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SHORT REPORT

Nuclear *vs* translocating steroid receptor models and the excluded middle

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Introduction

In response to a review by Gorski *et al.* (1994) which concluded that unoccupied glucocorticoid receptors are nuclear like the receptors for estrogens and progestins, Webster *et al.* (1994) have recently summarized the predominantly-held view that glucocorticoid receptors are two-step translocating receptors. In this model, the unoccupied receptors are located in the cytoplasm, and the occupied receptors actively translocate from that compartment to the nucleus after binding of the ligand and activation of the receptors. This interpretation is based mainly on immunocytochemistry of the glucocorticoid receptor because cell fractionation has been shown to involve extraction artifacts when used for localization of other steroid receptors. Our work using cell enucleation (Welshons *et al.*, 1985) wasn't consistent with a translocation model for the glucocorticoid receptors. We found instead that few ligand-binding glucocorticoid receptors were present in enucleated GH_3 cells (cytoplasts) and we proposed that the unoccupied glucocorticoid receptors were nuclear, as were estrogen receptors, progesterone receptors and the occupied glucocorticoid receptors (Welshons *et al.*, 1984, 1985).

Cell enucleation

Webster *et al.* cited two papers that reported glucocorticoid receptor localization results with cell enucleation (McDonald & Gelehrter, 1981; LaFond *et al.*, 1988) which they indicated instead supported the translocation model. In LaFond *et al.* which was the more detailed of the two for receptor localization, immunocytochemical signal for unoccupied glucocorticoid receptors was observed in both the nucleus and the cytoplasm of intact L-cells and intact GH_3 cells before enucleation. After enucleation, glucocorticoid receptor immunoreactivity was observed in L-cell cytoplasts and nucleoplasts. However, very nearly all of the glucocorticoid receptor immunoreactivity in GH_3 cells was lost upon enucleation, so that it is not clear what can be inferred from their enucleated GH_3 preps.

LaFond *et al.* (1988) specifically reported that they could not show any ligand-induced translocation of L-cell glucocorticoid receptor immunostaining from the cytoplasm to the nucleus, and unconvincing translocation is often a feature to current glucocorticoid receptor immunocytochemistry studies. Therefore, LaFond *et al.* might well be cited as evidence for a cytoplasmic, non-translocating receptor signal in addition to nuclear unoccupied receptors; however, it is not straightforward evidence for the translocating glucocorticoid receptor model. In the second study cited, McDonald and Gelehrter used cell enucleation to study glucocorticoid regulation of amino acid transport in HTC cells and secondarily measured a 'reduced' number of glucocorticoid receptors in their cytoplasts (McDonald & Gelehrter, 1981). From their results one might infer that at least 60–70% of the unoccupied glucocorticoid receptors were nuclear. However, only limited results were reported with respect to controls for receptor localization questions, and there was no indication, for example, whether the population of receptors they found in the cytoplasts was translocatable in the intact cell upon incubation with ligand.

Our own studies (Welshons *et al.*, 1985) were primarily oriented towards steroid receptor localization and were extensively controlled for related questions. We used ligand binding to measure glucocorticoid receptors, where we could show that we were describing the localization of high affinity, ligand-binding receptors and where we could determine accurate receptor numbers for recovery, which was 75% to 100% recovery of glucocorticoid receptors across the experiments; our results that the unoccupied receptors were nuclear therefore applied to most of the glucocorticoid receptors in the cells, not to a remnant fraction. For these reasons and others below, we feel that our experiments yielded particularly reliable results, at least for GH_3 cells.

Artifactual relocation of unoccupied receptors to the nucleus was cited by Webster *et al.* (1994) as a possible factor which may have affected our findings that the unoccupied glucocorticoid receptors were nuclear as assessed by cell enucleation. But relocation is a factor both ways, and relocation has already been documented in the other direction. For estrogen and progesterone receptors, the unoccupied forms of which are accepted to be nuclear, Milgrom's group has reported in work performed with several cell types, that incubation of cells with inhibitors of ATP synthesis can lead to the (artifactual) accumulation of the unoccupied receptors in the cytoplasm of the cell (Guiochon-Mantel *et al.*, 1991); occupied wild type receptors were not affected. Both of the enucleation experiments cited above by Webster *et al.* used cytochalasin B, which blocks glucose uptake and lowers intracellular ATP, therefore, low-ATP-induced relocation of nuclear glucocorticoid receptors to the cytoplasm could conceivably have been a factor in those experiments. We enucleated GH_3 cells in our experiments *without* cytochalasin B (Welshons *et al.*, 1985).

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An additional relocation issue, that was not raised by Webster *et al.*, is the report that the pH indicator phenol red interfered with glucocorticoid receptor localization studies in COS-7 cells (Picard & Yamamoto, 1987). If confirmed, this potential activity would add to the estrogenic and other activities that have been reported for contaminants of this indicator dye (Berthois *et al.*, 1986; Grady *et al.*, 1991). Although the effect of phenol red on glucocorticoid receptor distribution was not assessed in our studies, we found that there was too little of the indicator present to produce estrogenic effects in GH_3 cells under our culture conditions (discussed in Welshons *et al.*, 1988), nor did it interfere with the localization of estrogen receptors in MCF-7 cells (Welshons *et al.*, 1988).

As Webster *et al.* (1994) cited, we have recently reported that 15% of the estrogen receptors in enucleated MCF-7 human breast cancer cells were found in the cytoplasts (Welshons *et al.*, 1993a). However, these receptors were not translocatable by ligand (Welshons *et al.*, 1988, 1993a,b), and these receptors may not be part of either steroid receptor model – this is discussed further below. Webster *et al.* indicated that care should be taken in the interpretation of enucleation studies, which is of course true, but the same care should also be applied to the interpretation of immunocytochemistry.

Immunocytochemistry

Gorski *et al.* (1994) commented on the reduced immunostaining of the unoccupied receptors compared to the liganded receptors in glucocorticoid receptor immunocytochemistry (Fuxe *et al.*, 1985; Wikström *et al.*, 1987, and many others) and questioned whether the unoccupied receptors were in fact localized in those studies. Despite some exceptions, most of the published glucocorticoid receptor localization studies display a weak and sometimes patchy distribution of unoccupied glucocorticoid receptor signal in cytoplasm and nucleus, which contracts with the strong, discrete localization of the occupied glucocorticoid receptors in the nuclei of the cells.

The weak immunostaining of unoccupied glucocorticoid receptors also contrasts with the abundant cytoplasmic signal seen in the immunocytochemistry of nuclear localization signal (NLS) mutants of progesterone receptors which are cytoplasmic (Guiochon-Mantel *et al.*, 1989, 1991), or of estrogen and progesterone receptors made cytoplasmic by incubation of cells with ATP inhibitors (Guiochon-Mantel *et al.*, 1991). For the translocatable mutants in those experiments, the cytoplasmic immunocytochemical signal also showed unambiguous translocation out of the cytoplasm and into the nucleus of the cell upon incubation with ligand, while, as noted by Gorski *et al.* (1994), demonstration of ligand-dependent translocation of cytoplasmic glucocorticoid receptor immunoreactivity has been problematic.

Immunocytochemistry is a plastic technique in that for a given antigen, immunostaining is typically adjusted by variation of fixative, detergent, fixation time and antibody to obtain the optimal signal consistent with an accepted positive control or an expected result. Occupied steroid receptors are accepted as nuclear and remain there during homogenization of unfixed cells; therefore adequate fixation of the occupied receptors may not be critical. However, fixation of the unoccupied receptors, adequate to prevent their relocation or extraction, is the crucial question in their localization by immunocytochemistry. If fixation is not sufficient to immobilize the unoccupied glucocorticoid receptors, then they may be to varying degrees relocated or extracted and lost during tissue processing, while the occupied receptors may require little or no fixation to remain nuclear. Webster *et al.* (1994) cite a number of studies in support of the two-step translocation model. However, these studies rely on the interpretation of immunostaining of glucocorticoid receptors, and the evidence that is cited in support of the translocation model would equally well fit with relocation or extraction of nuclear unoccupied glucocorticoid receptors.

These questions would be addressed by the use of firm fixation where relocation or extraction were clearly prevented, and by verification of unoccupied glucocorticoid receptor recovery after fixation and processing through the immunocytochemical technique, preferably by ligand binding where high affinity, low capacity and ligand specificity can be demonstrated and where receptor numbers can be accurately determined. In a study where fixation was by strong cross-linking with glutaraldehyde, which gives proteins the mobility of sand in cement, Brink *et al.* (1992) reported that the unoccupied glucocorticoid receptors were found mostly in the nucleus, although a faint pattern of nontranslocatable staining was also observed in the cytoplasm. In the same study, extraction of the unoccupied receptors was demonstrated when weaker fixation was employed. Webster *et al.* raised several technical questions about the use of glutaraldehyde in this immunocytochemistry, and cited a report (Rossini & Malaguti, 1994) that glutaraldehyde caused translocation of glucocorticoid receptors to the nucleus (which could instead represent reduced extraction from the nucleus). But some immunocytochemistry that did not employ glutaraldehyde fixation has also localized the unliganded glucocorticoid receptors to the nucleus (Martins *et al.*, 1991), and if the results of Brink *et al.* are confirmed, it will be difficult to argue that in the live cell, most of the unoccupied glucocorticoid receptors are anywhere but in the nucleus.

The actual subcellular location of the unoccupied glucocorticoid receptors does not affect such experiments as those where immunocytochemistry has been used to assess steroid receptor activation, but it does affect the relative importance assigned to the unoccupied receptors. If the glucocorticoid receptors that activate transcription are in the cytoplasm when unoccupied, then it implies that the unoccupied receptors are biologically inactive and cannot play a role in transcriptional regulation. If unoccupied receptors are instead nuclear, then unoccupied glucocorticoid receptors may also function by binding to glucocorticoid response elements (GRE's) to regulate transcription, possibly to include negative regulation.

Non-nuclear steroid receptors (SR_{NN})?

In addition, and more to the point here, argument to interpret all cytoplasmic staining as evidence of two-step translocating receptors (even when translocation

of the signal is not clear) excludes other receptor models, the excluded middle as it were. Specifically, are there subpopulations of the steroid receptors that are cytoplasmic but nontranslocatable to the nucleus (SR_{NN}) and therefore would not participate in the genomic response? Most steroid receptor actions clearly involve the regulation of transcription in the nucleus, which is a slow response relative to transmembrane signaling, for example. However, there has been increased interest recently in the possibility of rapid steroid responses by mechanisms which do not directly involve transcription (summarized in Griffing, 1993). These responses have been termed 'nongenomic' and have been proposed to involve a different kind of steroid receptor or a different form of the steroid receptor. These include amphibian neuronal membrane-associated glucocorticoid receptors and a rapid glucocorticoid response to inhibit mating behavior, where both response and receptors differ in ligand specificity from the Type I glucocorticoid receptor and its genomic response (Orchinik *et al.*, 1991); membrane-associated mineralocorticoid receptors and rapid aldosterone effects on ion transport in human mononuclear leukocytes (Wehling *et al.*, 1992; Christ *et al.*, 1993); rapid estrogenic effects of Ca^{++} mobilization in chicken and porcine granulosa cells (Morley *et al.*, 1992), cytoplasmic estrogen receptor immunoreactivity in mammalian neuronal cell processes (Blaustein *et al.*, 1992), and identification of both plasma membrane-associated estrogen receptors and a rapid estrogenic response at approximately 1 min to stimulate prolactin secretion in GH_3/B6 cells (Pappas *et al.*, 1994).

Studies in our lab (Welshons *et al.*, 1993a,b, 1994) which yielded results we did not anticipate, have identified in cytoplasts of MCF-7 human breast cancer cells a subpopulation of estrogen receptors that are non-nuclear (ER_{NN}) and that constitute approximately 15% of the total number of estrogen receptors in intact cells. These receptors appear to be nontranslocating in intact cells, and are nontransforming (nondimerizing) on sucrose gradients, unlike the major form of the estrogen receptors (ER) in the cells. We have observed physical differences between ER and ER_{NN} in terms of aqueous two-phase partitioning, a measure of solution behavior and hydrophobicity/hydrophilicity, and perhaps in a 50% weaker affinity for estradiol by the ER_{NN}. These receptors do not appear to bind well to nonspecific DNA in solution. In this regard the ER_{NN} are similar to a reported alternative goat estrogen receptor form (Karthikeyan & Thampan, 1994). ER and ER_{NN} quantitatively bind both of the anti-estrogen receptor monoclonal antibodies D75 and H222. Therefore ER_{NN} do not appear to be an estrogen-binding protein different from the estrogen receptor. ER_{NN} show the same molecular weight by SDS/western blotting as do ER, approximately 65 kd. Additional characteristics of ER_{NN} such as whether they will form specific interactions with estrogen response elements (ERE's), whether they will bind an extended number of antibodies, and whether their aggregations with estrogen receptor-associated proteins are different remain to be determined.

As a non-nuclear, nontranslocating form of the estrogen receptor, ER_{NN} belong to neither a nuclear model nor a two-step translocating model. At this point in our work we cannot exclude that the ER_{NN} may be synthetic, degradative or modified intermediates, but the potential relation to nongenomic responses is particularly intriguing, and this is testable in enucleated cells, where no nucleus is present and where genomic responses are excluded. The intracellular location of potential SR_{NN} (nontranslocating glucocorticoid receptors in immunocytochemistry, for example) may shed light on possible functions. These intracellular locations might include: the plasma membrane in mediation of ion fluxes or interaction with transmembrane signaling; mRNA in regulation of their stability, and perhaps rough endoplasmic reticulum in regulation of stability of mRNA's for secreted proteins; as well as free ribosomes if related to partially synthesized steroid receptors.

Conclusions

There appears to be one structural estrogen receptor gene. The same protein product of this gene expressed by different cells induces different cell-specific responses. It may also be that a modified form of the protein, or the same receptor protein located in a different place in the cell, may also regulate different kinds of intracellular responses. The presence of non-nuclear, nontranslocating forms of other steroid receptors, including glucocorticoid receptors, should be considered in the interpretation of their immunocytochemistry, and these may contribute to better understanding of potential multiple mechanisms of steroid receptor action.

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