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Studies on the Non-Activated and Activated Forms of the Estrogen Receptor

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The activation of the steroid receptor is a necessary process for the biological role of the receptor. Many factors are involved in this mechanism; in addition to time, temperature, and salt concentration, RNA and RNAase can also affect the transformation of the non-activated to the activated form of the receptor. Using as a model the estrogen receptor of fetal uterus of guinea-pig, the studies of the interaction with three different monoclonal antibodies (D547, H222 and H226) reveal structural transformation during the process of the receptor activation. These conformational transformations suggest that a change in the exposure of the functional domains of the estrogen receptor occurs during activation.

Key words: Estrogen receptor, Monoclonal antibodies, RNA, Receptor activation.

In recent years there have been enormous advances in the knowledge of the structure of the steroid hormone receptors and the determination of the function of various domains of the steroid receptor. It is well documented that non-activated steroid hormone receptors, an oligomeric form with a 8-9S sedimentation coefficient, are bound to the 90 kDa heat shock protein (hsp 90) (15). Activation involves the dissociation of the 90 kDa heat shock protein and conformational changes of the oligomeric receptor with subsequent unmasking of the DNA binding domain (9). Many factors are involved in this transformation and *in vitro* studies for many years had well established that time, temperature and high salt concentration contribute to this transformation (8). Figure 1 gives the sequence of the different domains of the human estrogen receptor: the A/B region is considered to be responsible in part for the function of the activated receptor; the C region, rich in cysteine, is the DNA-binding domain (DBD) which interacts with the hormone regulatory elements (HRE) (10); the D region is an intermediate zone and E is the hormone-binding domain (HBD).

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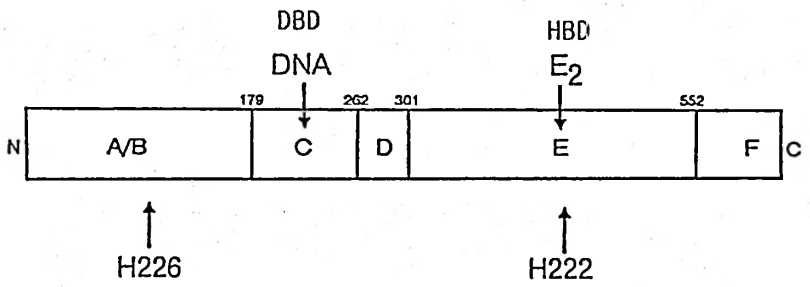

Fig. 1. *Localization of different epitopes in the human estrogen receptors (10).* DBD: DNA binding domain; HBD: hormone binding domain; E_2: estradiol; H-226: monoclonal antibody against ER which binds the AB region; H-222: monoclonal antibody to ER which binds the HBD domain.

Immunorecognition of the activated form of the estrogen receptor of fetal uterus of guinea pig using the monoclonal antibody D547. — The fetal uterus of guinea pig, which contains high quantities of estrogen receptors (ER) (13,14), is an interesting model to study the factors which are involved in the process of receptor activation, as well as the interaction of ER with different monoclonal antibodies. Using the monoclonal antibody D547 prepared against the estrogen receptor from the MCF-7 human breast cancer cells (7), two forms of the estrogen receptor were recognized in the cytosol fraction of fetal uterus of guinea pig. It was observed that 60-65 % of the total cytosol estrogen receptor (the α form, activated) was bound to the antibody, increasing its sedimentation coefficient in a high ionic strength sucrose gradient (10-30 % w/v sucrose, 0.4 M KCl) from 4.5 S to 8 S. The remaining fraction (the β form, non-activated) has the classical sedimentation coefficient of 4.5 S. Dynamic studies of the translocation *in vitro* of the cytosol re-

Table I. *Nuclear and DNA-cellulose binding (% of total receptor) of the time- and temperature- activated estrogen receptor.*

Samples 1, 3, 4, 6 and 7 of cytosol (0.5 ml) were incubated with 10 nM-[^{3}H]estradiol and samples 2, 5 and 8 with 10 nM-[^{3}H]estradiol + 10 mM-Na_2MoO_4 (+Mo) for 2 h or 20 h (as indicated) at 4 °C. Samples 3, 4 and 5 were warmed at 25 °C for 15 min and the rest were kept at 4 °C. At the end of the incubation, 10 mM-Na_2MoO_4 was added to samples 4 and 7. The data are expressed as means ± S.E.M.; —: assays not performed. (Quoted from Ref. N° 1.)

	Experimental conditions		Nuclear binding	DNA-cellulose binding
1		2 h	10.2±1.9	8.6±1.3
2	+Mo	2 h	5.8±1.5	6.0±0.6
3		2 h → 25 °C	20.8±4.4	17.8±3.4
4		2 h → 25 °C + Mo	21.7±2.5	22.2±3.1
5	+Mo	2 h → 25 °C	6.5±1.2	6.6±0.5
6		20 h	—	18.6±2.7
7		20 h → +Mo	—	24.0±3.5
8	+Mo	20 h	—	6.5±1.2

ceptor to the nucleus as a function of time have shown that the α form decreases sharply while the β form is slightly affected when the cytosol is incubated with the nuclei. In contrast, only one form, which is bound totally to the antibody, is found in the nuclear fraction. In addition, the presence of these two forms of the cytosol estrogen receptor was also demonstrated in newborn and immature animals (6).

Activation of the estrogen receptor as determined by its binding to nuclei or DNA-cellulose. — For many years it was demonstrated that the activation of the estrogen receptor could be correlated with the increase of binding to nuclei or DNA-cellulose (17). Table I shows that after warming, the cytosol estradiol-receptor complex increases its binding to nuclei or DNA-cellulose and the sodium molybdate blocks this increase when added before heating (1).

Effect of ribonuclease on estrogen binding to DNA-cellulose. — It was demonstrated that RNA can modify the interaction of steroid receptors with DNA-cellulose (11) and that the treatment with RNAase increases the ability of the receptor to bind to DNA-cellulose (16). Figure 2 shows a significant increase of binding to DNA-cellulose of the estrogen receptors of the guinea-pig after treatment with RNAase (2).

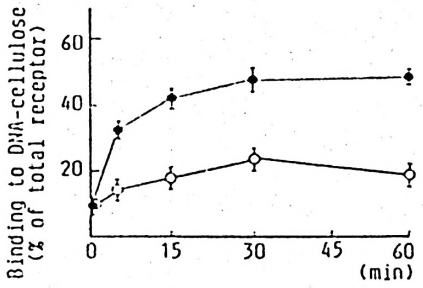

Fig. 2. *Effect of RNAase on estrogen receptor binding to DNA-cellulose.*
[^{3}H]Estradiol-receptor complexes were incubated with (●) or without (○) RNAase (1000 U/ml) for different times at 25 °C. Samples were then assayed for DNA-cellulose binding. Each point represents the average of 3-9 determinations ± S.E. (Quoted from Ref. 2).

Effect of RNA on the activation of the estrogen receptor. — Different exogenous RNAs (calf thymus RNA, yeast RNA and rabbit liver transfer RNA) are able to induce a transformation of the 9 S non-activated estrogen receptor of fetal guinea pig uterus to 4.5-7 S sedimenting forms in low salt sucrose density gradients, as an activating factor, and sodium molybdate inhibits this transformation. The monoclonal antibody D547 partially recognized the receptor treated with RNA, indicating that RNA induces a dissociation of the 9 S receptor and that at least one of the resulting forms is the activated receptor (3).

Interaction of the non-activated estrogen receptor with the D547, H222 and H226 monoclonal antibodies. — The estrogen receptor from fetal guinea-pig uterus is recovered in hypotonic cytosols as a non-activated (non-DNA-binding) oligometric complex that sediments at 9 S in low salt gradients containing sodium molybdate and dissociates to the 4.5 S monometric receptor when centrifuged through high salt gradients. Incubation with the antibody D547 does not modify the 9 S peak on low salt gradient, indicating that this antibody does not recognize the native form of the receptor (table II). However, incubation with the H222 antibody shifted the 9 S peak to 11 S and when this 11 S complex was centrifuged in high salt gradients it dissociated to a 8 S monomer-H222 complex (table II). The fact that a single 8 S peak was observed after high salt dissociation indicates that all the estradiol-binding subunits in the 9 S receptor were able to bind the H222 antibody. In contrast, the H226 antibody shifted the 9 S peak to 9.4 S and when this complex sedimented in high salt gradients,

Table II. *Interaction of the non-activated estrogen receptor from fetal guinea-pig uterus with the D547, H222 and H226 monoclonal antibodies.* Cytosol aliquots containing the [^{3}H]estradiol-receptor complex were incubated with the monoclonal antibodies and analyzed in low salt and high salt sucrose density gradients. LSSC and HSSC are low and high salt sedimentation coefficients, respectively. (Quoted from Ref. N° 4.)

Experimental conditions	LSSC	Hypothetical structure	HSSC	Hypothetical structure
Non-activated ER	9 ± 0.1 S	Heterooligomer	4.5 ± 0.1 S	Monomer
Non-activated ER + D547	9 ± 0.15	Heterooligomer	4.5 ± 0.15	Monomer
Non-activated ER + H222	11 ± 0.2 S	Heterooligomer-$[H222]_2$	8 ± 0.1 S	Monomer-H222
Non-activated ER ± H226	9.4 ± 0.2 S	Heterooligomer-H226	7 ± 0.2 S ± 4.5 S	Monomer-H226 + monomer

Table III. *Interaction of the activated estrogen receptor from fetal guinea-pig uterus with the D547, H-222 and H-226 monoclonal antibodies.* Cytosol aliquots containing the [^{3}H]estradiol-receptor complex were heated at 28 °C for 30 min, then incubated with the monoclonal antibodies and analyzed in high salt sucrose gradients. HSSC = High salt sedimentation coefficient. (Quoted from Ref. N° 4.)

Experimental conditions	HSSC	Hypothetical structure
Heat-activated ER	5.5 S + 4.5 S	Dimer + monomer
Heat-activated ER + D547	8 S + 4.5 S	Dimer-D547 + monomer-D547 + monomer
Heat-activated ER + H222	8 ± 0.1 S	Dimer-H222 + monomer-H222
Heat-activated ER + H226	9 ± 0.2 S + 7 S + 4.5 4	Dimer-$[H226]_2$ + monomer-H226 + monomer

Table IV. *Effect of the H222 monoclonal antibody on the estrogen receptor dimerisation.*
Cytosol aliquots containing the [^{3}H]estradiol-receptor complex were: A) incubated with both H222 and H226 antibodies; B) heated at 28 °C for 30 min and then incubated with both H222 and H226 antibodies; C) incubated with the H222 antibody, heated at 28 °C for 30 min and then re-incubated with the H226 antibody. Samples were analyzed in high salt sucrose density gradients. HSSC = High salt sedimentation coefficient. (Quoted from Ref. N° 4.)

Experimental conditions	HSSC	Hypothetical structure
A) Non-activated ER + H222 + H226	9 ± 0.1 S	Monomer-H222-H226
B) Heat-activated ER + H222 + H226	10.5 S + 9 ± 0.1 S	Dimer-H222-$[H226]_2$ + monomer-H222-H226
C) Non-activated ER + H222 → 28 °C + H226	9 ± 0.1 S	Monomer-H222-H226

it dissociated to yield a 7 S peak corresponding to the monomer-H226 complex, plus a 4.5 S peak which corresponds to the monomeric receptor not bound to the antibody (table II). The smaller shift of the 9 S receptor as compared to that provoked by the H222 antibody, together with the presence of estradiol-binding protein that did not react with the H226 antibody, suggests the existence of more than one estradiol-binding subunit in the non-activated receptor. These results also suggest that, while all the H222 epitopes located in the hormone-binding domain are accessible in the non-activated receptor, the H226 epitopes located in the A/B region appear to be partially masked. This might be due to the proximity of the H226 epitope with the DNA-binding domain (4, 5).

Interaction of the activated estrogen receptor with the D547, H222 and H226 monoclonal antibodies. — The estradiol-receptor complex can be partially activated *in vitro* by heating at 28 °C for 30 min, resulting in the dissociation of the 9 S receptor to slower sedimenting forms and the increase of its affinity for DNA (1,2). The high salt gradients show a broadening of the 4.5 S monomer receptor peak that seems to contain an additional ~ 5.5 S species. When the D547 antibody reacts with the activated receptor, an 8 S peak is observed (table III). This suggests that the dimer can bind only one D547 molecule. When this heat-activated receptor was combined with the H226 antibody a 9 S peak, in addition to the 7 S monomer-H226 complex peak, was observed in high salt gradients (table III). This 9 S peak apparently corresponds to the receptor complexed with two antibody molecules. The existence of a receptor form with two binding sites for the H226 antibody suggests the formation of a homodimer during the activation process; this dimeric receptor would correspond to the 5.5 S form observed in high salt gradients after activation.

On the other hand, even if a partial receptor dimerisation occurred during activation, only a single 8 S peak was observed after reaction with the H222 antibody, suggesting that the dimeric receptor binds only one H222 molecule. Therefore, the H222 epitope might be near to, or involved in, the dimerisation domain. In order to examine this hypothesis, we studied the effect of this antibody on the dimerisation process. As seen in table IV, when both H222 and H226 antibodies reacted with the heat-activated receptor, two peaks were detected in high salt gradients: one at 9 S corresponding to the monomer-H222-H226 complex and the other at ~ 10.5 S which appears to be a dimer-H222-$[H226]_2$ complex. However, when the receptor was combined with the H222 antibody prior to heating and then incubated with the H226 antibody, only a 9 S peak was observed, indicating that binding to the H222 antibody interfered with the receptor dimerisation. It is to be remarked that an inhibition of dimerisation by the H222 antibody has also been reported by LINSTEDT *et al.* (12).

Interaction of the nuclear estrogen receptor with the H222 and H226 mono-

Table V. *Interaction of the nuclear estrogen receptor from fetal guinea-pig uterus with the D547, H222 and H226 monoclonal antibodies.*
Nuclear extracts obtained after incubation of uterine slices with [^{3}H]estradiol at 28 °C for 30 min were incubated with the monoclonal antibodies and analyzed in high salt sucrose density gradients. HSSC = High salt sedimentation coefficient. (Quoted from Ref. N° 4.)

Experimental conditions	HSSC	Hypothetical structure
Nuclear ER	5.5 ± 0.1 S	Dimer
Nuclear ER + D547	8 ± 0.3 S	Dimer-D547
Nuclear ER + H222	8 ± 0.2 S	Dimer-H222
Nuclear ER + H226	9 ± 0.2 S	Dimer-$[H226]_2$

clonal antibodies. — The estradiol-receptor complexes formed in intact cells at 28 °C and extracted from nuclei were found to be homodimers sedimenting at 5.5 S and with similar immunological characteristics to the dimer obtained in cytosol after activation (table V). It is interesting to note that only the dimeric form of the receptor was found in the nuclei, suggesting that dimerisation is necessary for nuclear retention. This observation is in agreement with recent reports which propose that the estrogen receptor binds tightly to its regulatory elements as a homodimer.

Conclusions

The activation mechanism of the steroid receptor is a very complex process which involves many factors including time, temperature, salt concentration, RNA and RNAase, etc. Very probably, other factor(s) which can be involved in this process are still unknown. This is of capital importance because it is very well established that the steroid receptor must be activated before it can play a biological role. The use of monoclonal antibodies against estrogen receptor is very interesting in order to know conformational structures of estrogen receptor.

The study of the interaction of these three monoclonal antibodies provides direct evidence for a change in the availability of the specific epitopes recognized by the different antibodies D547, H222 and H226 as a result of the activation, suggesting that a change in the exposure of

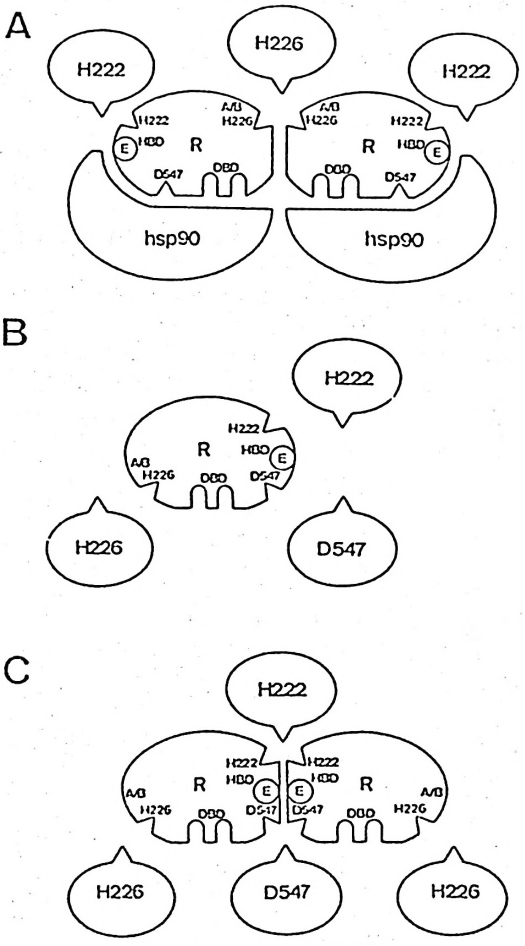

Fig. 3. *Hypothetical model for the conformation of the oligomer, monomer, and dimer estrogen receptor of the fetal uterus of guinea pig and their interaction with the three monoclonal antibodies D547, H222 and H226.*

A) In the non-activated oligomeric form of the estrogen receptor of fetal uterus of guinea pig. The epitope for the monoclonal antibody D547, between the hormone-binding (HBD) and the DNA binding (DBD) domains is completely masked. H222 epitope is totally exposed and the H226 epitope partially exposed. B) In the hypothetical intermediate monomeric receptors the three epitopes are exposed. C) In the activated dimeric receptor the epitopes for the monoclonal antibody H226 are totally exposed (A/B region) and those for the H222 antibody (hormone-binding domain, HBD) and the D547 antibody (intermediate region) partially masked. E: estrogen; R: hormone-binding unit; HSP: heat shock protein; A/B: NH_2-terminal region of the receptor. (Quoted from Ref. 5; with the permission of «*Endocrinology*»).

the functional domains of estrogen receptor occurs during the process of activation. Figure 3 gives a hypothetical model of these conformational transformations as suggested by the interaction of these three monoclonal antibodies with the estrogen receptor.

Resumen

La activación del receptor esteroide es un proceso necesario para la función biológica del receptor. Son muchos los factores implicados en este mecanismos: además del tiempo, temperatura y concentración de sal, la RNA y RNAasa también pueden afectar a la transformación de la forma no activada del receptor a la actividad. Utilizando como modelo el receptor estrogénico del útero fetal de cobaya, los estudios de la interacción con tres anticuerpos monoclonales diferentes (D547, H222 y H225) revelan la transformación estructural durante el proceso de la activación receptora. Estas transformaciones conformacionales sugieren que hay un cambio en la exposición de los dominios funcionales del receptor estrogénico durante la activación.

Palabras clave: Receptor estrogénico, Anticuerpos monoclonales, RNA, Activación del receptor.

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