meeting report

Looking at nuclear receptors from the heights of Erice

Workshop on nuclear receptor structure and function

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From May 12 to 15, 2001, the Center Ettore Majorana in Erice (Sicily) hosted the 4th EMBO Workshop on Nuclear Receptor Structure and Function, organized, as a now well-established tradition, by M. Beato, P. Chambon, J.-Å. Gustafsson, A. Maggi, M. Parker and W. Wahli.

Introduction

Since the initial cloning studies, steroid hormone receptors have appeared to belong to a large family of regulated transcription factors that has embraced receptors for vitamin D (VitD), thyroid hormones, retinoids and others of unknown ligand (the 'orphans'). The last decade has witnessed a rapid increase in the knowledge of the structures and molecular mechanisms adopted by this family of proteins to finely regulate gene transcription and interact with other signalling molecules. The Erice meeting covered topics ranging from chromatin remodelling to structural features and dynamics of nuclear receptor (NR) interactions with ligands, target DNA and co-regulators. Progress on two fronts was of particular relevance at this year's meeting: a wide range of activities of specific NRs or co-regulators had been determined through the use of genetically engineered mouse models, and novel mechanisms whereby a number of orphan receptors regulate metabolic pathways had been unravelled.

In this report we will summarize some of the recent findings presented at the workshop. Owing to space limitations, we were forced to select only a few of the many exciting data shown.

Spying into the intricacies of chromatin structure and function

A major question in understanding the mechanism of action of intracellular receptors relates to the dynamics of their binding to DNA to recruit and activate the transcription machinery. D. Reinberg's (Piscataway, NJ) task at this EMBO workshop was to prepare the ground for the discussion of this subject by presenting the latest investigations into chromatin effects on RNA polymerase II-dependent transcription (Kornberg and Lorch, 1999). The rules governing wrapping of DNA into the 30-nm nucleosome assembly, where it is inaccessible to transcription, or into the 11-nm fibres, where the transcription factors can gain access, are still unclear. However, it has been

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shown that modification of histones (such as acetylation, methylation and phosphorylation) take part in this mechanism. Using an *in vitro* transcription system consisting of chromatin fully reconstituted with unmodified histones, Reinberg demonstrated that the phosphorylation of histone 3 at Ser10 is a prerequisite for its subsequent acetylation. Acetylation of the core components of nucleosomes is structurally necessary to establish the activated state of the chromatin, since it neutralizes the positively charged lysine residues and disrupts the interactions among histones and between histones and DNA. In addition, histone 3 acetylation seems to be necessary for its interaction with the ATP-dependent helicase-containing complex (remodelling spacing factor), which gives chromatin the nucleosome phasing mandatory for transcription initiation. According to the model discussed, histone 3 phosphorylation at Ser10 could play a pivotal role in regulating the histone acetyltransferase (HAT) activity on DNA, an activity retained by several co-regulators recruited by NRs. What remains to be clarified is the nature of the signal leading to the phosphorylation of histone 3 and how the phosphorylated Ser10 favours acetylation.

What is the role of the NRs in this sequence of events? Remodelling of nucleosome structure is generally believed to precede the binding of other transcription factors and RNA polymerase II to the promoter. However, some members of the NR superfamily are known to be exceptions and bind to their responsive elements even if DNA is tightly wrapped into a nucleosome structure. Whether this can be considered a general feature of NRs is not yet known. Significant experimental evidence shows that NRs bind to DNA and then recruit the protein complexes that are capable of altering the chromatin structure through ATP-dependent mechanisms and post-translational modifications of histones. This disrupts the dynamic equilibrium of condensed and decondensed chromatin, which respectively favour or oppose the formation of the pre-initiation complex (PIC), the step necessary for transcription initiation. This view predicts that the protein complexes recruited by transcription factors contain enzymatic activities that are able to induce changes in chromatin topology. M. Beato (Marburg, Germany) showed that steroid hormone receptors recruit ATP-dependent chromatin remodelling activities to the mouse mammary tumour virus (MMTV) promoter, but that binding of other transcription factors, such as nuclear factor 1 (NF-1), can also directly contribute to the rearrangement of the chromatin architecture. Thus, NF-1 could act as a wedge to stabilize an open nucleosome conformation necessary for full progesterone receptor (PR) binding and full hormonal activation of the promoter.

Another important question in the elucidation of NR action is what happens after the ligand-activated receptor has bound its DNA responsive element, triggered PIC assembly and recruited RNA polymerase? Two groups at the meeting reported on this important topic. With a combined *in vitro*/*in vivo* study on the MMTV promoter, G. Hager (Bethesda, MD) provided convincing evidence that not only the glucocorticoid receptor (GR), but also the whole PIC, resides on the DNA only briefly. With chromatin reconstituted *in vitro*, Hager showed that the recruitment of the human Swi/Snf remodelling complex by GR onto the chromatin template led to NR displacement from DNA. The phenomenon was strictly dependent on ATP. Photobleaching experiments in a cell line carrying a tandem array of the MMTV promoter inserted into a chromosomal location

further confirmed the *in vitro* results. The use of GFP fusion variants of GR, PR, GRIP-1 (glucocorticoid receptor-interacting protein 1), NF-1 and RNA polymerase II permitted the visualization of their binding to the MMTV promoter in living cells and an estimation of their residence times on the promoter (~5–10 min). The molecular mechanism determining the release of the receptor from its DNA responsive element was discussed by K. Yamamoto (San Francisco, CA). His work in yeast and mammalian cells demonstrated that, after binding of the ligand-complexed GR to the MMTV promoter, a molecular chaperone complex appears on the response element. These and his previous data suggest a role for the p23 molecular chaperone in promoting dissociation of NRs from co-activators and the response element. In Yamamoto's view of the on/off cycle for the binding of steroid receptors to the DNA, chaperones play direct roles in generating the aporeceptor, thereby potentiating hormone binding, and in ejecting the receptor from the template once the chromatin remodelling factors and the PIC have been recruited to the promoter itself (Freeman and Yamamoto, 2001).

Co-regulators and co-factors: how necessary are they?

Co-regulators are co-activators or co-repressors required by NRs for efficient and tissue-specific transcription regulation (McKenna *et al.*, 1999). Generally, in type I (steroid) and type II (thyroid hormones and retinoic acid) NRs, the conformational changes induced by the ligand cause the association of co-activators with the AF1 or AF2 domains. Type II receptors, in the absence of their ligands, associate with repressors such as N-CoR (nuclear receptor co-repressor), SMRT (silencing mediator for retinoid and thyroid hormone receptor) and other factors. Ligand binding generally leads to the release of the co-repressor; this allows the formation of a complex with other molecules of the PIC (including co-activators) and the transcription of target genes. The involvement of co-regulators in NR activity is very important for the tissue specificity of their activities, their differential effects at selected promoters and the cross-coupling with other intracellular pathways responsible for signal transduction. The functional relevance of co-regulator binding was discussed by B.W. O'Malley (Houston, TX). Using an *in vitro* chromatin transcription system, he dissected PR-mediated transcriptional regulation, showing that steroid receptor co-activator 1 (SRC-1), a ligand-dependent co-activator, acts synergistically with the transcription factor p300; his data suggest an obligatory sequential recruitment of SRC-1 and p300 by PR, and this appears to be a common feature shared by the other members of the SRC family (SRC-2 and SRC-3). Moreover, he showed that, while the HAT domain of CBP is necessary for its transactivating function, this is not the case for the SRC-1 HAT domain. He also studied ligand-dependent estrogen receptor (ER) and PR transcription efficiency in the presence of increasing concentrations of co-regulators. The data shown directly substantiate the mechanism of selective receptor modulator (SRM) activity, revealing that mixed antagonist/agonist induces an intermediate receptor conformation that is exquisitely sensitive to local concentrations of co-regulators, thereby explaining the cellular specificity of mixed antagonist/agonist SRMs. Another player mediating the antagonist-dependent transcriptional repression is the repressor of

tamoxifen activity (RTA), characterized by D.P. McDonnell's laboratory (Durham, NC). McDonnell showed that, when the DNA recognition motif of RTA is mutated, all classes of selective ER modulators (SERMs) and ER antagonists function as agonists of ERα and proposed that the inhibitory activity of RTA can be overcome by agonists in cells in which the receptor is able to form transcriptionally active complexes.

The essential step that provided insight into the physiological effects of regulators was the generation and study of mice in which genes encoding co-regulators were deleted. The most well characterized co-repressors for NRs are N-CoR and SMRT, which appear to suppress the activities of certain receptors in the absence of their ligands and other receptors in the presence of antagonists. Mice devoid of N-CoR develop disorders in the central nervous system (CNS), thymus and in erythrocyte differentiation. In accord with the previous work that has shown this, M.G. Rosenfeld (San Diego, CA) reported that, in the absence of N-CoR, neural stem cells lose their undifferentiated phenotype and develop towards the astrocyte lineage, underlining the importance of N-CoR in neuronal and glial cell differentiation. A role for N-CoR was also demonstrated in lactotrope cell fate specification in the pituitary gland, where the pituitary specific transcription factor (Pit-1), thyroid receptor and other DNA-binding factors have been found to recruit an N-CoR co-repressor complex whose activation results in the long-term repression of the growth hormone gene in these cells.

The role of another repressor, NRIP1 (nuclear-receptor-interacting protein 1), previously called RIP140, has also been examined in knock-out (KO) mice. Malcolm Parker (London, UK) reported that NRIP1 is essential for female reproduction and plays a role in cell growth and abdominal fat accumulation. The inability of mice lacking this protein to ovulate reflects a defect in the ovary itself, rather than in the hypothalamus–hypophysis axis, as had been previously thought. A similar ovarian phenotype has been noted in mice devoid of PR or the enzyme cyclooxigenase 2 (COX-2) responsible for prostaglandin synthesis, suggesting that there might be a common basis for the defect. Interestingly, although PR expression was maintained in the NRIP1 mice, COX-2 expression was reduced, suggesting a link between prostaglandin and NRIP1-regulated signalling.

Lots left to learn about steroid receptors

The structure. The molecular details of the interactions between SERMs and the two ERs, ERα and ERβ, were thoroughly discussed in an attempt to define their structural characteristics and their pharmacological implications. SERMs are known to alter the affinity and/or selectivity of the ERs for their co-regulators, thus generating tissue-specific responses (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998). Previous reports from the laboratories of G. Greene (Chicago, IL) and others had shown that tamoxifen (OHT) promotes an inhibitory conformation of the ER by its specific positioning of one particular ER domain, termed helix 12 (Figure 1). This is achieved through two distinct mechanisms. The first is linked to OHT's bulky side chain and the second to its occupancy of the ligand hydrophobic pocket, which induces a series of local structural distortions that change the lengths of helices 3, 8 and 11 and hinders any association among them. Pursuing his studies on the structural features induced by the
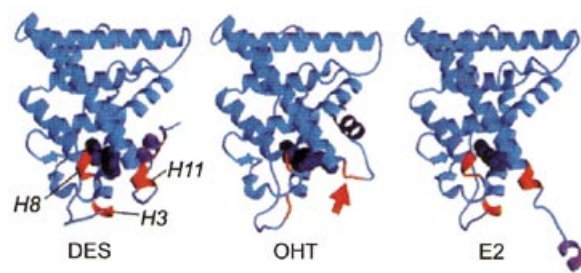

Fig. 1. Structures of the ERα LBD complexed to the agonists diethylstirbesol (DES) and estradiol (E2) and the antagonist tamoxifen (OHT). The ligands are shown in space-filling representations. In each complex, helix 12 is in magenta. Helices 3, 8 and 11 (H3, H8 and H11, respectively) are labelled in the DES complex (from Shiau *et al.*, 1998). The red arrow points to the sequence mainly responsible for helix 12 positioning in the antagonist conformation. This positioning, which is distinct from that of the agonist conformation, prevents the access of co-activators to their ER binding sites.

binding of specific ligands to ERα, Greene showed the crystallographic structure of the ERs bound to a novel synthetic SERM, *cis*-R,R-diethyl-dihydroxy-tetrahydrochrysene (THC), focusing on the role of helix 12 in the interaction. The image that he presented suggests that a bulky side chain is not, in fact, a strict requirement for antagonist behaviour. Instead, THC ERβ antagonist activity is achieved by filling the ligand-binding pocket of ERβ suboptimally and by allowing helix 12 to assume a position that, although in dynamic equilibrium with the agonist conformation, is shifted more towards the antagonist conformation. In contrast, when bound to the ERα ligand-binding domain (LBD), THC is able to stabilize the agonist conformation of helix 12 by making most of the same contacts as estradiol. To gain insight into the complexity of the protein–protein interactions affecting transcription, crystals of the ligand-bound ERβ LBD in combination with an LxxLL-containing co-activator peptide were generated, and their study showed that the presence of the peptide stabilizes helix 12 in its agonist conformation. An intriguing observation was reported by D. Moras (Illkirch, France), who presented the structure of a triple cysteine to serine mutant of the ERα LBD complexed with estradiol. In this case, in spite of the presence of the tightly bound agonist, the protein exhibits an antagonist-like conformation, similar to that observed with OHT or raloxifene (Ral, also an antagonist), i.e. helix 12 is positioned to block co-receptor binding. This mutant has the same binding affinity as wild-type (WT) ERα for estradiol, but its transcriptional activity upon estradiol binding is reduced by 50%. Moras' observation could reflect a more dynamic role for helix 12, with the control of the equilibrium between two stable locations relative to the rest of the protein determining the partial agonist character of a given ligand.

Novel functions. New insights were provided by studies in the classical estrogen target organs as well as in the less investigated estrogen-regulated tissues such as the CNS. The availability of selective ER KO mice allows a better understanding of the complex relationship between the two ERs, which in certain physiological contexts appear to operate in concert and in others as antagonists. The presentation by J.-Å. Gustafsson (Huddinge,

Sweden) emphasized the anti-proliferative effects of estrogen-activated ERβ in the uterus and prostate by showing that, in the uteri of βERKO (ERβ –/–) mice, ERα-regulated growth factors are hypersensitive to estrogen treatment and that βERKO males show prostatic hyperplasia (similar to what is observed after ablation of the estrogen synthetic enzyme aromatase). The finding, by K. Korach (Research Triangle Park, NC), that ERβ levels in ERKO (ERα –/–) mice are identical to those in WT mice would suggest that ERβ expression is not dependent on ERα. Korach also reported on the uterotrophic effects of estrogens that seem to be mediated by ERα. In fact, estradiol, as well as insulin-growth factor 1, failed to affect uterine growth in ERKO mice.

O. Conneely (Houston, TX) demonstrated a dual role for the two PR isoforms, PR-A and PR-B, on proliferative versus anti-proliferative effects using KO mice. During decidualization in the uterine epithelium, PR-B has a pro-proliferative effect, whereas PR-A is anti-proliferative. This finding could be a consequence of the fact that PR-B contains transcriptional activating properties that are lacking in PR-A (as shown *in vitro*) and that PR-A has been shown to act as a dominant repressor of the B form.

Non-classical targets for estrogen action were described by the groups of J.-Å. Gustafsson and A. Maggi (Milan, Italy). Neurodegeneration and astrogliosis were reported in adult brains of βERKO mice. Analysis of neural cell proliferation and apoptosis during embryogenesis showed increased cell death and down-regulation of genes relevant for the migration of neurons in selected areas of the βERKO cortex (with respect to WT). These observations, from Gustafsson's laboratory, point to a role for ERβ in the development of the CNS cortex and provide very strong evidence for the importance of estrogen signalling for neuronal integrity *in vivo*. A novel potential mechanism for the alleged neuroprotective effect of estrogens was provided by Maggi's studies, showing that estradiol, via ERs, blocks microglia activation induced by strong inflammatory compounds like bacterial lipopolysaccharide. By blocking the chronic inflammatory process known to be associated with neurodegeneration, estrogen might in fact reduce the oxidative damage to neurons and thus increase their lifespan (Vegeto *et al.*, 2001).

An innovative mouse model for the evaluation of estrogen activity *in vivo* was generated by P. Ciana (Milan, Italy). Using a luciferase reporter that is driven by a promoter responsive to activated ER (ERE-TK, the ER responsive element thymidine kinase promoter), he generated a mouse in which the reporter is modulated by estrogen in virtually all tissues expressing ERα or ERβ. Considering the rapid turnover rate of the luciferase protein and the ubiquitous expression of the transgene, this tool will make an important contribution to the *in vivo* analysis of the consequences of ER activation under physio-logical conditions and after pharmacological manipulations.

Orphans: the ugly duck became a swan

After two magnificent overviews, by D. Mangelsdorf (Dallas, TX) and Ron Evans (La Jolla, CA), that showed several orphan receptors to be key transcriptional regulators in cholesterol homeostasis (Lu *et al.*, 2001) and xenobiotic metabolism, W. Wahli (Lausanne, Switzerland) presented a novel function for the PPARs (peroxisome proliferator-activated receptors), orphans

known to be targets of hypolipidemic drugs. The observation that the three PPAR isotypes are highly expressed in mouse epidermis during fetal development but disappear from the inter-follicular epithelium after birth, only to reappear upon the application of stimuli that induce keratinocyte proliferation and differentiation, led to investigations into their role in the healing of skin wounds. PPARα and PPARβ were shown to be essential for the rapid epithelialization of a skin wound, and PPARβ was implicated in keratinocyte proliferation and differentiation. Nevertheless, the major role in skin development is played by retinoic acid receptors (RARs) and the retinoid X-receptors (RXRs), as elegantly demonstrated by P. Chambon (Strasbourg, France), who described conditional somatic mutants for RXRs and double KOs for RARα and RARγ. He had previously shown that ablation of RXRα is not compatible with the full develop-ment of the embryo, leading to *in utero* death. On the other hand, RARα or RARγ KO mice do not exhibit an abnormal skin phenotype because of the functional redundancy of these isoforms. RXRα conditional mutants (Li *et al.*, 2000) developed alopecia (hair loss) upon the blockade to receptor synthesis, and this phenotype was significantly more dramatic in females than in males. The primary cause of this phenomenon was linked to degeneration of the hair follicle. In addition, RXR conditional mutants showed hyperplasia of the epidermis and an inflamma-tory process in the dermis. The alopecia has analogies to the phenotype reported for the VitD KO mouse. However, in the latter model, neither inflammatory process nor interfollicular hyperplasia was observed. Reasoning that VitD receptor and RXR are known to heterodimerize, Chambon concluded that another partner of RXR must be responsible for the hyperplasia and inflammatory reactions observed in the RXRα mutant mouse.

With regard to orphans having a major role in the develop-ment of the CNS, T. Perlmann (Stockholm, Sweden) presented an update of his studies on the involvement of Nurr1 in the development of the midbrain dopaminergic system. In spite of the efforts made so far, the growth factors interacting with or regulated by Nurr1 are still elusive. However, of great interest is the observation that, in mature brain, Nurr1 contributes to the regulation of key enzymes involved in dopamine biosynthesis: this prompted speculation that Nurr1 malfunction could contribute to neurological disorders linked to defective dopaminergic trans-mission. Supporting this view are the findings of mutations of the human *nurr1* gene in several cases of schizophrenia and manic depressive disorder. The other orphan found to be of relevance for the differentiation and development of the nervous system is COUP-TF1 (chicken ovalbumin upstream promoter transcription factor 1). Disruption of this gene by homologous recombination in M.-J. Tsai's laboratory (Houston, TX) showed that the COUP-TF1 null mice die perinatally due to several defects in the peripheral and central nervous systems. The most impressive of these lesions is characterized by the absence of a developed cortical layer IV and by severe hypomyelination. The explanation for the absence of layer IV is provided by a series of experiments showing that inappropriate differentiation of subplate neurons results in improper thalamocortical axonal projection and inner-vation, leading to apoptosis of layer IV neurons. The hypo-myelination is due to improper differentiation of oligodendrocytes of the COUP-TF1 mutants. The molecular mechanism leading to the reported phenotype still awaits

elucidation; an interesting lead, however, might be the observation that COUP-TF1 controls the expression of POU (Pit-Oct-Unc) domain genes such as *tst-1/scip/oct6*. Interestingly, the ablation of another member of the COUP family, COUP-TFII, causes a dramatically different phenotype (S. Tsai). These homozygous COUP-TFII null mutants die during early embryo development due to aberrant heart development and a lack of angiogenesis. Remarkably, heterozygotes show growth retardation, lower body weight and severe fertility problems. These results clearly show the distinct roles played by the members of the COUP-TF family, as had been anticipated by the localization studies carried out by Tsai's group.

Conclusions

The 2001 workshop on nuclear receptor structure and function was marked by large amounts of new information on the biological functions of intracellular receptors combined with novel insights into the basis of their mechanisms of action. We are now looking forward to even more exciting progress being described at future EMBO workshops.

Acknowledgements

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