Heterodimerization, trafficking and membrane topology of the two proteins, *Ost* α and *Ost* β , that constitute the organic solute and steroid transporter

Na LI, Zhifeng CUI, Fang FANG, Jin Young LEE and Nazzareno BALLATORI¹

Department of Environmental Medicine, University of Rochester School of Medicine, Rochester, NY 14642, U.S.A.

Co-immunoprecipitation studies using mouse ileal proteins and transfected HEK-293 (human embryonic kidney-293) cells revealed that the two proteins, *Ost* α and *Ost* β , which generate the organic-solute transporter are able to immunoprecipitate each other, indicating a heteromeric complex. Mouse ileal *Ost* α protein appeared on Western blots largely as bands of 40 and 80 kDa, the latter band consistent with an *Ost* α homodimer, and both of these bands were sensitive to digestion by the glycosidase PNGase F (peptide:N-glycosidase F). *Ost* β appeared as bands of 17 and 19 kDa, and these bands were not sensitive to PNGase F. Both the 40 and 80 kDa forms of *Ost* α , and only the 19 kDa form of *Ost* β , were detected among the immunoprecipitated proteins, indicating that the interaction between *Ost* α and *Ost* β is associated with specific post-translational processing. Additional evidence for homodimerization of *Ost* α and for a direct interaction between *Ost* α and *Ost* β was provided by BiFC (bimolecular fluorescence complementation) analysis of HEK-293 cells transfected with *Ost* α and *Ost* β tagged with yellow-fluorescent-protein fragments.

BiFC analysis and surface immunolabelling of transfected HEK-293 cells also indicated that the C-termini of both *Ost* α and *Ost* β are facing the intracellular space. The interaction between *Ost* α and *Ost* β was required not only for delivery of the proteins to the plasma membrane, but it increased their stability, as noted in transfected HEK-293 cells and in tissues from *Ost* α -deficient (*Ost* α ^{−/−}) mice. In *Ost* α ^{−/−} mice, *Ost* β mRNA levels were maintained, yet *Ost* β protein was not detectable, indicating that *Ost* β protein is not stable in the absence of *Ost* α . Overall, these findings identify the membrane topology of *Ost* α and *Ost* β , demonstrate that these proteins are present as heterodimers and/or heteromultimers, and indicate that the interaction between *Ost* α and *Ost* β increases the stability of the proteins and is required for delivery of the heteromeric complex to the plasma membrane.

Key words: bimolecular fluorescence complementation (BiFC), co-immunoprecipitation (co-IP), heterodimer, membrane topology, organic-solute transporter (*Ost*), *Ost* α ^{−/−} mouse.

INTRODUCTION

Regulation of bile acids and other steroid-derived molecules is important for cellular homeostasis. The recently discovered *Ost* α –*Ost* β , a heteromeric organic solute and steroid transporter, appears to be a major basolateral transporter of bile acids and conjugated steroids in a variety of tissues, including kidney, intestine, adrenal gland and liver [1–6]. This transporter comprises a predicted seven-transmembrane (TM)-domain protein, *Ost* α , and a single TM-domain ancillary polypeptide, *Ost* β , but the roles of the two proteins in generating the functional transporter is unknown. *Ost* α and *Ost* β are encoded by genes on different chromosomes, they exhibit no sequence identity with each other and appear to lack paralogues in the mouse or human genomes [2,5]. *Ost* α –*Ost* β mediates transport of bile acid and conjugated steroids, including taurocholate, oestrone 3-sulfate and dihydroepinandrosterone sulfate by a facilitated diffusion mechanism [4] and thus can mediate either efflux or uptake, depending on the electrochemical gradient of a given substrate. Expression of both *Ost* genes is positively regulated by bile acids through the bile-acid-activated FXR (farnesoid X receptor) [6–10].

Although the mechanism by which *OST* α and *OST* β interact is unknown, some insight has been provided by the studies of Dawson et al. [3] and the very recent studies of Sun et al. [11]. Dawson and co-workers [3] demonstrated that, when mouse *Ost* α or *Ost* β are expressed individually, they display largely an endoplasmic-reticulum-staining pattern, with little

detectable plasma-membrane staining. In contrast, when they are co-expressed, they are found largely at the plasma membrane. Dawson et al. [3] also demonstrated that co-expression of *Ost* α and *Ost* β is required to convert the *Ost* α subunit into a mature N-glycosylated, Endo H (endoglycosidase H)-resistant form, indicating that co-expression facilitates the movement of *Ost* α through the Golgi apparatus. Thus stable association of both subunits may be required for transporter function, or the *Ost* β subunit may function as a chaperone to promote the egress of *Ost* α from the endoplasmic reticulum. This question was also recently examined by Sun et al. [11], who also showed that membrane targeting of the human OST proteins requires their co-expression, but the mechanism was not identified.

Additional insight into how *OST* α and *OST* β may interact has been provided by a comparison of the predicted membrane topologies of *OST* α and *OST* β with those of other heteromeric membrane proteins. In particular, the overall predicted membrane architecture of the *OST* α –*OST* β transporter is similar to that of GPCRs (G-protein-coupled receptors) that interact with the single-TM domain RAMPs (receptor activity-modifying proteins)-1, -2, or -3 [12–14]. A growing body of evidence supports the existence of GPCR–GPCR homodimers and heterodimers, and of GPCR–RAMP heterodimers and hetero-oligomers [15,16]. The similarity in membrane topology between *OST* α and the GPCRs, and between *OST* β and the RAMP proteins, raises the possibility that *OST* α may also be found in cells as a homodimer (i.e., *OST* α –*OST* α), and as hetero-oligomers with *OST* β (i.e., *OST* α –*OST* β or *OST* α –*OST* α –*OST* β).

Abbreviations used: BiFC, bimolecular fluorescence complementation; Endo H, endoglycosidase H; ES, embryonic stem; FXR, farnesoid X receptor; GPCR, G-protein-coupled receptor; IP, immunoprecipitation; MRP1/Mrp1, multidrug-resistance-associated protein 1; Neo, neomycin; Ost, organic-solute transporter; PNGase F, peptide:N-glycosidase F; RAMP, receptor activity-modifying protein; TM, transmembrane; YFP, yellow fluorescent protein.

¹ To whom correspondence should be addressed (email Ned_Ballatori@urmc.rochester.edu).

The goal of the present study was to test this hypothesis and to probe for possible interactions between mouse *Ostα* and *Ostβ* using IP (immunoprecipitation), BiFC (bimolecular fluorescence complementation) and by analysis of the fate of *Ostβ* in *Ostα*-deficient mice.

MATERIALS AND METHODS

Materials

HEK-293 cells were obtained from the A.T.T.C. (CRL-1573) and grown as monolayers at 37°C under an atmosphere of 5% (v/v) CO₂. Cells were maintained in medium consisting of Dulbecco's modified Eagle's medium (GIBCO; 10-013-CV) with 10% (v/v) fetal-calf serum, and antibiotics. Antibodies to mouse *Ostα* (mA315) and *Ostβ* (mB91) have been described previously [3,4]. The anti-FLAG M2 monoclonal (F 3165) and anti-c-Myc antibodies clone 9E10 (M 5546) were purchased from Sigma-Aldrich, the anti-MRP1 [anti-(multidrug-resistance-associated protein 1)] antibody (ALX-210-841, which is known to cross-react with mouse Mrp1) was from Alexis Biochemicals, and the anti-β-Actin (JLA20) antibody was purchased from Calbiochem. PNGase F (peptide:N-glycosidase F; P0704L) was purchased from New England Biolabs. [³H(G)]Taurocholic acid (1 Ci/mmol) was purchased from NEN. pDsRed2-ER (6982-1) plasmid was purchased from Clontech. All other chemicals and reagents were purchased from Ambion, Amersham Biosciences, Bio-Rad, Fermentas, Integrated DNA Technologies, Invitrogen, Jackson ImmunoResearch Laboratories, J.T. Baker Inc., New England Biolab, PerkinElmer, Qiagen Roche, Sigma-Aldrich or Stratagene. The *Ostα*-IRES-*Ostβ*-c-Myc construct was kindly provided by Dr Paul Dawson (Wake Forest University School of Medicine, Winston-Salem, NC, U.S.A.). C57BL/6 mice were purchased from Charles River Laboratories (Kingston, NY, U.S.A.) and were kept under a 12h-light/12 h-dark cycle at room temperature (20–22°C). All experimental protocols were approved by the local Animal Care and Use Committee, according to criteria outlined in [16a].

Generation of *Ostα*^{-/-} mice

A targeting vector was designed to replace exons 3–9 of *Ostα* (cDNA nucleotides 490–1545 or a deletion of ~4.3 kb) with a Neo (neomycin)-containing cassette (Caliper Life Sciences/Xenogen, Cranbury, NJ, U.S.A.). The 5' homologous arm (2.4 kb) was generated by PCR using BAC (bacterial artificial chromosome) clone rp23-12319 and proofreading LA Taq DNA polymerase (TaKaRa) and cloned into the pCRXL-TOPO vector. The 3' homologous arm (7.5 kb) was generated by RED cloning/gap repair and cloned into the FtNwCD vector. Both plasmids were confirmed by restriction digestion and end sequencing. The final FtNwCD vector contained Neo and diphtheria toxin fragment A expression cassettes for positive and negative selection in ES (embryonic stem) cells respectively, and the sequence was confirmed by both restriction digestion and end-sequencing analysis. The vector was linearized with NotI before electroporation into C57BL/6 ES cells (Caliper Life Sciences/Xenogen, Cranbury, NJ). G418-resistant ES clones were analysed by Southern-blot analysis, the selected clones injected into FVB blastocysts, and the blastocysts implanted into the uteri of pseudo-pregnant females for the generation of chimaeras. Male chimaeric mice were bred, and the resulting progeny were genotyped until heterozygous germline transmission was achieved. Heterozygous animals (*Ostα*^{+/-}) were bred to generate *Ostα*^{-/-} mice, and all animals were maintained on a standard laboratory diet (LabDiet, PMI Nutrition International, Saint

Louis, MO, U.S.A.) at the University of Rochester School of Medicine and Dentistry Vivarium. Genotyping was performed by PCR analysis of DNA isolated from tail biopsies. Genomic DNA was isolated from 0.5 cm of tail using DirectPCR-Tail lysis reagent (Viagen Biotech, Inc., Los Angeles, CA, U.S.A.). A 1 μl portion of crude lysate was used in a one-step genotyping PCR using primers for amplification of a ~600 bp segment of the Neo cassette (forward: 5'-CTGTGCTCGACGTTGTCAGT-3'; reverse: 5'-GATCCCCCTCAGAAGAACTCGT-3'), and a ~800 bp region of *Ostα* genomic DNA containing exons 4 and 5 (forward: 5'-ATTTGTGGTGTCAAGTTCTCTGTCT-3'; reverse: 5'-TATTATTGGCTTTGCCCTACACAAG-3') designed with Primer Express 1.5.

Protein and total mRNA isolation from various mouse tissues

Wild-type and *Ostα*^{-/-} mice were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). For protein isolation, liver, kidney and ileum were homogenized in buffer A [10 mM Tris/HCl, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 200 μg/ml EDTA, 0.25 M sucrose, 0.625% protease-inhibitor cocktail (Sigma-Aldrich P8340) and 2 mM PMSF], and then centrifuged at 800 g for 20 min at 4°C. The supernatant was the protein lysate for each tissue. For RNA isolation, liver, kidney, duodenum, jejunum and ileum were collected. Tissue samples not immediately processed for RNA isolation were stored in RNeasy Lysis Buffer (Qiagen). Total mRNA was isolated using RNeasy Midi Kit (Qiagen).

Isolation of a membrane fraction from mouse ileum

Ileum from C57BL/6 mice was homogenized in buffer A and then centrifuged at 800 g for 20 min at 4°C. The supernatant was centrifuged at 32 000 g for 20 min at 4°C. The mouse ileum membrane fraction was obtained by resuspending the pellet of the second centrifugation in buffer B (10 mM Tris/HCl, pH 7.4, 200 μg/ml EDTA, 0.125 M sucrose, 0.625% protease-inhibitor cocktail and 2 mM PMSF).

Co-IP and immunoblotting

IPs in mouse ileum membrane fractions and in transfected HEK-293 cells were performed using the Protein G IP kit (IP-50) from Sigma-Aldrich. After pre-clearing with Protein G-agarose, mouse ileum membrane fraction or proteins extracted from transfected HEK-293 cells (~300 μg) were solubilized in 250 μl of RIPA buffer (50 mM Tris/HCl, pH 7.4, 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and 0.625% protease-inhibitor cocktail). Proteins were incubated with 2.5 μg of anti-*Ostα* (mA315), anti-*Ostβ* (mB91), anti-Mrp1, anti-FLAG or 2 μl of anti-c-Myc antibodies overnight at 4°C. Immunocomplexes were isolated by incubation with 50 μl of Protein G-agarose for 2 h at room temperature. The agarose beads were washed four times with PBS, and the product of the IP was eluted by adding 90 μl of 1 × SDS sample buffer (40 mM Tris/HCl, 4% glycerol and 2% SDS) with 2-mercaptoethanol, incubating for 30 min at 37°C. The protein in the eluate was separated on SDS/4–20%-(w/v)-PAGE gels (Bio-Rad). The separated polypeptides were electrotransferred on to a PVDF membrane (Bio-Rad) for 90 min at 95 V using a wet-transfer apparatus (Bio-Rad). The membranes were blocked overnight in 5% (w/v) dried milk in TBST (20 mM Tris, 140 mM NaCl, 0.05% Tween-20, pH 7.4) at 4°C. Antibodies were used at 1:1000 [anti-*Ostα* (mA315) and anti-*Ostβ* (mB91)] and at 1:3000 [horseradish-peroxidase-conjugated anti-rabbit secondary antibody (Amersham Biosciences)]. Antibody binding was then detected using an enhanced-chemiluminescence technique (Perkin-Elmer).

Immunofluorescence staining

HEK-293 cells were grown on lysine-coated four-well chambered coverslips (Lab-Tek II) and transiently transfected with 0.3 μ g each of the following plasmids: Ost α -FLAG or Ost β -c-Myc (negative controls), both Ost α -FLAG and Ost β -c-Myc, or both Ost α -FLAG and c-Myc-Ost β . For surface antigen immunofluorescence, the cells were not permeabilized. Cells were fixed for 15 min in 4% (w/v) formaldehyde in PBS²⁺ (PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂), blocked for 30 min with 5% (v/v) normal goat serum in PBS²⁺, and then incubated at room temperature for 1 h with 1:50-diluted anti-c-Myc (M 5546) or 1:20-diluted anti-FLAG (F 3165) antibody. Subsequently, the cells were incubated at room temperature for 1 h with rhodamine-conjugated anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories) at a concentration of 30 μ g/ml and then mounted in Prolong[®] gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole; Invitrogen P36935). Labelled cells were analysed with an Olympus AH-2 microscope using 20 $\times$ or 40 $\times$ air-immersion objective lenses. When the cells were permeabilized, a similar procedure was used, except that the blocking and the antibody incubation solutions were supplemented with 0.1% saponin.

Protein stability assay

HEK-293 cells were transfected with Ost α -FLAG, Ost β -c-Myc or both (0.1 μ g of each plasmid per 3-cm-diameter plate). At 24 h after transfection, the cells were changed to medium with or without 100 μ g/ml cycloheximide (Sigma-Aldrich), a protein-synthesis inhibitor. Cells were harvested at different time points and processed for immunoblotting using anti-FLAG or anti-c-Myc antibodies.

Real-time quantitative reverse-transcriptase PCR analyses

Gene-specific primers for Ost β were as previously described [4,6]. Relative gene expression was determined on a Corbett Rotor-Gene 3000 real-time cycler. Samples were analysed using an iScript One-step reverse-transcriptase PCR kit with SYBR[®] Green (Bio-Rad).

Production of fluorescent fusion proteins

The sequences encoding YFP (1–154) (yellow fluorescent protein-1–154-peptide) and YFP (155–238) were kindly provided by Dr Tom K. Kerppola (University of Michigan Medical School, Ann Arbor, MI, U.S.A.) [17] and cloned into pCMV-HA (Clontech) and pCMV-FLAG2 (Sigma-Aldrich). The sequences encoding YFP (1–158) and YFP (159–238) were generously provided by Dr Catherine H. Berlot (Weis Center for Research, Geisinger Clinic, Danville, PA, U.S.A.) [18] and cloned into pDNAI/Amp. To construct mammalian expression vectors for BiFC analysis, the mouse Ost α sequence was fused to the gene coding for the N-terminal end of YFP (1–154), YFP (155–238) or the C-terminal end of YFP (1–158) to produce Ost α -YN, Ost α -YC, and YN-Ost α . Mouse Ost β was fused to the gene coding for the N-terminal end of YFP (155–238) or C-terminal end of YFP (159–238) to produce Ost β -YC or YC-Ost β . The constructs were verified by DNA sequencing.

Transient expression and assay for bile-acid-transport activity

HEK-293 cells were maintained under the conditions recommended by the A.T.T.C. Cells grown in six-well plates to 70–80% confluence were transfected with 0.1–1 μ g of the plasmids expressing the proteins indicated in each experiment using FuGENE[®] 6 (Roche). After 24 h of culture, the cells

were incubated at 37°C for 30 min in KH (Krebs–Henseleit) buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 0.6 mM MgSO₄, 1.25 mM CaCl₂ and 5 mM Hepes/Tris, pH 7.5) containing 25 μ M [³H]taurocholic acid. After incubation, the culture medium was removed and each cell monolayer was washed three times with ice-cold KH buffer containing 0.2% (w/v) BSA and 1 mM taurocholate, and then once more with ice-cold KH buffer alone. The cell monolayer was dissolved in 0.1 M NaOH and aliquots were taken to determine cell-associated protein and radioactivity.

Imaging of fluorescent fusion proteins

HEK-293 cells grown in 35-mm-diameter glass-bottomed dishes (MatTek P-35G-0-14-C) to 50% confluence were transfected with 0.25–0.5 μ g of various plasmids using FuGENE[®] 6. Transfected cells were incubated at 37°C for 24 h and then switched to 30°C for 2 h to promote fluorophore maturation. The cells were observed under a Leica TCS SP Spectral confocal microscope equipped with an inverted DMIRBE microscope. YFP fluorescence emission was measured at 555/15 nm. The fluorescence of DsRed2, a red fluorescent protein from the coral *Discosoma*, was measured at 610/30 nm.

Statistical analyses

Results are given as means $\pm$ S.E.M. Mean values were considered to be significantly different ($P < 0.05$) when one-way ANOVA followed by Bonferroni's multiple comparison test were used.

RESULTS

Mouse ileal Ost α and Ost β proteins appear as multiple bands on Western blots

In the mouse, Ost α and Ost β proteins are most abundant in kidney and small intestine, especially in the ileum [3]. To determine the relative molecular sizes of Ost α and Ost β in the ileum and to test for possible protein complexes, affinity-purified polyclonal anti-peptide antibodies for mouse Ost α (mA315) and Ost β (mB91) were used in Western-blot analysis of mouse ileum membrane proteins. Ost α was detected as a predominant protein band of $\sim$ 40 kDa, as well as bands of $\sim$ 50 and $\sim$ 80 kDa, although the 50 kDa band was faint and not always detectable (Figure 1A). Comparable results were observed when the gel was run under non-reducing conditions (results not shown). The predicted molecular mass of mouse Ost α is 37.8 kDa, and thus the 40 kDa band is likely to represent the monomer form of Ost α [3]. The 80 kDa band, on the other hand, may represent Ost α that is either post-translationally modified or complexed with another protein, including perhaps Ost α itself (i.e., the Ost α homodimer).

To test whether the higher-molecular-mass band of Ost α is a glycosylated form, mouse ileum membrane proteins were treated with PNGase F. As expected [3], PNGase F treatment led to a decrease in the 40 kDa Ost α band and the appearance of a 36 kDa band (Figure 1A, lane 2). Interestingly, the size of 80 kDa band also decreased after treatment with PNGase F, and a new band was formed at about $\sim$ 60 kDa (Figure 1A, lane 2). The 80 kDa band was not sensitive to Endo H treatment (results not shown).

For Ost β , bands were detected at about 19 and 17 kDa, although these bands often overlapped (Figure 1B). The predicted molecular mass of mouse Ost β is about 14.7 kDa. The Ost β antibody was not able to detect the $\sim$ 50 and 80 kDa bands stained by anti-Ost α , indicating that these higher-molecular-mass bands are not heteromeric complexes of Ost α and Ost β . The 17 and

Figure 1 Glycosidase sensitivity of Ost α and Ost β in mouse ileum, and co-IP of these proteins in mouse ileum and transfected HEK-293 cells

Mouse ileum membrane proteins (40 μ g) were incubated in the presence or absence of PNGase F and then subjected to Western Blotting using the anti-Ost α (A) or anti-Ost β (B) antibodies. Mouse ileum membrane protein samples were subjected to IP using anti-Ost α , anti-Ost β or Mrp1 antibodies, and immunoblotting (IB) using anti-Ost α (C) or anti-Ost β (D) antibodies. HEK-293 cells transfected with 1 μ g of plasmid DNA of Ost α -FLAG, Ost β -c-Myc or Ost α -FLAG-IRES-Ost β -c-Myc sequences were lysed and immunoprecipitated with anti-FLAG or anti-c-Myc antibody, and the immunoprecipitated fractions were collected by Protein G-agarose chromatography and immunoblotted using anti-Ost α (E) or anti-Ost β (F) antibodies (IRES is internal ribosome entry site). In (A) the open arrows indicate the bands that are sensitive to PNGase F, and the solid arrows indicate the new bands formed after PNGase F treatment. In panels (C)–(E), the arrows denote the bands that were co-immunoprecipitated. The asterisk in (C) indicates the band that was generated by the antibody that was used to immunoprecipitate the proteins.

19 kDa bands of Ost β were minimally affected by PNGase F (Figure 1B).

Co-immunoprecipitation of Ost α and Ost β

To examine whether Ost α and Ost β associate directly, IP and immunoblot analyses were carried out using mouse ileum membrane proteins. The mA315 (anti-Ost α) antibody was used to immunoprecipitate the Ost α subunit, and the precipitated proteins were probed with mA315 and mB91 antibodies. The experiment was also performed in the opposite direction using the anti-Ost β (mB91) to immunoprecipitate Ost β . Mrp1, a transport protein that is also located in the basolateral membrane, was chosen as a negative control. IP of Ost β from mouse ileum resulted in the co-IP of a 40 kDa protein that was detected by the anti-Ost α antibody (Figure 1C, lane 2). Likewise a 19 kDa Ost β -reactive band was co-immunoprecipitated by the Ost α antibody (Figure 1D, lane 1), indicating that the two proteins are associated with each other. Anti-Mrp1 antibody did not pull down any band corresponding to Ost α (Figure 1C, lane 3, open arrow), and only a faint signal was detected with the anti-Ost β antibody (Figure 1D, lane 3,

open arrow). Because the antibodies used to immunoprecipitate and immunoblot were both rabbit polyclonal antibodies, some extra bands were created that obscured the $\sim$ 50 and $\sim$ 80 kDa bands. Thus the higher-molecular-mass Ost α bands which were detected by mA315 (Figure 1A) were not distinguishable in this experiment.

To confirm these findings and examine whether the higher-molecular-mass forms of Ost α also interact with Ost β , co-IP studies were also performed in transfected HEK-293 cells. Mouse Ost α was labelled with the FLAG epitope and mouse Ost β was tagged with the c-Myc epitope on their C-termini. The epitope tags allowed the use of different antibodies for IP and immunoblotting. When Ost α -FLAG was expressed by itself at relatively high levels (1 μ g of DNA per 3-cm-diameter plate), bands at $\sim$ 36, 60 and 80 kDa were formed (Figure 1E, lane 2); however, when co-expressed with Ost β -c-Myc, a new $\sim$ 40 kDa mA315-antibody-reactive band was formed (Figure 1E, lane 4), consistent with glycosylation of Ost α [3]. When the same samples were blotted with anti-Ost β antibody, a prominent $\sim$ 19 kDa band was seen only in cells co-expressing Ost α -FLAG and Ost β -c-Myc (Figure 1F, lane 4), indicating that precipitation of Ost α resulted in the co-IP of Ost β . When the anti-c-Myc antibody was used to precipitate Ost β -c-Myc, bands were detected with the mA315 antibody at $\sim$ 40 kDa and $\sim$ 80 kDa, whereas the 36 kDa and the $\sim$ 60 kDa were not detected (Figure 1E, lane 8). These results support the findings obtained in mouse ileum, and suggest that the $\sim$ 80 kDa form of Ost α also interacts with Ost β .

Visualization of Ost α -Ost β heterodimers or heteromultimers in living HEK-293 cells

To confirm the IP results and to localize the putative Ost α -Ost β heterodimer or heteromultimers in living cells, BiFC analysis was performed. Although HEK-293 cells express human OST α and OST β mRNA, the levels are quite low (approx. 36 and 49 copies/ng of total RNA respectively) and did not interfere in the analysis of the heterologously expressed mouse genes. DNA coding for an N-terminal YFP fragment (residues 1–154) was fused to the C-terminus of mouse Ost α to produce Ost α -YN, and DNA coding for a C-terminal YFP fragment (residues 155–238) was fused to the C-terminus of mouse Ost β to produce Ost β -YC. The pDsRed2-ER plasmid (Clontech) was co-transfected as a marker of the endoplasmic reticulum, and the bJun-YN and bFos-YC plasmids were used as a BiFC-positive control. As reported previously [17], bJun-YN-bFos-YC associated preferentially in nuclei (Figures 2A–2C). When Ost α -YN was co-expressed with untagged Ost β , or when Ost β -YC was co-expressed with untagged Ost α , no fluorescence signal was detected, as expected (results not shown); however, when Ost α -YN and Ost β -YC were co-expressed, a fluorescence signal was detected at both the plasma membrane and intracellularly (Figures 2E and 2F). The intracellular Ost α -YN-Ost β -YC green staining partially co-localized with the red endoplasmic reticulum staining, suggesting that Ost α -YN and Ost β -YC interact in the endoplasmic reticulum and that the resulting complex(es) then proceed to the cell membrane.

To confirm that the putative Ost α -YN-Ost β -YC complex is functionally active, [3 H]taurocholate transport activity was measured in the transfected cells. As expected [2,3], co-expression of untagged Ost α -Ost β generated taurocholate transport activity (Figure 2G). Note that roughly comparable taurocholate transport activity was observed when Ost α -YN and Ost β -YC were co-expressed (Figure 2G), indicating that the C-terminal YFP fragment constructs are able to reach the plasma membrane and generate a functional transporter.

Figure 2 Heterodimerization and functional activity of *Ost* α -YN and *Ost* β -YC in HEK-293 cells

HEK-293 cells were transfected with bJun-YN, bFos-YC, and pDsRed2-ER (A–C) or *Ost* α -YN, *Ost* β -YC and pDsRed2-ER (D–F) and cultured for 24 h. The pDsRed2-ER product was visualized using 543 nm excitation and 610/30 nm emission filters (shown in red) (A and D). The BiFC complexes were visualized using 488 nm excitation and 555/15 nm emission filters (shown in green) (B and E). The images were superimposed to evaluate the distribution of the complexes in the same cell (C and F). Functional activity of the expressed proteins were examined by measuring the uptake of [³H]taurocholate (G). Cells were washed in KH buffer and incubated in 25 μ M [³H]taurocholate for 30 min at 37°C. Cells were then washed and lysed to determine cell-associated radioactivity and protein. Values are means $\pm$ S.E.M., $n = 3$. *Significantly different from control (pDsRed2-Mem-transfected cells), $P < 0.05$.

***Ost* α forms homodimers or homomultimers, and *Ost* β is required for delivery of the complex to the plasma membrane**

To directly test the possibility that *Ost* α is forming a homodimer and/or homomultimers, BiFC analysis was performed using two different *Ost* α constructs: one tagged with the YFP C-terminal fragment (*Ost* α -YC) and the other with the N-terminal fragment (*Ost* α -YN). When these two constructs were co-expressed in HEK-293 cells, the total amount of YFP signal was relatively low, but was detected in a number of cells, indicating that *Ost* α -YN interacts with *Ost* α -YC (Figures 3B and 3C). In contrast with the plasma-membrane localization of the heteromeric *Ost* α -YN–*Ost* β -YC complex (Figure 2E and F), the YFP signal of *Ost* α -YN–*Ost* α -YC largely overlapped that of the endoplasmic-reticulum marker and there was no significant plasma-membrane staining (Figure 3C).

On the other hand, when *Ost* β was transfected along with *Ost* α -YN and *Ost* α -YC, a strong YFP signal was now observed at the plasma membrane (Figures 3D–3F). In this experiment, pDsRed2-Mem, a marker of the plasma membrane, was used.

This plasmid encodes a fluorescent fusion protein that is targeted to plasma membrane by the N-terminal 20 residues of neuro-modulin [19] (Figure 3D). As illustrated in Figures 3(E) and 3(F), the fluorescence signal overlapped with that of the pDsRed2-Mem signal, confirming that the complex localizes to the plasma membrane.

Measurements of bile acid transport activity revealed that cells transfected with *Ost* α -YN and *Ost* α -YC showed no significant increase in taurocholate transport activity; however, when *Ost* β was co-transfected along with *Ost* α -YN and *Ost* α -YC, taurocholate transport activity was now comparable with that generated by untagged *Ost* α and *Ost* β (Figure 3G).

Membrane topology of *Ost* α and *Ost* β

On the basis of the evolutionarily conserved seven-TM-domain-predicted architecture of *Ost* α , this protein is thought to have an extracellular N-terminus and an intracellular C-terminus [2]. On the other hand, *Ost* β is thought to have a single TM domain [2], but its membrane topology is more difficult to predict. To

Figure 3 *Ostα* homodimer formation in HEK-293 cells

Cells were transfected with *Ostα*-YN, *Ostα*-YC and pDsRed2-ER (A–C) or *Ostα*-YN, *Ostα*-YC, *Ostβ* and pDsRed2-Mem (D–F), and, 24 h later, the pDsRed2-ER and pDsRed2-Mem products were visualized using 543 nm excitation and 610/30 nm emission filters (shown in red) (A and D). The BiFC complexes were visualized using 488 nm excitation and 555/15 nm emission filters (shown in green) (B and E). The images were superimposed to compare the distribution of the complexes in the same cell (C and F). Functional activity was assessed by measuring the uptake of 25 μ M [³H]taurocholate for 30 min at 37 °C (G), ($n = 3$, Results are means $\pm$ S.E.M., $n = 3$; * $P < 0.05$ significantly different from control (pDsRed2-Mem-transfected cells),

gain insight into the membrane topology of *Ostα* and *Ostβ*, immunofluorescence studies were carried out in HEK-293 cells transfected with C-terminally tagged *Ostα* (*Ostα*-FLAG) along with *Ostβ* tagged at either its C- or its N-terminus with the c-Myc epitope (*Ostβ*-c-Myc or c-Myc-*Ostβ* respectively) (Figure 4). Both of these combinations (i.e., *Ostα*-FLAG with either *Ostβ*-c-myc or c-myc-*Ostβ*) generated taurocholate transport activity in transfected cells (results not shown). When cells were permeabilized with 0.1% saponin, both of the combinations were labelled by the anti-c-Myc antibody (Figures 4A and 4C); however, when non-permeabilized cells were incubated with anti-c-Myc antibody, only cells transfected with the N-terminally tagged *Ostβ* construct were labelled (Figure 4D), supporting the conclusion that the C-terminus of *Ostβ* is intracellular. Likewise, when cells were incubated with anti-FLAG antibody, only cells permeabilized with saponin showed staining (Figures 4E and 4F), indicating that the C-terminus of *Ostα* is also intracellular.

To confirm this membrane topology, BiFC analysis was performed with various combinations of constructs, including YN-*Ostα* and YC-*Ostβ*, in which the two YFP fragments were

placed on the N-terminal portions of *Ostα* and *Ostβ*. HEK-293 cells were transfected with either (a) *Ostα*-YN and *Ostβ*-YC, (b) *Ostα*-YN and YC-*Ostβ*, (c) YN-*Ostα* and *Ostβ*-YC or (d) YN-*Ostα* and YC-*Ostβ*, along with pDsRed2-ER as a control for transfection and as a marker of the endoplasmic reticulum (Figures 5A–5D). Figures 5(A)–5(D) show the results of the merged YFP and pDsRed2 fluorescence signal, and Figure 5(E) provides measurements of their functional activity (taurocholate transport).

Of these four combinations of BiFC constructs, only the *Ostα*-YN and *Ostβ*-YC pair was able to elicit a YFP signal (Figure 5A), indicating that the C-termini of both proteins are on the same side of the membrane and most likely the cytoplasmic one. This combination also elicited taurocholate transport activity (Figure 5E). No fluorescence signal was detected when one YFP fragment was placed on the N-terminal portion of *Ostα* and one on the C-terminal portions of *Ostβ*, and vice versa (Figure 5). It should be noted, however, that the addition of N-terminal epitope tags to *Ostα* invariably abolished taurocholate transport activity (Figure 5E; results not shown), indicating that such modifications

Figure 4 *Ost* β is a type Ia transmembrane protein

Cells that had been transfected with either *Ost* α -FLAG and *Ost* β -c-Myc (**A**, **B**, **E** and **F**) or *Ost* α -FLAG and c-Myc-*Ost* β (**C** and **D**) were permeabilized (**A**, **C** and **E**) or not permeabilized using 0.1% saponin (**B**, **D** and **F**) and labelled with anti-c-Myc antibody (**A**–**D**) or anti-FLAG mouse antibody (**E** and **F**). The secondary antibody was anti-mouse IgG conjugated to rhodamine. The cells were visualized using an Olympus AH-2 microscope using a 20 $\times$ air-immersion objective lenses.

to *Ost* α generate products that are either unstable, unable to be targeted properly to the plasma membrane or lack transport activity. Of significance, although the *Ost* α -YN and YC-*Ost* β pair failed to generate a YFP signal, it generated taurocholate transport activity (Figure 5E), indicating a functional interaction. These observations provide additional direct support for the conclusion that the C-termini of both *Ost* proteins are on the same side of the plasma membrane, most likely the intracellular one.

Heterodimerization of *Ost* α and *Ost* β increases stability of the proteins

To examine whether the interaction between *Ost* α and *Ost* β influences the turnover rate of these proteins, HEK-293 cells were transfected with either *Ost* α -FLAG, *Ost* β -c-Myc, or both, and protein abundance was assessed at different time intervals by Western blotting (Figure 6). For these studies, a lower level of DNA was used for the transfection, namely 0.1 μ g of DNA per 3-cm-diameter dish as against 1 μ g of DNA per 3-cm-diameter dish for the studies illustrated in Figures 1(E) and 1(F). When

Figure 5 Membrane topology of *Ost* α and *Ost* β as assessed by BiFC analysis

At 24 h before observation, HEK-293 cells were transfected with DNA coding for both C-terminal fusion proteins *Ost* α -YN and *Ost* β -YC (**A**) or that coding for N- and C-terminal fusion proteins *Ost* α -YN and YC-*Ost* β (**B**) or YN-*Ost* α and *Ost* β -YC (**C**) or both N-terminal fusion proteins YN-*Ost* α and YC-*Ost* β (**D**). All cells were also transfected with the plasmid pDsRed2-ER. Images of transfected HEK-293 cells under YFP and DsRed2 filters were superimposed to evaluate the distribution of the putative *Ost* complex in the same cells (**A**–**D**). Functional activity was assessed by measuring the uptake of 25 μ M [³H]taurocholate for 30 min at 37°C (**E**). Results are means $\pm$ SEM, $n=3$. * $P < 0.05$ (significantly different from control (pDsRed2-Mem-transfected cells)).

Ost α or *Ost* β were expressed individually, the proteins were not detectable (Figure 6A, lane 2, and 6B, lane 3), but were easily detected when *Ost* α and *Ost* β were co-expressed (Figures 6A and 6B, lanes 4). To assess relative protein stability, the levels of transiently expressed proteins were compared at different time points in the presence (to inhibit protein synthesis) or absence of cycloheximide. In *Ost* α -*Ost* β -co-expressing cells, the *Ost* α and *Ost* β proteins continued to accumulate with time in culture (Figures 6C and 6D). In the presence of cycloheximide, protein levels decreased only slightly during the 24 h period (Figures 6E and 6F), but they were not detectable when expressed individually (Figures 6G and 6H), suggesting that the proteins are relatively stable when co-expressed.

If heterodimerization is required for protein stability, one would predict that the interacting partner should be present at lower levels in cells that are deficient in either *Ost* α or *Ost* β . To test this possibility, our study took advantage of an *Ost* α -deficient mouse model recently developed in our laboratory. *Ost* α -null mice are viable and fertile, but exhibit growth retardation (F. Fang, W. Christian, N. Li, C. Hammond and N. Ballatori, unpublished work). Interestingly, *Ost* β mRNA levels were maintained in tissues of *Ost* α ^{-/-} mice (Figure 7A); however,

Figure 6 Relative turnover rates of the *Ostα* and *Ostβ* proteins in HEK-293 cells

Cells were transfected with 0.1 μ g of plasmid DNA containing either *Ostα*-FLAG, *Ostβ*-c-Myc, or both, and, after 48 h, protein abundance was probed by Western blotting using anti-FLAG or anti-c-Myc antibodies (**A** and **B**). Proteins levels were also examined at 2, 8 and 24 h without (**C** and **D**) or with (**E–H**) treatment with 100 μ M cycloheximide. Actin was used as a loading control.

Ostβ protein was not detected in any of the tissues examined (Figure 7B). Taken together with the findings in HEK-293 cells (Figure 6), these results suggest that, in the absence of their heterodimerization partner, *Ostα* and *Ostβ* proteins are rapidly degraded.

DISCUSSION

The present observations provide important insights into *Ostα* and *Ostβ* membrane topology, trafficking and protein stability. *Ostα* and *Ostβ* are known to be expressed in parallel in most tissues, such that cells which express high levels of *Ostα* also express relatively high levels of *Ostβ*, and vice versa [2–5]. *Ostα* and *Ostβ* are both localized to the basolateral plasma membrane of polarized intestinal, renal and biliary epithelial cells, and *in vitro* transfection studies show that co-expression of *Ostα* and *Ostβ* is required for delivery of the individual proteins to

Figure 7 In tissues from *Ostα*^{-/-} mice, *Ostβ* mRNA levels are maintained, yet *Ostβ* protein is not detected

Total RNA and proteins isolated from *Ostα*^{-/-} and wild-type mice were analysed by real-time quantitative reverse-transcription PCR (**A**) and Western blotting (**B**) using anti-*Ostβ* (mB91) antibody. Results are means $\pm$ S.E.M. using five or six mice for each analysis. *Ostβ* mRNA data are reported relative to β -actin mRNA levels each tissue. Proteins from *Ostα*+*Ostβ*-co-transfected HEK-293 cells were used as a control in the Western blot (lane 1). Abbreviation: BD, below detection limit. Actin was used as a loading control.

the plasma membrane [3,4]. Dawson et al. [3] demonstrated that co-expression is also required to convert the *Ostα* subunit into a mature N-glycosylated Endo H-resistant form, suggesting that co-expression facilitates the movement of *Ostα* through the Golgi apparatus [3]. The present results extend these findings by showing that an *Ostα*–*Ostβ* heteromeric complex is apparently formed in the ER, is modified as it transits through the Golgi apparatus, and is then targeted to the plasma membrane. By contrast, when *Ostα* and *Ostβ* are expressed individually, the proteins appear to be targeted for degradation.

Evidence for a direct interaction between *Ostα* and *Ostβ* was obtained from co-IP and BiFC analyses, which are two powerful approaches with which to examine protein–protein interactions [20–22]. The use of primary antibodies to immunoprecipitate the native proteins in mouse ileum provides direct evidence for a physiologically relevant interaction, and the results of the BiFC analysis of transfected cells provides insights into the subcellular localization of the complex in intact cells.

The amino acid regions that are important for the interaction between *Ostα* and *Ostβ* have not yet been identified, although a recent report by Sun and co-workers [11] suggests that the N-terminal 50 amino acid residues of *OSTα* may be involved. These investigators demonstrated that truncation of this stretch of amino acids in human *OSTα* led to the intracellular accumulation of *OSTα* and *OSTβ* and to only background levels of taurocholate-transport activity. However, this truncation may have also affected *OSTα* biogenesis and folding, but these possibilities were not examined by Sun and co-workers [11]. Studies in our laboratory have revealed that although epitope tags are well tolerated on the C-terminus of *Ostα* (e.g., see Figures 2–5), tags placed on the N-terminus abolished taurocholate-transport activity (Figure 5), indicating that such modifications result in proteins that are either unstable, cannot be targeted properly to the plasma membrane

or lack transport activity. Studies are currently in progress to distinguish among these possibilities.

Co-expression of Ost α and Ost β was required not only for membrane targeting, but also to protect the proteins from degradation. Studies with other multimeric protein complexes have established that folding and assembly of such complexes are tightly controlled processes that ensure the expression of an appropriate number of fully assembled complexes on the cell surface. The assembly of multimeric protein complexes is often directly coupled to the trafficking of their individual components, preventing incompletely assembled complexes from reaching the cell surface [23]. In the present study, when Ost α -YN and Ost α -YC were co-expressed in the absence of Ost β , the resulting homodimer was retained within the cell, and mainly in the ER. However, in the presence of Ost β , the fluorescence was stronger and was mainly distributed at the plasma membrane (Figure 3). The altered subcellular YFP localization after transfection with Ost β indicates that the trafficking of Ost α is regulated by Ost β . Moreover, the smaller YFP signal from Ost α -YN-Ost α -YC indicates that this homodimer is not as stable in the absence of Ost β , and it suggests that Ost β somehow facilitates the movement of the Ost α homodimer through the endoplasmic-reticulum checkpoint. As demonstrated by Dawson et al. [3], co-expression facilitates the glycosylation of Ost α . Another possibility is suggested by studies showing that individual subunits of some ion channels or receptors complexes contain a discrete endoplasmic-reticulum retention signal that is hidden or overcome by forward trafficking signals in the correctly assembled protein complexes [24–26]. The unassembled proteins would be retained in the endoplasmic reticulum. Interestingly, mouse Ost β has an RXR (Arg-Xaa-Arg) motif in its C-terminus, whereas Ost α has an RRK (Arg-Arg-Lys) sequence, and both have dileucine motifs that have been demonstrated to function as endoplasmic-reticulum retention/retrieval signals, which prevent the cell surface expression of individual subunits and partially assembled complexes [23,27–29]. Thus one possibility is that Ost β functions to sequester this retrieval signal in the correctly assembled complex, but studies are needed to test this possibility.

Additional evidence that co-expression of Ost α and Ost β increases protein stability was provided by studies with transfected HEK-293 cells and with Ost $\alpha^{-/-}$ mice. When Ost α and Ost β were expressed individually using moderate levels of DNA for the transfections (0.1 μ g of plasmid DNA per 3-cm-diameter plate) the proteins were not detected, and were only seen when co-expressed. Interestingly, in Ost $\alpha^{-/-}$ mice the Ost β protein was also not detected, although the Ost β mRNA levels were comparable with those of wild-type mice, indicating that the absence of Ost β protein in the knockout mouse occurs post-transcriptionally and that Ost α expression is required for Ost β protein stability.

The present study also advances our understanding of the membrane topology of Ost α and Ost β by showing that C-termini of both Ost α and Ost β are located intracellularly. This overall topology of Ost α -Ost β is similar to that of the GPCR–RAMP complexes [12–14]. Ost α -Ost β and GPCR–RAMP both consist of a seven-helix TM protein (i.e. Ost α and GPCRs) and a single-TM accessory polypeptide (i.e. Ost β and RAMPs) with a cytosolic C-terminus. GPCRs are known to be present as homodimers and heterodimers, whereas GPCR–RAMP complexes are present as heterodimers and hetero-oligomers [15,16]. The present results indicate that the Ost α -Ost β complex is also likely to be a hetero-oligomer consisting of two Ost α and one or more Ost β proteins, although the actual stoichiometry has yet to be defined.

Taken together, these findings propose a model in which Ost β functions both as a chaperone and regulator of transport

activity. Formation of the heteromeric complex is coupled to the trafficking and post-translational modification of the proteins and may be required for the formation of the functional transporter. However, additional studies are necessary to confirm this model and to characterize the amino-acid regions that are important for dimerization and transport function.

This work was supported in part by National Institute of Health Grants DK067214 and DK48823, and National Institute of Environmental Health Sciences Training Grant ES07026 and Center Grants ES03828 and ES01247. We thank Dr Tom K. Kerppola for providing C-terminal BiFC plasmids, Dr Catherine H. Berlot for providing N-terminal BiFC plasmids, Dr Paul Dawson (Wake Forest University School of Medicine, Winston-Salem, NC, U.S.A.) for providing the Ost α -FLAG-IRES-Ost β -c-Myc construct, and Mr Michael Madejczyk, Mr Whitney Christian and Dr Sylvia Notenboom, all of this Institution, for their assistance with some of the analyses.

REFERENCES

- Wang, W., Seward, D. J., Li, L., Boyer, J. L. and Ballatori, N. (2001) Expression cloning of two genes that together mediate organic solute and steroid transport in the liver of a marine vertebrate. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 9431–9436
- Seward, D. J., Koh, A. S., Boyer, J. L. and Ballatori, N. (2003) Functional complementation between a novel mammalian polygenic transport complex and an evolutionarily ancient organic solute transporter, OST α -OST β . *J. Biol. Chem.* **278**, 27473–27482
- Dawson, P. A., Hubbert, M., Haywood, J., Craddock, A. L., Zerangue, N., Christian, W. V. and Ballatori, N. (2005) The heteromeric organic solute transporter α - β , Ost α -Ost β , is an ileal basolateral bile acid transporter. *J. Biol. Chem.* **280**, 6960–6968
- Ballatori, N., Christian, W. V., Lee, J. Y., Dawson, P. A., Soroka, C. J., Boyer, J. L., Madejczyk, M. S. and Li, N. (2005) OST α -OST β , a major basolateral bile acid and steroid transporter in human intestinal, renal and biliary epithelia. *Hepatology* **42**, 1270–1279
- Ballatori, N. (2005) Biology of a novel organic solute and steroid transporter, OST α -OST β . *Exp. Biol. Med.* **230**, 689–698
- Boyer, J. L., Trauner, M., Mennone, A., Soroka, C. J., Moustafa, T., Zollner, G., Lee, J. Y. and Ballatori, N. (2006) Upregulation of a basolateral FXR-dependent bile acid efflux transporter OST α -OST β in cholestasis in humans and rodents. *Am. J. Physiol. Gastrointest. Liver Physiol.* **290**, G1124–G1130
- Frankenberg, T., Rao, A., Chen, F., Haywood, J., Shneider, B. L. and Dawson, P. A. (2006) Regulation of the mouse organic solute transporter α - β , Ost α -Ost β , by bile acids. *Am. J. Physiol. Gastrointest. Liver Physiol.* **290**, G912–G922
- Zollner, G., Wagner, M., Moustafa, T., Fickert, P., Silbert, D., Gurnhold, J., Fuchsichler, A., Halilbasic, E., Denk, H., Marschall, H. U. and Trauner, M. (2006) Coordinated induction of bile acid detoxification and alternative elimination in mice: role of FXR-regulated organic solute transporter α / β in the adaptive response to bile acids. *Am. J. Physiol. Gastrointest. Liver Physiol.* **290**, G923–G932
- Landrier, J. F., Eloranta, J. J., Vavricka, S. R. and Kullak-Ublick, G. A. (2006) The nuclear receptor for bile acids, FXR, transactivates human organic solute transporter α and β genes. *Am. J. Physiol. Gastrointest. Liver Physiol.* **290**, G476–G485
- Lee, H., Zhang, Y., Lee, F. Y., Nelson, S. F., Gonzalez, F. J. and Edwards, P. A. (2006) FXR regulates organic solute transporters α and β in the adrenal gland, kidney and intestine. *J. Lipid Res.* **47**, 201–214
- Sun, A. Q., Balasubramanian, N., Xu, K., Liu, C., Ponamgi, V. M., Liu, H. and Suchy, F. (2007) Protein–protein interaction and membrane localization of human organic solute transporter (hOST). *Am. J. Physiol. Gastrointest. Liver Physiol.* **292**, G1586–G1593
- Morris, M., Christopoulos, A. and Sexton, P. M. (2003) RAMPs: 5 years on, where to now? *Trends Pharmacol. Sci.* **24**, 596–601
- Hay, D. L., Poyner, D. R. and Sexton, P. M. (2006) GPCR modulation by RAMPs. *Pharmacol. Ther.* **109**, 173–197
- Bockaert, J. and Pin, J. P. (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J.* **18**, 1723–1729
- Angers, S., Salahpour, A. and Bouvier, M. (2002) Dimerization: an emerging concept for G protein-coupled receptor ontogeny and function. *Annu. Rev. Pharmacol. Toxicol.* **42**, 409–435
- Bouvier, M. (2001) Oligomerization of G-protein-coupled transmitter receptors. *Nat. Rev. Neurosci.* **2**, 274–286
- National Academy of Sciences (1985) Guide for the Care and Use of Laboratory Animals, NIH publication 86–23, National Institutes of Health, Bethesda

- 17 Hu, C. D., Chinenov, Y. and Kerppola, T. K. (2003) Visualization of interactions among bZIP and Rel Family proteins in living cells using bimolecular fluorescence complementation. *Mol. Cell* **9**, 789–798
- 18 Hynes, T. R., Tang, L., Mervine, S. M., Sabo, J. L., Yost, E. A., Devreotes, P. N. and Berlot, C. H. (2004) Visualization of G protein $\beta\gamma$ dimers using bimolecular fluorescence complementation demonstrates roles for both β and γ in subcellular targeting. *J. Biol. Chem.* **279**, 30279–30286
- 19 Zuber, M. X., Strittmatter, S. M. and Fishman, M. C. (1989) A membrane-targeting signal in the amino terminus of the neuronal protein GAP-43. *Nature* **341**, 345–348
- 20 Phizicky, E. M. and Fields, S. (1995) Protein–protein interactions: methods for detection and analysis. *Microbiol. Rev.* **59**, 94–123
- 21 Kerppola, T. K. (2006) Visualization of molecular interactions by fluorescence complementation. *Nat. Rev. Mol. Cell Biol.* **7**, 449–456
- 22 Kerppola, T. K. (2006) Complementary methods for studies of protein interactions in living cells. *Nat. Methods* **3**, 969–971
- 23 Margeta-Mitrovic, M., Jan, Y. N. and Jan, L. Y. (2000) A trafficking checkpoint controls GABA_A receptor heterodimerization. *Neuron* **27**, 97–106
- 24 Letourneur, F. and Klausner, R. D. (1992) A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. *Cell* **69**, 1143–1157
- 25 Letourneur, F., Hennecke, S., Démollière, C. and Cosson, P. (1995) Steric masking of a dilysine endoplasmic reticulum retention motif during assembly of the human high affinity receptor for immunoglobulin E. *J. Cell Biol.* **129**, 971–978
- 26 Teasdale, R. D. and Jackson, M. R. (1996) Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and Golgi apparatus. *Annu. Rev. Cell Dev. Biol.* **12**, 27–54
- 27 Ellgaard, L., Molinari, M. and Helenius, A. (1999) Setting the standards: quality control in the secretory pathway. *Science* **286**, 1882–1888
- 28 Zerangue, N., Schwappach, B., Jan, Y. N. and Jan, L. Y. (1999) A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K_{ATP} channels. *Neuron* **22**, 537–548
- 29 Ma, D. and Jan, L. Y. (2002) ER transport signals and trafficking of potassium channels and receptors. *Curr. Opin. Neurobiol.* **12**, 287–292

Received 29 May 2007/11 July 2007; accepted 24 July 2007

Published as BJ Immediate Publication 24 July 2007, doi:10.1042/BJ20070716