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Review

Subcellular Distribution of the Glucocorticoid Receptor and Evidence for its Association with Microtubules

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The cellular distribution of the glucocorticoid receptor (GR) has not yet been firmly established. The extensive literature indicates that GR is present both in the cytoplasm and the cell nucleus, however, some studies have failed to detect cytoplasmic GR. It is still controversial as to whether GR is randomly diffusing in the cytoplasm and nucleus, or if the GR-distribution is organized or controlled in some way, which may be of importance for the transduction of glucocorticoid effects to cells. There is evidence that both non-activated and activated GR is associated with the plasma membrane, a number of cytoplasmic organelles and the nucleus. Both morphological and biochemical evidence show that GR is associated with microtubules during different stages of the cell cycle, i.e. GR co-localizes, co-purifies and co-polymerizes with tubulin. This indicates that GR is structurally linked to the intracellular MT-network which may be of importance in the mechanism of action of glucocorticoid hormones. The literature in this field is reviewed including the reported data on subcellular GR-localization.

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BIOCHEMICAL CHARACTERISTICS OF THE GLUCOCORTICOID RECEPTOR

The glucocorticoid receptor (GR) is a ligand-activated transcription factor belonging to the steroid hormone receptor superfamily. Protease degradation of GR defined three distinct, functional domains [1, 2] which were later confirmed at the GR cDNA level using site-directed mutagenesis [3].

The transactivating (N-terminal, immunogenic) domain is the least conserved domain among the various members of the steroid receptor superfamily. Most mono- and polyclonal antibodies against GR recognize this domain. The DNA-binding domain is highly conserved in all steroid receptors. The core region of this domain contains two zinc fingers, analogous to the zinc-finger regions of TFIIIA and other transcription factors responsible for sequence specific DNA-binding [4, 5]. The hormone binding (C-terminal) domain is required for high affinity steroid binding [6] and there is also evidence that this domain binds the hsp90 dimer [7]. Deletions within this domain of GR results in a protein with constitutive transactivating capacity [8]. The hormone-binding and DNA-binding domains are separated by a "hinge" region, containing a short stretch of highly basic amino acids [9]. This region has been implicated in nuclear localization of the rat GR [10] and is homologous to sequences required for nuclear localization of other proteins [11]. Further analysis of different parts of GR by deletion mutagenesis has revealed other functional regions as well. There are e.g. two separate transactivation regions within GR, localized in the N-terminal domain ($\tau 1$) and between the DNA- and steroid binding domains ($\tau 2$), respectively [12]. The amino acid sequence of the human GR, deduced from sequence analysis of cDNA clones, revealed the existence of two major isoforms of GR, α and β, 777 and 742 amino acids in length, respectively, generated by alternative splicing of an mRNA encoded from a single gene on human chromosome 5 [12]. In the rat GR (795 amino acids), the three domains contain

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409, 108 and 278 amino acids, corresponding to ≈51, 14 and 35% of the primary sequence, respectively [13].

During basal conditions, the non-activated GR is present in the cell as a multimolecular, hetero-oligomeric complex together with several other proteins, e.g. a dimer of the heat shock protein M_R90,000, hsp90 [14], the immunophilin hsp56 [15, 16] and p23 [17]. There is also evidence that the complex may contain other components, e.g. RNA [18] and phosphate [19]. Regarding tubulin, see below. Treatment of cells with glucocorticoid hormones in the presence of heat causes activation of GR, i.e. the receptor acquires DNA-binding ability [20]. Thus, at +4°C, the hormone only binds to, but does not activate GR. It is unclear why heat (+20°C, 30 min) is needed along with the ligand. This heat-requirement for activation represents an *in vitro* phenomenon and can not be of physiological significance in mammals *in vivo*. Some features of the activated GR are presented below.

Features of the activated human GR:
—Macromolecule, M_R94,000 [21]
—777 amino acids [12]
—Sedimentation coefficient ≈4S [21]
—Asymmetric protein, with a length of approx. 0.12–0.15 nm [22]
—Negative net charge [19]
—Acidic protein, pI 5.7 [23]
—Phosphoprotein [19]
—One molecule of glucocorticoid aporeceptor binds one molecule of glucocorticoid hormone [24]
—The activated GR is able to bind both to non-specific DNA and glucocorticoid response elements (GREs) [2].

TISSUE LOCALIZATION OF GR

GR has been detected in many different mammalian tissues, however, there are reports that certain tissues (rat) are devoid of GR, e.g. the intermediate lobe of the pituitary [25], liver Kupffer cells and liver endothelial cells [26], uterus, prostate gland, seminal vesicles, bladder, adipose tissue and jejunum [27], kidney glomeruli and proximal convoluted tubules [28] and acinary cells in submaxillary glands [29]. It has also been claimed that neither rat neurons nor rat lymphocytes contain GR [30], but this is in contrast to other reports [31, 32].

MODELS OF GLUCOCORTICOID HORMONE LOCALIZATION AND ACTION

Three commonly discussed models of steroid hormone mechanism of action (Fig. 1) have all been applied to GR. Early studies on steroid hormone receptor localization focused on the estrogen receptor (ER) and were based on cell fractionation experiments

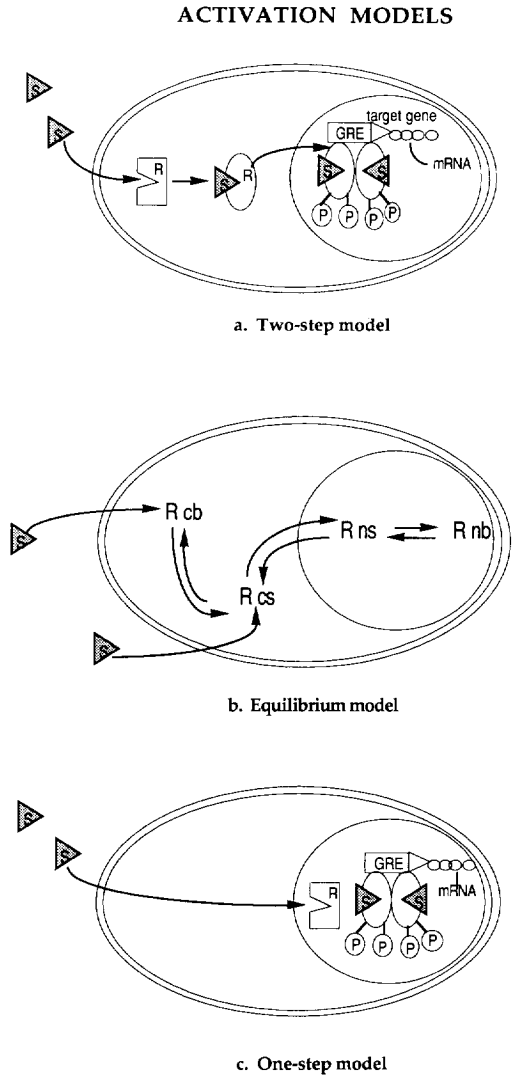

Fig. 1. Activation models. Three common, hypothetical models of steroid hormone action. R, receptor; GRE, glucocorticoid response element; p, phosphate; c, cytoplasm; n, nuclear; s, soluble; b, bound. The glucocorticoid hormone is represented by a grey triangle containing an "s" for steroid. See also text.

supported morphologically be cellular autoradiography [33]. These studies led to the development of "the two-step model" of estrogen hormone action [33] and later to a unified theory encompassing all steroid hormone receptors including GR [34] [see Fig. 1(a)]. The model stated that the non-liganded steroid hormone receptor is soluble in the cytoplasm/cytosol of the cell. After binding of a specific steroid ligand, the receptor complex undergoes a temperature-sensitive process denoted "activation" or "transformation", during which the receptor complex acquires an increased affinity for chromatin or DNA to alter gene expression.

However, the model was soon challenged [35]. New techniques for autoradiographic sample processing [36] lead to new conclusions and also to reinterpretation of some of the previous autoradiographic data. The two-step model was thus replaced by an "equilibrium

Table 1. Classification of non-liganded steroid hormone receptors according to their affinity for nuclear components (Adapted from Sanchez et al., 1990 [42])

Group 1	Tightly bound to nuclei even in the absence of ligand. High salt required for extraction from nuclei. e.g. Thyroid hormone receptor, retinoic acid receptor
Group 2	Relatively weakly bound to nuclei in the absence of ligand. Recovered in cytosolic fraction after lysis of cells in hypotonic buffer. Become tightly associated to nuclei after treatment with ligand and high salt is then required for extraction. e.g. Estrogen receptor, progesterone receptor, androgen receptor (?)
Group 3	Located in the cytoplasm in the absence of ligand. Recovered in cytosolic fraction after lysis of cells in hypotonic buffer. Become tightly associated to nuclei after treatment with ligand and high salt is then required for extraction. e.g. GR, 1,25-dihydroxy-vitamin D3-receptor (?)

model", originally for the ER and progesterone receptors (PR) [36, 37] and then expanded to all steroid receptors [38] [see Fig. 1(b)]. This model postulated an equilibrium between the cell bound and soluble forms of steroid receptors both in the nucleus (bound to chromatin) and the cytoplasm (bound to ?) based on partitioning according to the free water present in each compartment, fixed charges, pH, ionic strength etc. These parameters were expected to vary between tissues and species. Available data was interpreted such that for both ER and PR, at least part of the unbound receptor resided in the nucleus, and that it was extracted into the cytosolic fraction during homogenization and separation [38]. Recent studies using PR deletion mutants indicate that the nuclear localization of PR is due to such a dynamic equilibrium: PR diffusing into the cytoplasm is constantly and actively being transported back into the nucleus [39]. This nuclear–cytoplasmic shuttle energy-dependent mechanism is not dependent on an intact cytoskeleton [40]. Immunolocalization experiments have provided evidence that several of the members in the steroid hormone rceptor superfamily are localized in the cell nucleus at all times [41]. This led to the "one step model", implying that both non-liganded and liganded steroid hormone receptors are nuclear proteins and that the lipophilic ligands traverse both the plasma membrane and the nuclear envelope and bind to their respective receptors in the nucleus directly [see Fig. 1(c)]. The older biochemical data have then been reinterpreted and the cytosolic localization of receptors in cell extracts is claimed to be due to artifactual redistribution of receptors during cell- or tissue-homogenization. Attempts have even been made to group the steroid receptors according to their various degree of extractability [42] (see Table 1 for a summary).

NUCLEAR TRANSLOCATION

The concept of ligand induced nuclear translocation of GR has been widely accepted, even though several studies have failed to demonstrate such a process [43–47]. In any case, it seems clear that a part of the total GR-population must undergo at least one nuclear translocation, i.e. after being synthesized in the endoplasmic reticulum in the cytoplasm.

Macromolecules of the size of GR probably enter the nucleus in a controlled fashion [48] and require specific signal sequences for nuclear uptake [49]. By analogy to mitochondrial and endoplasmic proteins, controlled passage of large proteins across the nuclear envelope should require (i) a signal for nuclear migration within the protein itself and (ii) a mechanism at the nucleus to respond to the signal. There is evidence suggesting that nuclear envelope proteins, with M_R60,000 and 76,000, respectively, interact with nuclear localization signals of GR [50].

By experiments using deletion mutants and fusion proteins, Picard and Yamamoto found that GR contains two independent nuclear localization signals [10]. The first signal, NL1, is 50% homologous to the SV40 large T antigen nuclear localization sequence and located just at the C-terminal side of the DNA-binding domain (the "hinge"-region). The signal is functionally repressed when the steroid binding domain is present, but becomes constitutively active when this GR-domain is truncated. Nearly identical sequences are found in GR, PR and in androgen and mineralocorticoid receptors. In contrast, sequences in this region of the ER and 1,25-dihydroxy-vitamin D3 and thyroid hormone receptors do not exhibit strong homology to the T-antigen NLS [51]. The second signal, NL2, lies within the steroid binding domain and has not been separated from hormonal control. The nuclear translocation of GR is reported to be fast with a $T_{1/2}$ of 1–5 min at $+37^{\circ}$C [10].

GR-RECYCLING

The fate of GR after having exerted its gene regulatory effect(s) is unclear. Theoretically, GR may be degraded or reutilized. $T_{1/2}$ of the GR-mRNA is 4.5 h and is unaffected by hormone [52]. $T_{1/2}$ of the GR-

Table 2. Classification and composition of the three main cytoskeletal networks (Adapted from Alberts et al., 1989 [56])

Cytoskeletal network	Polymer subunit	Ø	M_R
Microtubules (MTs)	Tubulin (α- and β-isoforms)	25 nm	55,000
Intermediate filaments (IFs)	Several different IF-proteins	8–11 nm	40,000–210,000
Microfilaments (MFs)	Actin	5–7 nm	42,000

protein in the absence of hormone is 20–25 h, in the presence of hormone 9–11 h [52, 53]. Glucocorticoid hormones have dissociation half-times off GR of 2 min (cortisol, corticosterone) and 10–30 min (dexamethasone, triamcinolone acetonide), respectively [54]. There is evidence supporting a recycling mechanism of GR: activated nuclear GR is recycled back to the cytoplasm where it is deactivated [54, 55] followed by a net synthesis of GR [55]. Evidence has also been presented that e.g. PR is recycling between the nucleus and cytoplasm (see above).

INTERACTION BETWEEN GR AND THE CYTOSKELETON

There are three main cytoskeletal networks in mammalian cells, divided according to the diameter (Ø) of the skeletal "fibers" [56]. They are all polymers of different protein subunits (see Table 2).

Several observations suggest that GR may be linked to the microtubule (MT) part of the cytoskeleton (see below) and there is evidence that centrioles can bind steroid hormones such as 17β-estradiol, progesterone and testosterone [57]. There are also reports that glucocorticoids and GR may act through the microfilament (actin) system. GR binds to actin filaments through hsp90 [58] and treatment of cells with glucocorticoids stabilizes actin networks [59, 60]. It is thus conceivable that glucocorticoid hormones transduce some effect(s) to cells through an interaction between GR and the intracellular cytoskeleton.

CHARACTERISTICS OF MICROTUBULES

Microtubules (MTs) are ubiquitously distributed throughout the animal and plant kingdoms. They are hollow, unbranched, tubular cellular organelles of variable length and with an outer diameter of about 25 nm and are found in all nucleated eukaryotic, but not procaryotic, cells [61, 62]. The MT-cylinder contains a central core with unknown composition or function [62]. The walls of cytoplasmic MTs are composed of 13 subunits (protofilaments), which are aligned parallel to the long axis and folded into a cylinder. Each protofilament is built up by polymerized heterodimers of α- and β-tubulin.

The MT-structure is similar in cytoplasmic MTs, mitotic apparatus MTs, centriolar/basal body MTs and ciliar/flagellar MTs. In interphase cells, associations have been observed between cytoplasmic MTs and most intracellular organelles and membranes [62]. Such interactions may be direct links between the tubulin polymer and the respective organelle or indirect links via microtubule associated proteins (MAPs, see below).

CHARACTERISTICS OF TUBULIN

The basic subunit of MTs is tubulin, a 6S heterodimer of two globular polypeptide chains (α- and β-tubulin) with an M_R of 100 kDa, each monomer having an M_R of 50 kDa [63]. Tubulin exhibits very strong species homology through evolution: antibodies against sea urchin egg tubulin cross-react with tubulin from most species and tissues, including humans [62]. Both human α- and β-tubulin have been cloned and deduced to consist of 452 and 444 amino acids, respectively. In humans, each of the genes encoding α- and β-tubulins, constitutes a large multigene family of about 15–20 members [63, 64] only a portion of which represents functional isoforms. The α- and β-tubulins exhibit around 50% DNA sequence homology, suggesting a common ancestral gene.

Tubulin constitutes one of the most abundant cellular proteins and comprises 2–3% of total cellular protein. Only 40% of the intracellular tubulin pool is polymerized during interphase [65]. It is not known how the pool of soluble tubulin α–β-dimers is distributed throughout the cell and/or whether it is compartmentalized. The MT-polymer contains a number of distinct binding sites, e.g. for various MAPs (MAP-1B, MAP-2, tau), nucleotides (GTP, cAMP), Ca^{2+} and drugs (colchicine, vinblastine, actinomycin D, taxol, neuroleptic drugs) [66].

MT-FORMATION

In the cell, MT-assembly is unidirectional originating from discrete foci known as MTOCs (microtubule organizing center), where MTs are nucleated proceeding towards the plasma membrane [67]. Mammalian cells contain several MTOCs, e.g. the centrosome, serving as a nucleation site for the interphase cytoplasmic MTs, and kinetochores, which nucleate MTs during cell division. Tubulin polymerization occurs from one or several organizing centers towards the cell periphery. Drug-induced net depolymerization, on the other hand, starts in the periphery and progresses inwards. Exposure of cells to temperatures $< +10$°C depolymerizes most cytoplasmic MTs in less than 30 min [62].

CHARACTERISTICS OF MICROTUBULE ASSOCIATED PROTEINS (MAPS)

MAPs may be classified into structural MAPs (e.g. MAP-1, MAP-2, tau, hsp70), translocator MAPs (e.g. kinesin, dynein) and other MAPs (e.g. various enzyme activities, calmodulin, ankyrin) [66, 68–71].

The following criteria have been used to define MAPs [72]: (1) they induce assembly of purified tubulin *in vitro* in the presence of GTP and Mg^{2+}; (2) they co-polymerize with purified tubulin during repeated cycles of temperature-induced polymerization/

depolymerization maintaining a constant stoichiometric relationship with tubulin; and (3) they co-localize with cellular MTs. The assembly-induction is now believed to constitute an *in vitro* phenomenon for many MAPs and is thus not an obligatory criterion. More emphasis is focused on structural interaction with tubulin. The term "MT-binding proteins" would be a more accurate general designation, considering that some of these proteins seem to interact only transiently with MT [66].

ASSOCIATION BETWEEN MAPS AND MTS

Structural MAPs and translocator MAPs interact with different parts of the tubulin molecule [71, 73]. MAP-2 and the tau-proteins bind to the MT-polymer through a cationic MT-binding motif characterized by 3–4 highly conserved repeats of 18 amino acids [74]. MAP-1 has a different repeating motif responsible for MT-binding [69]. There is often a characteristic molar ratio for MTs and MAPs, suggesting a fixed spacing between the MAPs and the tubulin heterodimers along the MT backbone. This is in line with ultrastructural observations indicating a regular interval between various MT-extensions. The average periodicity for both MAP-2 and tau is around 100 nm [75] corresponding to one MAP-2 per 14 tubulin dimers and one tau per 17 tubulin dimers [75]. In the living cell, MTs are probably totally saturated with MAPs [76]. Although each tubulin monomer has a capacity to bind e.g. MAP-2 and tau-proteins, the actual interaction is more infrequent. This may be due to e.g. steric hindrance.

ASSOCIATION BETWEEN TUBULIN/MAPS AND DNA

Purified tubulin alone does not bind to DNA, however, tubulin in the presence of MAPs as well as MAPs alone strongly bind to DNA *in vitro* [77]. The tubulin/MAP mixture binds preferentially to satellite DNA-sequences in the eukaryotic genome. Such sequences are usually not transcribed and are located in the chromatin associated with the centromeric regions of the chromosomes.

CHARACTERISTICS OF THE MITOTIC SPINDLE

The mitotic apparatus is composed of MTs organized as a bipolar spindle. There is biochemical and morphological evidence that the mitotic spindle, besides tubulin, consists of a number of different proteins, such as various MAPs (MAP-1 [78], tau [79], kinesin [80], dynein [81], heat shock proteins (hsp90 [82], hsp70 [83]), ankyrin [68], calmodulin [84], various enzymes [85–87], myosin [88], actin [89], proteasomes [90] and GR [91, 92]. Some of these components are also associated with the cytoplasmic MTs.

CHARACTERISTICS OF CENTRIOLES

Centrioles have been observed in three distinct locations within the cell: (i) in the interphase centrosome; (ii) at the mitotic spindle poles; and (iii) in basal bodies just beneath cilia and flagella. The centrosome of interphase animal cells is localized above or at the edge of the nucleus next to the nuclear envelope [93] and typically consists of a pair of orthogonally arranged centrioles surrounded by an osmiophilic matter called the pericentriolar material comprising the centrosomal MTOC. In some cells, the pericentriolar material is distributed primarily around the older of the two centrioles. The typical centriole is a small, hollow cylinder, open at both ends unless it is ciliated [94].

The centriole is mostly composed of the MT-protein tubulin, but also other proteins, e.g. MAP-1, calmodulin and actin [95]. Furthermore, various enzyme activities have been found to be associated with centrioles, i.e. a protein kinase [96] and mechanochemical ATPases such as kinesin [80] and dynein [81]. Some reports indicate the presence of nucleic acids [94, 97], however, no direct evidence is available and this issue is highly controversial.

SUBCELLULAR LOCALIZATION OF GR

Three different types of methods have commonly been used to localize GR in cells.

Cell fractionation

Intact cells, with or without treatment with radiolabelled hormones, are ruptured by any of a number of different procedures, e.g. piston-homogenization, ultrasonic sound (sonication), shearing (e.g. by a Polytrone®) or detergent-induced cell lysis. The homogenate is then ultracentrifuged at $\approx 100{,}000\,g$ to obtain a cytosolic and a nuclear preparation, sometimes with intermediate centrifugation steps. The various biochemical fractions are analyzed, e.g. by detection of a radiolabel or by immunochemistry.

A major drawback of all assays performed on tissue extracts lies in their inability to provide information about inter- and intra-cellular distributions of a certain component. It is often unclear whether various biochemical fractions really represent specific cellular compartments *in vivo*, or if there is leakage of water

Table 3. Summary of previous results of immunolocalization of GR including effect of ligand

GR in both cytoplasm and nucleus, in various proportions
Effect of added ligand:
1. No effect [43, 44]
2. Partial translocation of GR to the nucleus [106, 109, 111]
3. Complete translocation of GR to the nucleus [10]
GR only in the nucleus [45, 46]
Effect of added ligand:
No effect of ligand has been reported in studies that claim solely nuclear GR-localization

and/or molecules between the compartments during sample preparation. Even though the interaction between ligand–receptor on the one hand and receptor–acceptor sites on the other is strong, it is non-covalent and thus, both the steroid and its receptor are subject to possible displacement during experimental manipulation of receptor preparations.

The "cytosol" has unfortunately often been referred to or thought of as representing the "cytoplasm". Similarly, pellet fractions have often been referred to as "nuclear fractions", without realizing that several components of the cytoplasm will probably always accompany nuclear preparations: the rough endoplasmic reticulum is e.g. continuous with the outer nuclear lamina [56], and several components of the cytoskeleton, i.e. microtubules, are also closely associated with the nucleus [62]. It has been suggested that lysosomal macromolecules, e.g. estrogen receptor (ER) like proteins, may be extruded from fragile lysosomes during vigorous homogenization in hypoosmotic media and thus may contaminate the "cytosol" supposed to contain only "soluble" proteins [98]. Regarding ER it has been claimed that the amount of apparent nuclear receptor varies greatly depending upon the procedure used to prepare the nuclear and cytosolic fractions [37]. However, many such investigations of GR-distribution have been interpreted as providing support for GR operating through "two-step model of steroid hormone action" (see below).

Another fractionation technique is enucleation, accomplished by centrifugation of intact cells in the presence or absence of cytochalasin B followed by detection of the receptor in the resulting nucleoplasts and cytoplasts [99]. However, here also, cytoplasmic structures connected to the nucleus may contaminate the "nuclear" fraction.

Cellular autoradiography

Intact cells are incubated with radiolabelled hormone. After appropriate washes, the cells are exposed to a photographic film and examined by the microscope. There are several recognized problems with this localization technique: (i) the added hormones may alter the distribution of receptor molecules after binding to their receptors; (ii) there may be redistribution of hormones during sample preparation for autoradiography; and (iii) long film exposure times are usually required (months to years) in order to obtain strong enough ^{3}H-signals for visualization. Studies using ^{3}H-cortisol, ^{3}H-dihydrotestosterone and ^{3}H-17β-estradiol have shown that there is both nuclear and cytoplasmic localization of ^{3}H-cortisol [100, 101], but mainly nuclear distribution of the receptors for the sex steroids [38, 102].

Immunocytochemistry

This term encompasses immunological localization procedures using antibody-based detection techniques. Both immunocytological and immunohistological studies are included under this heading. Fixed cells are labelled with specific mono- or polyclonal antibodies and detected by labelled secondary antibodies, representing an indirect detection. Direct immuno-detection implies the use of labelled primary antibodies. Monoclonal antibodies against GR became available during the mid 1980s [103–105]. This localization technique has several advantages. (i) High specificity, especially when using several different monoclonal antibodies. (ii) Antibody-based detection constitutes a ligand-independent detection system. This eliminates the problem of putative hormone-induced redistribution of receptors and receptors may be detected, even at low concentrations, in tissues taken from animals or patients with high endogenous hormone concentrations. Furthermore, this method identifies the total immunologically reactive GR present, while hormone-binding techniques only label non-liganded GR. (iii) The technique is rapid, reproducible and allows the detection of several components in the same cells (double or triple staining) by using specific primary and secondary antibodies with different detection systems.

The major disadvantage is that the cells have to be fixed and permeabilized to allow access of the antibodies to the inner parts of the cells. There may be significant intracellular redistribution of substances during sample processing before immunostaining. One way to circumvent this inherent problem is to combine several different fixation/permeabilization techniques using different chemical principles. Taken together, this technique has become the method of choice for localization of cellular proteins. A large number of studies regarding the localization of GR in cells or tissues have been presented. The conflicting evidence regarding immunolocalization of GR are summarized in Table 3.

In many previous immunological studies, GR has been reported to be diffusely distributed in the cytoplasm [43, 44, 106]. GR has, however, also been reported to interact with subcellular organelles or proteins, based both on biochemical and morphological techniques, see Table 4. There is evidence that both non-activated and activated GR binds specifically, strongly and preferentially to histones H3 and H4 and this histone-bound GR may thus represent non-extractable forms of nuclear GR [107, 108]. The

Table 4. Evidence of interactions between GR and cell organelles. The evidence is both biochemical (b) and morphological (m) in nature

Plasma membrane: m, b [32, 135]
Microfilaments: b [58]
Microtubles: m [91, 92, 111], b [112–114]
Ribosomes, both free and membrane bound: m [136]
Endoplasmic reticulum: m [136]
Mitochondria: m [137], b [138]
Nuclear envelope: m [139], b [140]

functional significance of this interaction is unknown, but may be related to the changes in nucleosome conformation that occur during transcription [107, 109]. It is noteworthy that tubulin has also been shown to both localize in intranuclear spots [83] and to interact with histone proteins with a similar predilection for different histones as GR [110].

INTRACELLULAR ORGANIZATION OF GR

As outlined above, there is a confusion in the literature regarding the subcellular distribution of GR in cells in tissues and in culture. We consider it unlikely that a macromolecule like the GR aporeceptor would be freely diffusing in the cytoplasm; it would rather be connected to some intracellular structure(s) in order for the cell to transduce and regulate the receptor mediated function(s) adequately.

There are several indications in support of such an organized intracellular distribution of GR: (i) immunocytological studies have shown that GR colocalizes with tubulin during the whole cell cycle in cultured mammalian cells, with or without treatment with glucocorticoid hormones [91, 92, 111]; (ii) tubulin copurifies with liganded GR from rat liver [91]; (iii) activated GR in L-cell cytosol is converted from soluble to particulate form under conditions that favor MT polymerization [112–114]. The particulate material contains several cytoskeletal components including tubulin, actin and vimentin. The C-terminal half of the receptor was necessary and sufficient for this association of GR with the cytoskeletal complex and DNA-binding activity was not required [114].

Recently, it has been reported that another member of this receptor family, the vitamin D receptor, interacts transiently with MTs during a few minutes after ligand binding [115].

COMMENTS ON PREVIOUS LITERATURE REPORTS

There are several possible explanations for the conflicting literature reports regarding GR-distribution.

Conceptual

The biochemically defined "cytosolic" GR (i.e. water soluble supernatant after high speed centrifugation) on the one hand and the morphologically defined "cytoplasmic" GR on the other have been compared as if "cytosol" and "cytoplasm" were synonymous. The fact that GR is recovered in the cytosol *in vitro* does not necessarily mean that GR is a freely diffusing, water soluble macromolecule *in vivo*. Furthermore, the observed hormone induced change in GR-distribution *in vitro*, i.e. cytosolic in the absence and bound to the "nuclear pellet" in the presence of glucocorticoids, is not necessarily equivalent to a hormone induced compartment shift *in vivo*.

Methodological

Results from immunocytological and immunohistological studies, as well as results from various fixation/permeabilization techniques used in either cytological or histological studies have been compared directly, as if the various ways of processing a tissue- or cell-sample before localization analysis might not affect the apparent subcellular localization of GR. On the contrary, various processing techniques may influence both the actual intracellular distribution of antigens, as well as allowing detection of certain, but not other, antigens, due to variations in accessibility of antigens and/or antibodies.

Biological

It is conceivable that different cell- and tissue-types may present differences regarding the precise intracellular distribution and function of GR, depending on differences in e.g. germinal layer origin, developmental stage, degree of cellular differentiation and phase of the cell cycle. Morphological experiments constitute a necessary complement to biochemical studies for providing an understanding of the function of steroid receptors.

COMMENTS ON METHODOLOGY IN OUR OWN LOCALIZATION STUDIES

Cell Types

We have studied a number of different cell types, both primary cultures and established cell lines, representing different germinal layers and mammalian species/organs [47]. We chose to concentrate the studies mainly on fibroblasts because these cells: (i) are target cells for endogenous and exogenous glucocorticoid action; (ii) are large cells which make them well suited for subcellular localization studies; (iii) exhibit a characteristic and stable phenotype, easily visually determined in the microscope; and (iv) require relatively simple culture conditions.

Table 5. Subcellular distribution of GR in cultured cells

General distribution	n + c
Cytoplasmic pattern	fibrillar or diffuse
Centrosome (MTOC)	+ +
Granular pattern along fibrils	+
Nuclear pattern	diffuse and granular
Nuclear envelope	(+)
Plasma membrane (intermittent)	+
Vesicle membrane	+
Mitotic apparatus (all mitotic stages)	+ +, especially centriolar regions
Cellular protrusions induced by treatment with	
MT-depolymerizing drugs	+
Vinblastine induced paracrystals	+

+, present; n, nucleus; c, cytoplasm; MTOC, microtubular organizing center [47, 91].

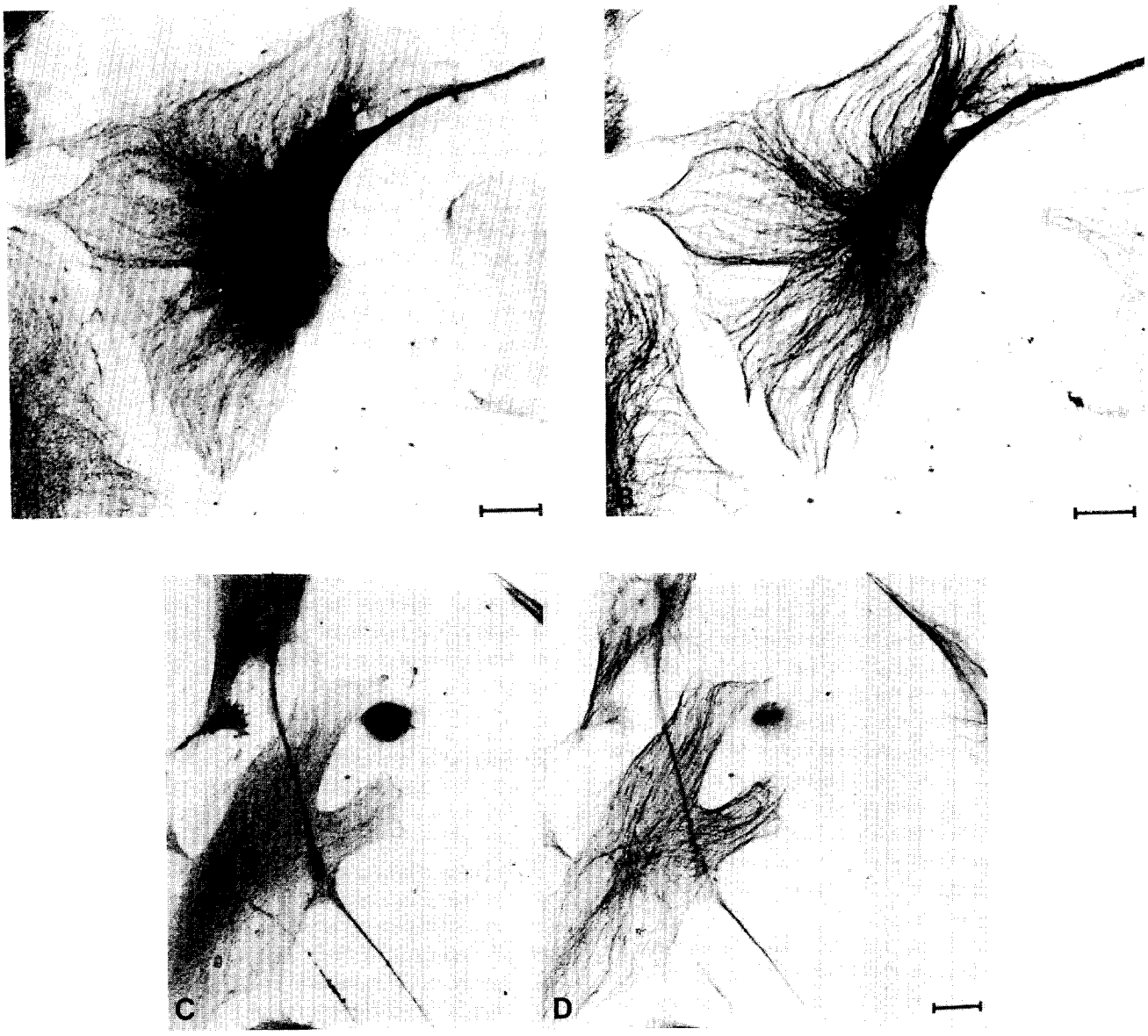

Fig. 2. Double staining of GR and tubulin. A, B, C, D: CLSM-produced transversal, 1 μm thin optical sections through two well spread human gingival fibroblasts (A/B and C/D) double stained for GR (A, C) and tubulin (B, D) using specific monoclonal antibodies and indirect immunofluorescence detection. GR is distributed both in the nucleus and the cytoplasm. There is much more GR than tubulin in the nucleus. Cytoplasmic GR is colocalized with microtubules. GR is distributed within the mitotic spindle apparatus (C; metaphase), where it is colocalized with tubulin (D). Bar corresponds to 20 μm (A, B) and 40 μm (C, D).

In order to achieve as "normal" a situation as possible, we focused on human, primary culture fibroblasts derived from explants of gingival mucosa. These cells exhibit a typical morphological fibroblast phenotype in the whole cell population in a monolayer, they remain stable for at least 30 subcultures and are generally easy to handle during cell culture and splitting.

Fixation/Permeabilization

We tried a number of different fixation/permeabilization techniques with principally different chemical mechanisms (crosslinking or precipitating fixation). We focused on a comparison between two techniques. (1) Cross-linking fixation: typically, we used 4% formaldehyde at +4°C, pH 7.4 for 10 min followed by 0.1% (v/v) Triton X-100® for 30 min. Formaldehyde at this concentration, pH and incubation time allows rapid crosslinking. At 5% (w/v) concentration, formaldehyde reacts preferentially with the ε-amino groups of the lysines, forming polymethylether crosslinks with imino-acetals at their reactive sites [116]. (2) Precipitating fixation: typically we used methanol at −20°C for 10 min.

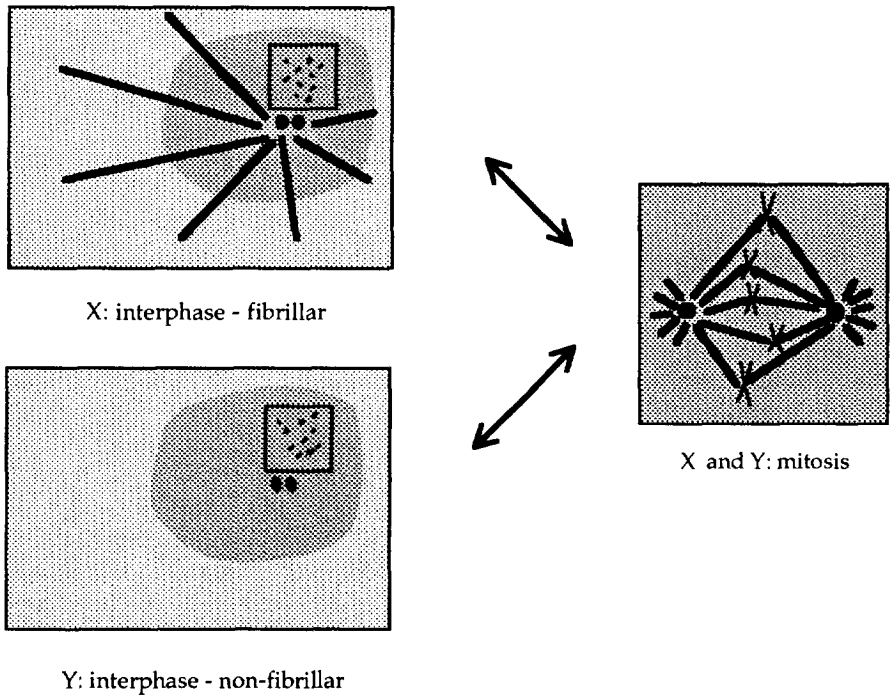

Fig. 3. Schematic drawing of differences in GR-distribution in mammalian cells. Different cell types, x and y indicated in the figure, exhibit a similar GR-pattern during mitosis, but different pattern during interphase, apart from centriolar and granular nuclear staining. GR is indicated with black and dark grey color. The nucleus is represented by a square.

It is important to keep the various staining conditions constant when comparing two methods. It has been reported that when the pH was dropped below 5, even for short periods of time, the staining of the glucocorticoid receptor became nuclear [106]. Therefore, our experiments were all performed at pH 7.35 in PBS-buffer [91].

Specificity

Ligand binding specificity

Ligand binding analysis *in vivo* and *in vitro* showed that the fibroblasts contained specific, saturable and high affinity GR-binding. Whole cell binding assays followed by Scatchard analysis revealed around 100,000 hormone binding sites/cell. Autoradiography after incubating intact monolayer fibroblasts with [^{3}H]dexamethasone mesylate, which covalently binds to GR [117], showed one band of M_R 94,000. Thus, we confirmed many previous investigations showing that human fibroblasts contain one GR-binding species.

Primary antibody specificity

We had access to several monoclonal mouse-anti-rat liver GR antibodies, previously produced in our laboratory and shown to recognize both non-activated and activated GR [104, 118]. These antibodies are highly specific for the glucocorticoid receptor and cross-react well with human GR [47, 91, 119].

Western immunoblot. A good control when using mono- or poly-clonal antibodies is to perform Western immunoblot experiments on crude cellular extracts. If only one band is detected, there is a fair probability that at least soluble or extractable contaminating antigens will not be detected by immunocytochemistry [120].

Four different monoclonal anti-GR antibodies, which recognize four different epitopes within the N-terminal regulatory domain of GR [104], showed the same M_R94,000-band on Western blots and very similar GR-distributions in cells [47]. This strongly indicates that the staining correctly depicts the actual cellular distribution of GR.

Preincubation. Preincubation of the anti-GR antibody "mab7" with purified rat liver GR blocked the immunostaining efficiently in fixed cells [10, 31, 44, 111]. Preincubation of anti-GR antibodies with molar excess of purified bovine brain MT-protein, containing 80% tubulin and 20% MAPs did not appreciably reduce the GR-staining intensity. Preincubation with nonimmune serum from the animal in which the second antibody was raised did not change the staining signal.

Staining procedure specificity

Antibody accessibility. It is necessary to test that the method of fixation/permeabilization used for localization studies in a particular cell-system really allows access of the antibodies to all cellular compartments and that it gives reproducible results. We therefore performed control experiments confirming the well established distributions of components in the cytoplasm (various cytoskeletal networks) and the nucelus (nuclear antigens labelled by polyclonal antibodies in

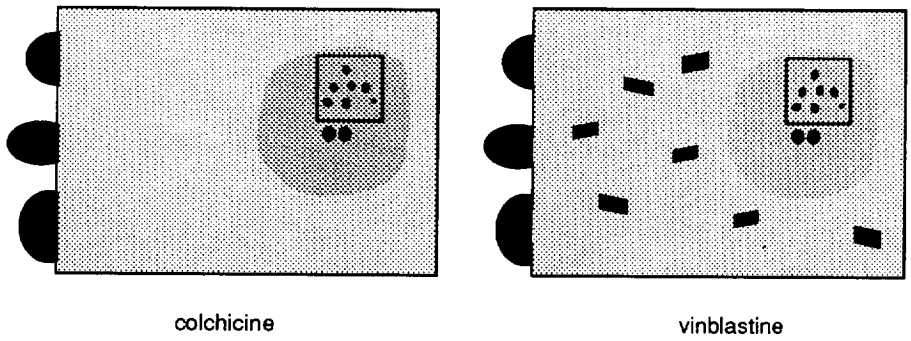

Fig. 4. Schematic drawing of parallel redistribution of GR and tubulin in mammalian cells. Redistribution of GR and tubulin during artificially induced MT-depolymerization. GR is found in cell protrusions and in vinblastine-induced paracrystals and is indicated with black and dark grey color. The nucleus is represented by a square.

pooled sera from patients with autoantibodies to nuclear antigens). Both fixation techniques revealed essentially the same results. These controls showed that the results of immunolocalizing GR would at least not be hampered by problems of antibody accessibility.

Substitution. Substitution of the first or second or both the first and second antibodies with buffer showed esentially no staining signal in any of the detection systems. This demonstrates that the immunosignal is dependent on the primary antibody.

Detergent. Including 0.1% Triton X-100® in antibody incubations and washes did not change the staining signal.

Limiting dilution. Dilution of the first anti-GR antibodies from 300 to 10 μg/ml gave rise to the same relative cellular GR-distribution, i.e. no part of the GR-signal could be selectively diluted away.

Fluorochrome separation

We focused part of the work on assuring reliable fluorochrome separation. Double staining experiments gave the same results as monostainings, both for conventional microscopy and confocal laser scanning microscopy.

Confocal Laser Scanning Microscopy (CLSM)

Compared to conventional transmission light microscopy, CLSM offers several advantages: (1) CLSM provides thin optical (single or serial) sections of the specimen, avoiding the risk of projection artifacts and also presenting all components in the particular focal plane in focus, regardless of size of the component (e.g. interphase and mitotic cells); (2) CLSM provides better resolution than conventional microscopy, especially along the optical axis, but also laterally; (3) CLSM presents data in a digitalized form which directly allows various kinds of image analysis such as subtraction of one image from another one or quantification, such as measurement of fluorescence intensities in various compartments or size of cells or individual compartments; (4) CLSM allows for two- or three-dimensional reconstruction of cells from serial sectioning data; (5) CLSM provides laser beam excitation of the specimen with a separate monochromatic wavelength for each fluorochrome, compared to a wavelength excitation interval in conventional microscopy. This may constitute an advantage in doublestaining experiments.

Using CLSM we quantified the immunoreactivity of GR in the cytoplasm and cell nucleus ± hormone treatment after different fixations on thin optical fibroblast sections and analysed the results statistically. This photometric quantification of GR represents the first attempt to actually measure GR immunoreactivity in a larger number of cells. This strongly reduces the bias regarding both the selection of cell-fields of view for presentation as well as the description of the visual analysis of GR-distribution. For a detailed description of the CLSM-photometry process see [47]. A summary of our results regarding GR-distribution is presented in Table 5 and in Figs 2–4.

LOCALIZATION OF GR DURING THE CELL CYCLE

Interphase

General. In all mammalian cell types examined (i.e. human primary culture fibroblasts from gingiva or skin, human isolated thymocytes and peripheral blood lymphocytes, mouse spleen lymphocytes and several cell lines, e.g. mouse 3T3, mouse L929, human HeLa, mouse MCF-7 and rat HTC), GR was distributed in both the cytoplasm and cell nucleus. Photometry on optical sections of human fibroblasts revealed that $\approx$10–12% of the whole cell GR was localized in the nucleus during control culture conditions [47]. This relation was independent of crosslinking or precipitating fixation. Regarding GR, the average GR-staining intensity was $\approx 2.5\times$ higher after crosslinking than precipitating fixation, presumably due to different degrees of fixation-induced extraction of GR. Similar results have been reported when these two types of fixations were compared regarding the immunostaining intensity for nuclear phosphoprotein p105 [121] and SV40 large T-antigen [122].

Nucleus. GR was found to display a diffuse distribution in most interphase nuclei, with an additional granular appearance in a fraction of the nuclei. GR was not present in the nucleoli.

Cytoplasm. In the cytoplasm, GR was found to exhibit a fibrillar or non-fibrillar staining pattern depending on cell type. In some cell types, e.g. human fibroblasts of different origin and mouse 3T3-cells, GR exhibited a fibrillar pattern which, in double-staining experiments, colocalized well with tubulin. GR was distributed in a granular pattern along individual fibrils and there was a predilection of GR for a subset of MTs. Subtraction analysis by CLSM further substantiated the close association between GR and tubulin. GR was also found in a more diffuse pattern in the perinuclear area in many cells. In other cell-types, e.g. rat liver HTC-cells and L-cells, the cytoplasmic GR was predominantly diffusely distributed in the interphase cell.

Plasma membrane. GR also stained parts of the plasma membrane, including vesicle (pinocytotic vacuoles?, lysosomes?) membranes of various sizes, often located along the leading edge of the cells.

Cell division

GR was located in the mitotic spindle apparatus, both in the pericentriolar area at the spindle poles and along the spindle MTs: kinetochore, astral and possibly also polar MTs. There was also a diffuse GR-staining outside the mitotic spindle throughout the mitotic cell. Double stainings showed that GR colocalized well with mitotic MTs during all stages of mitosis. Possibly, GR distributed in a larger zone than tubulin around the spindle poles.

HETEROGENEITY

We observed a strong inter- and intra-cellular GR-heterogeneity. Such heterogeneity has previously been described for several steroid hormone receptors, e.g. GR [43, 106, 123], ER [57, 124] and PR [120]. The heterogeneity concerns both GR-localization and GR-intensity and may represent both various cell cycle phases or genetic heterogeneity among the cells. This could probably serve as one explanation for the sometimes reported lack of good correlation between glucocorticoid dose, GR-quantity and cellular response.

DISTRIBUTION OF GR AND hsp90 AFTER DEPOLYMERIZATION OF MTS

Drug-induced depolymerization

10 μM colchicine induced an almost complete depolymerization of MTs within 1 h. Both α- and β-tubulin were distributed diffusely over the whole cell, leaving only occasional MTs intact. Both tubulin isoforms also localized in newly formed plasma membrane processes, known to contain cell organelles such as lysosomes, ribosomes and mitochondria [125]. Simultaneously, GR was reorganized in a very similar manner, indicating that GR is associated with individual tubulin dimers. The nuclear GR-staining remained unchanged after MT-depolymerization and GR was not observed in occasional intact MTs.

Depolymerization using 10 μM vinblastine or 10 μM nocodazole showed essentially the same effect as colchicine, but vinblastine induced GR-containing paracrystals as well [91, 92]. Treatment with 10 μM cytochalasin B for 1–2 h induced a strong arborization of the cells. Staining for GR showed a strong resemblance to the tubulin pattern, even though the individual MTs were not easily discernible after the strong morphological derangement. After cytochalasin B treatment, the actin staining pattern differed from that of GR and tubulin.

Cold-induced depolymerization

Exposure of cultured fibroblasts to +4°C for 2.5 h induced an almost complete depolymerization of cytoplasmic MTs with a parallel change from fibrillar to mainly diffuse GR-staining. However, the typical features of drug-induced MT-depolymerization and formation of new plasma membrane processes containing GR and tubulin, were not observed after cold-induced depolymerization. One possible explanation for this phenomenon may be that cold treatment reduces, whereas MT-inhibitors increase cytoplasmic mass flow [125].

TREATMENT WITH GLUCOCORTICOID HORMONES

We have never observed any distinct compartment shift of GR from cytoplasm to the cell nucleus after glucocorticoid hormone treatment in any of the mammalian cells tested, regardless of the type of fixation/permeabilization, cell culture conditions, or glucocorticoid administration. The rather large GR-heterogeneity, however, made it difficult to visually determine a possible, small hormone-induced change in GR-distribution.

We therefore quantified the photometric GR-intensities in the nucleus and cytoplasm on thin, CLSM-produced optical sections of human fibroblasts monostained for GR, using two standard fixations, with or without treatment with glucocorticoid [47]. This analysis revealed a hormone-induced significant increase in GR-immunoreactivity in both the nucleus and cytoplasm compared to controls. Since these effects were only detected after precipitating but not after crosslinking fixation, the results were interpreted as evidence in support of a hormone-induced increase in GR-affinity to existing docking sites in both nucleus and cytoplasm, without any sign of intracellular compartment shift. This change in GR-affinity may give rise to a visual impression of a partial nuclear translocation in some cells.

Similar results were obtained regardless of whether the GR-intensities in the nucleus and cytoplasm or the quotient between them were expressed per whole cell or per pixel, thereby excluding the possible influence by hormone induced change in the size of the whole cell or of the individual compartment(s). Similar results of GR-localization after hormone treatment were obtained after preceding drug-induced disassembly of MTs as well as after inhibition of energy synthesis by drugs such as oligomycin or Na-azide. However, these observations were not analysed by photometry.

TREATMENT WITH HEAT STRESS

Heat shock did not appreciably affect the cellular distribution of GR on visual analysis, but induced a reversible nuclear translocation of hsp90 [126]. This finding contrasts with a recent report claiming that heat shock induces a nuclear translocation of GR *in vitro*, i.e. GR is detected in the cytosol before and in the pellet fraction after heat shock treatment [127]. The discrepancy may be explained by a similar reasoning as applied to the glucocorticoid induced nuclear translocation *in vitro* described above.

ATTEMPTS TO ASSESS A FUNCTIONAL SIGNIFICANCE OF A GR–MT INTERACTION

During the course of this study, we have tried a number of *in vivo* assays to analyse the putative functional significance of the observed interaction between GR and MTs. We hypothesized that glucocorticoid-regulated function(s) may depend on an intact interaction between GR and MTs. If this was the case, glucocorticoids would not be able to elicit their effects after MT-depolymerization. This hypothesis has been tested for the following glucocorticoid test-systems: (i) induction of tyrosine aminotransferase (TAT) enzyme activity in rat liver HTC-cells; (ii) induction of alkaline phosphatase (ALP) enzyme activity in human fibroblasts; (iii) inhibition of the release of [^{3}H]arachidonic acid from human fibroblasts preincubated with [^{3}H]arachidonic acid; and (iv) inhibition of uptake of [^{3}H]thymidine in human fibroblasts. For several tested assays, we observed that the MT-drugs in the tested doses (1–10 μM) by themselves affected the tested variable to a large degree, sometimes more than the glucocorticoids alone. None of these *in vivo* assays revealed any consistent difference between glucocorticoid-induced effects with intact or depolymerized MTs.

There are several possible reasons for this. (a) The tested parameters rely on GR-action at the nuclear genome and such effects may not depend on intact MTs. It is possible that other glucocorticoid functions, that do not involve nuclear genomic regulation, are MT-dependent. (b) Depolymerization of MTs using 10 μM colchicine or vinblastine leaves a small number of assembled MTs that are resistant to drug-treatment. Even though we have not observed any GR-staining in these drug-resistant MTs, this residual MT-population may be sufficient for transduction of the glucocorticoid/GR-effect(s). (c) GR and MTs are not colocalized in all cell types. (d) The interaction between GR and MTs is coincidental or non-functional.

SPECULATIONS ON FUNCTIONAL SIGNIFICANCE OF A GR–MT INTERACTION

Based on some of the observations described in this review, there are several hypothetical possibilities as to how the effect of glucocorticoids may be transduced to cells, involving the well described effects of GR in the nucleus alone as well as extranuclear GR.

Extranuclear GR

Glucocorticoid effects may be transduced through the GR indirectly or directly on site in the cytoplasm, without involving the nuclear genome.

Indirect effects

Glucocorticoids/GR may operate at the mitochondrial genome or at the putative centrosomal genome.

Direct effects

MTs. Glucocorticoids/GR may exert direct, extragenomic effects on MTs: (a) glucocorticoids may regulate cytoplasmic and/or mitotic MTs directly by GR being a MAP or by GR binding to a MAP; (b) MTs may regulate cytoplasmic and/or mitotic GR in some as yet unidentified way(s).

Centrosomes. Several steroid hormones are reported to be able to bind to the centrosome [57] possibly because the corresponding receptors are present in this organelle, in a similar way as GR described here. This may constitute a mechanism for steroids to directly affect the centriolar cycle and thereby e.g. cell growth.

Lysosomal membranes. Glucocorticoids have been reported to stabilize lysosomal membranes by a mechanistically unknown process [128]. It is possible that this effect is mediated directly in the lysosomal membrane via membrane-bound GR.

There is evidence that after glucocorticoid-induced treatment *in vitro*, only 35–60% of the dissociated GR is activated and thus exhibits DNA-binding capacity [129]. The remaining 40–65% non-DNA binding GR-pool has a more acid pI than the DNA-binding GR-pool. The authors proposed that these two GR-pools represent different energy states of folding after dissociation of hsp90. The function of the non-DNA binding but dissociated GR is unknown, but may hypothetically be related to some glucocorticoid effect(s) in the cytoplasm.

Nuclear GR

Besides nuclear GR participating in transcriptional regulation of specific target genes, granular nuclear GR

may be localized in small ribonucleoprotein (snRNP) particles. There is evidence from a confocal laser microscopic analysis that overexpressed heterologous GR is localized in a non-random manner in nuclei in a pattern resembling that of snRNPs [130]. Other steroid hormone–receptor complexes, i.e. ER and AR, have been reported to be associated with snRNPs [131]. Such snRNP-particles exhibit a granular distribution pattern in interphase nuclei [132, 133]. Several different snRNPs participate in the processing of newly formed mRNA [134]. Taken together, this might imply that GR is associated with snRNPs and thereby participates in post-transcriptional mRNA maturation.

GR In The Mitotic Spindle

Mitotic spindle GR may be involved in transducing glucocorticoid effect(s) directly to spindle MTs thereby exerting its well documented growth-modulating effects.

CONCLUDING REMARKS

There is evidence that GR is associated with the cytoskeleton, both the microfilament- and MT-networks. Regarding MTs, GR is colocalized with tubulin during mitosis in all and during interphase in some of the investigated mammalian cell types. Besides its well known nuclear distribution, GR seems to be unique among the various proteins in the steroid hormone receptor superfamily in also having a distinct cytoplasmic location, where it associates with several parts of the cytoskeleton as well as with different cytoplasmic organelles. Evidence for an interaction with MTs has also been presented for another member of the steroid receptor superfamily, i.e. the 1,25-dihydroxy-vitamin D receptor [115]. Studies are in progress in our laboratory to further analyse whether the structural GR–MT-interaction is physiologically relevant for cells and tissues.

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