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STEROIDS AND GROWTH PROMOTING FACTORS IN THE REGULATION OF EXPRESSION OF GENES AND GENE NETWORKS

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Summary—Steroid hormones and growth promoting factors regulate the expression of a number of genes involved in cellular growth and differentiation. In many cases, cellular proliferation and differentiation-specific pathways are mutually exclusive. As an example for the mechanism of mutual exclusion, transcription factors responsible for pathway-specific gene expression interact with each other. Steroid hormone receptors inhibit the action of the transcription factor AP-1 (Fos/Jun) and vice versa. This inhibitory interaction is predominantly effected at the level of protein–protein contact without the need for the interfering transcription factor to bind to DNA. In some cases the two pathways function additively or even synergistically resulting in cooperation of the transcription factors in regulation of gene expression. The examples to be discussed in this text document how elaborate and important cross-talks between signal transduction pathways are. This is particularly demonstrated by the fine tuning and reversibility of these processes.

Steroid hormones and growth factors play important roles in development, growth and differentiation. The steroid hormones exert their regulatory function by binding to specific intracellular receptors which in turn interact with defined sequences on regulatable genes to modulate their activity. The growth factors act through membrane bound receptors which in turn stimulate the activity of intracellular proteins including transcription factors such as c-Fos and c-Jun. Growth factors also stimulate transcription resulting in increased accumulation of gene products involved in cell growth. Obviously transition from proliferation to differentiation and the reverse require tight coordinate control mechanisms. The transcription factors for each of both type of programs (growth and differentiation) need to "talk" to each other. Often these programs are mutually exclusive.

Although transcription factors are influenced by various parameters and are parts of complex networks of control proteins, principles of regulation can be derived from the study of single transcription factors such as the glucocorticoid receptor (GR) and Fos/Jun (AP-1), and from the study of simple model reactions. The hormonal control of cellular proliferation and differentiation in human breast tissues is one example of the complex interplay of synergistic and antagonistic actions of steroid hormone and growth factors. Whilst epidermal growth factor (EGF) in the presence of estradiol and progesterone stimulates growth of the mammary gland during pregnancy, it also inhibits premature differentiation induced by glucocorticoids, insulin and prolactin [1–3]. Other examples of such dual function of steroid hormones and EGF are evident in human prostate epithelium, the mouse keratinocyte cell line MK or in rabbit cornea where glucocorticoids oppose EGF-induced growth [4–6], whilst in the rat mammary epithelium, rat submandibular gland and in human amniotic cells, glucocorticoids enhance EGF-induced cell growth, and prostanoid synthesis [8–10].

Against this background, we will present here a regulatory pathway involving a recently discovered cross-talk between differentiation-specific factors and factors involved in cell proliferation. This cross-talk involves the transcription factor AP-1 and members of the family of steroid hormone receptors. We will present mechanisms of antagonistic action of steroid receptors and AP1 and instances in which steroid receptors/AP1 interaction could lead to positive regulation of gene expression.

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REPRESSION OF GROWTH FACTOR-INDUCED GENE EXPRESSION BY STEROID HORMONES

The antagonistic action of steroid hormones on gene expression could occur by either direct interference with transcription or by the induction of a repressor. While there are no direct instances in which the latter mechanism has been identified, there are a number of examples of the former which fall within two mechanistic groups: overlapping binding sites and direct functional interference.

OVERLAPPING BINDING SITES

In this case, binding sites for transcription factors such as AP-1 overlap with steroid hormone receptor binding sites. In the presence of both transcription factors there is competition for the same binding site by the two factors, which leads to mutual inhibition of each other's response. This type of regulatory element occurs in a sequence spanning nucleotides −172 to −150 upstream of the transcriptional start site of the rat α-fetoprotein gene [11]. When linked to a heterologous promoter, this sequence mediates a glucocorticoid response in the presence of GR and hormone or, in the absence of hormone, responds to the activity of the heterodimeric proteins, Jun and Fos that make up AP-1, in transient transfection in CVI or F-9 cells. When plasmids expressing GR, c-*jun* and c-*fos* are transfected together they repress each other's activity mediated by the α-fetoprotein regulatory sequence [11].

Another example of down-regulation of gene expression by GR through the mechanism of overlapping DNA binding sites is exemplified by the regulatory sequences in the mouse proliferin gene in which a glucocorticoid response element (GRE) also overlaps an AP-1 binding site [12, 13]. Unlike the α-fetoprotein gene described above, the proliferin gene does not carry a classical GRE. Although *in vitro*, it binds highly purified GR, it cannot mediate a glucocorticoid response on its own and can only achieve this with the help of c-Jun [13]. This indicates that in some cases an encounter of steroid hormone receptor with a member of the AP-1 complex can lead to positive regulation of gene expression. This is not only observed with GR but also with other steroid hormone receptors as will be discussed below. The synergistic action of GR and c-Jun at the proliferin gene regulatory element is however repressed by the presence of c-Fos [13]. Thus, either the heterodimeric Fos/Jun complex dislodges the GR binding or the GR down-regulates the positive effects of the Fos/Jun complex. That the latter situation is possible is demonstrated by experiments that showed that c-Fos and c-Jun mediate an AP-1 dependent response at the chimeric proliferin gene promoter in F-9 cells and that this response is repressed by GR [13]. Several examples of shared occupancy of AP-1 sites and steroid hormone response elements have also been described. For instance, in the osteocalcin [14, 15] and alkaline phosphatase genes [15], a vitamin D_3 response element (the vitamin D_3 receptor is a member of the steroid hormone receptor family), overlaps an AP-1 binding site. In both cases, the vitamin D_3 response is antagonized by the action of AP-1 and vice versa.

A presumably related mechanism through which steroid hormones negatively regulate gene expression, is defined by the binding of the steroid receptors to DNA sequences known as negative GREs (nGREs) [16, 17]. These elements negatively regulate gene expression when bound by the activated GR and are involved in cell type-specific expression. In the absence of glucocorticoids, nGREs enhance promoter activity presumably through the action of the cell type transcription factor binding to the same sequence to which the GR binds. In the presence of activated GR, expression is repressed possibly through competition of the GR for the regulatory sequence bound by this cell type-specific factor. It is at present not clear which factor(s) bind to nGREs in the absence of hormone. It appears that this mechanism can only work in one direction.

INTERFERENCE BY PROTEIN–PROTEIN INTERACTION

The cross-talk between the two types of transcription factors described above is so important that still another mechanism exists to make it possible. Negative action of steroid hormones on gene expression is also achieved without the need for the steroid receptors to bind to DNA. An example of this type of regulation is illustrated by the down-regulation of expression of the rat prolactin gene [18]. Estrogen induces expression of this gene through *cis*-regulatory elements between positions −1582 and −1568 upstream of the start of transcription, and glucocorticoids repress expression, without an

obvious binding of the GR to the promoter. Interestingly when the estrogen response element was removed from the promoter, expression from this mutated gene construct was repressed by both estrogen and glucocorticoid hormones [18].

The receptors for these steroids inhibit the expression of the rat prolactin gene construct through DNA elements that bind a tissue-specific positive transcription factor called Pit 1 [18]. Deletion analyses of the ER showed that it is not the DNA binding domain (DBD) of this receptor but rather a region inbetween the DBD and the steroid binding domain (the hinge region) that is responsible for this inhibition [18]. Replacement of the hinge region of the ER with that of the GR, resulted in an ER/GR chimeric receptor that also inhibited the expression of the prolactin gene [18]. It is not clear how the hinge domains of the GR and ER repress gene expression. The 63 amino acid hinge sequence in the ER shares only 4.5% homology with the comparable region in the GR. The two regions do not bind DNA but they share structural similarity, with regions rich in basic amino acids and similar hydrophilicity profiles [18]. It is therefore thought that these amino acid sequences are involved in protein–protein interaction of the steroid hormone receptors and other transcriptional factors, possibly Pit 1, that regulate the prolactin gene expression. Physical interaction of these proteins have not yet been demonstrated.

Recently a number of laboratories have reported that GR and AP-1 inhibit each other's effect on gene expression through a mechanism that does not involve competitive DNA binding [19–22]. An AP-1 binding site is not recognized by the GR, as it is a different regulatory sequence. Similarly the Fos/Jun heterodimeric complex does not bind to a GRE. The fact that GR and AP-1 influence each other's activity without the need for the interfering factor to bind to DNA, suggests a mechanism involving interference through protein–protein interaction.

Four different models can be proposed for bringing about such a negative response:

(i) titration of a common positive factor required for AP-1-induced expression by the GR or vice versa;
(ii) protein–protein interaction of AP-1 and GR leading to the destruction of the DNA binding properties of the inducing factor by the interfering factor;
(iii) protein–protein interaction that does not destroy DNA binding but affects the *trans*-activation properties of the inducing factor; and
(iv) modification of the activating factors in response to ligands that activate the interfering factor.

In the first model, experiments on the glucocorticoid repression of AP-1 induced activity at the human collagenase promoter have shown that the repression occurs in the absence of protein synthesis. It has further been observed that the level of AP-1 does not change during the repression, arguing against titration of a factor required for AP-1 synthesis. Mutant GR lacking the amino-terminal sequences that do not *trans*-activate, are nevertheless able to negatively regulate AP-1-induced expression [19, 22]. This indicates that the repression by the GR is not due to the titration of factors required for the positive action of this receptor. A remote possibility exists however that a common preformed auxiliary factor required for both AP-1 and GR response could be titrated by GR and AP-1, respectively. Such a reasoning has so far not been supported by any experimental finding and would therefore not be discussed further. The hormone modification of proteins other than the steroid hormone receptor itself as suggested in model 4 is, so far, only hypothetical. The most likely models are 2 and 3 where GR and AP-1 interfere with each others activity through protein–protein interaction.

Although a number of researchers are seriously engaged in experiments aimed at proving or disproving models 2 and 3, no clear conclusions have so far been reached. Antibodies to c-Fos and c-Jun have been used to precipitate the GR in extracts of cells that express the GR, c-Fos and c-Jun [7] indicating that the GR is in contact with c-Fos and c-Jun. Association of c-Fos and the GR or c-Jun and the GR could be demonstrated by immunoprecipitation of these complexes after *in vitro* synthesis of the proteins [7]. Others could demonstrate the occurrence of such GR/c-Fos or GR/c-Jun complexes only after cross-linking the c-Fos and the GR [22] or of c-Jun and the GR [13, 22]. To investigate whether these interactions destroy DNA binding of the GR or AP-1, *in vitro* gel retardation experiments were performed. These showed

that preincubation of bacterially expressed Jun with GR destroys each other's DNA binding activity [13, 22]. Different results were, however, obtained by *in vivo* footprinting of the binding of AP-1 to the human collagenase gene which is not dislodged by the presence of the activated GR [König *et al.*, unpublished]. Although a possibility exists that the AP-1 bound in the absence of the activated GR could have been replaced by another inactive AP-1 complex, this possibility appears unlikely. *In vitro* studies have shown that the binding of heterodimeric c-Fos/c-Jun to an AP-1 binding site is not affected by the presence of the activated GR [König *et al.*, unpublished]. It is therefore not clear which of the two models (models 2 and 3) could at best explain the mutual inhibitory properties of AP-1 and GR in gene expression.

Deletion analyses of c-Jun have revealed the requirement of the "leucine zipper" region of this protein for the repression of GR-induced expression [19]. In the case of c-Fos, amino acids 40 to 111 are required for repressing the GR response [20]. Studies using different chimeric constructs of the GR have shown that its DNA binding domain (DBD) is necessary for repressing AP-1 induced expression [19, 20]. Thus, although DNA binding activity of the GR is not required, the DBD is nevertheless necessary for *trans*-repression. This indicates that this region of the GR has functions other than just the binding to DNA.

Direct evidence that the DBD of the GR is involved in the repression of AP-1 induced promoter action is demonstrated by a set of GR/mineralocorticoid receptor (MR) mutants. In terms of gene activation, GR and MR recognize and induce expression from identical response elements [23]. However, cotransfection of GR and MR expression vectors with a 5 × TRE CAT construct into CVI cells showed that the GR represses AP-1-induced expression of the 5 × TRE CAT construct, but the MR does not. The MR can however be made to repress expression of the 5 × TRE CAT construct by substituting its N-terminal and the DBD with the corresponding regions of the GR (GGM). Repression of the activity of the 5 × TRE CAT construct was not observed if only the N-terminal domain of the MR was substituted (GMM) (Cato *et al.*, unpublished). This shows that the DBD of GR is needed for the repression but not that of the MR.

The DBD of steroid hormone receptors contains two zinc atoms tetrahedrally co-ordinated by cysteine residues required for proper folding and DNA binding [24]. The DBD of the MR differs from that of the GR by four amino acid residues at positions 444, 455, 497 and 498 (with reference to the rat GR). Preliminary studies of individual amino acid exchanges at these positions show that a single change from a tyrosine at position 497 to a leucine (as in the MR) abolishes the activity of the mutant GR to *trans*-repress but has no effect on its *trans*-activating properties (unpublished).

From crystallographic analysis of the GR–DNA interaction, the GR assumes a compact globular form divided into two substructures, each nucleated by a zinc coordination center and followed by amphipathic α helixes [25]. These substructures are joined together mainly through the interaction of five aromatic side chains (including Tyr 497) to form an aromatic cluster [25]. It is conceivable that this cluster is important for the interaction with AP-1, and that change of Tyr 497 disrupts both the substructures and the receptor's ability to *trans*-repress.

INDUCTION OF GENE EXPRESSION BY STEROID HORMONES AND GROWTH PROMOTING FACTORS

Although steroid hormone receptors and transcriptional factors involved in cell proliferation mutually antagonize each other's activity, they do, in some instances, function additively or synergistically. The synergistic action of c-Jun and the GR in the proliferin gene has already been described [13]. The response of progestational steroids is potentiated by the growth factor EGF in the human mammary tumor cell line T47D [26]. However the mechanisms through which EGF affects progestin action, are not clear. EGF does not affect features of the progesterone receptor (PR) required for gene activation, such as its level of phosphorylation or its DNA binding activity [26]. It is however known from three different lines of evidence that the EGF receptor is involved in the enhancement of progestin response. These are derived from:

(1) the analysis of the amount of EGF that half maximally potentiates progestin action (this value is of the same order as the K_d of EGF for the EGF receptor);
(2) the ability of TGFα to bind to the EGF receptor to mimic the EGF response; and

(3) the inhibition of the enhancing action of EGF response through the use of inhibitors of the kinase activity of the EGF receptor.

Though the mechanism of the positive action of EGF on progestin action is not known, a possibility exists that it is achieved through changes in phoshorylation of auxiliary factors that are required for the action of the PR. Such factors are yet to be identified. An important prerequisite for the positive action of the PR and the growth factor EGF is for the steroid receptor to bind to DNA.

Another way whereby growth stimulating factors c-Jun and c-Fos and steroid hormone receptors function synergistically is through a mechanism that does not require binding of the receptor to DNA. This is illustrated by a direct cooperation of c-Fos and c-Jun with the ER in induction of expression of the chicken ovalbumin gene [27].

One of two regulatory elements that control the estrogen regulation of the chicken ovalbumin gene encompasses the sequence 5′TGGGTCA3′. Although this sequence shares some degree of homology with an estrogen response element, it does not bind the ER. Instead it binds c-Fos and c-Jun. In gel retardation experiments, the Fos/Jun heterodimeric complex is not altered by the presence of the activated ER. However *in vivo* the ER coactivates expression at the ovalbumin promoter with c-Fos and c-Jun. This coactivation does not require the DBD of the ER and can be classified under a mechanism of protein–protein interaction. Unlike the protein–protein interaction involving the GR and Fos/Jun, this interaction of Fos/Jun and the ER does not produce an antagonistic but rather a positive response. How the coactivation of the ER with c-Fos and c-Jun occurs is not known. Either the ER conformationally alters DNA binding of AP-1 (*in vivo* footprints have not been performed) or ER, c-Fos and c-Jun cooperate through protein–protein interaction. Alternatively, as suggested above, the coactivation functions through an auxiliary protein whose expression is induced by estrogen or a Fos/Jun inhibitor is titrated by the ER.

From the mechanisms of antagonistic and synergistic action of steroid hormone receptors and factors controlling cell proliferation that we have reviewed here, it is obvious that different signal transduction pathways communicate with one another. This is achieved through transcription factors of these different signal transduction pathway interacting with each other. Depending on the gene that is regulated or on a particular physiological situation, such interaction could lead to a positive or a negative response of the gene controlled by these transcription factors. In most cases the interaction of the transcription factors for the different pathways occur in the absence of direct binding to DNA. It is therefore likely that they exert their influences through protein–protein interactions involving direct physical interaction with each other or indirectly through auxiliary factors. Future studies on the details of this interaction will greatly increase our knowledge of signal transduction mechanisms.

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