

Translocation of Oxysterol Binding Protein to Golgi Apparatus Triggered by Ligand Binding

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Abstract. A cDNA encoding a cytoplasmic oxysterol binding protein was expressed at high levels by transfection in animal cells. This protein binds oxysterols such as 25-hydroxycholesterol that regulate sterol metabolism by transcriptional and posttranscriptional effects. In the transfected cells, some of the oxysterol binding protein (OSBP) was distributed diffusely in the cytoplasm, and some was bound to small vesicles near the nucleus, as revealed by indirect immunofluorescence. Upon addition of 25-hydroxycholesterol, most of the OSBP became concentrated in large perinuclear structures that stained with lentil lectin, a protein that stains the Golgi apparatus. The structures that contained OSBP were disrupted by brefeldin A,

confirming their identification as Golgi. A mutant OSBP lacking the COOH-terminal oxysterol binding domain localized to the Golgi spontaneously, suggesting that this domain normally occludes the domain that binds to the Golgi and that sterols relieve this occlusion. The previously noted potential leucine zipper sequence in OSBP was not required for Golgi localization, nor was it essential for homodimer formation. We conclude that OSBP is triggered to bind extrinsically to Golgi membranes when it binds oxysterols and speculate that this translocation may play a role in the transport, metabolism, or regulatory actions of oxysterols.

OXYSTEROLS, analogues of cholesterol with additional polar groups, regulate sterol metabolism by means of nuclear and cytoplasmic actions in animal cells (Taylor and Kandutsch, 1985; Goldstein and Brown, 1990). In the nucleus oxysterols repress transcription of genes encoding enzymes of sterol biosynthesis, including 3-hydroxy-3-methylglutaryl (HMG)¹ CoA reductase and HMG CoA synthase, and they also repress transcription of the gene for the low density lipoprotein (LDL) receptor (Goldstein and Brown, 1990). In the cytoplasm, oxysterols inhibit translation of the mRNA for HMG CoA reductase and accelerate the proteolytic degradation of this ER enzyme (Dawson et al., 1991; Peffley et al., 1988). In addition, oxysterols activate another ER enzyme, acyl-CoA: cholesteryl acyltransferase (ACAT), that facilitates the storage of excess sterols as steryl esters (Brown et al., 1975; Chang and Doolittle, 1983). Together, these actions limit the biosynthesis and uptake of cholesterol, and they are part of a coordinated mechanism that prevents the accumulation of unesterified cholesterol within cells.

The proteins that mediate the regulatory actions of oxysterols have not yet been identified, but one candidate has been proposed. This protein, designated oxysterol binding protein (OSBP), was first identified by Taylor and Kandutsch

(1985) in the cytosol of mouse cells. It binds a wide variety of oxysterols with affinities that are generally proportional to their potencies in regulating sterol metabolism (Taylor et al., 1984). The protein was purified from hamster liver cytosol by Dawson et al. (1989b), and the corresponding cDNA was cloned from the rabbit (Dawson et al., 1989a) and the human (Levanon et al., 1990).

The sequence of rabbit OSBP, as deduced from the sequence of the cDNA, revealed a protein of 809 amino acids with a calculated molecular weight of 89,489. The protein shows no significant identity to the sequence of any known protein. It lacks a nuclear localization signal or any other known organelle localization signal, but it does have two recognizable features: (a) a glycine/alanine-rich region of 90 residues at the NH₂-terminus that bears a general resemblance (although no sequence identity) to the NH₂-terminal sequence of a protein designated GAP, which stimulates the GTPase activity of p21^{ras} proteins and which is loosely adherent to the plasma membrane (Vogel et al., 1988); and (b) a potential leucine zipper region that contains five leucine residues and one isoleucine residue spaced at intervals of seven residues and that fits the other criteria proposed for α -helical sequences that mediate dimerization of certain DNA-binding proteins (Landschulz et al., 1988). However, OSBP lacks the other feature of leucine-zipper DNA binding proteins, namely, an adjacent basic domain that binds to DNA. Instead, OSBP contains a cluster of acidic residues in the corresponding position.

1. *Abbreviations used in this paper:* ACAT, acyl-CoA: cholesteryl acyltransferase; HMG, 3-hydroxy-3-methylglutaryl; LDL, low density lipoprotein; OSBP, oxysterol binding protein.

When the cDNA encoding OSBP was expressed by transfection in animal cells, it produced a cytosolic protein that bound oxysterols such as 25-hydroxycholesterol with high affinity (Dawson et al., 1989a), but there was no evidence that it entered the nucleus (our unpublished data). When OSBP was expressed at high levels in stably transfected CHO cells, there was no detectable effect on the regulated transcription of HMG CoA synthase, HMG CoA reductase, or the LDL receptor (our unpublished data). Moreover, we have been unable to demonstrate that OSBP binds specifically to DNA, or that it recognizes the nucleotide sequence (designated the sterol regulatory element) (Goldstein and Brown, 1990) that mediates the action of sterols on transcription.

OSBP mRNA is expressed in all hamster tissues ($n = 10$) and all animal cell lines ($n = 25$) so far examined (Dawson et al., 1989a and our unpublished observations). Moreover, its amino acid sequence in humans shows 98% identity with that of rabbits, further suggesting that OSBP performs some vital housekeeping function in animal cells (Levanon et al., 1990). If it does not play a role in nuclear regulation, then perhaps OSBP might mediate one of the cytoplasmic regulatory actions of oxysterols.

To gain further insight into the function of OSBP, in the current studies we have explored the subcellular localization of OSBP by means of indirect immunofluorescence microscopy of cells overexpressing OSBP as a result of transfection. We have also used mutagenesis techniques to dissect the regions of OSBP that are responsible for binding of oxysterols and for dimerization. Native OSBP was found free in the cytoplasm and associated with vesicles concentrated in the perinuclear region. Upon addition of 25-hydroxycholesterol to the transfected cells, OSBP moved to a highly concentrated juxtannuclear position that corresponded to the Golgi. Deletion of the 25-hydroxycholesterol binding domain resulted in a protein that localized constitutively to the Golgi even in the absence of sterols. These data suggest that some of the cellular actions of oxysterols are mediated by interaction of OSBP with components of the Golgi apparatus.

Materials and Methods

All sterols were obtained from Steraloids, Inc. (Wilton, NH) and added to the culture medium in ethanol (1 μ l per ml of medium). Brefeldin A was obtained from Epicentre Technologies (Madison, WI) and added to the culture medium in methanol (1 μ l per ml of medium). Control cells received comparable volumes of ethanol or methanol. FITC-conjugated lentil lectin (*Lens culinaris*) was obtained from E-Y Laboratories (San Mateo, CA). Rhodamine-conjugated wheat germ agglutinin was obtained from Vector Laboratories (Burlingame, CA). All other reagents were obtained from previously described sources (Dawson et al., 1989a; Metherall et al., 1989).

Cell Culture and Transfection

All cells were grown in monolayers in an atmosphere of 8% CO₂. CHO cells were grown in a 1:1 (vol/vol) mixture of DME and Ham's F-12 medium containing 100 U/ml penicillin, 100 μ g/ml streptomycin sulfate, and 5 or 10% (vol/vol) FCS (medium A). CHO-7 cells, a line of CHO-K1 cells adapted for growth in lipoprotein-deficient serum (Metherall et al., 1989), were cultured in medium identical to medium A except that it contained 10% (vol/vol) newborn calf lipoprotein-deficient serum in place of FCS (medium B). Lipoprotein-deficient serum (density > 1.215 g/ml) was prepared by zonal rotor ultracentrifugation (Cable, 1983).

Stable overexpression of OSBP in CHO and CHO-7 cells was achieved in the following manner: CHO and CHO-7 cells were plated on day 0 at a density of 2.5×10^5 cells/100-mm dish in medium A or medium B,

respectively. On day 1, cells were transfected by the calcium phosphate technique (Metherall et al., 1989) with 0.5 μ g/dish of pSV₂NEO or pSV₃NEO (Southern and Berg, 1982) and 9.5 μ g/dish of OSBP plasmid DNA. On day 2, medium A or B containing 700 μ g/ml of G418 (GIBCO-Life Technologies, Inc., Grand Island, NY) was added. Medium was changed every second day until well-defined colonies were evident (12–14 d). Colonies were picked, expanded in 24-well plates, and screened for expression of OSBP plasmids by immunoblotting (see below). Pure clones were obtained by dilution subcloning in the presence of 350 μ g/ml of G418. On day 0, cells from stock cultures of the stably transfected cloned cell lines were seeded onto coverslips at a density of 5×10^4 cells/60-mm dish in medium A (CHO cells) or medium B (CHO-7 cells). On day 2, both types of cells were washed once with Dulbecco's PBS and switched to medium B. On day 3, the coverslips were processed for immunofluorescence as described below. Approximately 20% of G418-resistant colonies showed stable overexpression of OSBP.

Transient overexpression of OSBP in CHO cells was achieved in the following manner. Cells were seeded on day 0 onto glass coverslips at a density of 1×10^5 cells/60-mm dish in medium A. Cells were transfected on day 1 with 15 μ g/dish of plasmid DNA by the calcium phosphate technique (Metherall et al., 1989). On day 2, fresh medium A was added. On day 3, the cells were washed once with Dulbecco's PBS, and medium B was added. On day 4, the coverslips were processed for indirect immunofluorescence microscopy as described below.

Transient expression of OSBP in simian COS-M6 cells was carried out as follows: The cells were grown in DME (high glucose) containing 100 U/ml penicillin, 100 μ g/ml streptomycin sulfate, and 10% (vol/vol) FCS. On day 0, cells were seeded at a density of 6×10^5 cells/100-mm dish. Cells were transfected on day 1 by the DEAE-dextran method (Esser et al., 1988). On day 2, the cells were switched to medium identical to the above medium except that it contained 10% (vol/vol) calf lipoprotein-deficient serum in place of FCS. The cells were used for biochemical experiments on day 3.

Indirect Immunofluorescence

CHO and CHO-7 cells transiently or stably expressing wild-type or mutant OSBP plasmids were seeded onto glass coverslips and transfected as described above. On day 3 (stable transfectants) or on day 4 (transient transfectants), the cells were fixed in 10 mM sodium phosphate (pH 7.4), 225 mM NaCl, and 2 mM MgCl₂ (PBS) containing 3% (wt/vol) formaldehyde for 15 min at 20°C. Following two washes in PBS containing 5 mM ammonium chloride, cells were permeabilized in PBS plus 0.05% (vol/vol) Triton X-100 for 10 min at -20°C. The Triton X-100 solution was removed, and 2 ml of PBS containing 1% (wt/vol) fatty acid-free BSA (PBS/BSA) was added. After a 15-min incubation at room temperature, the PBS/BSA was replaced with buffer containing 2 μ g/ml of mAb IgG-11H9 or 5 μ g/ml polyclonal antibody W980 (IgG fraction) (see below). After incubation for 1 h at 37°C, the coverslips were washed twice with 2 ml of PBS/BSA for 15 min each and then incubated with fresh PBS/BSA containing one of the following reagents: 5 μ g/ml of FITC-goat anti-rabbit IgG, 5 μ g/ml of FITC-rabbit anti-mouse IgG (both purchased from Zymed Labs Inc., San Francisco, CA), or 5 μ g/ml of rhodamine-rabbit anti-mouse IgG (Chemicon, Temecula, CA). FITC-lentil lectin, which was used to counterstain the Golgi apparatus, was incubated at 2 μ g/ml with coverslips that received the rhodamine-rabbit anti-mouse IgG. After 45 min at 37°C, the fluorescent antibody was removed, and coverslips were washed twice at room temperature with 2 ml of PBS/BSA for 15 min and mounted on microscope slides with 90% (vol/vol) glycerol, 50 mM Tris-HCl (pH 9.0), and 2.5% (wt/vol) 1,4-diazadicyclo-[2.2.2]-octane. Fluorescence microscopy was performed with a Zeiss Photo microscope III using a 63 \times Neofluar oil immersion objective and FITC and rhodamine filter packages. Photomicrographs were obtained with Kodak Ektachrome 400 film.

Glutaraldehyde Crosslinking

COS-M6 cells were transfected with OSBP plasmids as described above, and cytosol (centrifuged at 10^5 g for 30 min) was prepared as previously described (Dawson et al., 1989a). For glutaraldehyde crosslinking assays, 20–40 μ g of cytosolic protein was incubated in a final volume of 75 μ l of 75 mM sodium phosphate buffer (pH 7.0) containing 0–0.5 mM glutaraldehyde. After a 45-min incubation at 20°C, crosslinking was terminated by the addition of 50 μ l of buffer containing 0.25 M Tris-HCl (pH 6.8), 4% (wt/vol) SDS, 20% (vol/vol) glycerol, and 10% (vol/vol) 2-mercaptoethanol. Samples were boiled for 3 min, fractionated by SDS-PAGE, and transferred to nitrocellulose for immunoblot analysis of OSBP (see below).

[³H]25-Hydroxycholesterol Binding to OSBP

Binding of [³H]25-hydroxycholesterol (87 Ci/mmol, New England Nuclear-DuPont, Boston, MA) to cytosolic proteins from transfected COS-M6 cells was carried out using a dextran/charcoal assay as previously described (Dawson et al., 1989b).

Antibodies and Immunoblot Analysis

Polyclonal antibodies B27 and AB21 were raised against synthetic peptides corresponding to amino acids 2–15 (Multiple Peptide Systems, San Diego, CA) and 737–755 (MilliGen/Biosearch, San Rafael, CA), respectively, of rabbit OSBP (Dawson et al., 1989b). Peptides were coupled to purified protein derivative of tuberculin (Statens Seruminstitut, Copenhagen, Denmark) before immunization of New Zealand white rabbits (Lachmann et al., 1986). Antibody W980 is a rabbit polyclonal antibody directed against a fragment of a bacterial fusion protein that contains amino acids 61–260 of rabbit OSBP. The bacterial expression construct was prepared by ligating the 596-base pair SmaI-HincII fragment of the rabbit OSBP cDNA (Dawson et al., 1989b) into the SmaI site of pGEX 3X. pGEX 3X directs expression of OSBP as a fusion protein with *Schistosoma japonicum* glutathione S-transferase (Smith and Johnson, 1988). The fusion protein was cleaved with factor Xa, and the glutathione S-transferase was removed by glutathione-agarose affinity chromatography before immunization.

IgG-11H9, a mAb directed against the intact fusion protein described above, was prepared by fusion of Sp2/0-Ag14 mouse myeloma cells with splenic B-lymphocytes derived from a female Balb/c mouse immunized with three injections of 30 µg each of the fusion protein. Hybridoma culture supernatants were screened by enzyme-linked immunosorbent assay using OSBP cleaved from the fusion protein as the antigen. The positive hybridoma (IgG-11H9) produced antibodies belonging to IgG subclass 1. The hybridoma cells were subcloned three times by limiting dilution and injected into the peritoneal cavity of pristane-treated mice. All antibodies were purified on Protein G-Sepharose Fast Flow Columns (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Immunoblotting was performed as previously described (Dawson et al., 1989b).

Expression Vectors for Wild-type and Mutant Versions of OSBP

Standard techniques of recombinant DNA were used (Sambrook et al., 1989). The construction of the wild-type plasmid pOSBP was previously described (Dawson et al., 1989a). Deletion construct pΔ181–809 was made by ligating the EcoRI-Ball fragment of the rabbit OSBP cDNA to a universal translation terminator (GCTTAATTAATTAAGC, Pharmacia LKB Biotechnology, Inc., Piscataway, NJ), followed by insertion into EcoRI-SmaI-cut pCMV2 (Andersson et al., 1989). Deletion construct pΔ261–809 was prepared in an identical manner using the EcoRI-HincII fragment of the rabbit cDNA. Construct pΔ455–809 was prepared by cutting pOSBP with BglII, filling in the ends with the Klenow fragment of DNA polymerase I, and ligating a translation terminator. Plasmid pΔ578–809 was constructed by introducing stop codons at amino acids 578 and 579 by site-directed mutagenesis (Zoller and Smith, 1984). Similarly, plasmids pΔ209–244 and pLeu/Ile→Val were made by site-directed mutagenesis of pOSBP. Construct pΔ210–296 was made by digestion of pOSBP with PstI and religation of the isolated vector. Plasmid pΔ1–187 was constructed by cloning the entire λclone H20 (Dawson et al., 1989a) into pCMV2. Translation in pΔ1–187 initiated from the methionine at amino acid 188.

Results

Fig. 1 presents a diagrammatic summary of the plasmids encoding wild-type and mutant forms of rabbit OSBP that were used in these studies. Wild-type OSBP contains 809 amino acids. Residues 1–90 comprise the glycine/alanine-rich region and residues 209–244 encompass the potential leucine zipper. Fig. 1 also summarizes the results of studies of cellular distribution, ligand binding, dimerization, and immunofluorescence localization for wild-type OSBP and for each of the mutants. Examples of these results are given in detail below.

All of the recombinant plasmids produced proteins of the

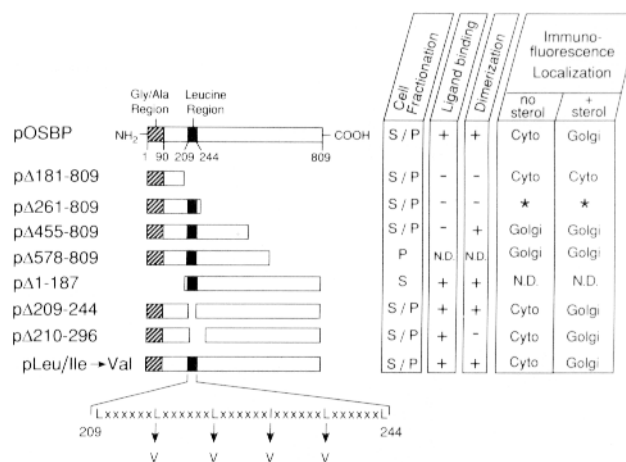


Figure 1. Structure of wild-type and mutant forms of OSBP and summary of experimental results. Assays for [³H]25-hydroxycholesterol binding and dimerization (by glutaraldehyde crosslinking) were carried out as described under Materials and Methods on COS-M6 cells that were transiently transfected with the indicated plasmid. The distribution of OSBP in the soluble or particulate fractions of transfected COS-M6 cell extracts was determined by centrifugation at 10⁵ g for 1 h at 4°C followed by immunoblot analysis. S/P denotes that approximately equal amounts of the expressed protein were found in the supernatant (S) and pellet (P) fractions. The cellular localization of OSBP by indirect immunofluorescence was determined in CHO cells transiently expressing each of the OSBP plasmids. The asterisk (*) for pΔ261–809 denotes that the mutant OSBP encoded by this plasmid shows a complex distribution, including nuclear and Golgi staining (discussed with experiment shown in Fig. 13). Before fixation, the cells were incubated for 5 h in medium B in the absence and presence of 1 µg/ml 25-hydroxycholesterol as described in Materials and Methods. Indirect immunofluorescence was also performed on stably transfected CHO cell lines expressing pOSBP, pΔ261–809, pΔ455–809, and pΔ209–296 following a 5-h incubation in medium B in the absence and presence of 1 µg/ml 25-hydroxycholesterol. The results for each plasmid were the same as those observed in the transiently expressing cells. Cyto, cytosol.

appropriate size when transfected into COS-M6 cells for transient expression, and the level of expression of mutant and wild-type plasmids was similar (Fig. 2) and was generally 20–100-fold over that of endogenous OSBP. Subcellular fractionation studies showed that ~50% of each of the proteins (except the pΔ578–809 mutant) was present in the cytosol, and 50% was found in the membrane pellet as estimated by ultracentrifugation (10⁵ g) and immunoblotting techniques (data not shown). Similar results were observed in CHO cells that stably overexpressed OSBP. When the 10⁵ g pellet from these CHO cells was resuspended in homogenization buffer containing 50 mM KCl and recentrifuged at 10⁵ g for 30 min, the majority of the membrane-bound OSBP was released into the supernatant fraction. However, ~15% of the total OSBP remained in the pellet fraction and was resistant to subsequent extraction by 2 M KCl. No changes in this distribution were observed when the transfected CHO cells were pretreated with 25-hydroxycholesterol. Thus, the majority of OSBP appears to be extrinsically associated with membranes, while a small portion (~15%) is bound more tightly (data not shown).

The wild-type OSBP in the cytosol of transfected COS-M6 cells bound [³H]25-hydroxycholesterol with high affinity

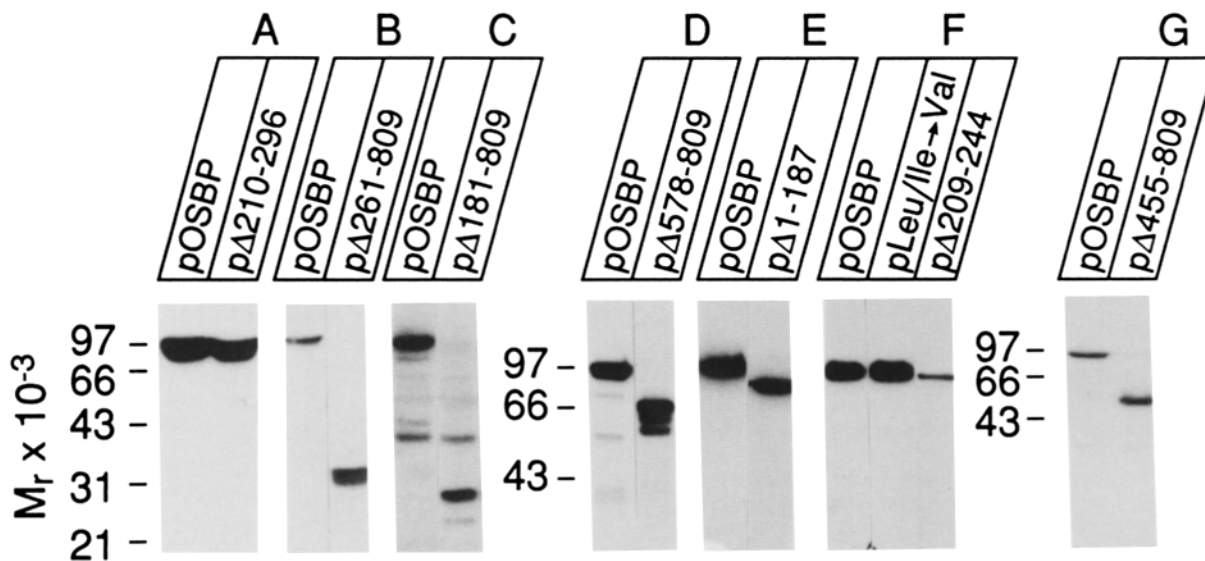


Figure 2. Expression of wild-type and mutant versions of rabbit OSBP in transiently transfected COS-M6 cells. COS-M6 cells were transfected with the indicated plasmid, and an aliquot of the cytosol (A-E, 25 μ g protein; F, 15 μ g; G, 30 μ g) was subjected to SDS-PAGE and immunoblot analysis as described in Materials and Methods. The following antibodies (IgG fractions) and Kodak XAR film exposure times at either -20°C or -70°C were employed: A, AB21 (2.7 μ g/ml), 3 h; B, B27 (3.3 μ g/ml), 3 h; C, B27 (6.5 μ g/ml), 8 h; D, IgG-11H9 (2.1 μ g/ml), 2 h; E, AB21 (2.7 μ g/ml), 12 h; F, AB21 (1.3 μ g/ml), 1 h; G, B27 (3.3 μ g/ml), 12 h. In D, the 10^5 g pellet fraction of p Δ 578-809-transfected cells was used for immunoblotting since this mutant protein is not recovered in the soluble fraction of the transfected COS-M6 cells. Molecular weight markers are indicated.

(Fig. 3). Binding was also observed with the deletion mutants Δ 1-187, and Δ 210-296 (Fig. 3, B and C). The Δ 455-809 mutant showed no evidence of [^3H]25-hydroxycholesterol binding (D), even though immunoblotting experiments showed that comparable amounts of this protein

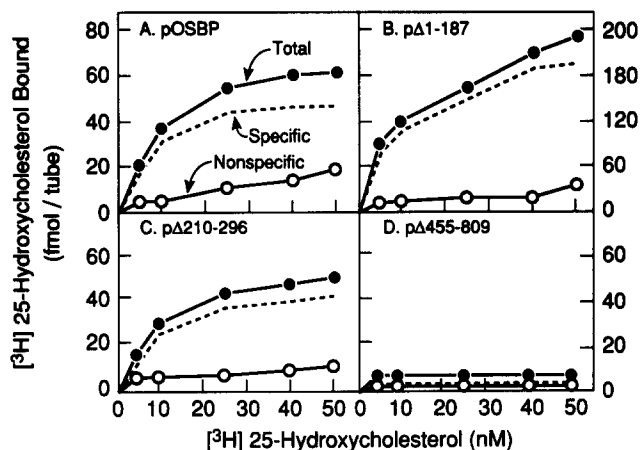


Figure 3. [^3H]25-hydroxycholesterol binding to cytosol of transiently transfected COS-M6 cells. Cytosols were isolated from cells transfected with the indicated plasmids as described under Materials and Methods. Each assay contained, in a final volume of 0.1 ml, 10 μ g cytosolic protein and the indicated concentration of [^3H]25-hydroxycholesterol (150–200 dpm/fmol) in the absence (\bullet) or presence (\circ) of 2 μM unlabeled 25-hydroxycholesterol. Specific binding (-----) was calculated by subtracting the value for total binding (\bullet) from the nonspecific value (\circ). Each value represents the average of duplicate assays. Expression of each mutant OSBP was verified by immunoblot analysis and found to be quantitatively similar to that of the wild-type OSBP.

and wild-type OSBP were present in the cytosol of the respective transfected cells (data not shown). These findings localize the oxysterol binding site to the COOH-terminal half of the protein.

Figs. 4–6 show a series of experiments in which we used glutaraldehyde crosslinking followed by SDS-PAGE and immunoblotting to test for dimerization of the OSBP molecules encoded by the various mutant plasmids. Preliminary studies showed that purified OSBP, which behaves on gel filtration

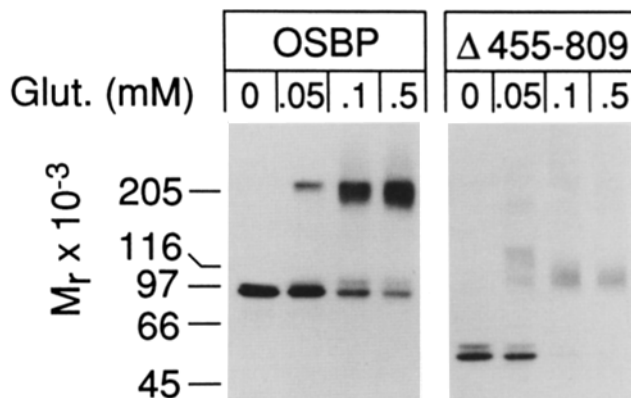


Figure 4. Glutaraldehyde-mediated crosslinking of OSBP dimers in cytosol from COS-M6 cells transiently transfected with pOSBP and p Δ 455-809. Cytosol (30 μ g protein) from the indicated transfected cells was incubated with the indicated concentration of glutaraldehyde (Glut.) as described in Materials and Methods. The reaction mixtures were subjected to 3–10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with 3.3 μ g/ml of antibody B27 as described in Materials and Methods. The immunoblot was exposed to Kodak XAR film for 5 h at -70°C .

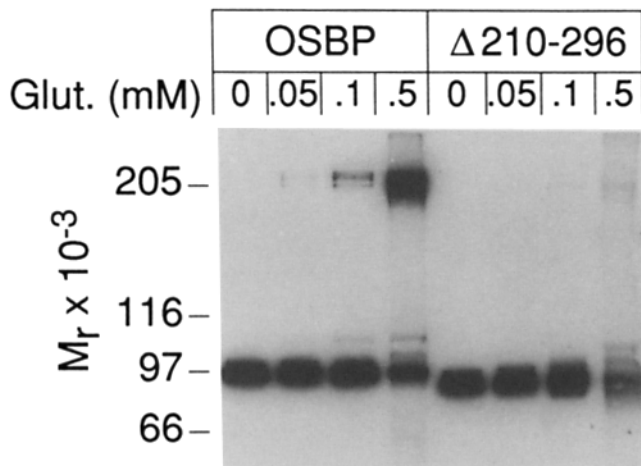


Figure 5. Glutaraldehyde-mediated crosslinking of OSBP dimers in cytosol from COS-M6 cells transiently transfected with pOSBP and p Δ 210-296. Cytosol (30 μ g protein) from the transfected cells was incubated with the indicated concentration of glutaraldehyde (Glut.). The reaction mixtures were subjected to 7% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with 2.7 μ g/ml of antibody AB21. The immunoblot was exposed to Kodak XAR film for 10 h at -20°C .

as a homodimer (Dawson et al., 1989b), can be crosslinked with glutaraldehyde into dimers that migrate as \sim 200-kD species on SDS-PAGE (data not shown). In the cytosol from COS-M6 cells producing wild-type OSBP, crosslinked dimers were observed with concentrations of glutaraldehyde exceeding 0.05 mM (Figs. 4-6). A similar degree of dimerization was observed for the COOH-terminal deletion mutant Δ 455-809 (Fig. 4). The internal deletion mutant Δ 210-296 showed very little tendency to dimerize as determined by glutaraldehyde crosslinking (Fig. 5). To determine whether the defect in the Δ 210-296 mutant is attributable to removal of the putative leucine zipper, we tested a smaller deletion, Δ 209-244, which precisely removes this region (Fig. 1). Surprisingly, the Δ 209-244 mutant showed nearly normal dimerization (Fig. 6). This experiment was repeated several times with similar results. Although the leucine zipper may contribute to dimerization, it is clearly not required for this function.

Studies of the dimerization of additional mutants, summarized in Fig. 1, confirmed the dispensability of the putative leucine zipper region. Thus, the substitution of valines for three of the leucines and one isoleucine, a type of mutation that was reported to abolish leucine zipper formation between DNA binding proteins (Ransone et al., 1989; Schuermann et al., 1989), did not affect dimerization of OSBP. We also observed that the protein that terminated at residue 261 did not dimerize (Fig. 1). Together, these data suggest that the crucial element for dimerization must lie in the interval between residues 261 and 296.

To confirm that the glutaraldehyde crosslinking reflected dimer formation, we performed glycerol gradient ultracentrifugation of COS-M6 cell cytosol and followed the OSBP by SDS-PAGE and immunoblotting (Fig. 7). In cytosol from cells transfected with the plasmid encoding wild-type OSBP, the immunoreactive material sedimented slightly faster than the 158-kD marker, at a position to be expected from an OSBP dimer. The quadruple leucine-valine substitution mu-

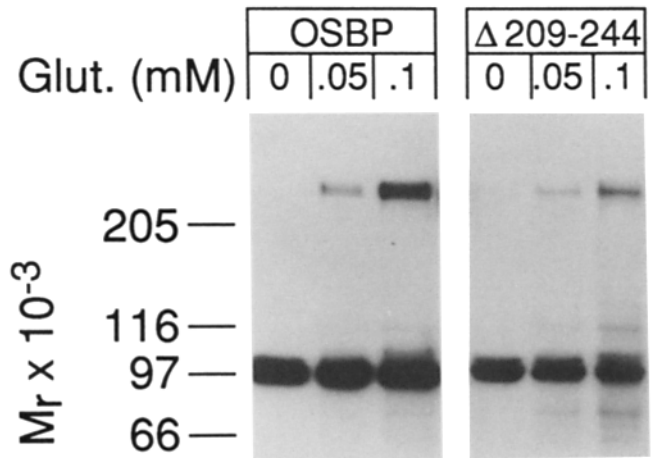


Figure 6. Glutaraldehyde-mediated crosslinking of cytosol from COS-M6 cells transiently transfected with pOSBP and p Δ 209-244. Cytosol (30 μ g protein) from the transfected cells was incubated with the indicated concentration of glutaraldehyde (Glut.). The reaction mixtures were subjected to 7% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with 2.7 μ g/ml of antibody AB21. The immunoblot was exposed to Kodak XAR film for 8 h at -70°C .

tant showed an identical sedimentation profile. On the other hand, the protein encoded by p Δ 210-296, which did not show glutaraldehyde crosslinking (Fig. 5), sedimented in the gradient in a position corresponding to a monomer (Fig. 7).

Fig. 8 shows the immunofluorescence localization of OSBP in CHO cells stably transfected with the plasmid encoding wild-type OSBP. Before the experiment, the cells were grown in lipoprotein-deficient serum to deprive the cells of exogenous sterols. When the cells were incubated in the absence of oxysterols, OSBP was visualized as small perinuclear dots suggesting association with small vesicles, together with a diffuse haze indicating a general cytosolic distribution (Fig. 8A). Addition of 25-hydroxycholesterol caused a remarkable redistribution of the protein, which now appeared as tightly clustered masses of perinuclear fluorescence, often concentrated at one or two poles (Fig. 8B). The addition of cholesterol, which does not bind to OSBP (Dawson et al., 1989a,b), produced no such redistribution (Fig. 8C). A control preimmune antibody gave no immunofluorescence (Fig. 8D). Nontransfected cells also showed no immunofluorescence with the anti-OSBP antibody, indicating that the level of expression of endogenous OSBP in CHO cells is too low to be detected by our indirect immunofluorescence assay.

The structures that stained for OSBP in the presence of 25-hydroxycholesterol had the appearance of the Golgi apparatus. To test this hypothesis, we assessed the sensitivity of these structures to disruption by brefeldin A. This agent blocks the recycling of membranes between the ER and Golgi, causing Golgi proteins to accumulate in the ER and in vesicles associated with the ER (Lippincott-Schwartz et al., 1990; Doms et al., 1989). Fig. 9 shows cells expressing wild-type OSBP that were incubated with 25-hydroxycholesterol either in the absence or presence of brefeldin A. In the presence of this agent, the OSBP immunofluorescence was no longer associated with compact masses corresponding to Golgi stacks, but rather it was found in short tubular and

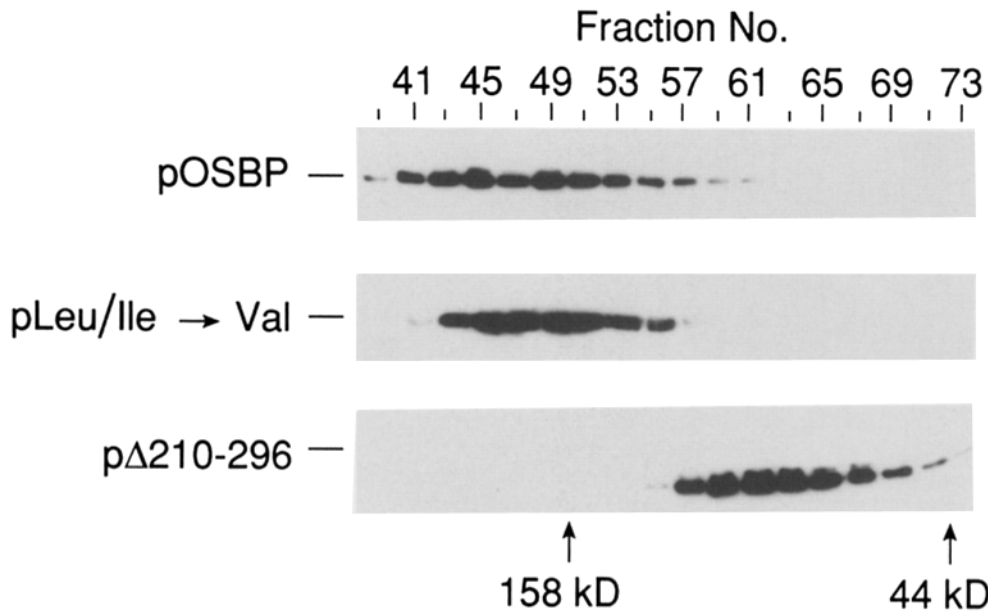


Figure 7. Glycerol gradient fractionation of cytosol from transiently transfected COS-M6 cells. Cytosol (0.2 ml containing 0.25–0.32 mg protein) from COS-M6 cells transfected with the indicated plasmid was loaded onto 5-ml 10–50% glycerol gradients in 10 mM Tris-HCl (pH 7.4) and 0.3 mM KCl. The three gradients were centrifuged at $2 \times 10^5 g$ for 18 h at 4°C. Fractions ($\sim 50 \mu\text{l}$) were collected from the gradients, subjected to 8% SDS-PAGE, and immunoblotted with antibody AB21 (1.3 $\mu\text{g/ml}$). The immunoblots were exposed to Kodak XAR film for 10 h at -70°C . Ovalbumin (44 kD) and ^{125}I -IgG (158 kD) were used as molecular mass standards for the glycerol gradients.

vesicular structures. Brefeldin A had no effect on the OSBP staining in the absence of 25-hydroxycholesterol (data not shown).

Fig. 10 shows a dual-label fluorescence experiment in which cells expressing wild-type or truncated OSBP

($\Delta 455$ –809) were incubated with 25-hydroxycholesterol and then simultaneously stained with an antibody against OSBP (visualized with a rhodamine-conjugated second antibody) and with fluorescein-conjugated lentil lectin. The latter binds to mannose-containing, fucosylated N-linked carbo-

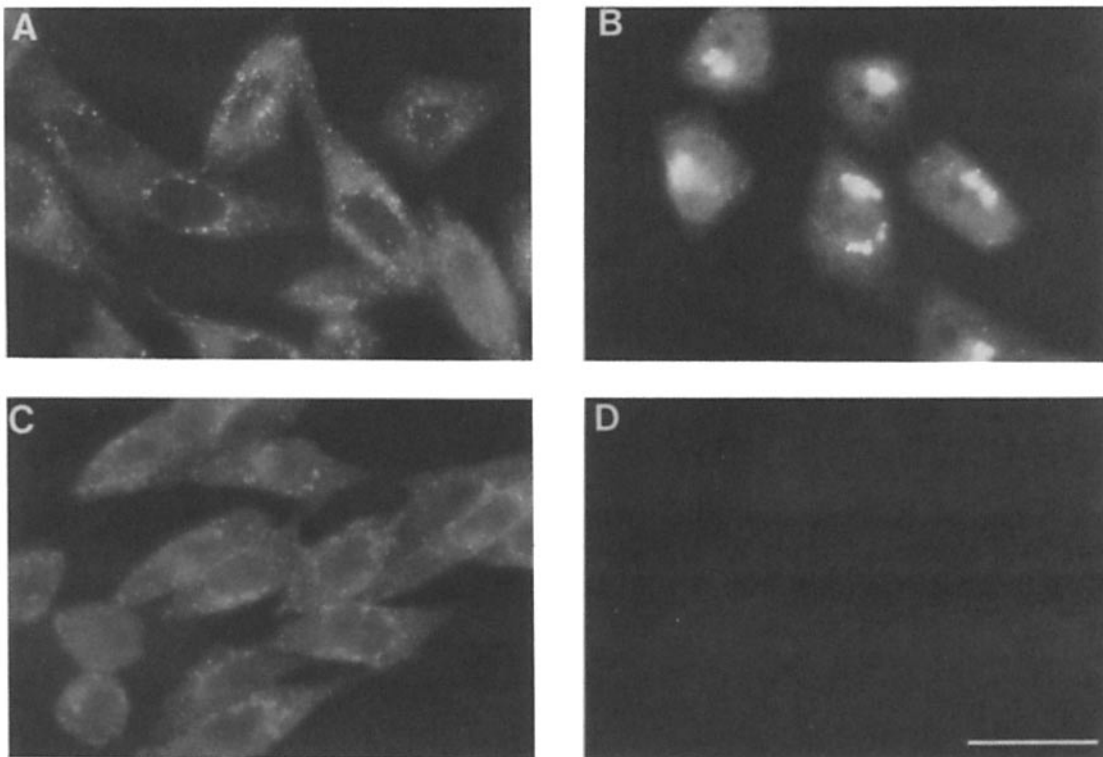


Figure 8. Localization of OSBP by immunofluorescence in stably transfected CHO cells. Transfected CHO cells overexpressing wild-type OSBP were set up for experiments as described in Materials and Methods. On day 3, each dish received medium B containing no sterol (A), 1 $\mu\text{g/ml}$ 25-hydroxycholesterol (B and D), or 5 $\mu\text{g/ml}$ cholesterol (C). After a 5-h incubation at 37°C, the cells were processed for indirect immunofluorescence microscopy as described in Materials and Methods. The primary antibody (5 μg of an IgG fraction) was either polyclonal anti-OSBP W980 (A–C) or a preimmune IgG fraction obtained before immunization (D). Bar, 10 μm .

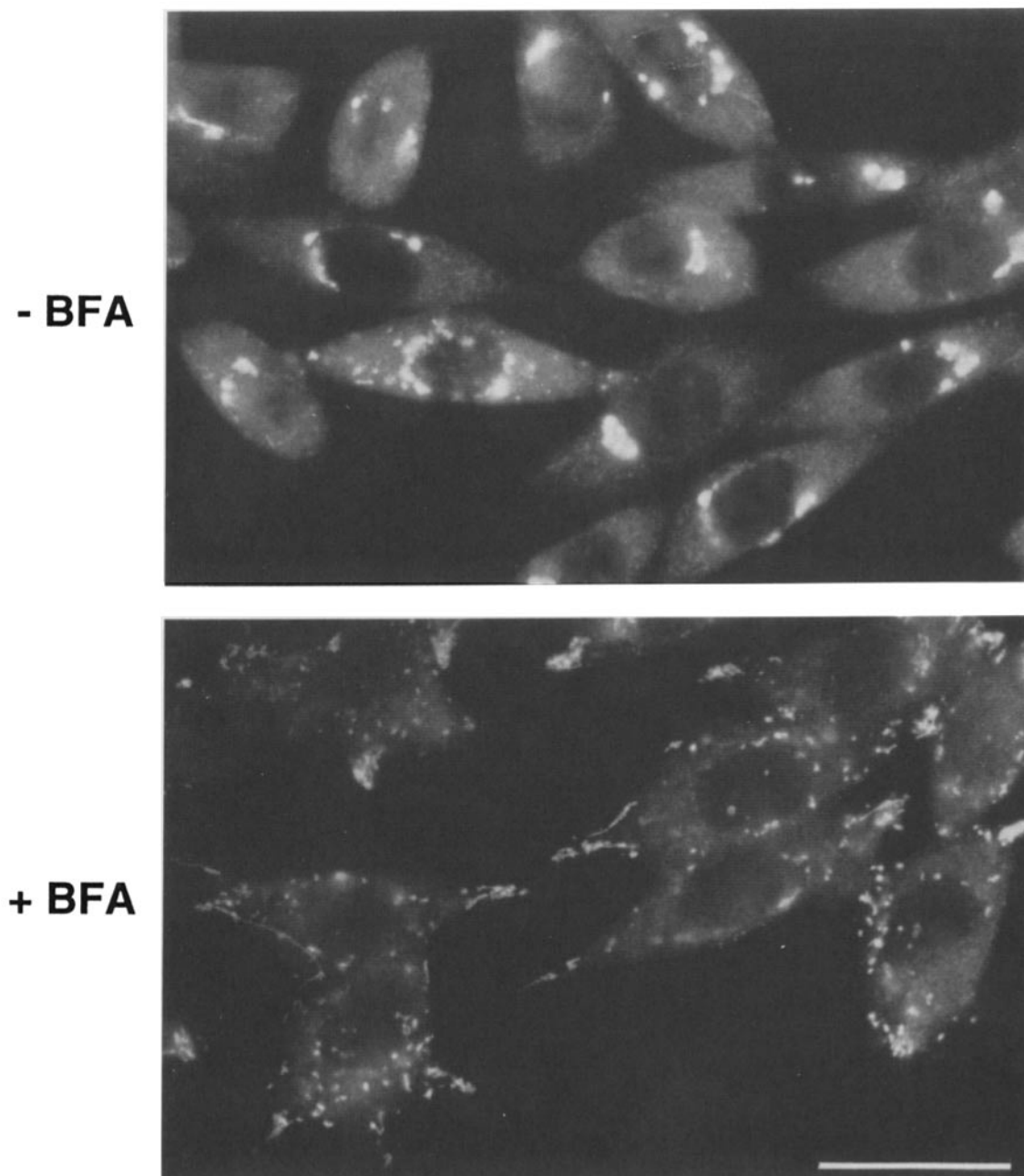


Figure 9. Disruption of 25-hydroxycholesterol-induced Golgi localization of OSBP by brefeldin A (*BFA*) in stably transfected CHO cells. Transfected CHO cells overexpressing wild-type OSBP were set up for experiments as described in Materials and Methods. On day 3, each dish received medium B containing 1 $\mu\text{g/ml}$ 25-hydroxycholesterol. After incubation for 5 h at 37°C, the medium was replaced with fresh medium B containing 1 $\mu\text{g/ml}$ 25-hydroxycholesterol in the absence (*top*) or presence (*bottom*) of 5 $\mu\text{g/ml}$ brefeldin A. After incubation for 1 h at 37°C, the cells were processed for indirect immunofluorescence as described in Materials and Methods. The primary antibody was 2 $\mu\text{g/ml}$ of monoclonal anti-OSBP IgG-11H9. Bar, 10 μm .

hydrate chains that terminate with galactose, N-acetylglucosamine, or sialic acid (Kornfeld et al., 1981). These types of chains are present in the Golgi, but not in the ER (Kornfeld and Kornfeld, 1985). The structures that contained the wild-type OSBP (Fig. 10 *A*) also stained with lentil lectin, confirming that they represented Golgi structures. The COOH-terminal deletion mutant $\Delta 455\text{--}809$, which does not bind 25-hydroxycholesterol (see Fig. 3), was also localized to the Golgi (Fig. 10, *C* and *D*). We used lentil lectin to visualize the Golgi apparatus instead of wheat germ aggluti-

nin because the lentil lectin gave a much brighter intensity of fluorescence in the CHO cells. In unpublished studies, Pathak and Anderson have shown by electron microscopy that lentil lectin localizes to the Golgi apparatus.

The overexpression of OSBP in the transfected CHO cells did not cause any gross change in the appearance of the Golgi as measured by the pattern of staining with lentil lectin (Fig. 11). Moreover, the addition of 25-hydroxycholesterol did not alter the appearance of the Golgi in either the nontransfected cells or the cells expressing OSBP (Fig. 11).

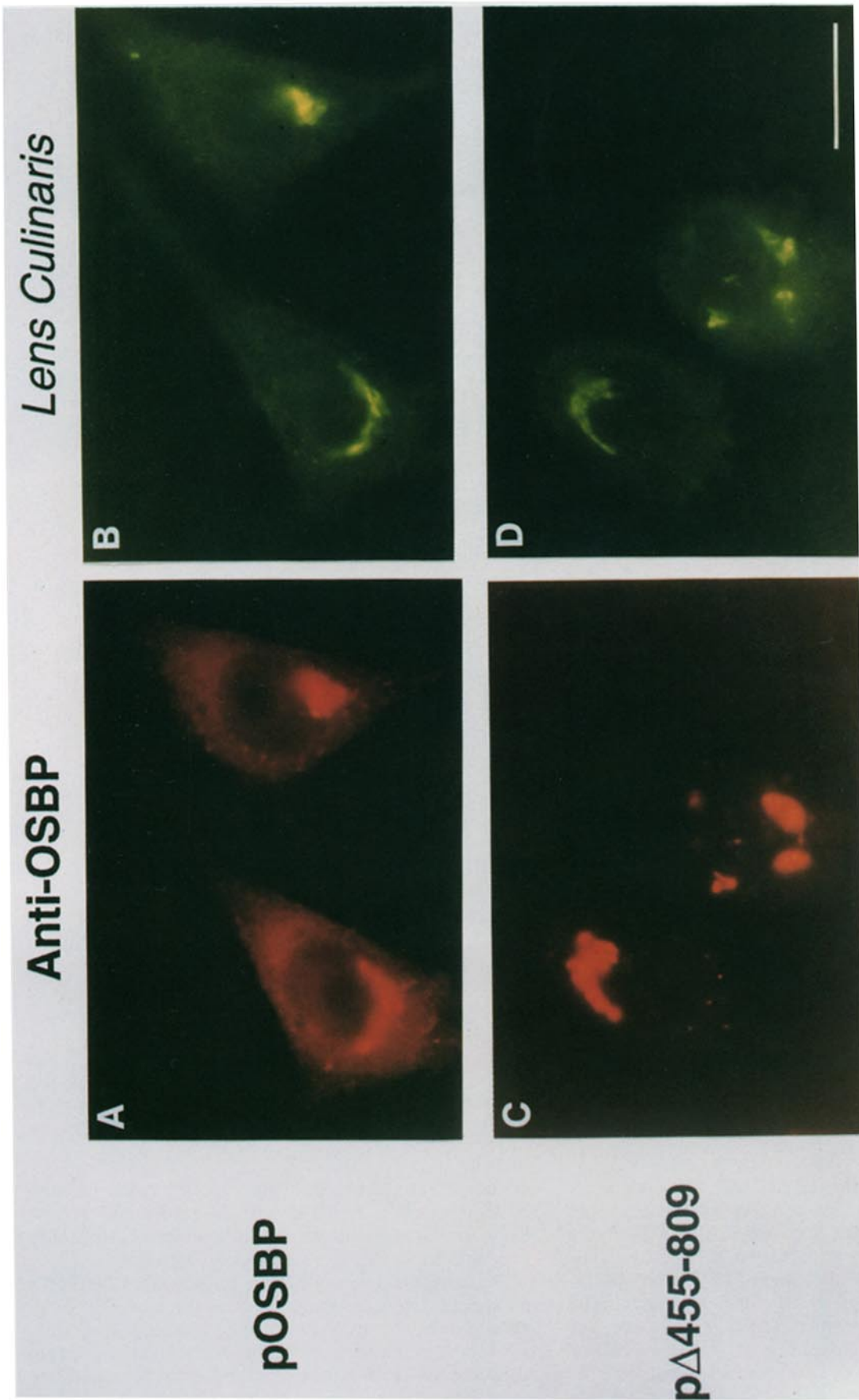


Figure 10. Colocalization of OSBP and a Golgi-specific lectin in 25-hydroxycholesterol-treated CHO-7 cells. Transfected CHO-7 cells stably overexpressing pOSBP (*A* and *B*) or p Δ 455-809 (*C* and *D*) were set up for experiments as described in Materials and Methods. On day 3, each dish was incubated for 5 h at 37°C with medium B containing 1 μ g/ml 25-hydroxycholesterol and then processed for double-label indirect immunofluorescence as described in Materials and Methods. OSBP was visualized with a rhodamine-labeled rabbit anti-mouse IgG after treatment with monoclonal anti-OSBP IgG-1IH9. The Golgi complex was stained with FITC-lentil lectin (*Lens culinaris*). Bar, 10 μ m.

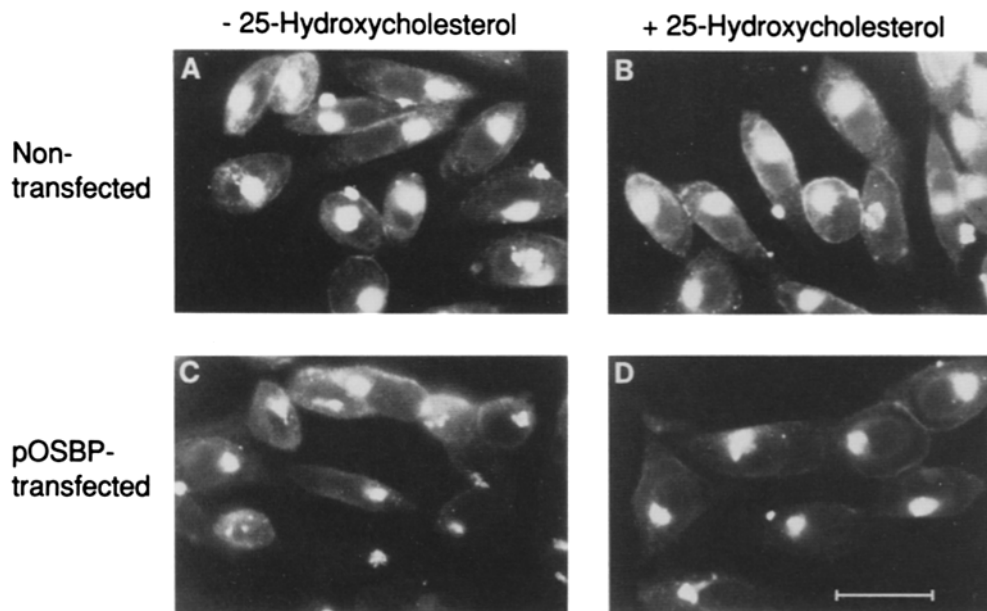


Figure 11. Immunofluorescence localization of FITC-lentil lectin in nontransfected and OSBP-transfected CHO cells. Non-transfected CHO cells (*A* and *B*) and CHO cells stably over-expressing pOSBP (*C* and *D*) were set up for experiments as described in Materials and Methods. On day 3, each dish received medium B containing either no sterol (*A* and *C*) or 1 $\mu\text{g/ml}$ 25-hydroxycholesterol (*B* and *D*). After incubation for 5 h at 37°C, cells were stained with FITC-lentil lectin. Bar, 10 μm .

We next tested a variety of deletion mutants to determine whether they localized to the Golgi either spontaneously or in response to 25-hydroxycholesterol (Fig. 12). These experiments were carried out in transiently transfected CHO cells under conditions in which $\sim 5\%$ of the cells expressed visible amounts of OSBP. As before, the wild-type OSBP moved to the Golgi only after the addition of 25-hydroxycholesterol (Fig. 12, *A* and *B*). The most extensive COOH-terminal deletion mutant, $\Delta 181-809$, failed to localize (Fig. 12, *C* and *D*). The $\Delta 210-296$ deletion, which does not form dimers, nevertheless localized to the Golgi in response to 25-hydroxycholesterol (Fig. 12, *E* and *F*), indicating that the dimerization domain and the leucine zipper region are not required. The most surprising result was obtained with the $\Delta 455-809$ mutant, which lacks the oxysterol binding domain. This protein was attached to the Golgi, even in the absence of 25-hydroxycholesterol (Fig. 12, *G* and *H*). This striking result was consistently observed in multiple other transfection experiments, involving both stably and transiently transfected CHO cells.

Experiments similar to those in Fig. 12 were conducted with the other mutant forms of OSBP, and the results are summarized in Fig. 1. In most cases, these localization studies were performed on permanently transfected cells as well as on cells that expressed the plasmids only transiently. The only other mutant that localized spontaneously to the Golgi was the COOH-terminal $\Delta 578-809$ deletion, which, like the $\Delta 455-809$ mutant, lacks the oxysterol binding domain. We have been unable to test [^3H]25-hydroxycholesterol binding to this mutant because we have been unable to release it from membranes in an active form. The observation that this mutant localizes to the Golgi apparatus in the absence of oxysterols supports the notion that the oxysterol binding site exerts a tonic inhibitory effect on Golgi binding, which is relieved when oxysterols bind.

One mutant gave results that were difficult to interpret with the techniques used, but it is included for completeness. As shown in Fig. 13, the protein with a stop codon at position 261 ($\Delta 261-809$) showed diffuse cytoplasmic and intranuclear staining in permanently transfected cells incubated in the absence of 25-hydroxycholesterol (Fig. 13 *C*). In the pres-

ence of 25-hydroxycholesterol, the amount of staining of the nucleus appeared reduced, and a small amount of protein became localized to the Golgi (Fig. 13 *D*). Although the amount of Golgi association was slight as compared with wild-type OSBP (Fig. 13 *B*), it was reproducible on several occasions in permanently transfected CHO cells. Moreover, in double-label experiments with lentil lectin, three independent observers were able to distinguish the 25-hydroxycholesterol-treated cells from the untreated cells based on a slight amount of fluorescence staining of the Golgi with the anti-OSBP antibody. The potential implications of these findings are discussed below.

In additional experiments not shown, we found that the 25-hydroxycholesterol-induced movement of wild-type OSBP to the Golgi apparatus is first apparent ~ 30 min after adding 25-hydroxycholesterol to the culture medium, but does not reach a maximum until 3–5 h. This translocation also occurred when we added 5–10 $\mu\text{g/ml}$ of 20-hydroxycholesterol or 7-ketocholesterol to the culture medium, although the magnitude of the movement was not as complete as observed with equivalent concentrations of 25-hydroxycholesterol. Both 20-hydroxycholesterol and 7-ketocholesterol are known to bind to OSBP and to regulate cellular sterol metabolism, albeit at higher concentrations than 25-hydroxycholesterol (Dawson et al., 1989*a,b*; Taylor et al., 1984). On the other hand cholesterol, pregnenolone, and β -sitosterol at concentrations of 10 $\mu\text{g/ml}$ did not promote detectable translocation of OSBP to the Golgi (data not shown). These sterols do not bind to OSBP *in vitro*, and they do not regulate sterol metabolism when added to culture medium at these concentrations. Incubation of the OSBP-transfected CHO cells with plasma LDL, plasma β -migrating very low density lipoproteins (β -VLDL), or mevalonate did not promote movement of OSBP to the Golgi when added at concentrations that effectively suppress HMG CoA reductase activity and stimulate ACAT activity (data not shown).

Discussion

The current experiments show that OSBP in transfected cells exists as a cytoplasmic protein that is partly soluble and

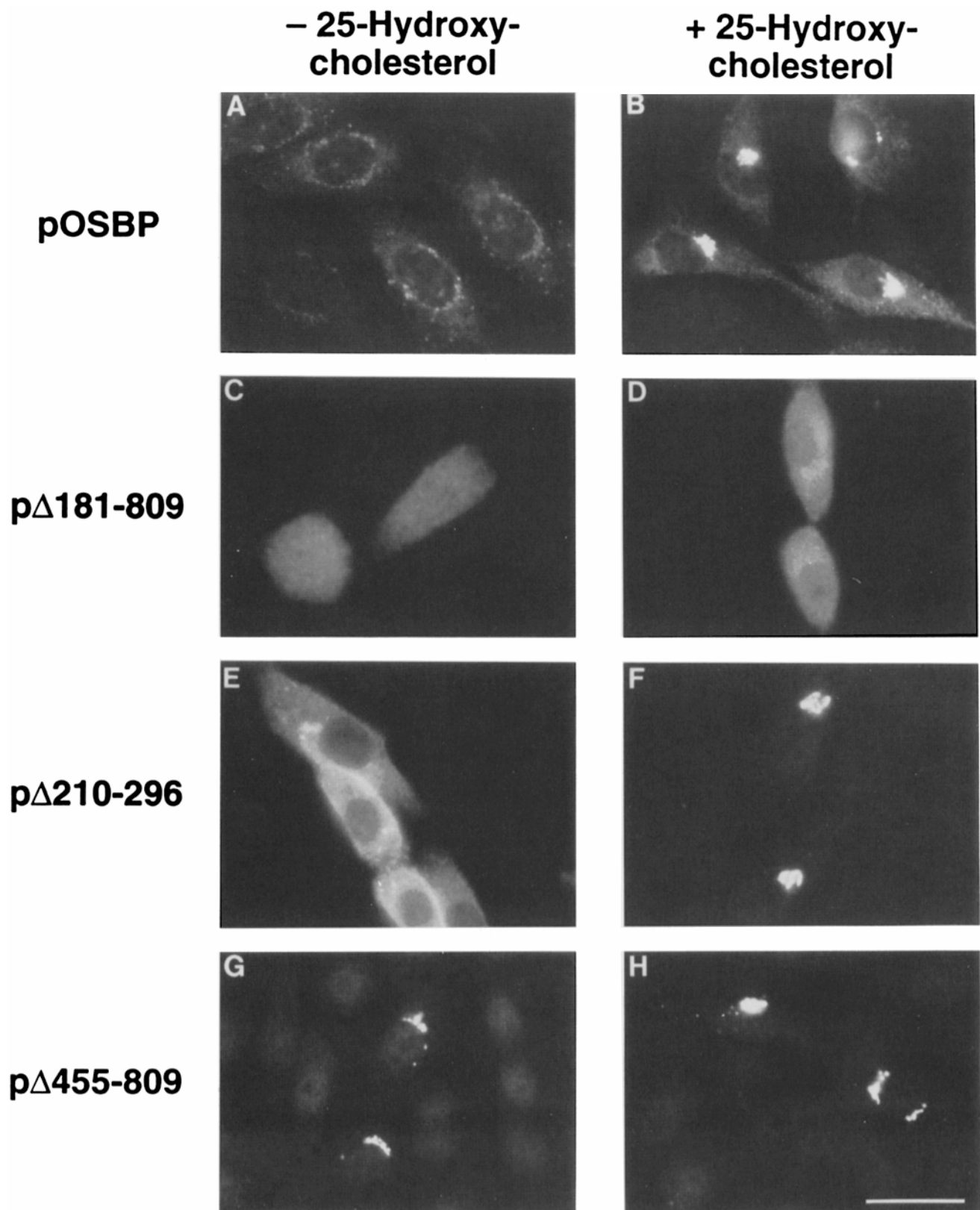


Figure 12. Immunofluorescence localization of mutant versions of OSBP in transiently transfected CHO cells. The indicated plasmids were transiently transfected in CHO cells as described in Materials and Methods. On day 4, each dish was incubated for 5 h at 37°C with medium B in the absence or presence of 1 $\mu\text{g/ml}$ 25-hydroxycholesterol as indicated and then processed for indirect immunofluorescence microscopy as described in Materials and Methods. The primary antibody was 2 $\mu\text{g/ml}$ of monoclonal anti-OSBP IgG-11H9. Bar, 10 μm .

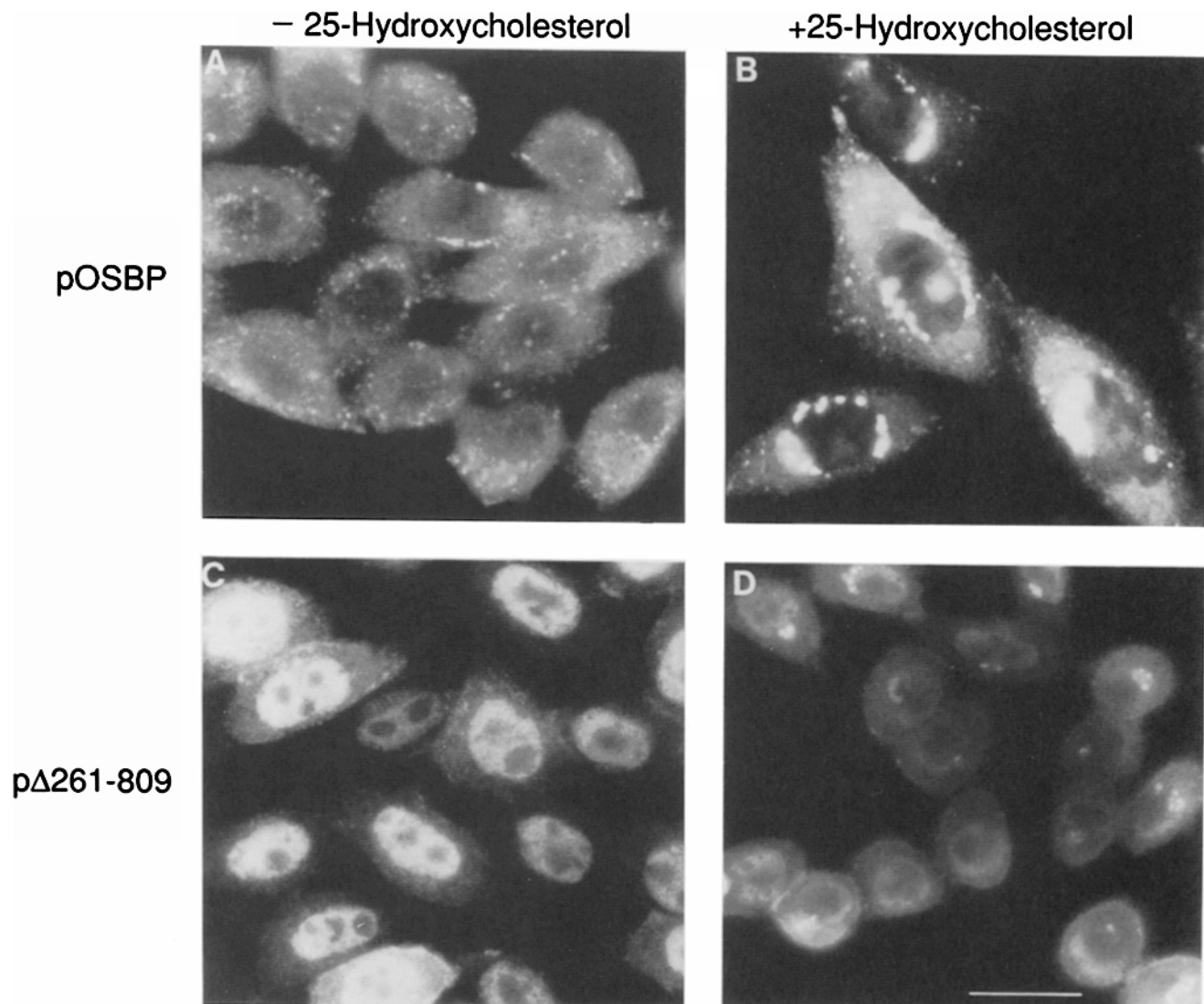


Figure 13. Immunofluorescence localization of wild-type and $\Delta 261-809$ OSBP in stably transfected CHO cells. Transfected CHO cells stably overexpressing pOSBP (*A* and *B*) or p $\Delta 261-809$ (*C* and *D*) were set up for experiments as described in Materials and Methods. On day 3, each dish received medium B in the absence (*A* and *C*) or presence (*B* and *D*) of 1 $\mu\text{g}/\text{ml}$ 25-hydroxycholesterol. After incubation for 5 h at 37°C, the cells were processed for indirect immunofluorescence as described in Materials and Methods. The primary antibody was 5 $\mu\text{g}/\text{ml}$ of polyclonal anti-OSBP W980 (IgG fraction). Bar, 10 μm .

partly bound to small perinuclear vesicles. Upon binding oxysterols, OSBP translocates to the Golgi apparatus where it remains attached as an extrinsic protein. This translocation appears to be triggered by an interaction between the oxysterol binding domain in the COOH-terminal half of the protein and a putative Golgi-binding domain in the NH₂-terminal half.

The experiments with deletion mutants suggest that the COOH-terminal half of OSBP exerts a tonic inhibitory effect upon Golgi localization that is relieved when oxysterols bind to this domain. Thus, in the stop 455 and stop 578 mutants, which lack the oxysterol binding domain, OSBP associated with the Golgi constitutively in the absence of sterols. This control mechanism resembles the mechanism that controls the nuclear entry of the glucocorticoid receptor, which regulates transcription of nuclear genes (Picard et al., 1988). The glucocorticoid receptor contains a COOH-terminal steroid binding domain that prevents nuclear entry unless steroids

are bound. By analogy, it seems likely that the COOH-terminal domain of OSBP physically interacts with the part of the protein that mediates Golgi attachment, obscuring this site unless sterols are bound.

The OSBP mutant that stops at residue 261 ($\Delta 261-809$) did not localize to the Golgi spontaneously even though it lacks the oxysterol binding domain. Moreover, despite the absence of this domain, a detectable fraction of the mutant protein appeared to localize in the Golgi in response to 25-hydroxycholesterol. The relative amount of such localization was small when compared with wild-type OSBP and with the stop 455 and stop 578 mutants. At present, we have no insight into the mechanism whereby oxysterols promote Golgi localization of the stop 261 mutant. It is possible that this localization occurs in response to some subtle change in the Golgi that is induced by 25-hydroxycholesterol administration. Alternatively, the p $\Delta 261-809$ protein may interact in some fashion with endogenous OSBP. We believe that the

stop 261 mutant cannot form a direct complex with native OSBP because it does not form a homodimer and appears to lack the dimerization domain. However, it is possible that this mutant protein forms a complex with another protein that binds to endogenous OSBP and that this complex then localizes to the Golgi by virtue of the action of the native OSBP. Against this hypothesis is our observation that the amount of endogenous OSBP is too low to be visualized by immunofluorescence, and hence we should not detect any stoichiometric complex between the mutant OSBP and endogenous OSBP.

The $\Delta 261-809$ mutant was also the only construct to give nuclear staining. The amount of nuclear staining appeared to be reduced when oxysterols were added, but no quantification was performed. It should be noted that the $\Delta 181-809$ mutant, which is shorter by 80 residues than the $\Delta 261-809$ mutant, failed to localize either in the nucleus or the Golgi. Thus, the residues between 181 and 261 may play a role in both localizations. Further studies will be necessary to define the puzzling properties of the $\Delta 261-809$ mutant.

The binding site on the Golgi membranes that serves as the recognition site for OSBP must be present in large excess since OSBP was localized quantitatively to the Golgi in transfected cells that overexpressed OSBP by 20–100-fold as compared to wild-type cells. This observation suggests that the binding site on the Golgi may also serve as the attachment site for other proteins that are more abundant than OSBP. Several other proteins have been shown to bind extrinsically to the Golgi apparatus, including a 110-kD protein that is released from the Golgi upon addition of brefeldin A (Donaldson et al., 1990) and a 58-kD protein that also binds to microtubules (Bloom and Brashear, 1989). When the sequences of these proteins are obtained, it will be of interest to determine whether they bear any resemblance to OSBP, which would suggest the existence of a common binding site for extrinsic Golgi-associated proteins.

When OSBP was purified to homogeneity, it was observed to be a homodimer as indicated by its behavior upon gel filtration (Dawson et al., 1989b). The presence in both rabbit and human OSBP of a sequence that fits the criteria for a leucine zipper immediately suggested that this was the region that mediated dimerization (Dawson et al., 1989a; Levanon et al., 1990). The current studies show that this region, which lies between residues 209 and 244, is not necessary for homodimer formation. Rather, dimerization appears to depend upon a sequence in the interval between residues 261 and 296. Leucine zippers represent examples of α -helices that interact with each other through the formation of a coiled coil (O'Shea et al., 1989). The leucine zipper in OSBP may act to stabilize dimers without being essential for their formation. Finally, it is possible that OSBP might be joined to another protein through leucine zippers, but so far we have been unable to find evidence for such heterodimer formation with OSBP. In particular, we have been unable to find evidence that OSBP associates with heat shock proteins, which have been shown to bind to some steroid hormone receptors in a steroid-regulated fashion (Beato, 1989).

If, as the current data indicate, OSBP is a protein that translocates to the Golgi upon binding oxysterols, what is its function? Oxysterols regulate three events that are believed to take place in the ER, namely, repression of translation of HMG CoA reductase, acceleration of degradation of HMG

CoA reductase, and activation of ACAT (Goldstein and Brown, 1990). If OSBP mediates any of the regulatory actions of oxysterols, it is likely that it affects one or more of these processes. It is possible that in the absence of oxysterols OSBP cycles between the cytoplasm, the ER, and the Golgi apparatus. Upon binding oxysterols, it may remain in the Golgi, and its absence from the ER might lead to one of the regulatory changes discussed above.

The current data also raise the possibility that OSBP is a transport protein that delivers oxysterols to the Golgi. This seems somewhat unlikely because OSBP is a trace protein in all cell types examined to date, and the amounts are insufficient for a bulk transport role. Moreover, once OSBP has translocated to the Golgi, it remains attached there for several hours, as determined by immunofluorescence, even when the 25-hydroxycholesterol is washed out of the culture medium (data not shown). This type of behavior would be more characteristic of a regulatory protein than a transport protein whose supply of substrate should be exhausted after the oxysterol is removed. Further studies will be necessary to determine whether OSBP plays a role in metabolism or transport of oxysterols in the Golgi apparatus, or whether its true role is regulatory.

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