

Evolving Trends in Estrogen Receptor Biology

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1. Introduction

Discussions on receptors involved in estrogen action (Stanišić et al, 2010) have so far focused on the two major forms of “classical” estrogen receptors, the estrogen receptor α (ER α) and estrogen receptor β (ER β). Both are DNA binding as well as hormone binding forms, with distinct, well-characterized functional domains. The differences between the two have mainly been in their respective molecular masses and shapes and in the target genes with which they interacted.

I have been involved in research in estrogen action for 4 decades and more. Since the focus of my work was chiefly on non-conventional estrogen receptors and receptor associated proteins, often the progress made was felt by me as slow. Nevertheless, it is my sincere belief that what has been unveiled in this direction over the years have not gone unproductive. The two proteins that have been identified in this context, one a non DNA binding estrogen receptor and the other a transcription factor that dimerises with this receptor in the nucleus, have pointed towards the existence of a unique system of receptor in estrogen action. For the first time ever, it has become clear that there is a form of estrogen receptor whose primary functional role is in post transcriptional regulatory mechanisms that include splicing, nucleocytoplasmic transport of RNA and finally, the translation of mRNA. Also, deeper insights into the functional biology of the transcription factor have unfolded certain experimental data hitherto unknown in the literature on steroid hormone action. What is being discussed in this chapter deals exclusively with these two proteins, one a plasma membrane localized estrogen receptor which moves into the nucleus to involve itself in gene regulatory events and the other a transcription factor with a parallel functional role in mitochondrial steroidogenesis

2. The concept of steroid hormone receptor activation

The favorite theme of the 60's and early 70's in descriptions of steroid hormone action, particularly with reference to estrogen action, used to be that the receptor primarily existed in the cytosol. Upon hormone binding and the consequent “receptor activation” the receptor entered the nucleus and interacted with the genetic elements. This “two-step mechanism”, independently proposed by the research groups led by Jensen and Gorski (Jensen & DeSombre, 1973; Shyamala & Gorski, 1969; Mohla et al, 1972) formed the basis for all subsequent discussions on intracellular movements of the receptor -steroid hormone

complex. It was proposed that the cytosolic receptor existed as a high molecular weight form that sedimented at 8-9S in low salt linear sucrose gradients. Later studies have revealed that in this cytosolic form, the receptor with an average sedimentation value of 4S, remained in association with heat shock protein 90(hsp 90) when there was no hormone bound to it (Pratt, 1990; Pratt & Toft, 1997). Hormone binding to the receptor initiated dissociation of the receptor from Hsp-90, which formed a key event in steroid receptor activation (Pratt, 1990). One of the major structural changes noticed in the receptor during its activation was the transformation of the 4S receptor to a form that sedimented at 5S in sucrose gradients containing 0.3M KCl (Shyamala & Gorski, 1969).

The 4S-5S conversion was the target of several hypotheses that attempted to explain the molecular event. In the Hsp-90 model, it was clear that association of the receptor with Hsp-90 prevented the nuclear migration of the receptor the reason for which was not clear at that time. It was the first ever report on the sequencing of amino acids of the human estrogen receptor α (ER α) by Chambon's group at Strasbourg that paved the way for a number of active studies in this direction (Green & Chambon, 1987 a, b). The identification of the nuclear localization signal (NLS) in ER α (Kumar et al., 1986; Kumar et al., 1987) was one such landmark observation. Thampan's group subsequently extended the studies using ER α isolated from goat uterus and purified and characterized a 55kDa protein (p55) that apparently recognized the nuclear localization signal (NLS) on ER α and initiated the nuclear entry of the receptor (Nirmala & Thampan, 1995 a,b). The studies reported by Thampan's group gave additional validity to the role of p55 in the nuclear entry of ER α .

3. The role of estradiol in the nuclear entry of ER α

Sai Padma et al (2000) and Sai Padma & Thampan (2000) observed that there were three nuclear proteins that contributed to the regulated entry of ER α into the nuclei. (a) the p55 that recognized the NLS on ER α (b) a 28kDa protein, p28 that bound to the NLS signal on ER α and thereby prevented the p55-ER α interaction; (c) a 73 kDa protein, p73 that bound to the hormone binding domain (HBD) on ER α . Under hormone free conditions, p28 remained bound to the ER α NLS, blocking the NLS recognition by p55. Estradiol binding to the HBD and the consequent conformational change in the HBD brought the HBD-bound p73 in close interaction with p28. This resulted in the dissociation of p28 from the NLS which was subsequently occupied by p55. The interaction culminated in the nuclear entry of ER α , also mediated by the cytoskeletal elements, actin and tubulin (14).

4. Search for the “receptor-activator” protein and the discovery of E-RAF

There was a line of thinking that originated from Notides' (Notides & Nielson, 1974) and Yamamoto's (Yamamoto, 1974) laboratories that in estrogen action there was a possibility for the involvement of a DNA binding X-protein in converting the non-DNA binding estrogen receptor to a DNA binding form. Based on these observations and consideration of a potential possibility that a non-hormone binding transcription factor could be involved in the “activation” process, Thampan and Clark (1981, 1983) presented the first ever experimental evidence for the existence of an estrogen receptor activation factor (E-RAF) in the rat uterus. A parallel thinking that contributed to the design of experiments was the already available information that many transcription factors were moderately basic proteins and also that such proteins failed to bind to DEAE cellulose. It was this information

that primarily led to the separation of E-RAF from the estrogen receptor that it dimerises with during DEAE-cellulose chromatography. Thampan and Clark (1981) reported that a 3S protein of the rat uterine cytosol, that appeared in the DEAE cellulose flow through fraction, promoted the DNA binding of a specific class of non-DNA binding estrogen receptor. Thampan (1987,1989) in his reports on the purification of E-RAF observed that E-RAF existed in two molecular forms, E-RAF II and I.

While both forms displayed identical molecular weight of 66kDa, their molecular shapes appeared to be different as displayed by the results of gel filtration chromatography and also in their dissimilar sedimentation behavior in linear sucrose density gradients. Functional assays were carried out in which the proteins were incubated with labeled DNA, which was subsequently exposed to S1 nuclease in order to digest the single stranded regions. The results showed that while E-RAF II destabilized DNA double helix and enhanced strand separation, the reverse property (stabilization of double helical structure) was found associated with E-RAF I. In vitro transcription assays involving isolated nuclear RNA polymerases also highlighted this differential behavior of the two molecular forms. While E-RAF II enhanced transcription, in a system containing nuclear RNA polymerase purified from goat uterine nuclei, E-RAF I inhibited transcription in a dose-dependent manner.

5. A vision into the molecular identity of the type I and type II nuclear estrogen binding sites

The report in which functional characterization of E-RAF was described (Thampan,1989), also presented a method for the assay for E-RAF in association with the nuclear RNA polymerases. Nuclear RNA polymerases were extracted from isolated rat uterine nuclei and subjected to partial purification through chromatography on DEAE Sephadex A-25 and elution with linear $(\text{NH}_4)_2\text{SO}_4$ gradient. Ovariectomized rats were used in this study. While control rats received injection of the vehicle alone, experimental animals were subjected to subcutaneous injections of $3\mu\text{g}$ estradiol- 17β for a duration of one hour. The RNA polymerase fractions derived from both control and experimental nuclei and eluted from DEAE-Sephadex A-25 column were subjected to the nuclear exchange assay that was developed earlier by Clark and coworkers (Clark & Peck,1979;Clark et al.,1979). It was through this nuclear exchange assay that Clark's group had demonstrated the existence of type I and type II estrogen binding sites in rat uterine nuclei (Eriksson et al.,1978).

Following DEAE Sephadex-A25 chromatography of nuclear sonicates, the fractions collected were subjected to the estradiol exchange assay as well as RNA polymerase assay with calf thymus DNA as the template .RNA polymerase peaks representing I, II, IIIa and IIIb were clearly demonstrated in the DEAE-Sephadex A-25column fractions. Also demonstrated was the estrogen binding function associated with all four peaks of RNA polymerase activity. The 'receptor' activity associated with the RNA polymerase II was subjected to further analysis. Sucrose density gradient analysis displayed two peaks of activity, a small peak at 5S and a large peak at 3S.While the 5S peak was distinctly DNA binding, the 3S peak which represented the major share of receptor activity, remained non DNA binding. Subsequent studies (Thampan,1989) have demonstrated that the DNA binding function of the 5S peak was due to the presence of E-RAF and an estrogen receptor that dimerised with E-RAF while the non DNA binding 3S fraction was represented by a receptor that did not dimerise with E-RAF.The same studies have concluded, subsequently that the receptor of the 5S peak

was the non activated estrogen receptor (naER), a glycoprotein and a tyrosine kinase sensitive to the presence of estradiol and primarily localized at the plasma membrane (Karthikeyan & Thampan, 1994). The naER was the only estrogen receptor that could dimerise with E-RAF. The 3S peak on the other hand, represented the nuclear estrogen receptor II (nERII), a tyrosine kinase insensitive to the presence of estradiol. The nuclear estrogen receptor II failed to dimerise with E-RAF, the obvious reason being the changes induced in naER conformation during its transformation to nERII (Karthikeyan & Thampan, 1995; Thampan et al., 1996). The naER to nERII transformation was accomplished by a 61kDa nuclear naER-transforming factor (naER-TF), originally reported by Jaya and Thampan (2000).

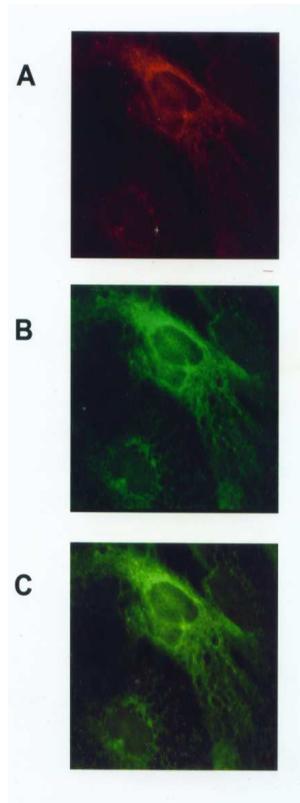
6. Factors regulating nuclear entry of E-RAF

Endoplasmic reticulum is the primary site of localization of intracellular E-RAF. A 55kDa anchor protein, ap55, that binds estradiol with high affinity retains E-RAF at the endoplasmic reticulum (Govind et al., 2003 a,b). Figure 1 displays the immunolocalisation of E-RAF in the endoplasmic reticulum of a goat uterine cell.

E-RAF remains anchored to ap55, through the mediation of a 66kDa nuclear transport protein, tp66. The tp66 recognizes the NLS in E-RAF. Within the E-RAF-tp66 complex, tp66 is anchored by ap55 in an estrogen dependent manner. Presence of saturating levels of estradiol maintains a specific conformation of ap55 that keeps tp66-E-RAF complex anchored to it. Lowering of estradiol concentration results in altered ap55 conformation that facilitates the release of tp66-E-RAF complex from ap55. The complex moves to the nucleus during which tp66 gets docked to a 38kDa nuclear pore-complex protein, npc38. E-RAF enters the nucleus.

E-RAF is a high affinity progesterone and cholesterol binding protein (Thampan et al., 2000). Under both conditions E-RAF dissociates from the ap55-tp66 complex and migrates to the nucleus (possibly also to the mitochondria as cholesterol bound form). Premkumar et al (1999) presented information on the functional domains of E-RAF. Nuclear run on transcription studies were carried out in order to identify the genes influenced by E-RAF. For this, subtractive hybridization approach was attempted (Jacob, 2006). Free E-RAF which can be transported to the nuclei by tp66, and progesterone bound E-RAF that gets transported to the nuclei on its own displayed totally distinct response patterns. It was a 55kDa nuclear pore complex protein (npc55) that docked progesterone bound E-RAF at the pore complex. On the contrary, the free E-RAF-tp66 complex was docked to npc38. While free E-RAF was found to enhance the expression of splicing factor(s) genes, a major gene that was shown to be influenced by progesterone bound E-RAF was the collagenase(s) gene(s). The gene(s) if any, that are under the regulatory influence of cholesterol bound E-RAF remain to be known. Also the nuclear pore complex protein that docks cholesterol bound E-RAF is to be identified.

It appears that cholesterol is a natural regulator of E-RAF mediated gene expression (Thampan et al, 2000). The presence of an inhibitor that prevented the dimerisation between E-RAF and naER in goat uterus was recognized early in E-RAF studies. The inhibition in the formation of E-RAF -naER heterodimer and the subsequent decline in the nuclear binding of the receptor was the assay target employed for the identification of this inhibitor. GC-MS analysis of the purified molecule showed its identity as unmetabolised cholesterol (Thampan et al, 2000).



(A) The cells were fixed, permeabilised and exposed to rabbit anti-goat E-RAF IgG, followed by Cy3 labeled anti rabbit IgG.

(B) The cells were also stained with DiOC6 (3) to highlight the endoplasmic reticulum.

(C) The merged figures created by confocal microscopy clearly showed that endoplasmic reticulum is the primary site of localization of E-RAF.

Fig. 1. Intracellular association of E-RAF with endoplasmic reticulum in goat endometrial cells in culture. A primary culture of goat endometrial cells was exposed to estradiol-free medium for 48 hours following which the cells were examined under a Leica confocal microscope.

7. E-RAF in pregnant rat uterus: significance of the findings

Premkumar and Thampan (1995) examined the level of E-RAF in the uteri of pregnant rats during a full term of pregnancy. It was noticed that from day 1 of pregnancy the E-RAF titer in the uterus registered a steady increase. It reached an all time peak towards mid-pregnancy following which E-RAF level began to decline. The rate of decline was found to be very fast; two days before parturition the uterine E-RAF titer became virtually undetectable. It is known that progesterone is essential for maintaining the functional integrity of the pregnant uterus. The possibility, therefore, exists that the E-RAF titer is a reflection of the progesterone requirement of the pregnant uterus. The decline in E-RAF titer

during the second half is again indicative of the need for progesterone withdrawal prior to parturition. The hypothetical presentation given in figure 2 takes into account the E-RAF titer in rat uterus during pregnancy.

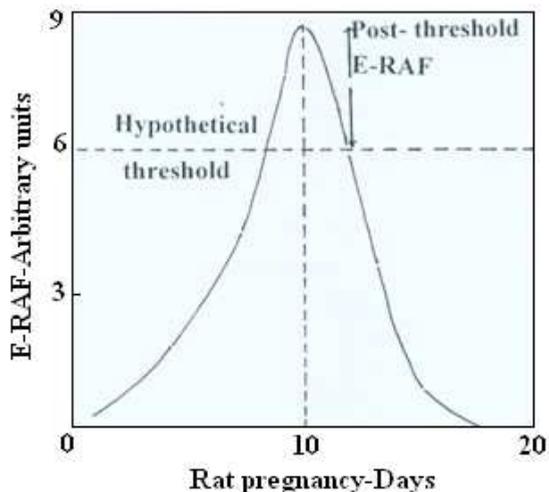
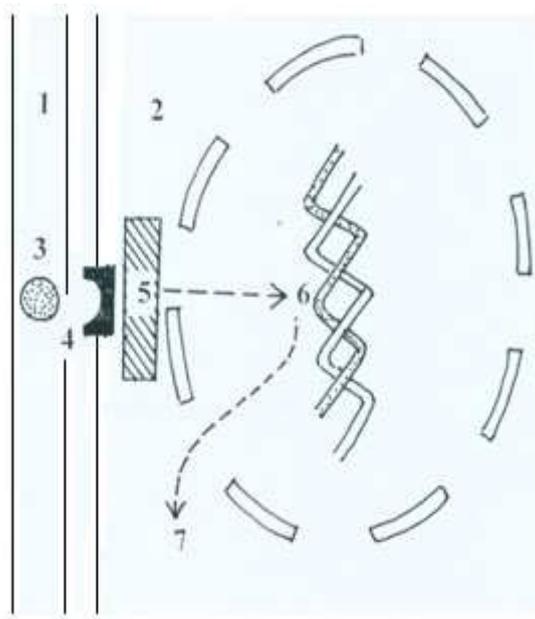


Fig. 2. A generalized plan of E-RAF titer in rat uterus during pregnancy. Uterine tissue was collected from a group of rats everyday during the 21-day pregnancy term. The tissue samples were homogenized in the coating buffer (10mM carbonate and 40mM bicarbonate buffer, pH 9.6), and the homogenates were centrifuged at 10,000Xg for 15 minutes. To the supernatant an equal volume of a suspension of DEAE cellulose in coating buffer was added. After 30 minutes of incubation in ice, the DEAE cellulose flow through fraction was collected. An aliquot of this fraction was used for E-RAF estimation through ELISA. The data takes into account the potential existence of an intracellular threshold level of E-RAF. It is being postulated that, beyond this threshold E-RAF enters the blood and gets transported to the specified site that, under conditions where E-RAF titer is low releases the hypothetical factor which, upon binding to its receptor on the uterine cell membrane, initiates the signal transduction events leading to enhanced E-RAF gene expression. During the negative regulatory mechanism the enhanced level of E-RAF could inhibit the release of the stimulatory factor, thereby suppressing the signal transduction process leading to E-RAF gene activation.

If one assumes that there is an upper limit (threshold) in the uterine level of E-RAF, beyond which the E-RAF enters the blood, it is possible to reconstruct a molecular event. E-RAF is probably transported by the blood to an anatomical site (brain?), which is responsible for enhancing uterine E-RAF gene expression through distinct signal transduction mechanisms mediated by a specific macromolecular agent. Possibly, the presence of E-RAF in circulation could inhibit the release of this mediator, the eventual result being the decline in E-RAF synthesis, leading to the final disappearance of the proteins prior to parturition. Figure 3 illustrates the proposed mechanism of action of this hypothetical regulator of E-RAF gene expression in uterine cells.



(1) Blood plasma (2) Target cell (3) E-RAF-GRA (E-RAF gene regulatory agent) (4) receptor for E-RAF-GRA (5) signal transduction events that succeed the receptor -GRA-interaction (6) E-RAF gene expression (7) synthesis of E-RAF in the cytoplasm.

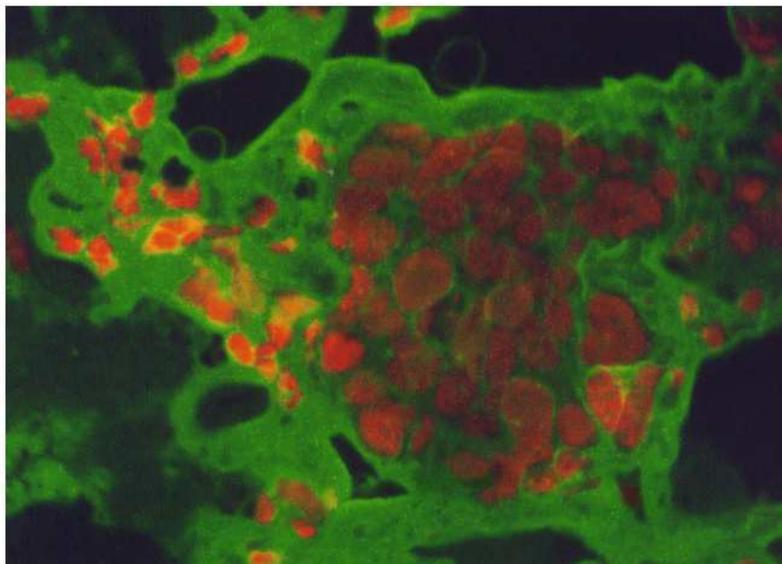
Fig. 3. A model that explains the role of an external factor, transported by the blood, on uterine E-RAF synthesis. The factor recognizes its receptor on the target cell plasma membrane and induces signal transduction events which eventually terminate in enhanced expression of E-RAF gene.

The model indicates that the regulatory factor, E-RAF gene regulatory agent (E-RAF-GRA) transported by the blood, binds to its receptor on the plasma membrane of the uterine cell. This interaction initiates a cascade of signal transduction events, ultimately leading to the enhancement in E-RAF gene expression in the nucleus and synthesis in the cytoplasm. The decline in the circulating presence of the factor, as it may happen during the second half of pregnancy, will be reflected in the decline in uterine E-RAF gene expression. Preliminary report has already been made on the presence of E-RAF in the goat uterine mitochondria (Praseetha & Thampan, 2009). Possibly one of the major functions of E-RAF is to transport cholesterol to the mitochondria where it will be converted enzymatically to pregnenolone and progesterone.

8. E-RAF and mammary cancer

E-RAF could play a role in the progression of mammary cancer. E-RAF II is a very active transcription factor and this molecular form of E-RAF represents more than 75% of total E-RAF population representing both E-RAFI and E-RAF II. If one assumes that the benign to malignant transformation of the mammary cancer is associated with enhanced expression of E-RAFI, that should be reflected in immunofluorescent detection of E-RAF in frozen

biopsies of mammary tissue. Figure 4 presents the results of a recent study carried out in this direction where the tissue sections were exposed first to anti E-RAF IgG and subsequently to FITC labeled secondary antibody. There is a dominating presence of E-RAF in the cytoplasm and also in the nuclei (primarily stained with propidium iodide).



A cryostat section of human breast cancer biopsy was fixed, permeabilised and exposed overnight to rabbit anti goat E-RAF IgG at 4°C. Further exposure of the section to goat anti rabbit IgG labeled with FITC was conducted in the dark for 1 hr following which the nuclei were stained with propidium iodide. The sections were examined using a Leica fluorescence microscope. The green fluorescence indicates the cytoplasmic presence of E-RAF. The nuclei are stained red with propidium iodide. However the presence of E-RAF in the nuclei is marked by the transition of the red colour to light orange and even yellow.

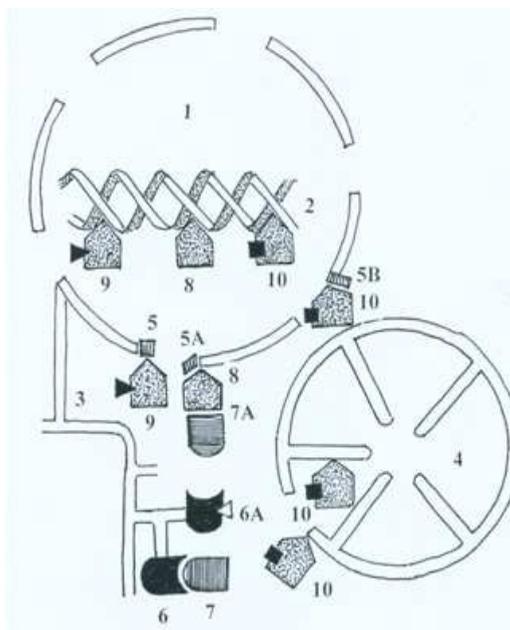
Fig. 4. Detection of E-RAF in human breast cancer biopsy.

If what I discussed in the previous paragraph regarding the brain derived regulator of E-RAF gene expression is proven correct, it is possible to suggest that defects in this regulatory protein mechanism and its action could lead to uncontrolled expression of the E-RAF gene. How does this enhanced expression of E-RAF gene influence mammary cancer progression remains to be seen. One of the major molecular targets of progesterone bound E-RAF is the collagenase gene. Whether the progesterone bound E-RAF mediated enhancement in collagenase gene expression has any role in mammary cancer metastasis is yet to be examined.

9. Multiple intracellular sites for E-RAF action

Recent observation regarding the positive presence of E-RAF in goat uterine mitochondria is indicative of a possible functional role for E-RAF in the mitochondria. Confocal microscopic studies conducted on goat endometrial cells in culture with exposure to varying concentrations of estradiol or progesterone showed that while 3-5nM concentrations of estradiol helped in the nuclear entry of E-RAF, progesterone mediated nuclear entry was

found to be effective only in the presence of 15-16nM progesterone. The corresponding effects produced in the presence of different concentrations of cholesterol remains to be seen. The postulate that the enhancement in E-RAF titer in the pregnant uterus is an indirect reflection of the progesterone production in the uterus during the first half of pregnancy takes into account the possibility that mitochondrial steroidogenesis in the uterine cell is under E-RAF control. As mentioned earlier, E-RAF may function as a cholesterol transporter to the mitochondrial steroidogenic site, eventually facilitating the conversion of cholesterol to pregnenolone and progesterone. The nuclear genes influenced by cholesterol-bound E-RAF could well be those the products of expression of which are constituents of the mitochondrial steroidogenic complex like cytochrome P450(Fig.5)



(1) Nucleus (2) Genes (3) Endoplasmic reticulum (4) Mitochondria (5) (5A) and (5B) Nuclear pore complex proteins that bind free E-RAF, progesterone-bound E-RAF and cholesterol bound E-RAF respectively. (6) ap55 (6A) Estradiol-bound ap55 (7) tp66 (7A) tp66-E-RAF complex (8) Free E-RAF (9) Progesterone bound E-RAF (10) Cholesterol bound E-RAF.

Fig. 5. **Mode of action of E-RAF in the target cell.**

The primary site of intracellular location of E-RAF is the endoplasmic reticulum where it remains anchored to the anchor protein 55(ap55) through the mediation of tp66 (transport protein 66) in an estrogen dependent manner. tp66 transports E-RAF to the nucleus, after dissociation from ap55, when the intracellular level of estrogen declines. When bound by cholesterol or progesterone, E-RAF dissociates from tp66 and moves independently to the nucleus. The nuclear entry of E-RAF again is regulated by nuclear pore complex proteins (npcp). Apparently it is the conformation of E-RAF that determines the identity of npcpc with which it should interact. There is a distinct possibility that within the nucleus free E-RAF, progesterone bound E-RAF and cholesterol bound E-RAF recognize

and regulate the expression of specific sets of genes, possibly influenced by specific acceptor proteins.

Mitochondria appears to be the other target of cholesterol bound E-RAF. The possibility exists that E-RAF functions as a cholesterol transporter to mitochondria, favoring the conversion of cholesterol to pregnenolone and progesterone. This higher titer of E-RAF should be reflected in higher production of uterine progesterone that could eventually contribute to the maintenance of the pregnant uterus during the first half of pregnancy.

10. Identification of the estrogen receptor that dimerizes with E-RAF

The experimental observations on the goat uterine E-RAF signals a clear indication that a special class of estrogen receptor dimerised with E-RAF within the nucleus. A DNA cellulose binding assay was developed in which the non-DNA binding estrogen receptor was labeled with ^3H -estradiol and the binding of the hormone-receptor complex to DNA cellulose in the presence of E-RAF was quantitated. Anuradha et al (1994) reported on the isolation and characterization of a 66kDa, high affinity estrogen binding protein from the goat uterus. The receptor displayed the same affinity to bind estradiol as that of the estrogen receptor α (ER α). In view of its inability to bind to DNA on its own, this new estrogen receptor was designated as non-activated estrogen receptor (naER).

A method was developed for the isolation of the non-DNA binding estrogen receptor that dimerized with E-RAF. The method involved preparation of goat uterine cytosol, collection of the DNA-Sepharose unadsorbed fraction, successive ion exchange chromatography over DEAE cellulose and phosphocellulose and finally Hsp 90 Sepharose chromatography in the presence of sodium molybdate, achieving final elution with zero molybdate buffer (Anuradha et al., 1994). While showing its distinctiveness over ER α , as a non DNA binding protein, the naER further demonstrated its function as a glycoprotein and a tyrosine kinase (Karthikeyan & Thampan, 1996). The tyrosine kinase property was sensitive to the presence of estradiol: the enzyme activity was totally inhibited in the presence of the hormone at concentrations which saturated its binding sites. The observation was a clear indication to the possibility that the naER tyrosine kinase activity can become functional only after naER undergoes a critical structural change within the cell.

Direct biochemical analysis showed that plasma membrane is the primary site of localization of naER (Karthikeyan & Thampan, 1996). The possibility of plasma membrane being a site of intracellular localization of estrogen receptor was first proposed by Pietras and Szego (1975, 1977) several years ago. Sreeja and Thampan (2004 a,b) demonstrated that naER dissociated from the plasma membrane following exposure to estradiol. This was shown to be an estrogen-specific phenomenon since non-estrogenic steroids failed to bring about the dissociation while the non steroidal estrogen, diethylstilbestrol was as effective as estradiol-17 β in inducing naER dissociation from the plasma membrane. What was unique in this observation was that the dissociation of naER appeared to be an energy dependent process. The involvement of a $\text{Ca}^{++}/\text{Mg}^{++}$ dependent ATPase in the process was evident. Enhancement of the ATPase activity was dependent on exposure of the membrane to estradiol and the activity was inhibited by the flavanoid, quercetin (Sreeja & Thampan, 2004 a).

11. Protein protein interactions during naER internalization following estradiol binding

The studies reported from our laboratory (Sreeja & Thampan, 2004 b) have indicated that the internalization of naER from the plasma, following estradiol binding to the receptor was a clathrin-coated vesicle (CCV)-mediated mechanism. A 55 kDa protein of the CCV, apparently carrying the internalization signal (Trowbridge et al., 1993) is the target protein for naER in CCV. The internalized naER interacts with a 58kDa nuclear transport protein, the actin binding p58, that recognizes the nuclear localization signal (NLS) on the receptor. Prior to recognizing p58, the site involved on the naER is bound by Hsp-90. Estradiol binding to naER promotes dissociation of Hsp-90 from the receptor (Anilkumar et al., 2010). Confocal microscopic studies presented in this study showed that in goat endometrial cells in culture exposure of the cells to estradiol resulted in the intracellular movement of both naER and Hsp-90. It was observed that both naER and Hsp-90 entered the nuclei within a matter of 3 hours following the exposure of the cells to estradiol. The functional significance of Hsp-90 in the nuclei remains to be known. naER is transformed into nuclear estrogen receptor II (nERII) within the nucleus. It is evident that this change in identity is associated with a distinct structural change in the protein. Possibly, this transformation that takes place within the nucleus is chaperoned by Hsp-90.

12. Nuclear estrogen receptor II (nERII)

Long before naER discovery became a reality, a nuclear receptor that was distinctly different from the classical estrogen receptors had come to my notice. It was observed that when uterine nuclei from ovariectomized rats were exposed to 10nM 3H-estradiol, at 30-37°C, the hormone-binding component moved out of the nuclei and reached the outer medium within a span of 5 minutes after hormonal exposure (Thampan, 1985; 1988). What became apparent in the subsequent studies was that the hormone was bound to a class of ribonucleoproteins (RNP) that moved out of the nuclei following exposure to estradiol. *In vivo* studies involving ovariectomized rats demonstrated that the RNP that moved out of the nuclei was found associated with cytoplasmic polysomes. The results gave a clear indication to the possibility that a new class of estrogen receptors existed whose primary functional role was in post-transcriptional control mechanisms like splicing, nucleocytoplasmic transport of RNP and the translation.

The subsequent studies reported by our group (Jacob et al., 2006) presented systematic observations on both naER and nERII and concluded that the latter was a transformed form of the former. The observed differences between the two proteins are being listed below (Table 1). The methods employed for purifying the two proteins were identical.

Going back to the observations related to E-RAF function (Thampan, 1989), it may be recalled that the estrogen receptor function detected in close proximity to rat uterine nuclear RNA polymerases displayed both naER and nERII characteristics with nERII representing the major share of this activity. The naER existed in dimerisation with E-RAF. Later reports by Karthikeyan and Thampan (1996) showed that nERII tyrosine phosphorylated three subunits of nuclear RNA polymerase II. A re-examination of the 1989 report (Thampan, 1989) will reveal that the naER/nERII interaction was not restricted to RNA polymerase II alone. There was very clear evidence to support the hypothesis that the receptor interacted with all four classes of nuclear RNA polymerases. Therefore, it may be speculated that nERII-mediated tyrosine phosphorylation involved specified subunits of all 4 categories of the enzyme.

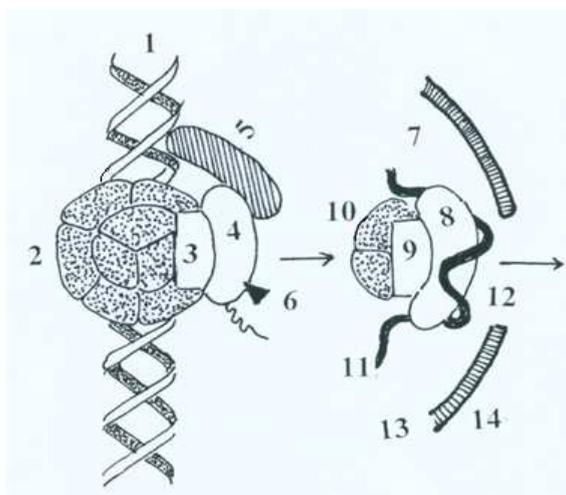
	naER	nERII
Sedimentation Value	4.6S	3.7S
Stokes radius	36A ⁰	23 A ⁰
Glycoprotein nature	Yes	No
Tyrosine kinase activity	Sensitive to exposure to estradiol	Insensitive to estradiol exposure
Dimerisation with E-RAF	Yes	No
Interaction with Hsp-90 in the presence of estradiol	No	Yes
nM estradiol needed for saturation binding	20	30

The factor responsible for this transformation was subsequently found to be a 61kDa protein, the naER transforming factor (Jaya & Thampan,2000).

Table 1. Comparison of molecular properties associated with naER and nER II of the goat uterus

13. Does tyrosine phosphorylation of a RNA polymerase subunit favour its dissociation from the core enzyme?

The two models presented here (figures 6 and 7) make an attempt to find an explanation for the observations mentioned above.



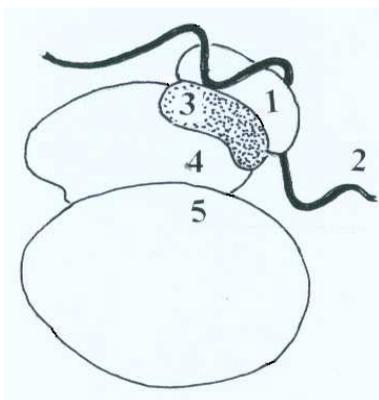
(1) Gene (2) RNA polymerase (3) RNA polymerase subunit that is recognized by naER (4) naER (5) E-RAF (6) estradiol-17 β (7) spliceosome (8) nERII (9) RNA polymerase subunit phosphorylated by nERII (10) other subunits dissociated from the RNA polymerase (11) RNA (12) Nuclear pore complex (13) Nucleus (14) cytoplasm.

Fig. 6. naER-nERII transformation during post transcriptional control of gene expression.

The figure on the left displays the interaction of naER-E-RAF heterodimer with genetic elements. While E-RAF recognizes the DNA, naER binds to nuclear RNA polymerase subunits. The figure on the right is a spliceosome -set -up in which the nERII -RNA complex is shown in association with subunits dissociated from RNA polymerase.

nERII is a RNA binding estrogen receptor. Whether naER-E-RAF heterodimer has its binding site on the target gene different from those of the ER α /ER β mediated gene regulation or whether the action of the heterodimer is independent of the classical estrogen receptor function remains to be clarified. The binding site on the estrogen responsive target gene for E-RAF-naER heterodimer has not yet been identified while there is every likelihood to suggest that it will be different from the estrogen responsive element(ERE).A candidate site could well be AP-1 site in view of an earlier observation that c-fos and E-RAF share immunological similarity. While E-RAF binds to the gene, naER interacts with the nuclear RNA polymerases. Possibly, the naER to nERII transformation could be an event that takes place at the end of the transcription process initiated by the heterodimer. At this stage, nERII dissociates from E-RAF and binds to the RNA (rRNA/mRNA/5S rRNA/tRNA). I wish to propose here that the phosphorylated subunits of the RNA polymerases might dissociate from the core enzyme and move along with nERII during the succeeding stages of gene regulation that witness splicing, nucleocytoplasmic transport and translation.

Sebastian and Thampan (2002 a,b)and Sebastian et al(2004) presented some fascinating observations in this context. Goat uterine nERII was found to be associated with ribonucleoproteins containing U-1 and U-2 snRNA's. Within the snRNP framework nERII interacted with three proteins with molecular masses 32kDa, 55kDa and 60kDa. While p55 and p60 were found to be RNA binding proteins, p32 was found to be involved only in protein-protein interactions with nERII. Whether this protein is the same as SC35 reported by Parnaik in the context of spliceosome assembly (Tripathi & Parnaik,2008) remains to be seen. It was interesting to observe that nERII in association with p32 and p55 formed an effective Ca⁺⁺/Mg⁺⁺ activated ATPase that appeared to be directly involved in the nucleocytoplasmic movement of RNP.



(1) nERII (2) rRNA (3) RNA polymerase I subunit(4)40S ribosomal subunit(5)60S ribosomal subunit.

Fig. 7. A hypothetical representation for the association of nERII with 40S ribosomal subunit.

The assumption is that nERII binding to subunits of RNA polymerase I, followed by nERII mediated tyrosine phosphorylation of those subunits results in the dissociation of the subunits from the enzyme along with the rRNA, remaining bound to nERII. The rRNA-nERII-RNA polymerase I subunit complex is shown here as forming an integral part of the 40S subunit of the ribosome.

I wish to speculate here that the subunits dissociated from the RNA polymerases following phosphorylation by nERII could continue their association with nERII and find their involvement in splicing reactions, nucleocytoplasmic transport of RNA and eventually, in translation. If this assumption is correct, future studies on ribosomal subunits should be able to confirm the presence of RNA polymerase I and III subunits in 40S ribosomes. It should also be possible to confirm whether the RNA polymerase II subunits are present in spliceosomes.

14. General conclusions and hypothetical possibilities

1. Discussions exclusively on the classical estrogen receptors, ER α and ER β will serve to uncover only limited information on the role of the receptors in regulating gene expression.
2. There is a distinct possibility that naER-E-RAF heterodimer has a parallel role to play in regulating transcriptional events as has been proposed for ER α and ER β .
3. nERII is undoubtedly the estrogen receptor that mediates post transcriptional events in gene expression in estrogen target cells.
4. The nERII-mediated events are related to gene expression protocols influenced by all 4 forms of nuclear RNA polymerases.
5. E-RAF targets both the nucleus and the mitochondria. Free E-RAF, progesterone-bound E-RAF and cholesterol bound E-RAF encounter distinct genes that are under regulatory influence. It might function as cholesterol transporter to the mitochondria and facilitate mitochondrial steroidogenesis leading to the production of progesterone. This is, in all possibility, a mechanism projected to take place during pregnancy.
6. The possibility exists that there is an "external" regulator of E-RAF gene expression and also that this regulatory agent is involved in the control of E-RAF gene expression in the pregnant uterus.
7. The possibility for a role for E-RAF in the progression of mammary cancer cannot be ruled out. Studies to be held in the near future are bound to enlighten this possibility.

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16. References

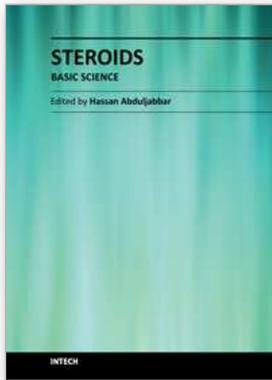
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This book explains the basic science of steroids and is targeted towards professionals engaged in health services. It should be noted that medical science evolves rapidly and some information like the understanding of steroids and their therapeutic use may change with new concepts quickly. Steroids are either naturally occurring or synthetic fat-soluble organic compounds. They are found in plants, animals, and fungi. They mediate a very diverse set of biological responses. The most widespread steroid in the body is cholesterol, an essential component of cell membranes, and the starting point for the synthesis of other steroids. Since the science of steroids has an enormous scope, we decided to put the clinical aspects of steroids in a different book titled "Steroids-Clinical Aspects". The two books complete each other. We hope that the reader will gain valuable information from both books and enrich their knowledge about this fascinating topic.

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