European Journal of Neuroscience, Vol. 11, pp. 1927–1936, 1999

Real-time imaging of glucocorticoid receptor dynamics in living neurons and glial cells in comparison with non-neural cells

Mayumi Nishi, Nobuyuki Takenaka,[1] Noriyuki Morita, Takao Ito, Hitoshi Ozawa and Mitsuhiro Kawata
Department of Anatomy and Neurobiology, Kyoto Prefectural University of Medicine, Kawaramachi Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan
[1]Department of Orthopedics, Kyoto Prefectural University of Medicine, Kawaramachi Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan

Keywords: CCD camera, CNS, nuclear translocation, primary culture, transcription factor

Abstract

To investigate the intracellular trafficking of glucocorticoid receptor (GR) in response to various conditions in a single living cell, a green fluorescent protein (GFP) and rat GR chimera construct (GFP-GR) was prepared. We transiently transfected GFP-GR into primary cultured rat hippocampal neurons, cortical glial cells, and non-neural cells, e.g. COS-1 cells and CV-1 cells, and compared the dynamic changes in subcellular localization of GFP-GR in these cells. When GFP-GR was expressed in the cells, GFP-GR efficiently transactivated the mouse mammary tumour virus promoter in response to dexamethasone (DEX). The cytoplasm-to-nuclear translocation of GFP-GR induced with 10^{-7} M DEX, a specific agonist of GR, at 37 °C was completed within 30 min in all cell types used, and the rate of nuclear translocation was dependent on the ligand dose. The translocation of GFP-GR into the nucleus from the cytoplasm was induced in a ligand-specific manner, similar to that of the native GR. The disruption of microtubules by colchicine or nocodazole showed no significant effect on the DEX-induced GFP-GR translocation from the cytoplasmic region to the nuclear region. The cells were not deteriorated during time-lapse imaging analysis for 1 h at 37 °C. The present findings suggest that the subcellular localization of GFP-GR is dynamically changed in response to extracellular and intracellular conditions, and that there are no conspicuous variations in the manner of trafficking of GR among different types of cells *in vitro*.

Introduction

Glucocorticoids exert markedly diverse effects on development, differentiation, ageing and regeneration in the nervous system (Funder & Sheppard, 1987; De Kloet, 1991; McEwen, 1991; Nishi *et al*., 1994; Hu *et al*., 1997). These effects are mediated via two receptor systems in the brain, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Reul & De Kloet, 1985; Evans & Arriza, 1989). The expression of GR occurs in most neurons and glial cells throughout the brain, while MRs are localized mainly in the hippocampus. GR and MR belong to the steroid receptor superfamily, which is comprised of hormone-dependent transcription factors. It is generally accepted that the unliganded GR resides predominantly in the cytoplasm, and that hormone activation leads to the translocation of GR from the cytoplasm to the nucleus and gene activation (Gasc & Baulieu, 1987; Wikstrom *et al*., 1987; Beato, 1989; Carson-Jurica *et al*., 1990; Akner *et al*., 1995). However, the mechanisms involved in the shuttling of GR between the nucleus and the cytoplasm, and targeting GR to regulatory sites in chromatin are poorly understood. The fundamental question, why unliganded GR resides in the cytoplasm waiting for ligands while many other steroid receptors constitutively reside in the nucleus, remains to be determined.

Correspondence: M. Nishi, as above.
E-mail: nmayumi@koto.kpu-m.ac.jp

Received 9 November 1998, revised 16 January 1999, accepted 20 January 1999

The subcellular localization of GR has commonly been studied by three different types of methods: cell fractionation; cellular autoradiography; and immunocytochemistry. The actual subcellular distribution of GR is quite controversial due to rather large heterogeneities of GR and methodological problems. In the case of cell fractionation, it is often unclear whether various biochemical fractions truly represent specific cellular compartments *in vivo*, and cytoplasmic structures connected to the nucleus may contaminate the nuclear fraction (Martin & Sheridan, 1980; Alberts *et al*., 1989). Because the cellular autoradiography method requires incubation with a radiolabelled hormone, the added hormones may alter the distribution of receptor molecules after binding to their receptors (Coutard *et al*., 1978; Stalker *et al*., 1989). Although the immunohistochemical method is a powerful tool for studying the receptor localization, it has a disadvantage that the cells have to be fixed and permeabilized to enable the antibodies to access the inner parts of the cells. In the previous immunohistochemical studies, GR has been reported to be distributed in both cytoplasm and nucleus without an effect of ligand (LaFond *et al*., 1988; Gasc *et al*., 1989) translocated to the nucleus upon binding with ligand (Antakly *et al*., 1990; Picard & Yamamoto, 1987). In contrast, Brink *et al*. (1992) showed that GR is localized only in the nucleus with or without ligand. Because these three methods cannot be used for living cells, it is difficult to examine dynamic changes in the subcellular localization of GR in single cells, especially to investigate shuttling of GR between the cytoplasm and the nucleus. In order to overcome these problems, recent studies have

employed a chimera of green fluorescent protein (GFP), a 27-kDa protein from the jellyfish *Aequorea victoria*, and GR (Ogawa *et al.*, 1995; Carey *et al.*, 1996; Htun *et al.*, 1996). Because tagging with GFP allows us to directly detect the chimera protein without fixing and staining the cells, it becomes possible to visualize a dynamic change in the subcellular localization of the desired protein in single living cells. These studies have mainly been examined in vertebrate cell lines, but no research has been performed in living neural cells.

In the present study, we investigated the mechanisms underlying the intracellular trafficking of GR in response to various extracellular and intracellular conditions both in single living neural cells and non-neural cells using GFP and rat GR chimera construct. We examined: (i) the time-course; (ii) ligand concentration and specificity; (iii) association with signal transduction system; (iv) effects of cytoskeletal elements; and (v) comparison of these parameters among different cell types in order to elucidate whether there is a difference in the dynamics of the subcellular localization of GR between living neural cells and non-neural cells. The findings of the present study clearly showed that the subcellular localization of GFP-GR is dynamically changed in response to extracellular and intracellular milieu, and that there are no conspicuous variations in the manner of trafficking of GFP-GR among different types of cells.

Materials and methods

Plasmid construct

The 6RGR vector (provided by Dr K.R. Yamamoto, Department of Biochemistry and Biophysics, University of California, San Francisco, USA) containing the rat liver GR cDNA was digested at the BamHI sites and subcloned into pGEM-4 vector (Promega, Madison, WI, USA). The rat cDNA with a truncated 5′ coding region was isolated from pGEM-4-GR by EaeI-BamHI digestion and ligated in frame to the multiple cloning sites (Bsp120I and BamHI) of pEGFPC1 (Clontech, Palo Alto, CA, USA), encoding the GFPmut1 (Cormack *et al.*, 1996) which contains the double-amino acid substitution of Phe-64 to Leu and Ser-65 to Thr (Fig. 1A). In the resulting fusion protein, the C terminus of pEGFPC1 was coupled to the 20th position of the N terminus amino acid of GR (Fig. 1B).

Cell culture and transfection

Dissociated hippocampal primary neuronal cultures and cortical mixed glial cultures were prepared from 18-day-old Sprague–Dawley rat foetuses according to the method of Azmitia & Hou (1994) and McCarthy & FeVellis (1980), respectively. Briefly, mothers were anaesthetized with CO_2 gas and the rat foetuses were removed from the placenta in a laminar flow hood and transferred to ice-cold dissecting solution (0.8% NaCl, 0.04% KCl, 0.006% $Na_2HPO_4 \cdot 12H_2O$, 0.003% KH_2PO_4, 0.5% glucose, 0.00012% phenol red, 0.0125% penicillin G and 0.02% streptomycin). The isolated hippocampus and frontal cortex were mechanically dissociated by triturating through a fire-polished glass pipette. The dissociated cells were plated on 16-well glass slides precoated with 0.1 mg/mL polyethylenimine (Sigma, St. Louis, MO, USA) at an initial plating density of 1×10^5 cells/well by adding 200 µL of the cell suspension to each well (area of 0.28 cm^2; Lab-Tek Chamber Slide, Nunc, Naperville, IL, USA). The cultures were maintained in complete neuronal medium (CNM), consisting of 92.5% (v/v) Eagle's minimum essential medium (MEM, Sigma), 1% (w/v) non-essential amino acids (Gibco-BRL-Life Technologies, Gaithersburg, MD, USA), 0.16% (w/v) glucose and 5% (v/v) foetal calf serum (FCS) (Sigma) in a CO_2 incubator at 37 °C with 5% CO_2/95% air. COS-1 cells and CV-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma), without phenol red, supplemented with 10% FCS overnight in 12-mm tissue culture wells.

Plasmid DNA was transiently transfected into cells by a liposome-mediated method using LipofectAMINE (Gibco-BRL) according to the manufacturer's instruction. Hippocampal cells were cultured in CNM for 48 h then treated with 1 mM cytosine-b-D-arabinofuranoside for 24 h to suppress the proliferation of glial cells. They were then cultured in serum-free medium (SFM) without steroids (MEM with 0.16% of glucose, 1% non-essential amino acids, 20 mM putrescine, 15 nM sodium selenite, 5 µg/mL insulin and 100 µg/mL transferrin) for 48 h before transfection. Cortical glial cells were cultured in CNM for the first 7 days and then subcultured with trypsin–EDTA. The obtained secondary glial cultures were maintained in CNM for another 14 days and in SFM for 1 day before transfection. Over 99% of the cells were glial fibrillary acidic protein-positive glial cells. For COS-1 cells or CV-1 cells, the medium was replaced with SFM 2 h before transfection. Cells were transfected with 200 µL of OPTI MEM (Gibco-BRL) containing 8 µL of LipofectAMINE solution and 200 ng of plasmid DNA per well of 1×10^5 cells for 5 h at 37 °C. After removing the transfection mixture, primary neuronal or glial cultures were left for 48 h in CNM, while COS-1 and CV-1 cells were left for 24 h in DMEM with 10% FCS. The medium was changed to SFM 24 h before observation or drug treatment.

In the case of ligand stimulation, cells were washed in SFM and treated with 10^{-9} or 10^{-7} M DEX (Sigma) at 37 °C. Cells were also treated with 10^{-7} M aldosterone (Ald, Sigma), 10^{-7} M 17β-oestradiol (E2, Sigma), 10^{-6} M forskolin (FK, Sigma) or 100 ng/mL brain-derived neurotrophic factor (BDNF, Promega). In order to analyse the role of cytoskeletal elements for GR trafficking, cells were pretreated with 1 µM or 10 µM colchicine (Sigma), or 1 µg/mL or 10 µg/mL nocodazole (Sigma) for the described period of time and then treated with 10^{-7} M DEX at 37 °C.

Immunocytochemistry and immunoblotting

The GR receptor antibody was raised against a part of the transcription modulation domain of the rat liver GR (Morimoto *et al.*, 1996). The antiserum was affinity purified using the antigen-coupled Sepharose 4B column.

The immunocytochemistry of the cultured cells was performed according to a previously described method (Nishi *et al.*, 1996). Briefly, after drug treatments, cultured cells were fixed for 15 min at 37 °C in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). After blocking with 2% bovine serum albumin (BSA) in PB including 0.2% Triton X-100 for 1 h at room temperature (RT), the fixed cells were incubated with rabbit antisera directed against GR (1/2000 dilution) for 48 h at 4 °C. The cultured cells were then rinsed five times with 2% BSA in PBS and then reacted with biotinylated goat antirabbit antibody (1/250 dilution; Boehringer Mannheim, Mannheim, Germany) for 1 h at RT. The cultures were rinsed five times with PBS and reacted with avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 1 h at RT. The cells were visualized with 0.02% 3,3′-diaminobenzidine (Sigma) and 0.006% H_2O_2 in Tris–HCl-buffered saline (pH 7.6). To confirm microtubules disruption, we used monoclonal antityrosinated α antibody (Sigma) at a dilution of 1:800.

For immunoblotting analysis, COS-1 cells transfected with GFP-GR for 24 h were scraped in ice-cold 100 mM PBS, and pelleted by centrifugation at 1000 ***g*** for 5 min at 4 °C. The pellet was resuspended in ice-cold 100 mM PBS and the same volume of 2 × sample buffer [200 mM Tris–HCl, 4% sodium dodecyl sulphate (SDS), 20% glycerol, 10% 2-mercaptoethanol and a small amount of bromophe-

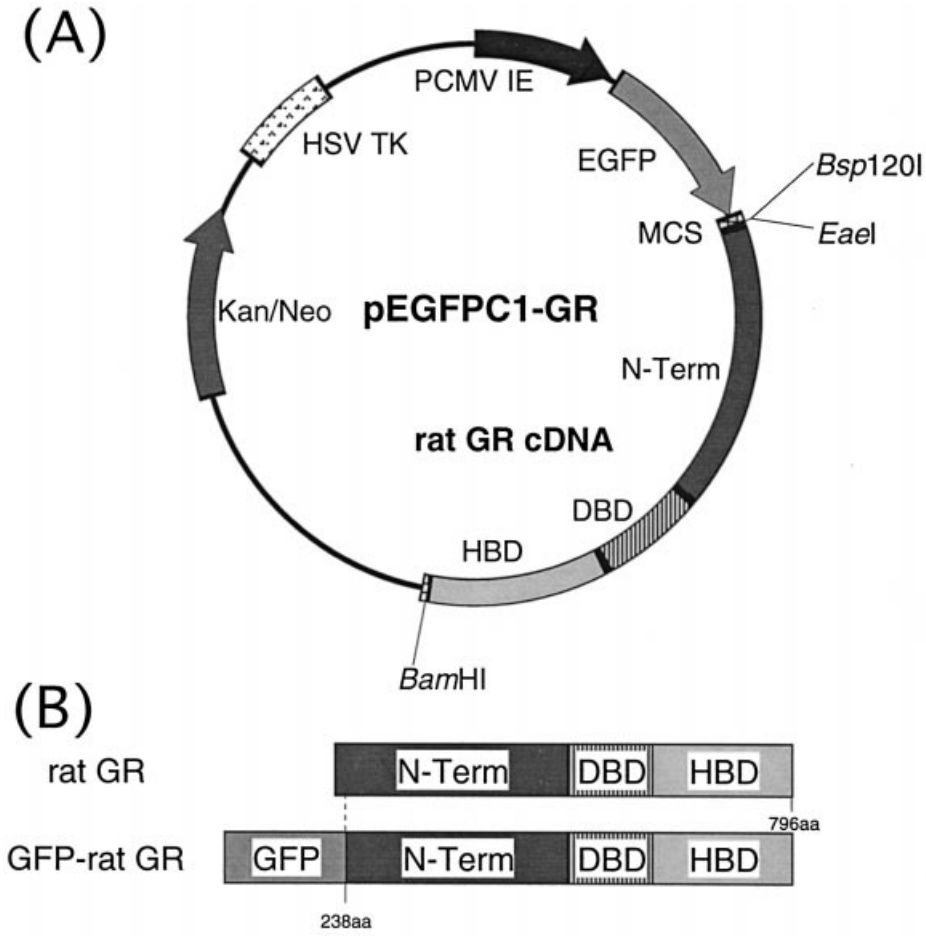


FIG. 1. Construction of GFP-GR expression plasmid. (A) Schematic structure of GFP-GR expression plasmid. pEGFPC1, encoding the GFPmut1 variant, was fused to the rat 6RGR. (B) Structure of wild-type rat GR and GFP-GR chimera protein. In GFP-GR, GFP was fused to the amino-terminal of rat GR that lacks the first 19 amino acid residues. N-term, N-terminal domain; DBD, DNA binding domain; HBD, hormone binding domain.

nol blue (BPB)]. Proteins were run on a 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using a Laemmli buffer system (Laemmli, 1970). Samples were electroblotted to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA) by using a semidry blotting apparatus (Transblot-SD, Bio-Rad, Hercules, CA, USA). Membranes were blocked in rinse buffer (20 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.6) containing 2.5% skimmed milk (Difco, Detroit, MI, USA) overnight at 4 °C. They were incubated with anti-GR antibody (1:5000 dilution) for 2 h at RT, and then washed 4 × for 5 min each in rinse buffer. Secondary goat-anti rabbit-horseradish peroxidase (HRP) (Bio-Rad) was added at 1:4000 for 1 h at RT. Blots were washed three more times in rinse buffer and visualized using enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK).

Examination of transcriptional activity

COS-1 cells plated on 35-mm dishes were cotransfected with 1 μg of DEX-inducible mouse mammary tumour virus promoter (MMTV)-Luc reporter (Ogawa *et al*., 1995) and 1 μg of GFP-GR by lipofection. One microgram of pCH110, a mammalian positive control vector for the expression of β-galactosidase (Hall *et al*., 1983) was also cotransfected as an internal standard. COS-1 cells were cultured in SFM for 12 h before transfection, and were transfected using 1 mL of OPTI MEM with LipofectAMINE–DNA complex for 5 h. After the removal of the transfection mixtures, cells were maintained in DMEM with 10% FCS for 18 h, and then the medium was changed to SFM. Twelve hours later, cells were treated with 10^{-7} M DEX for 4 h at 37 °C, and then harvested in lysis buffer. Cell lysates were centrifuged at 12 000 r.p.m. for 2 min at 4 °C, and the luciferase activity of the resulting supernatants was assayed at 25 °C using the luciferase assay system Pica Gene (Toyo Inki, Tokyo, Japan) and normalized to β-galactosidase activity. Maximum induction obtained with 10^{-7} M DEX for 4 h was taken as 100 after normalization by β-galactosidase activity, and the relative reporter luciferase activities were plotted.

Time-lapse image acquisition and analysis

For the living cell imaging experiments, the culture medium was replaced with SFM buffered with 20 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid] (HEPES, Sigma), and the image acquisition was performed in a room temperature-controlled at 37 °C. Images were acquired on a SenSys1400 high-resolution, cooled CCD camera (Photometrics, Tucson, AZ, USA) attached to a microscope (IXL70, Olympus, Tokyo, Japan) equipped with an epifluorescence attachment. In the observation of neurons or glial cells, a 60 × objective lens was used, while COS-1 cells or CV-1 cells were observed with a 40 × objective lens. For the identification of the nuclear position, the chromatins were stained with 100 ng/mL Hoechst33342 (Sigma). GFP fluorescence was observed with an excitation filter with peak transmission at 485 nm and an emission filter with peak transmission at 515 nm. Data were evaluated with the image analysis software program, IPLab Spectrum (Signal Analytical Corp., Vienna, Austria). For the time-course analysis, images were captured every 15 s using the time-lapse program of IPLab Spectrum. In order to measure nuclear : cytoplasmic ratios of GFP-GR fluorescence intensity, data were collected and quantified using a

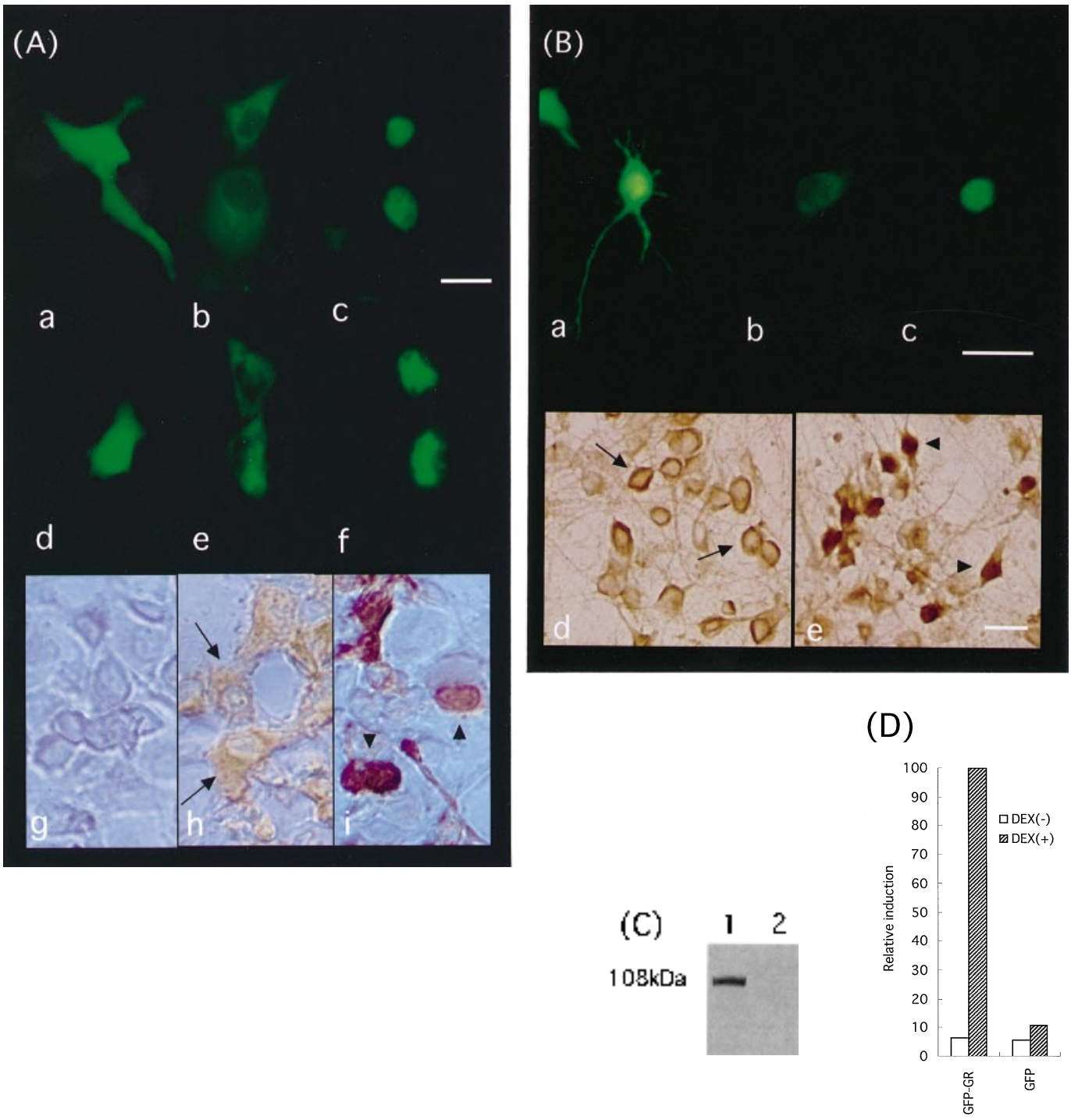
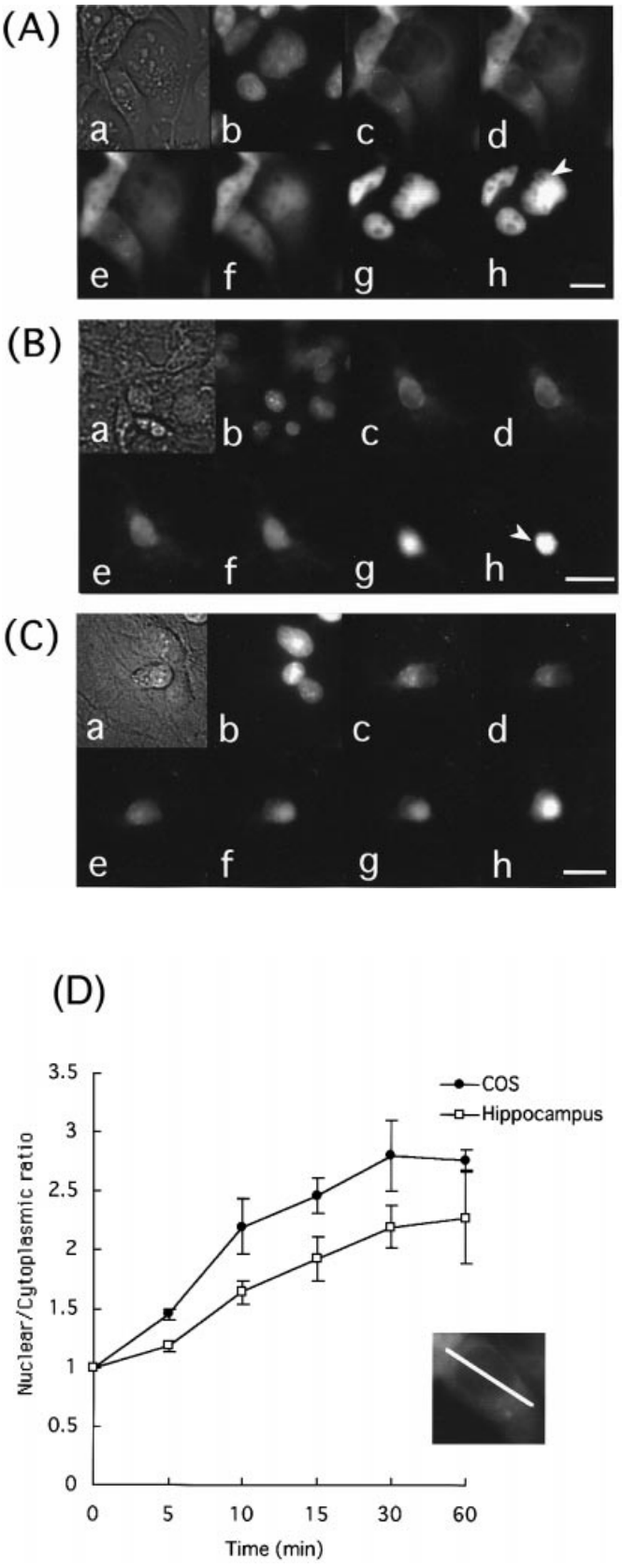


FIG. 2. Characterization of GFP-GR. (A) COS-1 cells (a–c, g–i) and CV-1 cells (d–f) were transfected with GFP alone (a, d and g) or GFP-GR (b, c, e, f, h and i), grown in SFM for 24 h and then treated with or without 10^{-7} M DEX for 30 min before observation. (a–f) Typical fluorescence images, (g–i) immunocytochemical stainings. (a) GFP-transfected COS-1 cells. (b) GFP-GR-transfected COS-1 cells without DEX. (c) GFP-GR-transfected COS-1 cells with DEX. (d) GFP-transfected CV-1 cells. (e) GFP-GR-transfected CV-1 cells without DEX. (f) GFP-GR-transfected CV-1 cells with DEX. (g) GFP-transfected COS-1 cells. No GR immunoreactivity. (h) GFP-GR-transfected COS-1 cells without DEX. GR immunoreactivity in the cytoplasm (arrow). (i) GFP-GR-transfected COS-1 cells with DEX. GR immunoreactivity in the nucleus (arrowhead). Bar, 20 µm. (B) Hippocampal neurons were grown for 48 h in the presence of CFS, and then for 48 h in SFM without steroids before transfection. After transfection, cultured cells were grown in serum containing media for 24 h, then left in SFM without steroids, and treated with or without 10^{-7} M DEX for 30 min before observation. (a–c) Representative fluorescence images, (d and e) immunocytochemical stainings. (a) GFP-transfected hippocampal neurons. (b) GFP-GR-transfected hippocampal neuron without DEX. (c) GFP-GR-transfected hippocampal neuron with DEX. (d) Hippocampal neurons without DEX. GR immunoreactivity in the cytoplasmic regions (arrow). (e) Hippocampal neurons with DEX. GR immunoreactivity in the nuclei (arrowhead). Bar: 20 µm. (C) Immunoblotting of transfected COS-1 cells stained with anti-GR antibody. Lane 1, cells transfected with GFP-GR exhibited GR staining at the predicted molecular mass of 108 kDa, whereas lane 2, cells transfected with GFP alone showed no GR staining. (D) Transcriptional stimulation by GFP-GR in COS-1 cells. The DEX-inducible reporter MMTV-Luc was cotransfected with expression plasmids encoding GFP-GR or GFP alone. As an internal standard, pCH110, a mammalian positive control vector for the expression of β-galactosidase was also cotransfected in each case. The maximum induction obtained with 10^{-7} M DEX for 4 h was taken as 100 after normalization by β-galactosidase activity, and the relative reporter luciferase activities were plotted.

line intensity profile across the cell. For each set of conditions, the intensities of pixels were summed within the individual nuclei and cytoplasms of at least five cells from three independent experiments. Nuclear : cytoplasmic fluorescence ratios were calculated and pooled for each time point. The results were normalized with the value at 0 min as 1.



Results

Properties of GFP-GR protein

The GFP-GR chimera construct was transiently transfected to various kinds of cells expressing fusion proteins which were detected by fluorescence imaging, immunocytochemistry and immunoblotting. Figure 2Aa–f showed representative fluorescence images of COS-1 cells and CV-1 cells transfected with GFP alone or transfected with GFP-GR. The GFP-transfected COS-1 cells and CV-1 cells exhibited strong green fluorescence in the whole soma area including both the cytoplasm and the nucleus (Fig. 2Aa and d, respectively) independent of a ligand effect. In contrast, in the GFP-GR-transfected COS-1 cells and CV-1 cells, green fluorescence was detected mainly in the cytoplasmic region without ligand (Fig. 2Ab and e, respectively), while green fluorescence was accumulated in the nuclear region with ligand (Fig. 2Ac and f, respectively). The results of the immunocytochemistry using anti-GR antibody are shown in Fig. 2Ag–i. As a control, cells transfected with only GFP showed no GR immunoreactivity (Fig. 2Ag), which demonstrates that COS-1 cells express no endogenous GR protein. In the GFP-GR-transfected cells, GR immunoreactivity was found in the cytoplasm in the absence of ligand (Fig. 2Ah), whereas treatment with DEX induced the localization of GR immunoreactivity in the nucleus (Fig. 2Ai). Typical fluorescence images of cultured hippocampal neurons transfected with GFP alone or GFP-GR are shown in Fig. 2Ba–c. In the GFP-transfected hippocampal neurons, strong green fluorescence was distributed not only in the soma area but also in many processes (Fig. 2Ba). The GFP-GR-transfected hippocampal neurons showed green fluorescence mainly in the cytoplasmic region in the absence of ligand (Fig. 2Bb), but only in the nuclear region in the presence of ligand (Fig. 2Bc). We also observed the immunocytochemical staining of native primary cultured hippocampal neurons without transfection. As shown in Fig. 2Bd, GR immunoreactivity was observed mainly in the cytoplasmic region in the absence of ligand (Fig. 2Bd), but incubation with DEX induced an accumulation of GR immunoreactivity in the nucleus in more than 70% of the cells (Fig. 2Be).

FIG. 3. (A) COS-1 cells transfected with GFP-GR were cultured in the absence of serum and steroids for 24 h before observation, and then the culture medium was replaced with SFM buffered with HEPES. (a) Nomarski differential interference contrast image of the cells. (b) Nuclei of the cells observed in (a) were visualized with Hoechst 33342. (c) Fluorescence image of COS-1 cells in (a) before treatment with 10^{-7} M DEX at 37 °C. (d) One minute after DEX; (e) 3 min after DEX; (f) 5 min after DEX; (g) 10 min after DEX; (h) 30 min after DEX. Arrowhead shows nucleolus. Bar, 20 µm. (B and C) Hippocampal neurons and cortical glial cells transfected with GFP-GR, respectively. Cells were cultured in SFM without steroids for 24 h, and then the culture medium was replaced with SFM buffered with HEPES before observation. In both B and C: (a) Nomarski differential interference contrast image of the cells; (b) Nuclei of the cells observed in (a) were visualized with Hoechst 33342; (c) Fluorescence image of the cells in (a) before treatment with 10^{-7} M DEX at 37 °C; (d) 1 min after DEX; (e) 3 min after DEX; (f) 5 min after DEX; (g) 10 min after DEX; (h) 30 min after DEX. Arrowhead shows nucleolus. Bar: 20 µm. (D) Quantitative analysis of the nuclear transport of GFP-GR in COS-1 cells and hippocampal neurons. Average nuclear : cytoplasmic ratios were quantified for at least five transfected cells for each data point as described in Materials and methods.

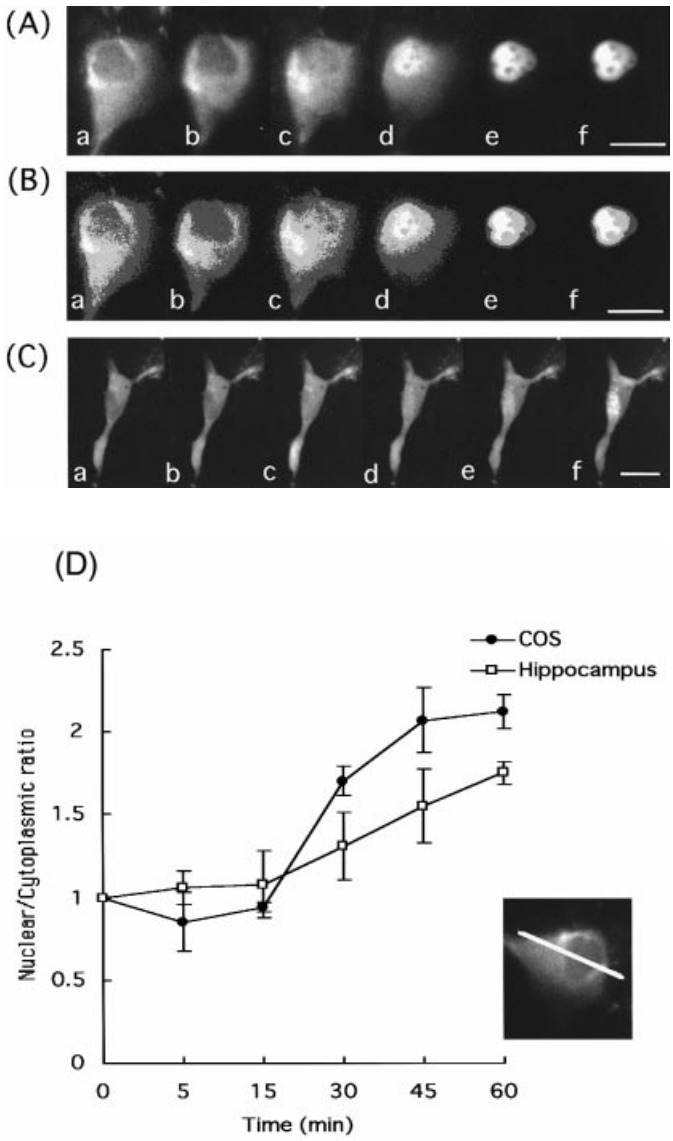


FIG. 4. Ligand concentration-dependent nuclear translocation. COS-1 cells (A and B) and hippocampal neurons (C) transfected with GFP-GR were treated with 10^{-9} M DEX at 37 °C for: (a) 0 min; (b) 5 min; (c) 15 min; (d) 30 min; (e) 45 min; and (f) 60 min. (B) Fluorescence intensity of GFP-GR observed in A presented by a gradation-pattern grey scale. A brighter colour indicates higher intensity; white spots represent the highest intensity. In each figure: (a) before DEX treatment; (b) 5 min after DEX; (c) 15 min after DEX; (d) 30 min after DEX; (e) 45 min after DEX; (f) 60 min after DEX. The distribution pattern of GFP-GR in the nucleus became more heterogeneous during the course after exposure to ligand, accumulating in some discrete regions, and finally concentrated into one cluster represented by white (Be and f). Bar, 20 μm. (D) Quantitative analysis of nuclear transport of GFP-GR in COS-1 cells and hippocampal neurons. Average nuclear/cytoplasmic ratios were quantified for at least five transfected cells for each data point as described in Materials and methods.

Immunoblots of GFP-GR-transfected COS-1 cells showed a major band at 108 kDa labelled by anti-GR antibody, but no specific band was seen in cells transfected with GFP alone (Fig. 2C).

The GFP-GR construct was cotransfected with DEX-inducible reporter MMTV-Luc into COS-1 cells. As an internal standard, β-galactosidase gene was also cotransfected. When activated with 10^{-7} M DEX, GFP-GR was sufficient to induce the significant activation of MMTV-Luc reporter DNA (Fig. 2D). These results demonstrate that the transfection of the GFP-GR chimera construct was effectively achieved and functionally active in DEX-mediated transcriptional activation of the transiently transfected reporter plasmid DNA.

Ligand-specific cytoplasm-to-nuclear translocation of GFP-GR

Time-course and dose dependency

Exposure to DEX caused the nuclear localization of GFP-GR in 100% of the fluorescing cells, with the rate of translocation from the cytoplasm to the nucleus dependent on the concentration of ligand. We observed that 10^{-7} m DEX induced a complete cytoplasm-to-nuclear translocation of GFP-GR within 30 min at 37 °C in both of the two vertebrate cell lines, COS-1 cells and CV-1 cells, which express no endogenous GR (Figs 3A and 2Af, respectively), and primary cultured neurons and glial cells, which express endogenous GR (Fig. 3B and C, respectively). Before the DEX treatment, fluorescence was observed homogeneously in the cytoplasm. The average nuclear : cytoplasmic ratios of GFP-GR fluorescence intensity for each time point under 10^{-7} M DEX are shown in Fig. 3D. Each resulting curve reflected a relative increase in the nuclear localization of GFP-GR. The nuclear : cytoplasmic ratio was increased after exposure to ligand and reached a plateau in 30 min. According to these quantitative data and fluorescence images, the cytoplasmic perinuclear accumulation of GFP-GR was detected at 1 min, nuclear entry at 3 min, and half-maximal accumulation at about 10 min (Fig. 3A–D). The rate of nuclear translocation was reduced by 10^{-9} M DEX, requiring about 45 min for complete nuclear localization in COS-1 cells (Fig. 4A and B). The average nuclear : cytoplasmic ratios of GFP-GR fluorescence intensity for each time point obtained at 10^{-9} M DEX are also exhibited in Fig. 4D. Each resulting curve reflected a relative increase in the nuclear localization of GFP-GR. Under this concentration, cytoplasmic perinuclear accumulation was observed at about 5 min, nuclear entry at 15 min and half-maximal accumulation at about 30 min. This time-course of nuclear translocation can be recognized more clearly in Fig. 4B, which shows a fluorescence intensity of GFP-GR with a gradation-pattern grey scale in which a brighter colour indicates higher fluorescence intensity. In the GFP-GR-transfected hippocampal neurons stimulated with 10^{-9} M DEX, although the time-course of the initial nuclear translocation of the cytoplasmic GFP-GR was almost the same as that in COS-1 cells, the nuclear accumulation rate of GFP-GR after entering the nucleus became slow, and GFP-GR remained in the cytoplasmic region for over 60 min (Fig. 4C). These results were confirmed by the quantitative analysis shown in Fig. 4D. Cortical glial cells transfected with GFP-GR showed the same time-course of the nuclear translocation under 10^{-9} M DEX. The states of cells after taking images were almost the same as those observed before taking images. The cells were not deteriorated during time-lapse imaging for 1 h.

Ligand specificity of translocation

The ligand specificity of the nuclear translocation of GFP-GR was investigated by treating the cells with various drugs. Other classes of ligands for steroid receptor, i.e. Ald and E2, were employed. We found that 5×10^{-7} M Ald, which is a ligand for MR and has a affinity for GR, induced nuclear accumulation of GFP-GR in COS-1 cells (Fig. 5A), hippocampal neurons and cortical glial cells. The time-courses and cytological distribution patterns were essentially the same as those in the case of DEX. In contrast, 10^{-7} M E2, which is a ligand for oestrogen receptor and does not bind to GR, did not induce the nuclear translocation of GFP-GR in COS-1 cells (Fig. 5B), hippocampal neurons or cortical glial cells.

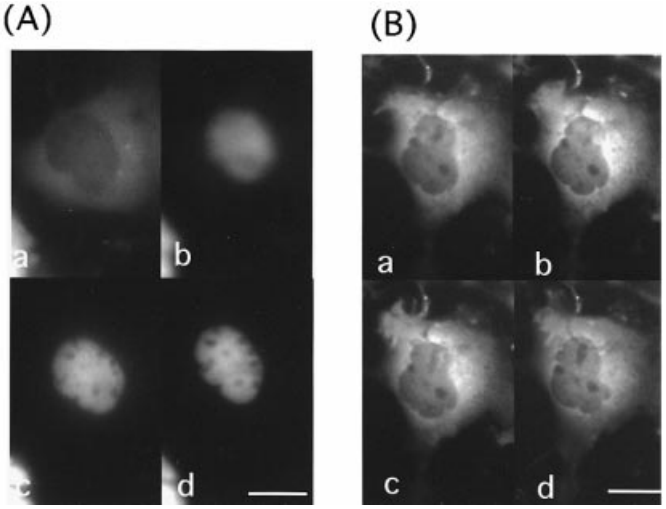


FIG. 5. GFP-GR-transfected COS-1 cells treated with 5×10^{-7} M Ald (A) or 10^{-7} M E2 (B) at 37 °C. In each treatment, cells were incubated with each substance for: (a) 0 min; (b) 15 min; (c) 30 min; or (d) 60 min. Note that Ald induced the cytoplasmic-nuclear translocation of GFP-GR, whereas E2 had no effect on the changes of GFP-GR subcellular localization. Bar, 20 μm.

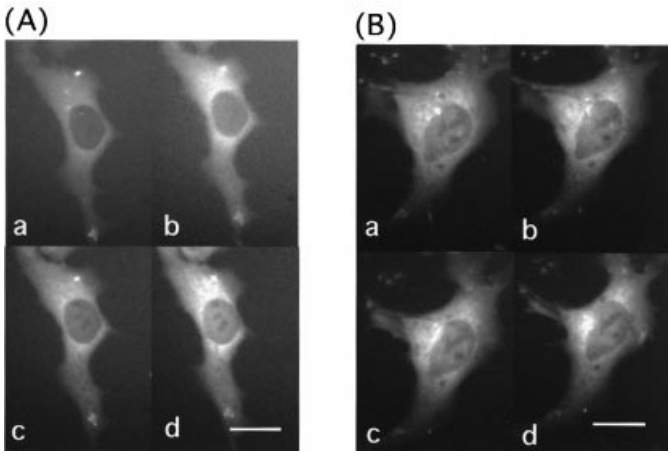


FIG. 6. GFP-GR-transfected COS-1 cells treated with 10^{-6} M FK (A) or 100 ng/mL BDNF (B) at 37 °C. In each treatment, cells were incubated with each substance for: (a) 0 min; (b) 15 min; (c) 30 min; or (d) 60 min. These two treatments caused no changes in the GFP-GR subcellular localization. Bar, 20 μm.

Effects of signalling molecules on translocation

It has been shown that cAMP can promote the transcriptional activation of GR induced with DEX (Gruol *et al*., 1986; Jewell *et al*., 1995). This promotion may be obtained by a direct effect on GR or through an indirect mechanism. In order to elucidate whether cAMP alone affects the nuclear translocation of GR, cells were treated with FK, an activator of cAMP-dependent protein kinase, and subjected to a time-lapse imaging study. We saw that 10^{-6} M FK had no effect on the nuclear translocation of GFP-GR in COS-1 cells (Fig. 6A), hippocampal neurons or cortical glial cells. We also examined the effects of BDNF, one of the MAP kinase activators. Because BDNF shows a kinase activity as does FK, we investigated whether BDNF affects the subcellular localization of GR. BDNF (100 ng/mL) induced no nuclear accumulation of GFP-GR in COS-1 cells (Fig. 6B), hippocampal neurons or cortical glial cells.

Effects of cytoskeletal elements on cytoplasm-to-nuclear translocation of GFP-GR

We addressed the question of whether a certain pathway is required for receptor trafficking between the cytoplasm and the nucleus, or for targeting the receptor to specific transcription sites. We focused on microtubules in the present study. Pre-treatment with colchicine (10 μM) or nocodazole (10 μg/mL) (Fig. 7A and B, respectively), which are microtubule-disrupting agents, for 3 h did not cause significant changes in the DEX-induced cytoplasm-to-nuclear translocation pattern of GFP-GR in hippocampal neurons and COS-1 cells. In both cells, GFP-GR was accumulated in the nuclear region around 40 min after DEX treatment. After treatment with 10 μM colchicine or 10 μg/mL nocodazole for 3 h, both hippocampal neurons and COS-1 cells showed morphological changes, and tubulin disruption was confirmed by immunocytochemistry using antityrosinated α tubulin antibody. COS-1 cells showed rough-surfaced cellular morphology and more round-shaped nuclei (Fig. 7A and Cb), and the fibrous staining of tyrosinated α tubulin immunoreactivity observed in the control cultures (Fig. 7Ca) disappeared in colchicine-treated COS-1 cells, with only amorphous staining observed (Fig. 7Cb). In hippocampal neurons, tyrosinated α tubulin immunoreactive processes shrinked after treatment with colchicine (Fig. 7Db).

Discussion

In the present study, we employed a GFP-GR chimera system to examine dynamic changes in the subcellular localization of a steroid hormone receptor in a single living cell. We confirmed that our GFP-GR fusion protein is transcriptionally active using the MMTV-Luc reporter gene. Although recent studies used a similar approach to examine the subcellular localization of GFP-GR in living vertebrate cell lines (Ogawa *et al*., 1995; Htun *et al*., 1996), no study has been performed in living neurons or glial cells using this approach.

The time-course study of GFP-GR trafficking showed that the rate of translocation is dependent on the concentration of ligand. This result indicates that the rate of translocation plays an important role in GR function. Shortly after GFP-GR enters the nucleus, the fluorescence appearance became accumulated in some specific regions, and finally concentrated into one cluster. Htun *et al*. (1996) suggested that such nuclear clusters observed with GFP-GR correspond to activated target genes. In the study of GFP-MR (Fejes-Toth *et al*., 1998), the authors reported that agonist-activated MRs accumulate in discrete clusters in the nucleus, and this phenomenon occurs only with transcriptionally active mineralocorticoids. In contrast to these studies using GFP-receptor fusion protein, Van Steensel *et al*. (1995, 1996) demonstrated the spatial distribution of GRs and MRs in clusters in specific nuclear domains using immunofluorescence technique with confocal microscopy. They indicated that these clusters are not directly involved in active transcription. One of the possible explanations for these discrepancies is that the observed focal distribution of receptors represents a primary step leading to transcriptional activation. The exact nature and function of the nuclear clusters remain to be determined.

In the present study, we analysed both cell lines expressing no endogenous GR, and primary cultured neural cells, hippocampal neurons and cortical glial cells, expressing endogenous GR. The results showed no significant difference in the rate of cytoplasm-to-nuclear translocation of GFP-GR between these two different cell types at an excessive concentration of ligand (10^{-7} M DEX). GFP-GR was mostly accumulated within 30 min in all types of cells. These data were confirmed with the previous studies (Ogawa *et al*., 1995;

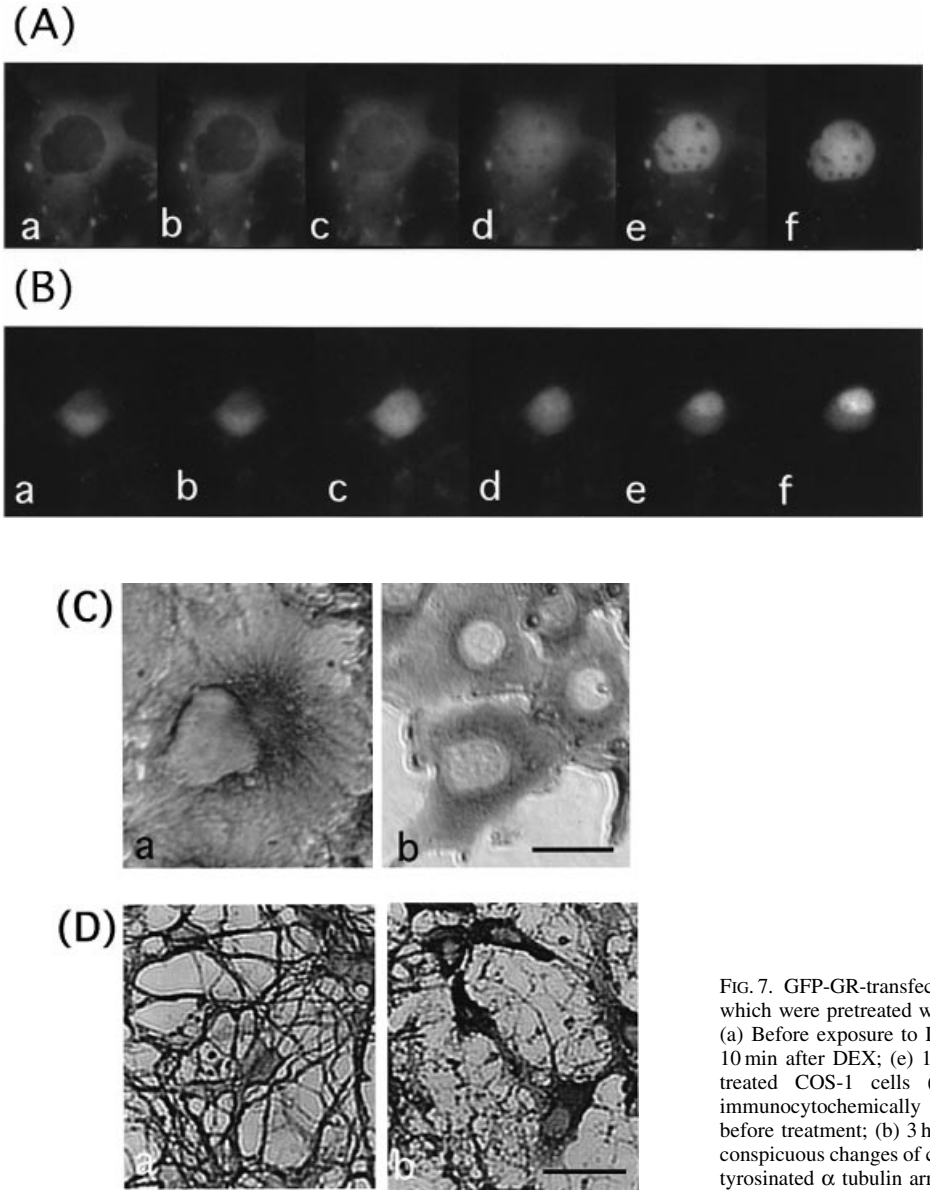


FIG. 7. GFP-GR-transfected COS-1 cells (A) and hippocampal neurons (B) which were pretreated with 10 μM colchicine for 3 h exposed to 10^{-7} M DEX. (a) Before exposure to DEX; (b) 5 min after DEX; (c) 8 min after DEX; (d) 10 min after DEX; (e) 15 min after DEX; (f) 40 min after DEX. Colchicine-treated COS-1 cells (C) and hippocampal neurons (D) which were immunocytochemically stained with antityrosinated α tubulin antibody: (a) before treatment; (b) 3 h after treatment with 10 μM colchicine. Note that the conspicuous changes of cell morphology were accompanied with turbulence of tyrosinated α tubulin arrangement. Bar, 20 μm.

Htun *et al*., 1996). Conversely, at the concentration around the K_d value (10^{-9} M DEX), the nuclear translocation of GFP-GR was completed in about 45 min in COS-1 cells, while some of the fluorescence of GFP-GR remained in the cytoplasm of hippocampal neurons for over 60 min. Because more than 90% of primary cultured hippocampal cells have endogenous GR (Bohn *et al*., 1994; Nishi & Kawata, 1997), and an *in vivo* study showed that both neurons and glias in hippocampal and cortical regions express GR protein (Morimoto *et al*., 1996), GFP-GR may compete with endogenous GR for ligand at a concentration around the K_d value, while the kinetics of the cytoplasm-to-nuclear translocation of GFP-GR could be independent of the basal level of endogenous GR at an excessive concentration of ligand (Webster *et al*., 1997). In the native hippocampal cultured cells, the rate of the nuclear accumulation of GR induced with the same concentration of DEX detected by GR immunocytochemistry was slightly slower (Nishi & Kawata, 1997) than that detected here in living hippocampal cultures transfected with GFP-GR. It is not clear whether this discrepancy observed in primary cultures is due to technical differences, e.g. fixation and/or permeabilization in the process of immunocytochemistry, the effects of tagging with GFP or the overexpression of GFP-GR. Another possible explanation is that in the fixed cells it is hard to detect the transient and subtle changes in the distribution of receptor immunoreactivity, whereas the time-lapse imaging system is suitable for capturing such changes. The present findings also revealed that there is no marked difference in the nuclear translocation of GFP-GR between hippocampal neurons and cortical glial cells. This observation suggests that there are no particular cell types or region specificities for GR regarding nuclear translocation in the central nervous system (CNS). These findings have prompted us to speculate that there is a common intrinsic trafficking mechanism of GR for regulating gene expression in order to respond to diversified stress stimuli in the brain.

In addition to DEX, we employed treatment with Ald and E2 to examine the ligand specificity of GFP-GR. Ald, a typical agonist for MR, has a chemical structure similar to that of DEX (Joels & De

Kloet, 1994). Therefore Ald shows binding affinity for GR, although the K_d value of Ald for GR is about 100-fold higher than that of DEX. We observed that 5×10^{-7} M Ald induced the nuclear translocation of GFP-GR in essentially the same manner as that observed with DEX. In contrast, E2 has a different structure at the ligand binding site and cannot bind with GR. We found that GFP-GR was not translocated into the nuclear region with 1×10^{-7} M E2. Taken together, the present findings confirm that GFP-GR retains its native receptor structure and exhibits high specificity for ligands.

Although it is known that cAMP promotes transcription induced with GR (Gruol *et al*., 1986), the present findings indicate that activation of GR with cAMP alone cannot induce nuclear translocation of GR, the primary step for GR to activate specific transcription sites. We also found that BDNF, which has a kinase activity, fails to cause the nuclear accumulation of GFP-GR. These findings suggest that this type of intracellular signal alone is not sufficient for promoting nuclear translocation of GR, whereas unmasking of the nuclear localization signal (NLS) in GR induced by ligand binding (Cadepond *et al*., 1992) could be essential for nuclear translocation. Our results also indicate that GR phosphorylation induced by a protein kinase may not induce the nuclear translocation of GFP-GR, in agreement with a previous study (Jewell *et al*., 1995).

Extensive studies have recently been focused on the nucleocytoplasmic transport of macromolecules including transcription factors (Carey *et al*., 1996; Corbett & Silver, 1997; Nakielny & Dreyfuss, 1997; Nigg, 1997). These studies investigated mainly nucleopore complexes and shuttling proteins, all of which are elements involved in transport just around the nuclear membrane. In contrast, little is known about the pathway of GR transport from the cytoplasmic region to the perinuclear site. Previous morphological and biochemical studies indicated that GR is associated with intracellular microtubule networks (Scherrer & Pratt, 1992; Akner *et al*., 1995). An immunocytochemical study showed the colocalization of GR with tubulin in the cytoplasmic regions of fibroblast cells (Akner *et al*., 1995). The present study examined effects of microtubule disruption on the nuclear translocation of GFP-GR in living cells. We pretreated cells with 10 μM colchicine or 10 μg/mL nocodazole for 3 h before exposure to DEX. These doses of drugs were shown to induce an almost complete depolymerization of microtubules within 1 h of treatment (Sazapary *et al*., 1994; Akner *et al*., 1995). We confirmed the microtubule disruption by immunocytochemistry using antityrosinated α tubulin (Kreis, 1987), whereas the functional disruption cannot be fully determined by morphological changes alone. Under these conditions, although the rate of cytoplasm-to-nuclear translocation was slightly reduced as compared to that observed in the absence of colchicine, the GFP-GR was completely translocated into the nuclear region. These results suggest that the microtubules are not essentially involved in transporting GFP-GR from the cytoplasm to the nucleus. However, we cannot entirely rule out the possible interactions of microtubules and GR in the cells. Future studies will examine more precise relations between GR and specific organelles including cytoskeletal elements.

In conclusion, the present study using a GFP-GR chimera system has revealed a dynamic alteration in the subcellular localization of GFP-GR in response to various extracellular and intracellular environments. Although there are still problems in tagging proteins with GFP and overexpressing the receptors, this approach enables the observation of events in living cells which have never been detected in fixed cells. GFP-tagged GR may thus be a powerful tool for answering important questions, e.g. how the receptor enters the nucleus, binds to specific transcriptional sites and interacts with other cofactors at the molecular level, not only in non-neural cells, but also in neural cells.

Acknowledgement

The authors are grateful to Dr Yasunori Hayashi for valuable comments and discussion, and Dr Toyoshi Hosokawa for kind support during the course of this study. We also would like to thank Dr Kazuhiko Umesono for providing MMTV-Luc. This work has been supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

Abbreviations

Ald, aldosterone; BDNF, brain-derived neurotrophic factor; BPB, bromophenol blue; BSA, bovine serum albumin; CNM, complete neuronal medium; CNS, central nervous system; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; E2, 17β-oestradiol; FCS, foetal calf serum; FK, forskolin; GFP, green fluorescent protein; GR, glucocorticoid receptor; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]; HRP, horseradish peroxidase; MEM, minimum essential medium; MMTV-Luc, mouse mammary tumour promoter-luciferase; MR, mineralocorticoid receptor; NLS, nuclear localization signal; PBS, phosphate buffered saline; PFA, paraformaldehyde; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SFM, serum-free medium.

References

Akner, G., Wikstrom, A.C. & Gustafsson, J.-A. (1995) Subcellular distribution of the glucocorticoid receptor and evidence for its association with microtubules. *J. Steroid Biochem. Mol. Biol.*, **52**, 1–16.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, J.D. (1989) *Molecular Biology of the Cell*. Gerland, New York, NY.

Antakly, T., O'Donnell, D. & Thompson, E.B. (1990) Immunocytochemical localization of the glucocorticoid receptor in steroid-sensitive and -resistant human leukemic cells. *Cancer Res.*, **50**, 1337–1345.

Azmitia, E.C. & Hou, X.P. (1994) Steroid regulation of neurotrophic activity: primary microcultures of midbrain raphe and hippocampus. In De Kloet, E.R. & Sutanto, W. (eds), *Methods in Neuroscience 22; Neurobiology of Steroids*. Academic Press, San Diego, CA, pp. 359–371.

Beato, M. (1989) Gene regulation by steroid hormones. *Cell*, **56**, 335–344.

Bohn, M.C., O'Banion, M.K., Young, D.A., Giuliano, R., Hussain, S., Dean, D.O. & Cunningham, L.A. (1994) In vitro studies of glucocorticoid effects on neurons and astrocytes. *Ann. NY Acad. Sci.*, **746**, 243–259.

Brink, M., Humbel, B.M., De Kloet, E.R. & Van Driel, R. (1992) The unliganded glucocorticoid receptor is localized in the nucleus, not in the cytoplasm. *Endocrinology*, **130**, 3575–3581.

Cadepond, F., Gasc, J.-M., Delahaye, F., Jibard, N., Schweizer-Groyer, G., Segard-Maurel, I., Evans, R. & Baulieu, E.-E. (1992) Hormonal regulation of the nuclear localization signals of the human glucocorticosteroid receptor. *Exp. Cell Res.*, **201**, 99–108.

Carey, K.L., Richards, S.A., Lounsbury, K.M. & Macara, I.G. (1996) Evidence using green fluorescent protein-glucocorticoid receptor chimera that the RAN/TC4 GTPase mediates an essential function independent of nuclear protein import. *J. Cell Biol.*, **133**, 985–986.

Carson-Jurica, M.A., Schrader, W.T. & O'Malley, B.W. (1990) Steroid receptor family: structure and functions. *Endocrinol. Rev.*, **11**, 201–220.

Corbett, A.H. & Silver, P.A. (1997) Nucleocytoplasmic transport of macromolecules. *Microbiol. Molec. Biol. Rev.*, **61**, 193–211.

Cormack, B.P., Valdivia, R.H. & Falkow, S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene*, **173**, 33–38.

Coutard, M., Osborne-Pellegrin, M.J. & Funder, J.W. (1978) Tissue distribution and specific binding of tritiated dexamethasone in vivo: autoradiographic and cell fractionation studies in the mouse. *Endocrinology*, **103**, 1144–1152.

De Kloet, E.R. (1991) Brain corticosteroid receptor balance and homeostatic control. *Front. Neuroendocrinol.*, **12**, 95–164.

Evans, R. & Arriza, J.L. (1989) A molecular frame work for the action of glucocorticoid hormones in the nervous system. *Neuron*, **2**, 1105–1112.

Fejes-Toth, G., Pearce, D. & Naray-Fejes-Toth, A. (1998) Subcellular localization of mineralocorticoid receptors in living cells: effects of receptor agonists and antagonists. *Proc. Natl Acad. Sci. USA*, **95**, 2973–2978.

Funder, J.W. & Sheppard, K. (1987) Adrenocorticosteroids and the brain. *Annu. Rev. Physiol.*, **49**, 397–411.

Gasc, J.-M. & Baulieu, E.E. (1987) In Clark, C.R. (ed.), *Steroid Hormone*

Receptors: Their Intracellular Localization. Ellis Horwood, Chichester, UK, pp. 233–250.
Gasc, J.-M., Delahaye, F. & Bauieu, E.E. (1989) Compared intracellular localization of the glucocorticoid receptor and progesterone receptors: an immunocytochemical study. *Exp. Cell Res.*, **181**, 492–504.
Gruol, D.J., Campbell, F. & Bourgeois, S. (1986) Cyclic AMP-dependent protein kinase promotes glucocorticoid receptor function. *J. Biol. Chem.*, **261**, 4909–4914.
Guiochon-Mantel, A., Delabre, K., Lescop, P. & Milgrom, E. (1996) Intracellular traffic of steroid hormone receptors. *J. Steroid Biochem. Mol. Biol.*, **56**, 3–9.
Hall, C.V., Jacob, P.E., Ringold, G.M. & Lee, F. (1983) Expression and regulation of Escherichia coli lacZ gene fusion in mammalian cells. *J. Mol. Appl. Genet.*, **2**, 101–109.
Htun, H., Barsony, J., Renyi, I., Gould, D. & Hager, G.L. (1996) Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera. *Proc. Natl. Acad. Sci. USA*, **93**, 4845–4850.
Hu, Z., Yuri, K., Ozawa, H., Lu, H. & Kawata, M. (1997) The in vivo time course for elimination of adrenalectomy-induced apoptotic profiles from the granule cell layer of the rat. *J. Neuroscience*, **17**, 3981–3989.
Jewell, C.M., Webster, J.C., Burnstein, K.L., Sar, M., Bodwell, J.E. & Cidlowski, A. (1995) Immunocytochemical analysis of hormone mediated nuclear translocation of wild type and mutant gluococorticoid receptors. *J. Steroid Biochem. Mol. Biol.*, **55**, 135–146.
Joels, M. & De Kloet, E.R. (1994) Mineralocorticoid and glucocorticoid receptors in the brain. Implication for ion permeability and transmitter systems. *Prog. Neurobiol.*, **43**, 1–36.
Kreis, T.E. (1987) Microtubules containing detyrosinated tubulin are less dynamic. *EMBO J.*, **6**, 2597–2606.
Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **277**, 680.
LaFond, R.E., Kennedy, S.W., Harrison, R.W. & Villee, C.A. (1988) Immunocytochemical localization of glucocorticoid receptors in cells, cytoplasts and nucleoplasts. *Exp. Cell Res.*, **175**, 52–62.
Martin, P.M. & Sheridan, P.J. (1980) Intracellular distribution of estrogen receptors: a function of preparation. *Experimentia*, **36**, 620–622.
McCarthy, K.D. & FeVellis, J. (1980) Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell Biol.*, **85**, 890–902.
McEwen, B.S. (1991) Non-genomic and genomic effects of steroids on neural activity. *Trends Pharmacol. Sci.*, **12**, 141–147.
Morimoto, M., Morita, N., Ozawa, H., Yokoyama, K. & Kawata, M. (1996) Distribution of glucocorticoid receptor immunoreactivity and mRNA in the rat brain: an immunocytochemical and in situ hybridization study. *Neurosci. Res.*, **26**, 235–269.
Nakielny, S. & Dreyfuss, G. (1997) Import and export of the nuclear protein import receptor transportin by a mechanism independent of GTP hydrolysis. *Current Biol.*, **8**, 89–95.
Nigg, E.A. (1997) Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature*, **386**, 779–787.
Nishi, M. & Kawata, M. (1997) State dependent subcellular localization of glucocorticoid receptor in rat hippocampal culture. *Soc. Neurosci. Abstr.*, **23**, 1152.
Nishi, M., Ueda, S., Morita, N. & Kawata, M. (1994) The effects of glucocorticoid on neurons of rat dentate gyrus in dissociated culture. *Neuroreport*, **5**, 2446–2448.
Nishi, M., Whitaker-Azmitia, P.M. & Azmitia, E.C. (1996) Enhanced synaptophysin immunoreactivity in rat hippocampal culture by 5-HT1A agonist, S100b, and corticosteroid receptor agonists. *Synapse*, **23**, 1–9.
Ogawa, H., Inoue, S., Tsuji, F., Yasuda, K. & Umesono, K. (1995) Localization, trafficking, and temperature-dependence of the Aequorea green fluorescent protein in cultured vertebrate cells. *Proc. Natl. Acad. Sci. USA*, **92**, 11 899–11 903.
Picard, D. & Yamamoto, K.R. (1987) Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO J.*, **6**, 3333–3340.
Reul, J.M.H.M. & De Kloet, E.R. (1985) Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. *Endocrinology*, **117**, 2505–2511.
Sazapary, D., Barber, T., Dwyer, N.K., Blanchette-Mackie, E.J. & Simons, S.S. Jr (1994) Microtubules are not required for glucocorticoid receptor mediated gene induction. *J. Steroid Biochem. Mol. Biol.*, **51**, 143–148.l.
Scherrer, L.C. & Pratt, W.B. (1992) Association of the transformed glucocorticoid receptor with a cytoskeletal protein complex. *J. Steroid Biochem. Mol. Biol.*, **41**, 719–721.
Stalker, A., Hermo, L. & Antakly, T. (1989) Covalent affinity labeling, radioautography, and immunocytochemistry localize the glucocorticoid receptor in rat testicular Leydig cells. *Am. J. Anat.*, **186**, 369–377.
Van Steensel, B., Brink, M., van der Meulen, K., van Binnendijk, E.P., Wansink, D.G., de Jong, L., De Kloet, E.R. & van Driel, R. (1995) Localization of the glucocorticoid receptor in discrete clusters in the cell nucleus. *J. Cell Sci.*, **108**, 3003–3011.
Van Steensel, B., Van Binnendijk, E.P., Hornsby, C.D., Van der Voort, T.M., Krozowski, Z.S., De Kloet, E.R. & Van Driel, R. (1996) Partial colocalization of glucocorticoid and mineralocorticoid receptors in discrete compartments in nuclei of rat hippocampal neurons. *J. Cell Sci.*, **109**, 787–792.
Webster, J.C., Jewell, C.M., Bodwell, J.E., Munck, A., Sar, M. & Cidlowski, J.A. (1997) Mouse glucocorticoid receptor phosphorylation status influences multiple functions of the receptor protein. *J. Biol. Chem.*, **272**, 9287–9293.
Wikstrom, A.-C., Bakke, O., Okret, S., Bronnegard, M. & Gustafsson, J.-A. (1987) Intracellular localization of the glucocorticoid receptor: evidence for cytoplasmic and nuclear localization. *Endocrinology*, **120**, 1232–1242.