Biochem. J. (1989) 263, 519–532 (Printed in Great Britain)

Indirect immunofluorescence localization of β-adrenergic receptors and G-proteins in human A431 cells

Hsien-yu WANG, Miguel BERRIOS and Craig C. MALBON*
Department of Pharmacology, Diabetes & Metabolic Diseases Research Program, Health Sciences Center, The State University of New York, Stony Brook, NY 11794-8651, U.S.A.

Polyclonal antibodies directed against (i) rodent lung β_2-adrenergic receptor, (ii) a synthetic fragment of an extracellular domain of the receptor, and (iii) human placenta G-protein β-subunits, were used to localize these antigens *in situ* in intact and permeabilized human epidermoid carcinoma A431 cells. Antibodies directed against β_2-adrenergic receptors showed a punctate immunofluorescence staining throughout the cell surface of fixed intact cells. Punctate staining was also observed in clones of Chinese hamster ovary cells transfected with an expression vector harbouring the gene for the hamster β_2-adrenergic receptor. The immunofluorescence observed with anti-receptor antibodies paralleled the level of receptor expression. In contrast, the β-subunits common to G-proteins were not stained in fixed intact cells, presumably reflecting their intracellular localization. In detergent-permeabilized fixed cells, strong punctate staining of G_β-subunits was observed throughout the cytoplasm. This is the first indirect immunofluorescence localization of β-adrenergic receptors and G-proteins. Punctate immunofluorescence staining suggests that both antigens are distributed in clusters.

INTRODUCTION

Transmembrane signalling pathways mediate the effects of many hormones (Gilman, 1987; Neer & Clapham, 1988) and other sensory inputs, including photoreception (Stryer & Bourne, 1986) and olfaction (Snyder *et al.*, 1988). These seemingly diverse pathways share a common feature in their composition. Each is composed from three classes of membrane proteins: (i) receptors, such as the β-adrenergic receptors or the photopigment rhodopsin, are exposed to the external surface of the cell membrane and are the primary elements of signal discrimination (Stiles *et al.*, 1984; Findlay & Pappin, 1986); (ii) effector units, such as adenylate cyclase and phospholipase C, are exposed to the cytoplasm and generate second messenger molecules; and (iii) guanine-nucleotide-binding, regulatory G-proteins are transducers mediating the signal from activated receptors to effector unit(s) (Lochrie & Simon, 1988). The catecholamine-sensitive activation of adenylate cyclase, too, exemplifies this organization. Binding of catecholamines to β-adrenergic receptors activates the G-protein G_s, which, in turn, stimulates adenylate cyclase. β-Adrenergic stimulation of this enzyme increases intracellular cyclic AMP (Levitzki, 1988).

Our understanding of the biochemistry and molecular biology of G-protein-linked receptors and G-proteins has expanded greatly (for reviews see Stiles *et al.*, 1984; Lochrie & Simon, 1988). Recently, molecular cloning techniques have revealed the primary sequence of β-adrenergic receptors from hamster, human and avian species (Dixon *et al.*, 1986; Yarden *et al.*, 1986; Kobilka *et al.*, 1987). The G-proteins are heterotrimeric membrane proteins (α,β,γ; 1:1:1), distinguished by unique α-subunits but sharing common β-subunits (Gilman, 1987). The protein sequences of the α- and β-subunits of several G-proteins have also been determined by molecular cloning (Sugimoto *et al.*, 1985; Robishaw *et al.*, 1986; Sullivan *et al.*, 1986). In contrast to the advances in our understanding of the biochemistry and molecular biology of these transmembrane signalling elements, little is known of their cell biology. Indirect immunofluorescence is an invaluable tool with which one can begin to elucidate the cell biology of G-protein-linked receptor pathways. Recently Strader *et al.* (1987) and Kaveri *et al.* (1987) reported on the use of indirect immunofluorescence to study β-adrenergic receptors. These two studies focused upon the effects of chronic treatment of cells with agonist on receptor levels. The current work, in contrast, focuses upon the cellular localization of β-adrenergic receptors and G-proteins.

Specific antisera for β-adrenergic receptors and G-protein β-subunits have been developed and characterized in detail by immunoprecipitation, immunoblotting, e.l.i.s.a., and functional assays (Rapiejko *et al.*, 1986; Moxham *et al.*, 1986, 1988; Evans *et al.*, 1987; Ros *et al.*, 1988; Hadcock & Malbon, 1988; Alho *et al.*, 1988). Immunofluorescence localization *in situ* of G-protein-linked receptors and G-proteins was explored using human epidermoid carcinoma A431 cells. A431 cells are replete with β-adrenergic receptors, display β-adrenergic agonist stimulation of adenylate cyclase, and demonstrate agonist-promoted desensitization of transmembrane signalling (Delavier-Klutchko *et al.*, 1984; Guillet *et al.*, 1985; Kashles & Levitzki, 1987). These features and the ability of the cells to grow well on glass surfaces prompted our studies using A431 cells.

Abbreviations used: G-protein; guanine-nucleotide-binding protein; CHO, Chinese hamster ovary; KLH, keyhole-limpet haemocyanin; ICYP, iodocyanopindolol; DIC, differential interference-contrast; PAGE, polyacrylamide-gel electrophoresis.

* To whom all correspondence should be addressed.

EXPERIMENTAL

Cells and culture conditions

Four subclones of A431 human epidermoid carcinoma cells were obtained and designated as follows: A431-ATCC cells from the American Type Cell Collection (A.T.C.C.) (Bethesda, MD, U.S.A.); A431-O from Dr. Roger Davis (University of Massachussetts Medical Center, Worcester, MA, U.S.A.); A431-N from Dr. Gary Johnson (National Jewish Center for Immunology, Denver, CO, U.S.A.); and A431-Y from Dr. John Perkins (Yale University, New Haven, CT, U.S.A.). Chinese hamster ovary (CHO) cells were obtained from the A.T.C.C. CHO cells co-transfected with expression vectors pSV2BAR (harbouring the cDNA encoding the hamster β_2-adrenergic receptor) and pHOMER (harbouring the neomycin-resistance gene) were isolated in this laboratory. These clones stably express β-adrenergic receptors at levels ranging from 200 to 2 million receptors per cell (George *et al.*, 1988).

A431 cells were routinely plated at a density of 1×10^6 cells/150 mm^2 dish (for membrane preparation) or a density of 0.5×10^6 cells/100 mm^2 Petri dishes containing one glass microscope slide in Dulbecco's modified Eagle's medium supplemented with streptomycin (100 mg/l), penicillin (60 mg/l) and 5% heat-treated fetal bovine serum. CHO cells were grown in RPMI 1640 media containing 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). For transfectant CHO clones, the serum-containing RPMI media was supplemented with G418 (300 μg/ml).

Antigens and antibody fractions

β-Adrenergic receptors used to generate anti-receptor antibodies in rabbits were purified by affinity chromatography and h.p.l.c. steric-exclusion, as described earlier (Cubero & Malbon, 1984; Benovic *et al.*, 1984; Graziano *et al.*, 1985). Characterization of the anti-receptor antiserum 6EN-1 was reported earlier (Moxham *et al.*, 1986, 1988; Weiss *et al.*, 1987; Ros *et al.*, 1988; George *et al.*, 1988). For the preadsorption studies, the purified β_2-adrenergic receptors employed were isolated from S49 mouse lymphoma cells (George & Malbon, 1985). The receptor displayed a specific activity of approx. 9 nmol of ligand binding per mg of protein and behaved as monodispersed 65000-M_r peptide species on silver-stained polyacrylamide gels following chemical reduction, alkylation and electrophoresis in the presence of SDS (see the Results section). The peptide CM-2 (YHKETC), which corresponds to the amino-acid sequence 185–190 of the hamster β_2-adrenergic receptor, is located at the second extracellular loop of the model of the β-adrenergic receptor predicted from hydropathy analysis (Dixon *et al.*, 1986). The peptide was synthesized by the Center for Analysis and Synthesis of Macromolecules at the State University of New York, Stony Brook. The composition of the peptide was determined by amino-acid analysis and the sequence was verified by gas-phase microsequencing. Purity of the peptide preparation was greater than 87% as determined by separation by reverse-phase h.p.l.c. Early termination products of the synthesis lack the terminal cysteine residue and failed to couple to keyhole-limpet haemocyanin (KLH).

The G_β-subunits (M_r 35000 and 36000) were isolated from human placenta (Evans *et al.* 1987) or rabbit heart (Malbon *et al.*, 1985). Based upon polyacrylamide-gel analysis (see the Results section) and functional reconstitution (Evans *et al.*, 1987) the G_β-subunit preparation was essentially pure. Antiserum G_β-6 was characterized previously (Rapiejko *et al.*, 1986; Evans *et al.*, 1987; Watkins *et al.*, 1987; Ros *et al.*, 1988).

Anti-peptide antibodies were raised as follows: 5 mg of peptide CM-2 was coupled through its native cysteine residue to a carrier protein KLH (4 mg) using *m*-maleimidobenzoyl *N*-hydroxysuccinimide (Liu *et al.*, 1979; Green *et al.*, 1982). The coupling efficiency was 21%, as determined by the use of ^{125}I-labelled peptide as a tracer. An aliquot of peptide–KLH conjugate (0.2 mg) was emulsified in 0.2 ml of a mixture of monophosphoryl lipid A and trehalose dimycolate before injection. New Zealand rabbits (Hazelton, Denver, PA, U.S.A.) were immunized with the KLH–peptide conjugate by injecting into the Popliteal lymph node (Sigel *et al.*, 1983). At 2 weeks after the first immunization, the rabbits were injected again and every week thereafter. The rabbits were bled 4 days after a boost injection and the sera analysed for anti-receptor antibody titre by immunoblotting. Anti-peptide antiserum CM8-3 was employed in the experiments described below. Calf alkaline phosphatase-conjugated goat anti-rabbit IgG fraction was from Cappel (West Chester, PA, U.S.A.); affinity-purified rhodamine-conjugated goat anti-rabbit IgG fraction was from Boehringer Mannheim (Indianapolis, IN, U.S.A.).

Membrane fractions

Cells were harvested from 150 mm^2 Petri dishes with an ice-cold buffer composed of 10 mM-Hepes, pH 7.2, 150 mM-NaCl, 1 mM-EDTA, and a mixture of protease inhibitors. The protease inhibitors used were leupeptin (5 μg/ml), aprotinin (5 μg/ml) and phenylmethanesulphonyl fluoride (50 μM). The cells were collected by centrifugation at 1000 *g* for 5 min. The cell pellets were homogenized in an ice-bath with 20 full strokes using a tightly-fitted 7 ml Dounce homogenizer (Wheaton no. 357542). Membrane fractions were prepared as described earlier (George & Malbon, 1985). Protein was determined by the method of Lowry *et al.* (1951).

Measurement of β-adrenergic receptor

The density of β-receptors was measured in intact cells and in membrane fractions prepared from cells using equilibrium binding analysis and a high-affinity antagonist ligand [^{125}I]iodocyanopindolol ([^{125}I]ICYP), exactly as described (Bahouth & Malbon, 1987). Non-specific binding was less than 10%. Binding assays were performed in triplicate and the variance among values was typically less than 5%.

Immunoblotting

A431 cell membrane proteins were chemically reduced with 20 mM-dithiothreitol in Laemmli (1970) sample solution, alkylated with 50 mM-2-iodoacetamide and subjected to electrophoresis on 10% polyacrylamide gels in the presence of 0.1% (w/v) SDS. The separated membrane proteins were transferred to nitrocellulose using a Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories, Rockville Centre, NY, U.S.A.), following the procedure of Towbin *et al.* (1979), as modified by Erikson *et al.* (1982). Nitrocellulose blots of the separated proteins were probed with primary and then secondary

antibodies and the immune complexes were made visible as described (Moxham *et al.*, 1986, 1988; Rapiejko *et al.*, 1986).

Indirect immunofluorescence

A431 cells grown on glass slides were fixed at 25 °C for 3 min with freshly prepared 3% (w/v) paraformaldehyde in modified Shields' medium buffered with Pipes [MSM/Pipes (Smith *et al.*, 1987)], without detergents, composed of 18 mM-$MgSO_4$, 5 mM-$CaCl_2$, 40 mM-KCl, 24 mM-NaCl and 5 mM-Pipes, pH 6.8. Permeabilization of A431 cells was achieved by supplementing the fixative buffer with 0.1% (v/v) Triton X-100 and 0.1% (v/v) Nonidet P40. The conditions that yield permeabilization with minimum distortion of cell morphology were determined empirically using increasing concentrations of detergent and indirect immunofluorecence with antibodies against nuclear antigens (Smith *et al.*, 1987). After fixation, the slides were washed twice with MSM/Pipes for 5 min. The slides were then incubated with antisera 6EN-1 (1:50–1:400 dilution), CM8-3 (1:50–1:400 dilution), G_β-6 (1:200 dilution) or pre-immune serum (1:50–1:400 dilution) for 30 min at 37 °C in a humidified chamber. Preadsorption of antisera with antigen was performed with antisera at 1:400 dilution that had been incubated overnight at 22 °C in a volume of 0.2 ml with excess antigen. The following amounts of antigen were used in the preadsorption experiments: 5 μg of purified β-adrenergic receptor for 6EN-1; 0.1 μmol of 8-3 peptide for CM8-3; and 0.5 μg of purified human placental β-subunits (Evans *et al.*, 1987) for G_β-6.

Slides were washed three times for 10 min each with MSM/Pipes buffer and then incubated with rhodamine-conjugated goat anti-rabbit IgG (1:1000 dilution) at 37 °C for 30 min. After incubation with the second antibody, slides were washed in a buffer composed of 10 mM-KH_2PO_4, pH 7.5, and 560 mM-NaCl. A coverslip was then mounted and the stained specimens were examined by phase-contrast and epifluorescence microscopy using a Zeiss Axiophot microscope. Photographs were taken using Kodak 2415 film, hypersensitized at 25–30 °C with forming gas at 62 kPa for 4 days. Differential interference-contrast (DIC) microscopy was performed at the Marine Biological Laboratory (Woods Hole, MA, U.S.A.).

Materials

The radiochemicals [^{125}I]ICYP, ^{125}I-Bolton–Hunter reagent and ^{125}I-labelled goat anti-rabbit IgG were from New England Nuclear (Boston, MA, U.S.A.). All other reagents were obtained from sources identified earlier (Moxham *et al.*, 1986, 1988; George *et al.*, 1988).

RESULTS

β-Adrenergic receptor expression in four A431 cell subclones

Fig. 1 shows four different A431 cell subclones obtained for these studies. A431-N and A431-O cells (Figs. 1*a* and 1*b*) display very similar morphology with a pronounced spindle-like shape and large nuclei. A431-Y and A431-ATCC cells (Figs. 1*c* and 1*d*), in sharp contrast, display a distinctly different morphology appearing quite flat and thin, and non-spindle-like in shape. For this reason, the size of the A431-ATCC and A431-Y cells appears to be much greater than that of either A431-N or A431-O cells. More detailed structural information on cells from the A431 clone selected for most of the subsequent work was obtained in images from DIC microscopy (Figs. 1*e* and 1*f*). Protruding nuclei were highlighted by the clear, rugose edges of the nuclear envelope. Interestingly, many large vesicle-like structures were observed about the nuclear periphery. These vesicle-like structures were previously observed in scanning electron microscopy (Cohen *et al.*, 1982). The phase-contrast and DIC microscopy provided a useful framework for interpretation of the images obtained by indirect immunofluorescence.

The β-adrenergic receptor complement of each of the four subclones was determined by equilibrium binding assay with the high-affinity β-adrenergic antagonist ligand [^{125}I]ICYP. Expressed in fmol of β-adrenergic receptor/10^6 cells, the receptor concentration in the A431 subclones was found to vary from a low of 77 (A431-N) to a high of 160 (A431-ATCC), i.e. 48200–96000 sites per cell respectively. These data agree well with the levels reported by others for various A431 clones (Delavier-Klutchko *et al.*, 1984; Kashles & Levitzki, 1987). Measurements of receptor levels in membranes obtained from the cells provided comparable data. Specific activities of ICYP binding (fmol/mg of membrane protein) of membrane fractions isolated from sucrose-density gradients were as follows: 390 (A431-O), 690 (A431-N), 770 (A431-Y) and 750 (A431-ATCC). In spite of the differences in cell morphology, the results of radioligand-binding assays as well as immunoblotting and indirect immunofluorescence using the four A431 subclones were remarkably similar (results not shown). The results shown were obtained using subclone A431-Y and are representative of all four subclones.

Identification of β_2-adrenergic receptors in A431 cell membranes using antibodies directed against rodent lung β_2-receptors

Identification of A431 β_2-adrenergic receptors was performed by immunoblotting. A crude membrane fraction prepared from A431 cells was subjected to SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) and transferred to nitrocellulose. Immunoblots probed with anti-receptor antiserum 6EN-1 displayed a prominent staining of a 65000-M_r peptide species (Fig. 2*a*, lanes 1 and 2; 0.2 and 0.4 mg of protein per SDS/PAGE lane, respectively). This 65000-M_r peptide from A431 cells co-migrated with β_2-adrenergic receptors isolated from a mouse source (Fig. 2*a*, lane 3). The purified mouse receptor displayed a specific activity of > 9 nmol of ligand binding/mg of protein, near theoretical purity (George & Malbon, 1985), and co-reconstituted receptor-mediated activation of purified G_s in defined liposomes (Moxham *et al.*, 1988). Minor bands with M_r values of 55000–63000 were also observed on heavily-loaded and overstained blots of A431 membrane proteins (Fig. 2*a*, lanes 1 and 2). These peptides and other minor bands represent either proteolytic fragments or incompletely reduced forms of the receptor (Cubero & Malbon, 1984; Benovic *et al.*, 1984; Moxham & Malbon, 1985; Graziano *et al.*, 1985). When blotting was performed under standard conditions (0.1 mg of protein per SDS/PAGE lane), prominent immunoreactive staining was confined to a 65 000-M_r peptide in blots probed with 6EN-1 antibody at various dilutions (Fig. 2*b*). These results demonstrate that these antibodies directed against rodent β_2-

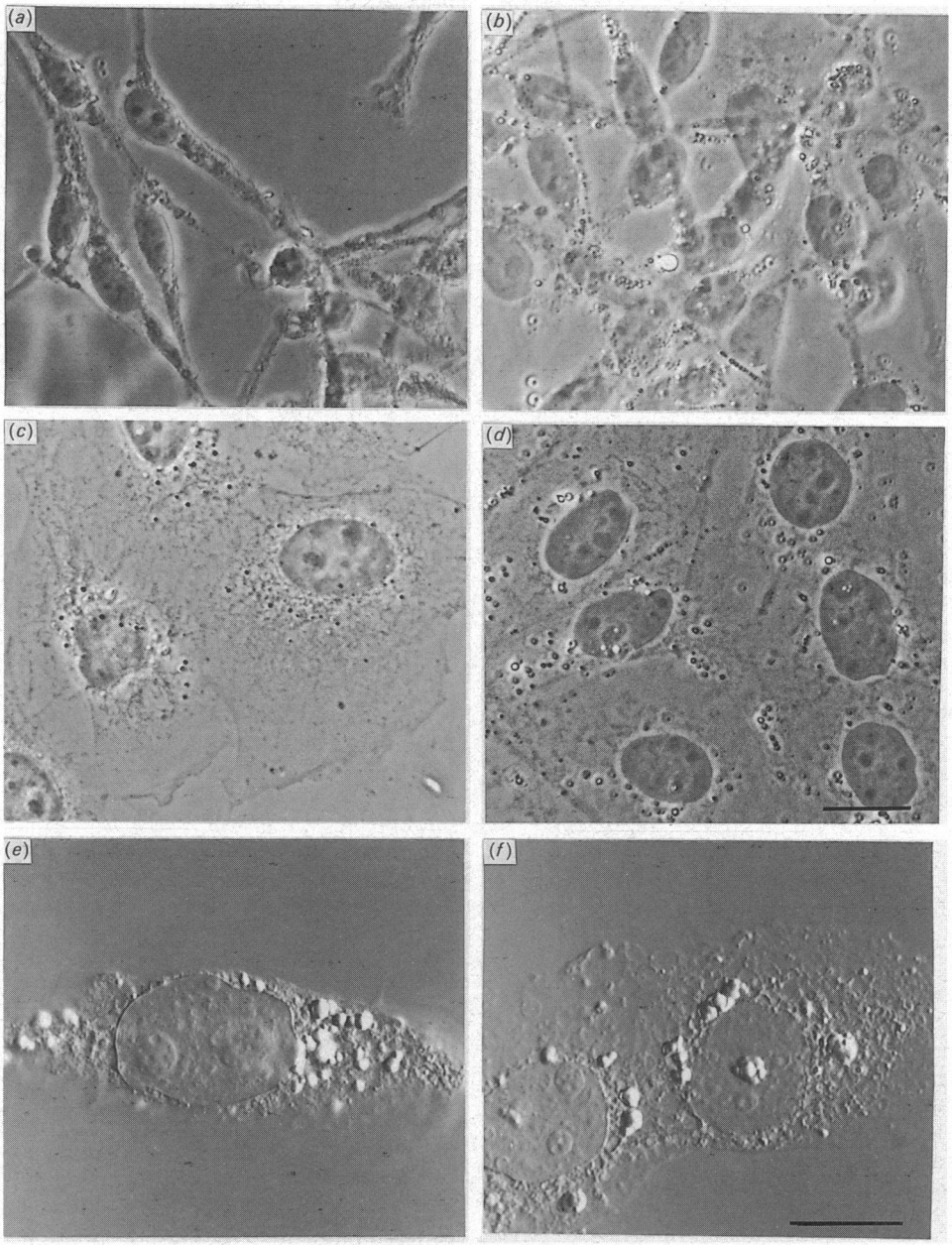


Fig. 1. Phase-contrast and DIC microscopy of A431 cells

Phase-contrast images of different cell types are as follows: (*a*) A431-N, (*b*) A431-O, (*c*) A431-Y and (*d*) A431-ATCC (bar 25 μm). (*e*) and (*f*) are DIC images of A431-Y cells (bar 30 μm).

adrenergic receptor specifically recognize their human counterparts. The immunological data support the prediction from molecular cloning studies for the existence of a high degree of similarity between human and rodent β_2-adrenergic receptors (Kobilka *et al.*, 1987).

Immunoblot analysis of β_2-adrenergic receptors of A431 cell membranes with anti-peptide antibodies directed against an extracellular domain of the hamster receptor

Independent confirmation of the 65000-M_r species as the β-adrenergic receptor in A431 cells was provided by probing blots of A431 cell membrane-fractions with a site-directed, anti-peptide antibody. This antiserum (CM8-3) was raised against a synthetic peptide that corresponds to the second extracellular loop of the hamster β_2-adrenergic receptor (Dixon *et al.*, 1986). Immunoblots of A431 membrane proteins probed with anti-peptide antiserum CM8-3 at various dilutions (Fig. 2*c*) revealed immunoreactive staining identical to that obtained with antiserum 6EN-1 (Figs. 2*a* and 2*b*). Prominent staining of a 65000-M_r species was observed. Immunoblots of A431 membrane proteins probed with antibodies directed against KLH as well as against an actin–KLH conjugate displayed no immunoreactive staining (results not shown). Thus, prominent staining of A431 membrane proteins by antibodies raised against the purified receptor or a synthetic peptide fragment of the receptor was confined to a 65000-M_r peptide. Highly purified β-adrenergic receptors isolated from a variety of mammalian sources have been shown to migrate

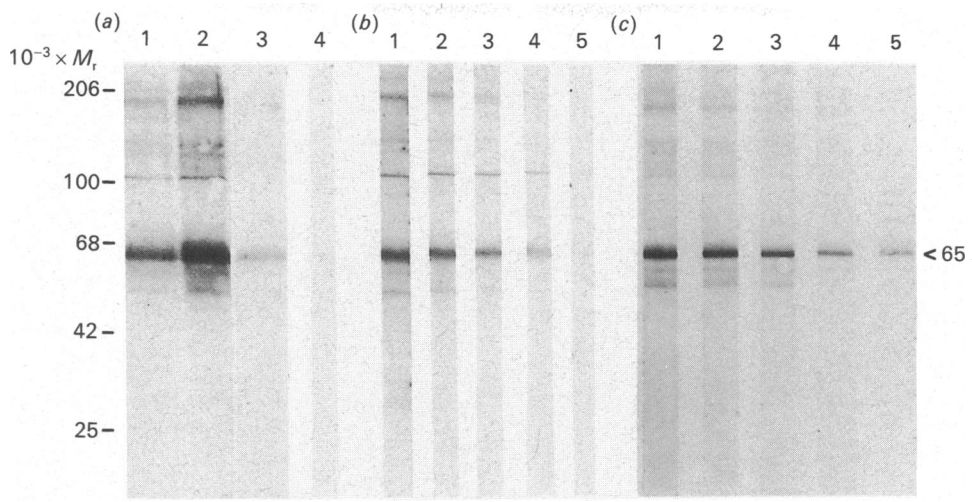


Fig. 2. Immunoblotting of β-adrenergic receptors using antisera 6EN-1 and CM8-3

6EN-1 antiserum was raised against β-adrenergic receptors purified from guinea pig lung (Moxham *et al.*, 1986). CM8-3 antiserum was generated against a synthetic peptide of the hamster lung β_2-receptor. Purified β-adrenergic receptors isolated from S49 cells and membrane-bound receptors (from A431 cells) were subjected to chemical reduction by dithiothreitol (20 mM), to alkylation with excess 2-iodoacetamide, and then to electrophoresis on SDS/10%-polyacrylamide gels. The separated proteins were transferred electrophoretically (Bio-Rad Transblot) to nitrocellulose and probed with the anti-receptor antisera. (*a*) Highly purified membranes of A431 cells (200, 400 and 85 μg of protein in lanes 1, 2 and 4 respectively) and purified β-adrenergic receptor (100 ng; lane 3) were subjected to immunoblotting. Antisera dilutions were as follows: 6EN-1, 1:100 (lanes 1, 2 and 3); preimmune 1:100 (lane 4). (*b*) Each lane contains 85 μg of highly purified membranes from A431 cells. Dilutions of 6EN-1 anti-receptor antiserum were as follows: 1:50 (lane 1), 1:100 (lane 2), 1:200 (lane 3), 1:500 (lane 4) and 1:1000 (lane 5). (*c*) Each lane contains 85 μg of highly purified membranes from A431 cells. Anti-receptor antiserum CM8-3 was used at a dilution of 1:50 (lane 1), 1:100 (lane 2), 1:200 (lane 3), 1:500 (lane 4) or 1:1000 (lane 5). Immune complexes were made visible by autoradiography using ^{125}I-labelled second antibody (goat anti-rabbit IgG).

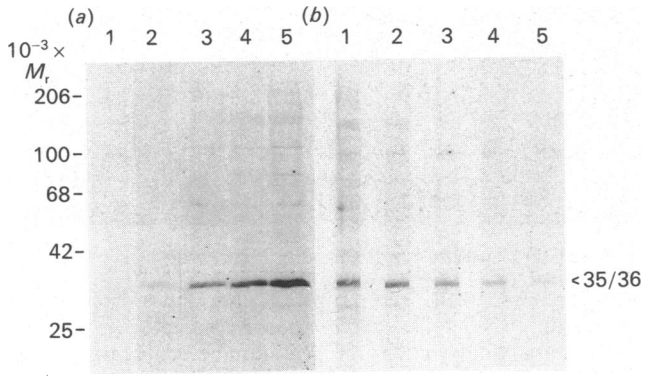


Fig. 3. Immunoblotting of β-subunits of G-proteins using G_β-6

Pure β-subunit of G-protein (from human placenta) and highly purified A431 membranes were subjected to chemical reduction by dithiothreitol (20 mM) and were alkylated with excess 2-iodoacetamide prior to being subjected to electrophoresis on SDS/10%-polyacrylamide gels. The separated proteins were transferred electrophoretically to nitrocellulose and probed with G_β-6. (*a*) A sample of 100 ng of pure β-subunit (lane 2) as well as 100 μg (lane 1 and lane 3), 200 μg (lane 4) and 400 μg (lane 5) of A431 cell membrane protein were subjected to immunoblotting. Blots of A431 cell membrane proteins were probed with 1:200 dilution of pre-immune serum (lane 1) or 1:200 dilution of G_β-6 (lanes 2–5). (*b*) Each lane contains 85 μg of A431 cell membrane protein. G_β-6 was used at a dilution of 1:50 (lane 1), 1:100 (lane 2), 1:200 (lane 3), 1:500 (lane 4) or 1:1000 (lane 5). Immune complexes were made visible using ^{125}I-labelled second antibody (goat anti-rabbit IgG) in tandem with autoradiography.

uniformly as single polypeptides with M_r values of 65000–67000 on SDS/PAGE, as made visible by silver staining, immunoprecipition, photoaffinity radiolabelling, and immunoblotting (Benovic *et al.*, 1984; Cubero & Malbon, 1984; Graziano *et al.*, 1985; George & Malbon, 1985; Moxham *et al.*, 1986).

Immunoblot analysis of G_β-subunits of A431 cell membranes with antibodies directed against human placenta G_β-subunits

Identification of G_β-subunits of A431 cells was performed by probing immunoblots of membrane fractions with antibodies (G_β-6) raised against the G_β-subunits purified from human placenta (Fig. 3). Immunoreactive 35000- and 36000-M_r peptides stained prominently in the blots. Mammalian G_β-subunits have been shown to migrate on SDS/PAGE as M_r-35000–36000 species, as made visible by silver staining of the purified peptides and by immunoblotting of membrane fractions with specific antisera (Sternweis & Robishaw, 1984; Roof *et al.*, 1985; Andigier *et al.*, 1985; Evans *et al.*, 1987; Watkins *et al.*, 1987). A few minor slower-migrating species were observed in A431 membrane fractions, but only in overstained blots prepared from heavily loaded gels. Under standard conditions (0.1 mg of protein per SDS/PAGE lane), prominent staining of immunoblots of A431 cells with antiserum G_β-6 over a range of dilutions was confined to peptides of M_r-35000–36000 (Fig. 3*b*).

Localization of β-adrenergic receptors in A431 cells *in situ*

To determine the subcellular localization of β-

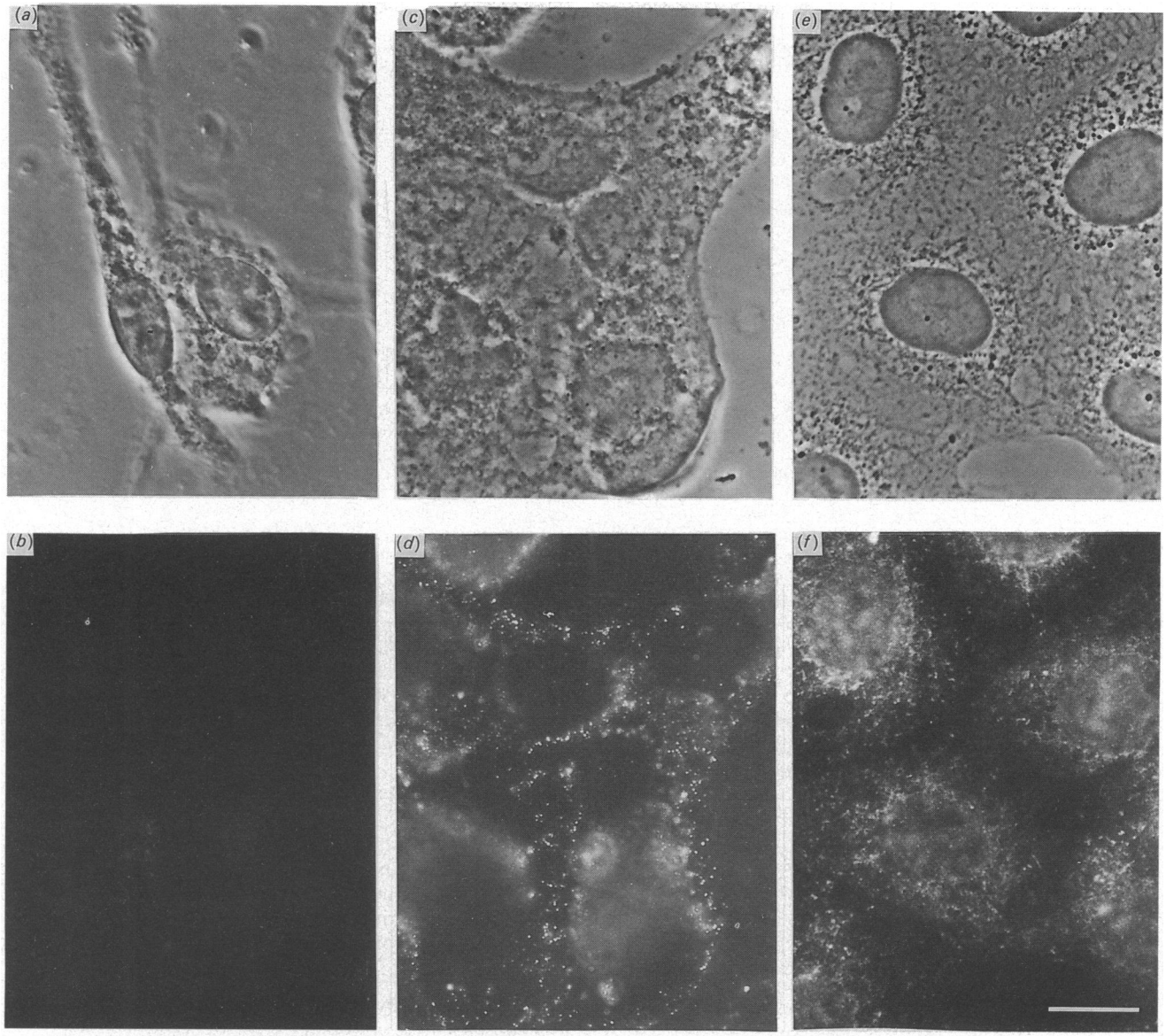


Fig. 4. Indirect immunofluorescence of fixed intact A431 cells stained with antisera specific for β-adrenergic receptors

Phase-contrast (*a*, *c*, *e*) and epifluorescence (*b*, *d*, *f*) micrographs are shown. A431 cells were grown on glass slides, fixed with paraformaldehyde, and probed with preimmune serum (*a* and *b*), antiserum 6EN-1 raised against purified receptor (*c* and *d*) or anti-peptide antiserum CM8-3 (*e* and *f*). Primary antisera were used at a final dilution of 1:100. Rhodamine-conjugated goat anti-rabbit IgG was used as a second antibody, diluted 1:1000. Bar, 25 μm.

adrenergic receptors in A431 cells, we performed indirect immunofluorescence on fixed intact as well as detergent-permeabilized cells. No significant fluorescence signal was obtained with either intact cells (Fig. 4*b*) or detergent-permeabilized cells (Fig. 5*b*) probed with preimmune antiserum. Specific immunostaining of intact A431 cells with anti-receptor antiserum 6EN-1, in contrast, revealed a striking 'punctiform' pattern of epifluorescence (Fig. 4*d*). These punctate images were not uniform in apparent size, nor were they uniformly distributed over the entire surface of the cells. Patterns of fluorescent staining of intact cells nearly identical to those obtained with antiserum 6EN-1 were also observed using the anti-peptide antiserum CM8-3 (Fig. 4*f*)

Upon permeabilization of the A431 cells with detergents, the epifluorescence signal obtained using anti-receptor antiserum 6EN-1 increased (compare Figs. 4*d* and 5*d*). The minimum concentration of Nonidet P40 and Triton X-100 required to permeabilize the A431 cells was found to be 0.1% (v/v). Permeabilization, evaluated at concentrations of detergents ranging from 0.005 to 0.5% by the simultaneous immunofluorescent staining of β-adrenergic receptor and nuclear envelope antigens, was empirically established to be complete at 0.1% detergent concentration (results not shown). Minimal distortion of cell morphology was observed under these conditions of permeabilization. Examined at the basal level of the cell surface, the indirect immunofluorescence shows specific staining with those anti-receptor antisera in most regions of the cell, with the exception of the nucleus itself. The increased immunofluorescence observed in detergent-permeabilized cells probably

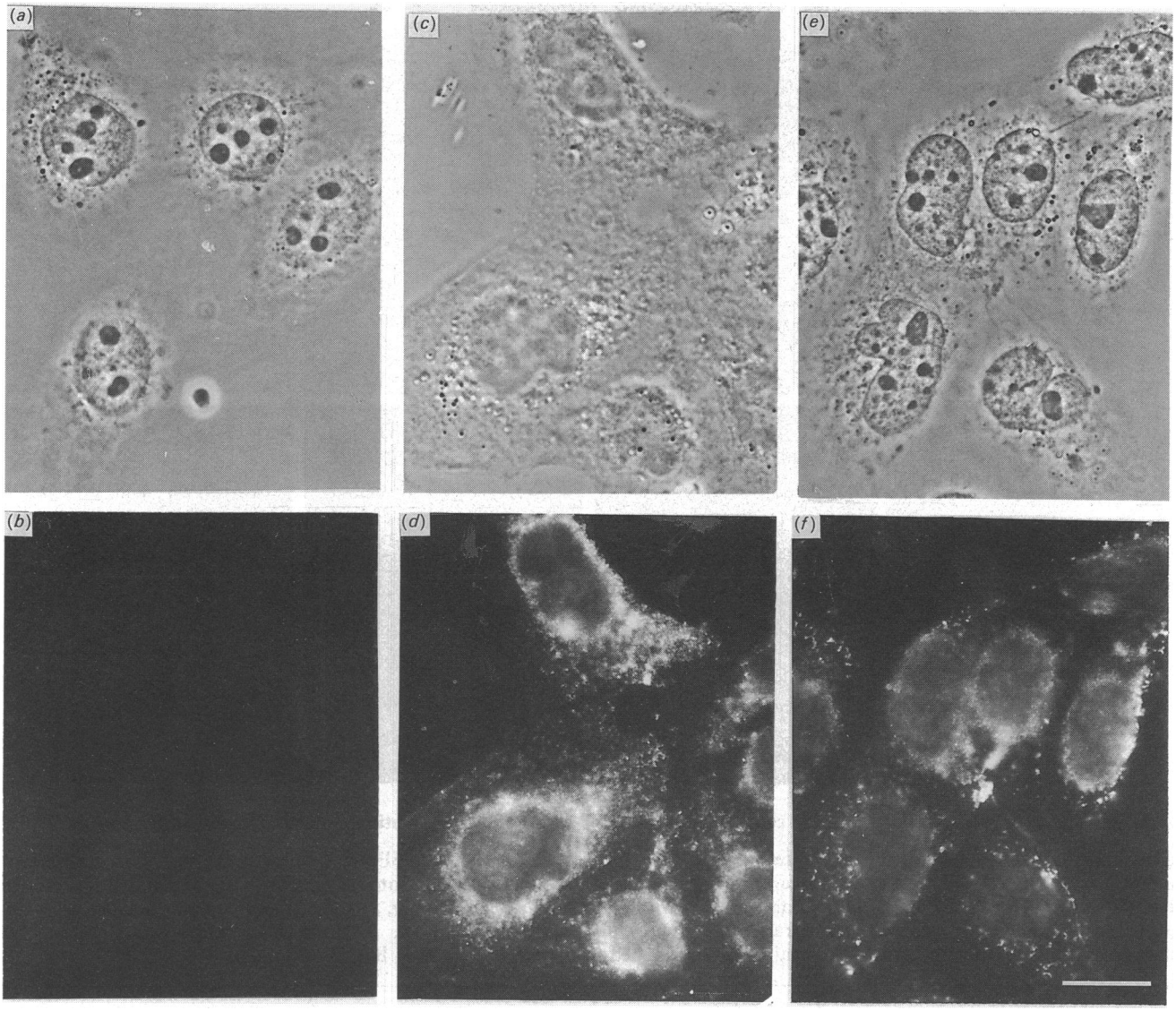


Fig. 5. Indirect immunofluorescence of fixed permeabilized A431 cells stained with antisera specific for β-adrenergic receptors

Phase-contrast (*a*, *c*, *e*) and epifluorescence (*b*, *d*, *f*) micrographs are shown. A431 cells were grown on glass slides, fixed with paraformaldehyde, permeabilized with detergents, and probed with preimmune serum (*a* and *b*), antiserum 6EN-1 raised against the purified receptor (*c* and *d*), or anti-peptide antiserum CM8-3 (*e* and *f*). Primary antisera were used at a final dilution of 1:100. Rhodamine-conjugated goat anti-rabbit IgG was used as a second antibody, diluted 1:1000. Bar, 25 μm.

reflects the staining of a cytoplasmic complement of antigen as well as the staining of cytoplasmic domains of cell-surface antigens that were not accessible to the antibodies in fixed, intact cells. An intracellular complement of β-adrenergic receptors has been identified by radioligand binding and has been estimated to range from 20 to 40 % of the cell complement, depending upon the cell type (Limas & Limas, 1987). Measurements of receptor binding in intact cells using the water-soluble radioligand CGP-12177 suggest that about 30 % of β-adrenergic receptors in A431 cells are intracellular (results not shown).

The fluorescence signal of A431 cells was found to be higher in permeabilized than non-permeabilized cells probed with anti-receptor antisera 6EN-1 or CM8-3. However, the magnitude of the increase in epifluorescence observed upon permeabilization was found to be much less for those cells stained with antiserum CM8-3 (Fig. 5*f*) than those stained with 6EN-1 (Fig 5*d*). This result with CM8-3 would be expected since the antiserum was generated against a synthetic peptide fragment corresponding to a predicted extracellular domain of the receptor. The increase in fluorescence signal of permeabilized cells stained with CM8-3 antiserum is probably due to exposure of the intracellular complement of receptors unavailable for staining in intact cells. Staining with 6EN-1 antiserum increases in permeabilized cells for this same reason and also as a result of the exposure of intracellular domains of both cell-surface and intracellular receptors.

The specificity of the immunofluorescence signal of A431 cells stained with 6EN-1 and CM8-3 antisera was examined further by preadsorbing these antibodies with antigens prior to immunofluorescence. Immuno-

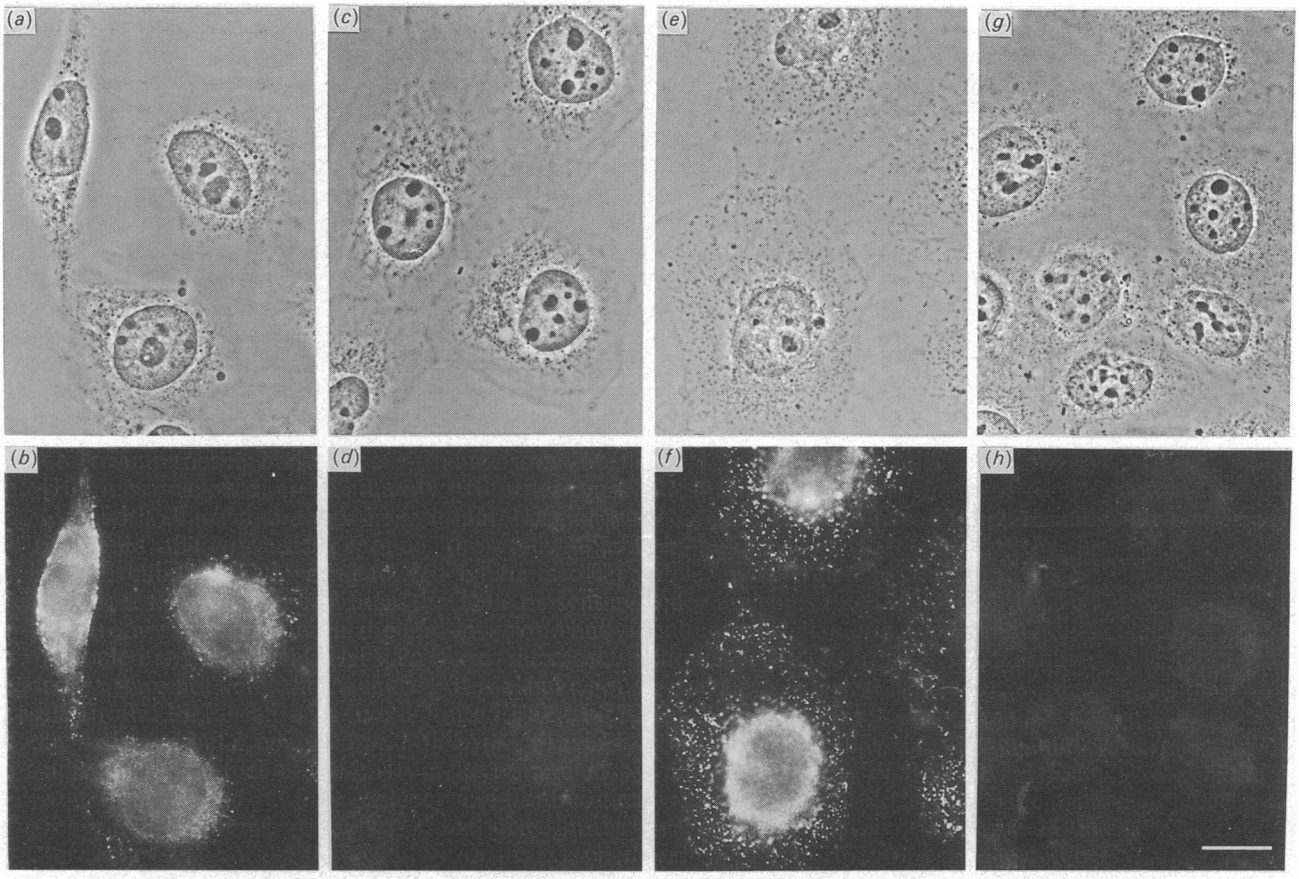


Fig. 6. Indirect immunofluorescence of A431 cells: preadsorption of anti-receptor and anti-G_β-subunit antisera with antigens

Fixed and permeabilized A431 cells were stained with anti-receptor antiserum 6EN-1 (panels *a*, *b*) and with antiserum preadsorbed with purified β-adrenergic receptor (*c*, *d*). Details of the preadsorption protocol appears in the Experimental section. Fixed and permeabilized A431 cells were also stained with anti-G_β-antiserum G_β-6 (*e*, *f*) and with antiserum preadsorbed with purified G_β-subunits (*g*, *h*). Phase-contrast (*a*, *c*, *e*, *g*) and epifluorescence (*b*, *d*, *f*, *h*), microscopy are shown. The immunofluorescence protocol was identical to that described in the legend to fig. 4. Bar, 25 μm.

fluorescence staining of A431 cells with anti-receptor antibodies (Fig. 6*b*) was abolished by preadsorption of the antiserum 6EN-1 with purified antigen (Fig. 6*d*). Fig. 7 is an SDS/PAGE analysis of the antigens employed for these preadsorption studies. The proteins were made visible by silver staining. Preadsorption of antiserum CM8-3 with the peptide used to prepare the immunogen also abolished the specific antibody staining of A431 cells (results not shown).

The epifluorescence staining of fixed, intact A431 cells stained with anti-receptor antiserum 6EN-1 was analysed at different planes of focus. Optical sectioning revealed the punctate immunofluorescence staining extending from the basal to the apical surfaces of A431 cells (Fig. 8). Punctate staining varied slightly at different planes of focus.

Although the striking patterns of immunofluorescence staining of β-adrenergic receptors were observed with two antisera (one against the receptor, the other against a synthetic peptide of the receptor sequence) and could be abolished by preadsorption with highly purified antigens, we capitalized on the availability for study of CHO transfectant clones expressing various levels of β-adrenergic receptor (George *et al.*, 1988). The immunofluorescence of β-adrenergic receptors was examined by CM8-3 staining of wild-type CHO cells and CHO clones transfected with an expression vector harbouring the gene for the hamster β_2-adrenergic receptor. These stably transfected CHO clones express β-adrenergic receptors that are properly processed and tightly coupled to adenylate cyclase. Both the sensitivity and the magnitude of the cyclic AMP response in these cells increased with increasing levels of receptor expression (George *et al.*, 1988). The epifluorescence signal was greatest in the CHO clone 32f expressing the highest level of β-adrenergic receptor (Fig. 9*h*), least in the wild-type CHO cells (Fig. 9*d*), and intermediate in transfectant clone 30b expressing moderate levels of receptor (Fig. 9*f*). The levels of fluorescence signal (Fig. 9) paralleled both specific antibody staining of immunoblots of cell membrane proteins and β-adrenergic receptor expression measured by ICYP binding (George *et al.*, 1988). Indirect immunofluorescence and the preadsorption experiments (Fig. 6) demonstrate the specificity of these antibodies for β-adrenergic receptors.

Localization of G_β-subunits in A431 cells *in situ*

The distribution and localization of G-proteins were probed using indirect immunofluorescence in tandem

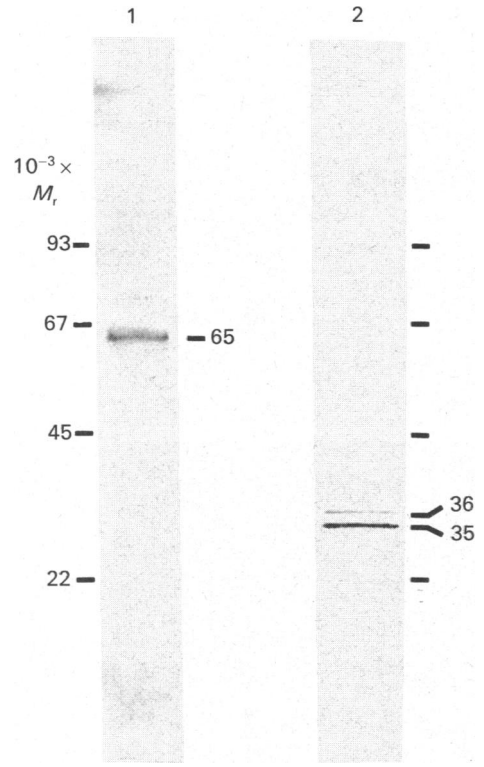


Fig. 7. SDS/PAGE analysis of β-adrenergic receptors and G_β-subunits

Samples of the antigens used in the preadsorption studies were subjected to electrophoresis on 10% polyacrylamide gels in the presence of SDS as described in the Experimental section. the gels were stained with the silver reagent. Lane 1, 50 ng of β-adrenergic receptor isolated from S49 mouse lymphoma cell membranes; lane 2, 100 ng of G_β-subunits isolated from human placenta.

with antisera against purified human G_β-subunits. Fig. 10 displays immunofluorescence staining of A431 cells with anti-G_β-subunit antiserum. No fluorescence signal was observed in intact fixed A431 cells that were stained with antiserum G_β-6 (Fig. 10*b*). In contrast, when cells were first fixed and permeabilized, the immunofluorescence signal was intense (Fig. 10*d*). Adsorption of anti-G_β-subunit antibodies with purified G_β-subunits markedly attenuated the specific antibody staining of fixed and permeabilized A431 cells (Fig. 6*h*). The nature of the antigen used in these preadsorption studies is shown in the PAGE analysis (Fig. 7*b*). The patterns of staining indicate that these cellular antigens are distributed throughout the cytoplasm and on the cytoplasmic face of the plasma membrane. Fluorescent staining of G_β-subunits in fixed, permeabilized A431 cells was punctate, like that of the β-adrenergic receptor.

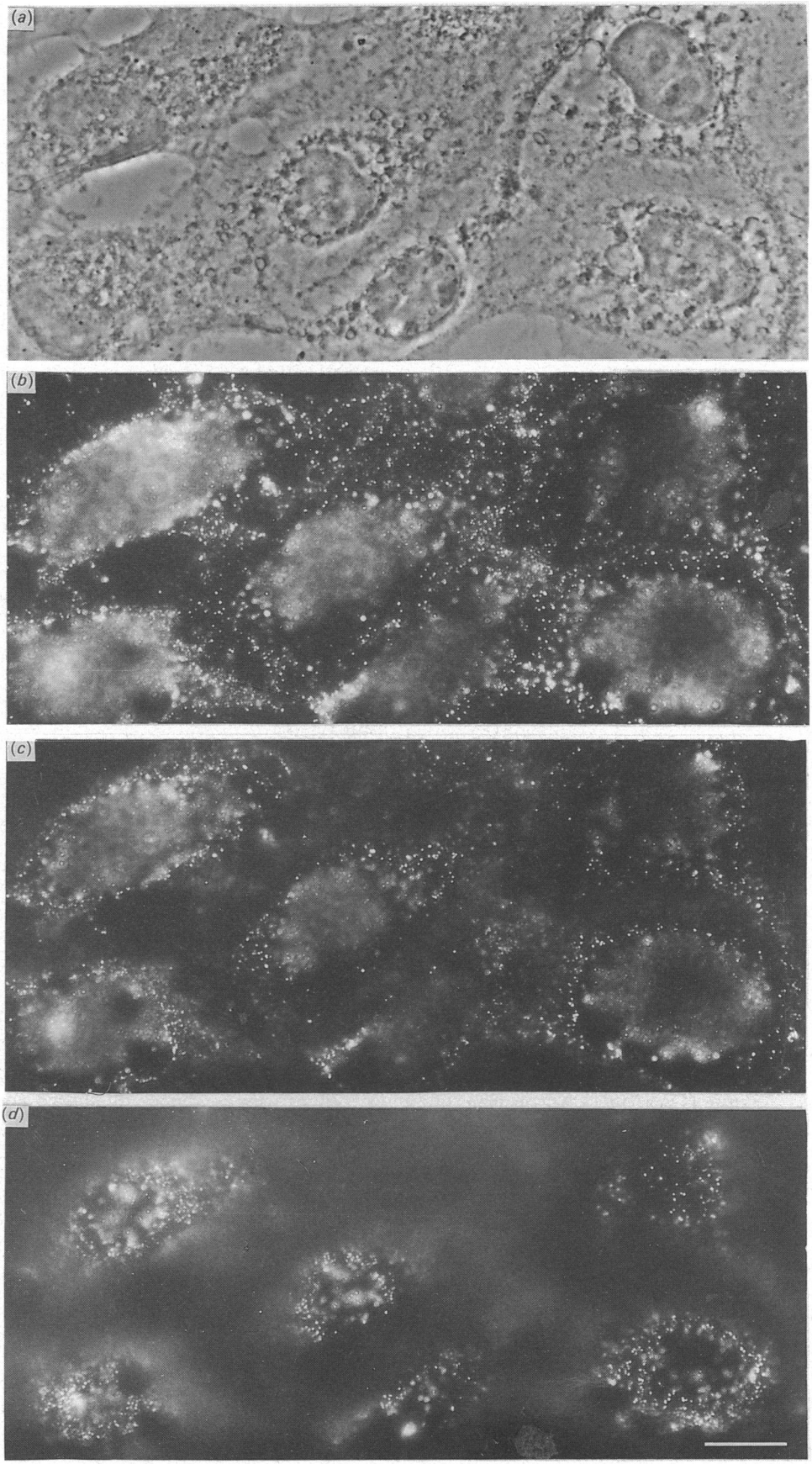
DISCUSSION

The present study takes advantage of several new antibodies to study two components of a G-protein-linked receptor pathway, the β-adrenergic receptor and the β-subunits of G-proteins in a human epidermoid carcinoma cell line. The A431 cell line was adopted for these studies for the following reasons: (i) antibodies were either generated against or recognized these human antigens; (ii) A431 cells grow well on glass slides and have a high density of β-adrenergic receptors; and (iii) A431 cells have previously been used in the immunocytochemical detection of other cell surface receptors (Warren *et al.*, 1984; Weigant *et al.*, 1986).

Since the success of an immunocytochemical analysis critically depends upon the availability of specific antibodies, detailed characterization of antibody cross-reactivity with the A431 cell antigens was a primary goal. The antibodies raised against the purified β-adrenergic receptor (6EN-1) as well as those directed against a synthetic peptide domain (CM8-3) corresponding to a region of the β_2-adrenergic receptor (predicted to be in the second extracellular loop) both recognized a single 65000-M_r peptide in immunoblots of A431 cell membrane fractions. These results agree with those of others demonstrating the mammalian β-adrenergic receptor to be a polypeptide with an M_r of 65000 on SDS/PAGE (Cubero & Malbon,1984; Benovic *et al.*, 1984; Graziano *et al.*, 1985; Moxham *et al.*, 1986; Bahouth & Malbon, 1987; George *et al.*, 1988). The antibody raised against purified human placenta β-subunits of G-proteins (G_β-6) recognized 35000–36000-M_r peptides in A431 cell membrane fractions. This antiserum has previously been shown to recognize the β-subunits of G-proteins from a wide range of cell types (Rapiejko *et al.*, 1986; Evans *et al.*, 1987; Watkins *et al.*, 1987; Ros *et al.*, 1988). The data presented here are thus truly the immunocytochemical localization of a G-protein-linked receptor and G-proteins.

Although detection of β-adrenergic receptors by indirect immunofluorescence has been reported for several cell lines (Strader *et al.*, 1987; Ventimiglia *et al.*, 1987), including A431 cells (Kaveri *et al.*, 1987), these previous studies did not provide a complete characterization of the antibodies employed and did not evaluate the staining of cells with either preimmune sera or with fluorochrome-linked second antibody alone. Thus, comparison of the present work with these earlier studies is difficult (Kaveri *et al.*, 1987; Strader *et al.*, 1987; Ventimiglia *et al.*, 1987). Our study, rather than focusing solely upon the effects of desensitization on receptor staining, focuses on providing high-resolution immunofluorecence images to localize *in situ* both a G-protein-linked receptor and G-proteins. In spite of morphological differences, the four A431 cell subclones used in this study shared common properties such as (i) similar receptor abundance as detected by radioligand binding analyses, (ii) identical patterns of antigen recognition in immunoblots of membrane fractions probed with antibodies specific for β-adrenergic receptors and the β-subunits of G-proteins; and (iii) similar immunocytochemical staining patterns of receptor and G-protein subunits.

Using anti-receptor antisera 6EN-1 and CM8-3 we observed a punctate staining decorating the surface of fixed intact A431 cells. In an attempt to provide further information on the topological organization of β-adrenergic receptors, immunofluorescent localization of receptor was also examined in cells permeabilized with non-ionic detergents. Using 6EN-1, the specific epifluorescence signal increased significantly in permeabilized as compared with intact cells. Since 6EN-1 was raised against the receptor holoprotein, this observation was not unexpected. The increase in signal from permeabilized cells reflects staining of an intra-


(a)
(b)
(c)
(d)

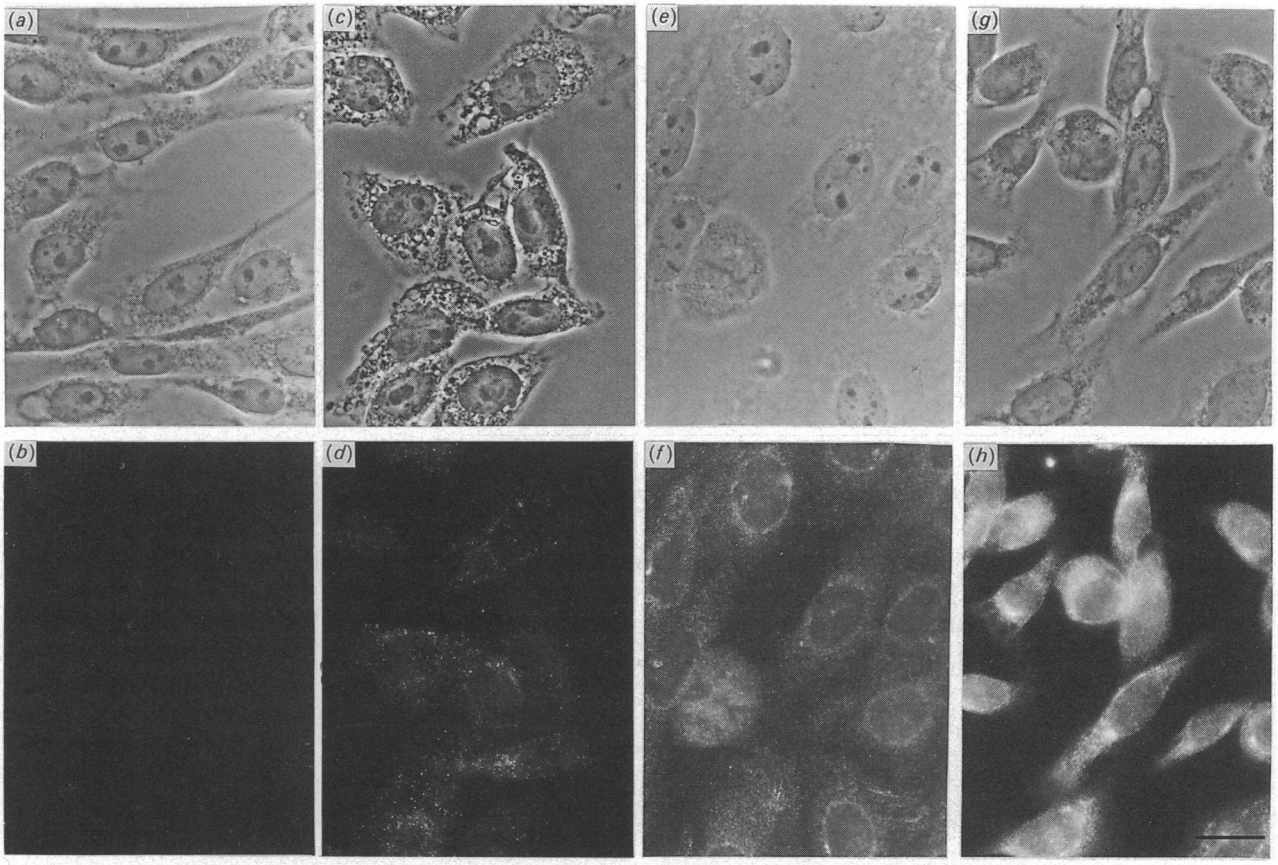


Fig. 9. Indirect immunofluorescence of β-adrenergic receptors in CHO cells expressing different amounts of receptor

CHO cells were grown, plated, fixed and permeabilized as described earlier (George *et al.*, 1988). The cells were stained either with preimmune sera (*a*, *b*) or with anti-peptide antiserum CM8-3 (*c–h*) and the indirect immunofluorescence was performed as described in the legend to Fig. 4. Wild-type CHO cells and CHO cells transfected with an expression vector harbouring the cDNA for hamster β_2-adrenergic receptor under control of the simian-virus-40 early promoter (George *et al.*, 1988) were fixed and permeabilized. Phase-contrast (*a*, *c*, *e*, *g*) and epifluorescence (*b*, *d*, *f*, *h*) micrographs are shown. Membranes from these cells demonstrate β-adrenergic receptor levels (specific ICYP binding: fmol/mg of protein) as follows: wild-type 15 (*a–d*); CHO clone 30f, 795 (*e*, *f*); and CHO clone 32f, 2600 (*g*, *h*). Bar, 25 μm.

cellular complement of receptors previously identified by radioligand binding analyses with membrane-permeant and impermeant ligands (Barovsky & Brooker, 1980; Staehelin *et al.*, 1983) and also reflects recognition of cytoplasmic domains of the receptor not accessible to antibodies in intact cells. Further support for this notion was provided by similar experiments performed with the anti-peptide antisera directed against a receptor domain predicted to be extracellular. The increase in epifluorescence signal in permeabilized cells stained with CM8-3 as compared with 6EN-1 presumably reflects the ability of CM8-3 antibodies to stain the intracellular complement of receptors. The intracellular staining by CM8-3 may be restricted largely to receptors being synthesized and processed. These data provide visible evidence for the existence of an intracellular complement of a G-protein-linked receptor heretofore hypothesized to exist (see Sibley & Lefkowitz, 1985, and references cited therein). In addition, these data provide preliminary evidence in support of the model for the membrane organization of the mammalian β-adrenergic receptor, placing the receptor fragment used to raise antibodies as an extracellular domain (Dixon *et al.*, 1986).

We took advantage of the availability of antibodies raised against purified human G_β-subunits to initiate an immunocytochemical analysis of G-proteins in a human cell line. Whereas no specific signal was obtained when fixed intact A431 cells were probed by indirect immunofluorescence with G_β-6 antiserum, in permeabilized cells an intense, specific staining of the intracellular compartment of the cells was observed. Excluded from the staining were the cell nuclei, suggesting widespread,

Fig. 8. Indirect immunofluorescence of A431 cells stained with anti-receptor antisera: analysis at different planes of focus

Phase-contrast (*a*) and fluorescence (*b*, *c* and *d*) microscopy are shown. The epifluorescence images were obtained at different depths of focus with a 63X Zeiss Planapochromat objective lens (numerial aperture = 1.40). (*b*) The focal plane is on the basal level of the cell surface, (*c*) the focal plane is on the more distal regions of the cell surface; (*d*) the focal plane is on the apical regions of the cell surface. Bar, 25 μm.

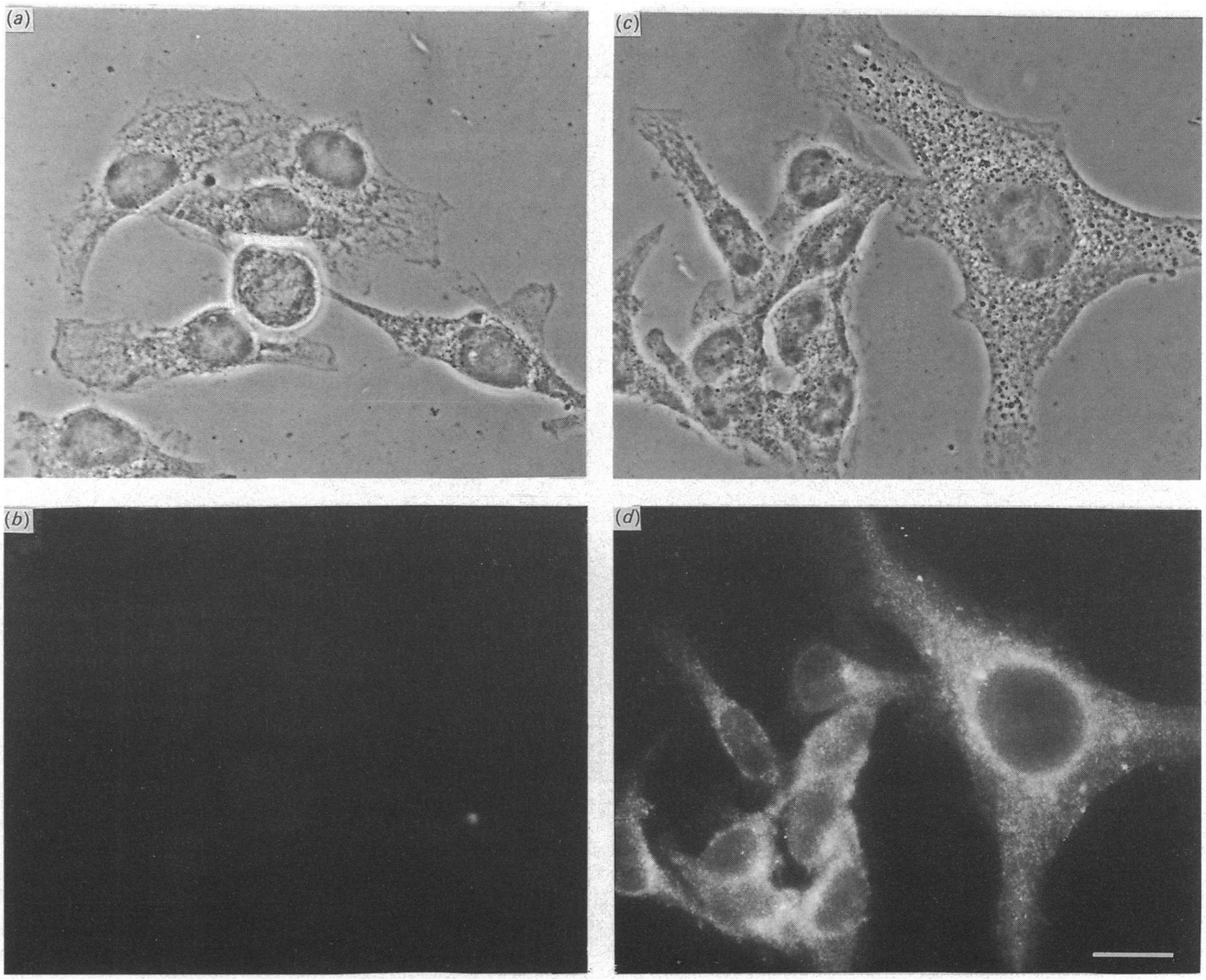


Fig. 10. Indirect immunofluorescence of A431 cells stained with anti-G_β-subunits antisera

Cells were grown on glass slides and fixed with or without detergent permeabilization, and probed by anti-β-subunit antisera (G_β-6) diluted 1:200. Rhodamine-conjugated goat anti-rabbit IgG was used as the second antibody. Phase-contrast (*a*, *c*) and epifluorescence (*b*, *d*) micrographs are shown. Fixed, non-permeabilized cells (*a*, *b*) or permeabilized cells (*c*, *d*) were probed with G_β-6, anti-β-subunit of G-protein antiserum. Bar, 25 μm.

non-nuclear localization of G-proteins. Staining of G-protein β-subunits was punctate, like that for β-adrenergic receptors.

The present study provides the first immunofluorescence localization *in situ* of both the G-protein-linked β-adrenergic receptor and G-protein β-subunits. The images suggest that both transmembrane signalling elements appear to be organized as aggregates, thus giving images of punctate fluorescence. The organization of these membrane proteins is distinct; the β-adrenergic receptor displays extracellular as well as intracellular domains. Specific staining of receptors was achieved in both intact and permeabilized cells. For G-proteins, specific staining was demonstrated only in permeabilized cells, an observation supporting its presumed intracellular localization. The bulk of the receptors appears to be associated with the cell membrane. An intracellular complement of receptors, long hypothesized, was made visible using a site-directed anti-peptide antibody and detergent-permeabilized cells. G_β-subunits, in contrast, were distributed intracellularly and throughout the cytoplasm.

The solubilization of G-proteins by detergents and the stimulation of adenylate cyclase by hormones have been shown to be modulated by the status of the cytoskeleton (Insel & Kennedy, 1978; Cherskey *et al.*, 1980; Rasenick *et al.*, 1981; Sahyoun *et al.*, 1981). In addition, the β/γ-subunit complex of G-proteins has been demonstrated to be associated with cytoskeletal elements (Carlson *et al.*, 1986). The punctate fluorescent staining observed for G-protein β-subunits may be a reflection of G-protein–cytoskeletal interactions. The punctate images of receptors, in turn, may represent aggregates of receptors organized either by interactions with G-proteins, or perhaps by cytoskeletal elements.

The development of a capability to perform indirect immunofluorescence analysis of G-protein-linked receptors and G-proteins provides new opportunities for the

study of the cell biology of signal transduction (Malbon *et al.*, 1988). In a companion paper (Wang *et al.*, 1989), we examine the effects of chronic stimulation of A431 cells by β-adrenergic agonist upon the status and distribution of β-adrenergic receptors.

We thank Dr. John K. Northup (Department of Pharmacology, Yale University, New Haven, CT, U.S.A.) for the antibodies against G-protein β-subunits and for his critical reading of this work. In addition, we thank Ms. Jeanne Yokelson for her expert assistance in the photography. This work was supported by United States Public Health Services grants DK30111, DK25410, and K04-AM00786 from the National Institutes of Health (to C.C.M.), and by grant DCB-8615969 from the National Science Foundation (U.S.) (to M.B.).

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Received 13 February 1989/8 May 1989; accepted 16 May 1989