence (Fig. 3D) and GFP detected with secondary antibodies in Cy3 red fluorescence (Fig. 3E) properly colocalized in superimposition images (Fig. 3F). However, polyclonal antibodies against GFP did not recognize the OBR_l-GFP green fluorescent pattern in unpermeabilized cells (data not shown).

3.4. Intracellular localization of OBR_l-GFP and TfR in HEK293 cells upon monensin treatment

Cells expressing OBR_l-GFP (Fig. 4A) were labelled with antibodies against the human TfR for immunofluorescence. The TfR staining displayed a punctuate vesicular pattern in the perinuclear area (Fig. 4B) but was also found dispersed throughout the cytoplasm in vesicles positive for OBR_l-GFP (Fig. 4A,B). In parallel, transfected HEK293 cells were exposed to 50 μM monensin for 30 min before fixation. Monensin is a drug that prevents early endosomal trafficking by decreasing the pH of the endocytic vesicles, thus affecting proteins that eventually recycle not to return back to the surface but accumulate in early recycling endosomes. In monensin-treated cells, the TfR accumulated in distinct perinuclear structures characteristic of early recycling endosomes (Fig. 4D), while OBR_l-GFP fluorescence

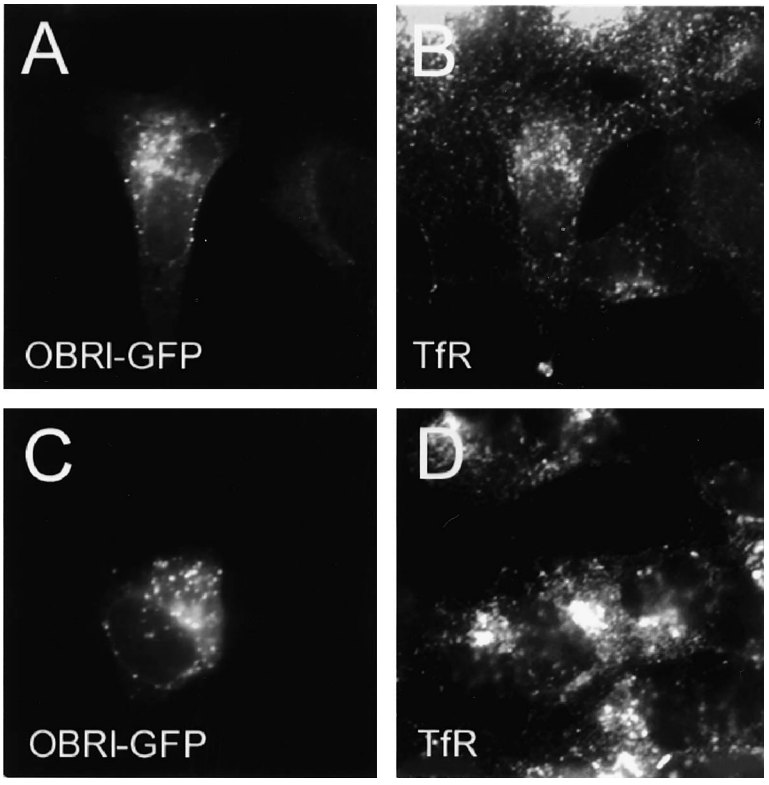

Fig. 4. Intracellular localization of OBR_l-GFP and TfR upon monensin treatment. HEK293 cells expressing OBR_l-GFP (A) revealed a bright punctuate vesicular staining. This staining pattern was partially in colocalization with the human TfR detected with monoclonal antibodies against TfR (B). In cells pretreated with 50 μM monensin for 30 min prior to fixation, TfR positive vesicles were accumulated in perinuclear vesicular regions recognized as early recycling endosomes (D), while the OBR_l-GFP vesicle distribution remained mainly unharmed in vesicles mostly negative for TfR (C).

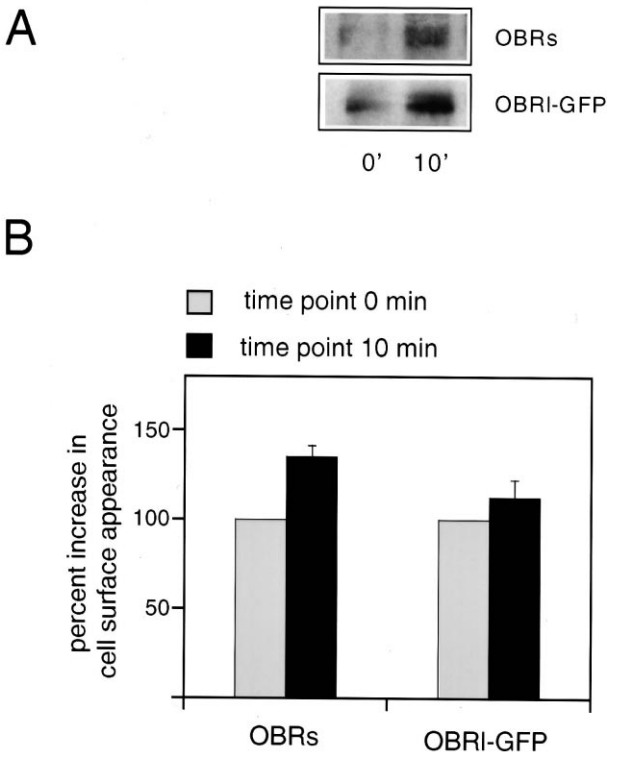

Fig. 5. Alternative cell surface appearance of OBR_s and OBR_l-GFP. HEK293 cells non-transfected and transfected with OBR_l-GFP were stimulated with 2 nM leptin for 0 and 10 min before cell surface biotinylation on ice for 30 min. Non-stimulated cells were used as 'time point 0 min'. Lysates were immunoprecipitated with antibodies against OBRs (K278) in non-transfected cells and with antibodies against GFP for OBR_l-GFP detection in transfected cells. Biotinylated proteins were detected with streptavidin-HRP in Western blotting (A). The amount of OBR_s at 'time point 0 min' was defined as 100%. The increase in cell surface biotinylated proteins after 10 min was calculated from time point 0 min and quantified by densitometric analysis using ImageQuant Software (B). Results shown in A are representative of three experiments. Results shown in B represent the mean ± S.E. of three independent experiments.

remained labelled with a more dispersed staining in vesicles mainly negative for TfR (Fig. 4C).

3.5. *Dissimilar receptor cell surface appearance of OBR_s and OBR_l-GFP*

For receptor transport studies, we took advantage of a method based on surface biotinylation to monitor differences in intracellular transport and cell surface appearance between OBR_s and OBR_l-GFP upon leptin stimulation. Quantitative biochemical analysis of OBR_s and OBR_l-GFP intracellular trafficking from the cell interior and the cell surface were performed by incubating cells with 2 nM leptin for 10 min prior cell surface receptor biotinylation. We estimated 10 min to be the most adequate period of time for studying rapid cell surface appearance of these receptors and to avoid interference of recycling abilities in the measurements. These experiments revealed that OBR_s was recruited faster at the cell surface as compared to OBR_l-GFP since an increase of 35% was found at the cell surface for OBR_s calculated from the initial amount at time point 0 (Fig. 5B 'OBRs'). The corresponding figure for OBR_l-GFP was only 12% (Fig. 5B 'OBRl-GFP').

4. Discussion

Hormone-receptor complexes may be taken up into the cells and degraded in order to terminate intracellular signalling. The existence of different intracellular routes for receptor trafficking may be an important device to regulate receptor cell surface appearance and ligand retrieval. It is furthermore general knowledge that for example insulin, insulin-like growth factor, and transferrin receptors mediate blood to brain transports through the brain capillary endothelium by receptor-mediated endocytosis. Moreover, receptor-mediated endocytosis has been examined as a tool for drug delivery aimed at targeting drugs to specific intracellular compartments [23,24]. Carrier-drug complexes may be formed with ligand or antibodies recognizing a certain receptor [25,26].

It has been shown that the short forms of the OBR are expressed throughout the body, whereas the expression of the long form has mainly been localized to particular nuclei within the hypothalamus [8,9,15]. Mutations in the OBR gene are believed to underlie the abnormalities in energy balance and plasma leptin concentrations in certain animal models of obesity and in some obese humans, possibly explained by a reduced sensitivity to leptin resulting from such mutations [27]. Recent studies on isolated human brain microvessels support the idea that the choroid plexa, sites of high expression of the short form of the OBR_s, serves as the site of leptin transport from the serum to the cerebrospinal fluid [20]. Steady state

levels of circulating leptin would require an efficient system for distribution of leptin into the brain. Furthermore, one may hypothesize that a massive increase in blood leptin levels would require a quick withdrawal of leptin from the plasma membrane into the cell interior for storage and/or degradation in order to prevent nonphysiological OBR_l signalling. OBR_s appears to be a suitable candidate for both these tasks. The receptor should then, in an efficient receptor recycling manner, after ligand dissociation, reappear at the cell surface for another turn of internalization.

A number of different ligand-receptor complexes are dissociated within the early endocytic compartment at a pH of 6.2–6.5 [28], allowing free receptors to recycle back to the plasma membrane and the ligand protein to shuttle to lysosomes for degradation. Two hypotheses have been proposed for the organization of the endocytic compartment. The 'maturation model' suggests a gradual maturation of clathrin-coated vesicles during their intracellular transport [29]. The 'vesicle shuttle model' suggests transport between preexisting endocytic organelles to be mediated by transport [30,31]. Independent of the organization of the endocytic compartment, it is evident that it contains sorting functions.

Since monensin is a cation ionophore with characteristics of decreasing the pH of endocytic compartments it is affecting receptor-ligand dissociation and degradation [32,33]. The TfR is traversing early sorting endosomes before transport to early recycling endosomes from where it cycles back to the cell surface [34,35]. This pathway has been proven to be monensin sensitive [32,33]. In immunofluorescence, we found OBR_l-GFP not to accumulate in TfR positive vesicles indicating that OBR_l-GFP probably shuttles to degrading compartments after cell surface internalization.

We have performed quantitative analysis of OBR cell surface appearance in cell surface biotinylation experiments. Total biotinylation (at 37°C), resulted in an intracellular accumulation of both OBR_l-GFP and OBR_s. After 10 min of leptin stimulation, we observed significant differences between the amount of biotinylated OBR_s and OBR_l-GFP at the cell surface compared to time point 0. One may hypothesize that the fast appearance of OBR_s at the cell surface upon leptin stimulation possibly may reflect a more pronounced recycling ability for this isoform. For certain receptors, internalization is driven by the presence of signal sequences, recognized by specific adaptor proteins. Studies on the subject of potential endocytic sequence motifs in the carboxyl-terminal tail of OBR_s and OBR_l are underway in our laboratories. It is thus obvious that the different isoforms of the OBR contain several signal sequences that are known to function in clathrin-coated endocytosis.

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