AN INSIGHT INTO THE FUNCTIONAL ROLE OF CHOLESTEROL AS AN INHIBITOR OF ESTROGEN ACTION.

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

By

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TO MY UNCLE AND MY PARENTS

DEPARTMENT OF BIOCHEMISTRY
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Dated: 18-12-1998

DECLARATION

1 hereby declare that the work presented in this thesis has been carried out by me under the supervision of Dr. R. V. Thampan, and has not been submitted for any degree or diploma in this University or any other University.

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CERTIFICATE

This is to certify that Mr.N. S. Imam, has carried out the research work embodied **in** the present thesis under my supervision and guidance for the full period subscribed under the Ph.D ordinance of this University. I recommend his thesis entitled **"An Insight Into The Functional Role Of Cholesterol As An Inhibitor Of Estrogen Action"** for the submission for the Degree of Doctor of Philosophy of this University.

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Imam

Abbreviations:

ATP		AdenosineTriPhosphate
CD	:	Circular Dichroism
CNBr	;	Cyanogen Bromide
DE-52		Diethylaminoethyl cellulose 52
DNA		Deoxynbonucleic Acid
EDTA		EthyleneDiamineTetraAceticAcid
ER	:	Estrogen Receptor
E-RAF		Estrogen Receptor Activation Factor
FITC		Fluorescein isothiocyanate
GC/MS	:	Gas Chromatography and Mass Spectrometry
MAP		Hydroxylapatite
HDL	:	High Density Lipoprotein
hERR 1		Human Estrogen Receptor Related Protein I
hERR 11		Human Estrogen Receptor Related protein II
Hsp 90	:	Heat Shock Protein 90
1DL		Intermediate Density Lipoprotein
1,DL		Low Density Lipoprotein
NaLiR		Non-Activated Estrogen Receptor
NBS	:	N-Bromosuccinimide
PEG	:	Polyethylene glycol
PMSF	:	Phenylmethylsulfonyl fluoride
SREBP	:	Sterol Regulatory Element Binding Protein
TLC	:	Thin Layer Chromatography
VLDL	:	Very Low Density Lipoprotein

CONTENTS

CHAPTER - I

INTRODUCTION

STEROID RECEPTOR MODULATORS

Steroid receptor 'activation' is a molecular mechanism that has received wide recognition by investigators working with different steroid receptor systems (Higgins et al, 1973; Bailly et al, 1980; Grody et al, 1982). The steroid receptor 'activation' is the term applied to the mechanism in which the receptors are transformed from a non-activated form that does not bind to the DNA to an activated form that can bind to DNA/nuclei. The receptors were found to get activated under high temperatures (20-37°C) and high salt concentration (0.4M KC1). Low molecular weight ligands inhibiting steroid hormone-receptor activation have been known for a long time. Milgrom and co-workers (1973) suggested that an inhibitor could be involved in receptor inactivation of glucocorticoid receptor. They observed that there was an enhanced nuclear binding of the glucocorticoid receptor after they partially purified the receptor from the cytosol (Milgrom et al., 1975).

Sato and co-workers (Sato et al., 1978, 1980) observed that a heat stable, low molecular weight and dialysable component endogenous to the mammalian uterus prevented the estrogen receptor activation process. They found that removal of this inhibitor by dialysis or by gel filtration enhanced the binding of ^{3}H-E_2-ER complex to the nuclei They also found that this inhibitor regulated the activation of the androgen and glucocorticoid receptors as well. During temperature-mediated activation of steroid receptors it is possible that high temperature causes dissociation of the inhibitor from the receptor.

Markaverich and colleagues identified a low molecular weight inhibitor of estradiol binding in rat uterine nuclei (Markaverich et al., 1983). This inhibitor did not

affect the type 1 binding sites. Dilution of uterine nuclear fractions from estrogen treated rats prior to the nuclear exchange assay resulted in a 3 to 4 fold increase in the measurable quantities of type II sites The number of type I sites was not affected by dilution The increase in type II sites following nuclear dilution occurred independent of protein dilution and it was observed to be due to the dilution of a specific endogenous inhibitor of estradiol binding to the sites The preliminary characterisation of this inhibitory activity resulted in the identification of two distinct activities, a and P, which displayed similar molecular weight and were heat stable. Clark and Markaverich (1982) postulated that this molecule represented an endogenous ligand for the nuclear type II sites and that the function of these binding sites is to bind the inhibitor instead of estradiol Probably this could be the reason for the lower measurement of the type II sites in the H-estradiol exchange assay. It was further found that the non-hyperplastic tissues (uterus, liver, lactating mammary gland) contained both a and (3 components of the inhibitor whereas the estrogen induced rat mammary tumours contained very low quantities of the (3 inhibitor activity. This endogenous ligand was found to regulate cell growth (Markaverich et al., 1987) and was characterised to be methyl p-hydroxyphenyllactate (MeHPLA). MeHPLA was shown to influence uterine cell growth and proliferation (Markaverich et al., 1988, 89, 90).

The involvement of a low-molecular weight inhibitor in glucocorticoid receptor (GR) activation in rat liver cytosol was also reported (Cake et al., 1976; Bailly **et** al., 1977). Bodine and Litwack (1988a and 1988b) purified and characterised this inhibitor and termed it as a 'modulator'. This modulator was found to be an 'ether amino phospho glyceride' which inhibits glucocorticoid receptor activation and stabilises **the** steroid

binding of the unoccupied glucocorticoid receptor. Exogenous sodium molybdate has also been shown to inhibit GR activation and stabilise the unoccupied GR (Leach et al., 1979; Dahmer et al., 1984). This suggested that the modulator was the endogenous factor that the sodium molybdate mimicked in the steroid receptor system. Bodine and Litwack (1988a) proposed that the modulator bound to the same site on the GR to which the molybdate was supposed to bind. The modulator was 100 times more potent than the molybdate. Further, large scale purification of the modulator (Bodine and Litwack, 1990) resolved the modulator into two isoforms, modulator-1 and modulator-2. These two modulators have similar chemical structures but differ in their molecular weights. Despite their structural similarity, the two modulators have different potencies for GR activation and receptor stabilisation. The action of these modulators is synergistic towards the occupied/unactivated receptor. These results suggest that the hormone occupied/unactivated receptor complex has two modulator binding sites, one site for each of the modulator isoforms. The unoccupied/unactivated receptor has only one modulator binding site. Bodine and Litwack (1990a) have proposed a hypothetical model for the interaction of modulator-1 and 2 with the GR. This model is based on the assumption that, like sodium molybdate, the modulator interacts directly with the receptor (Grandics et al., 1984; Schmidt et al., 1985). They proposed that the modulator-2 bound to the unoccupied receptor. Binding of the steroid to the receptor created a second modulator binding site, or increased the binding affinity of a pre-existing site, and that modulator-1 would then bind to this site. Once bound to the occupied/unactivated receptor, modulator-1 and 2 would act in a synergistic fashion to stabilise steroid binding and

inhibit activation. Finally, the dissociation of the two modulators and the hsp 90 would lead to receptor activation.

A second low-molecular weight inhibitor/steroid binding stabiliser was identified by Meshinchi et al., (1988, 1990). This modulator-like factor was also found in the rat liver and is believed to be a molybdenum containing component. This was shown to be important for the binding of hsp 90, to both occupied and unoccupied GRs (Meshinchi, et al., 1990).

In addition to the low-molecular weight inhibitors and modulators of steroid hormone action, macromolecular entities have also been shown to inhibit the action of steroid hormone receptors. A macromolecular inhibitor of the estrogen receptor was reported by Chamnes et al., (1974) and Nishizawa et al., (1981). Lerea et al., (1987) have characterised a factor present in bovine uterine cytosol that inhibits estrogen receptor binding to the nuclei *in vitro.* This heat stable macromolecule displays the properties of a heparin-like glucosaminoglycan.

The estrogen receptor activation process has been studied in our laboratory, in a totally different perspective. The ER under study is not the well-recognised receptor that binds as a heterodimer to the estrogen responsive element in the target gene. This alternate ER of our interest, non-activated estrogen receptor (naER) is not a DNA binding protein. It gains access to the DNA only after it dimerizes with the estrogen receptor activation factor (E-RAF), a DNA binding protein whose binding domain on the estrogen responsive gene still remains unresolved. The 'receptor activation' in our studies is, therefore the dimerization between the naER and E-RAF.

During the early attempts to purify goat uterine E-RAF, it was observed that an endogenous inhibitor was co-purified along with the E-RAF, which inhibited the E-RAF binding to DNA (Thampan, 1987 and unpublished data). It was essential, during the purification of E-RAF, to introduce a dialysis step without which the DNA binding of the protein remained totally undetected. It was therefore decided to isolate and identify this inhibitor from the goat uterus so that the material could be made available as yet another tool to study the mechanisms of action of estradiol in mammalian target organs.

THE ESTROGEN ACTION

The estrogen, estradiol-17β, has long been recognised as the primary hormone involved in the development and maintenance of the female sex organs, mammary glands, and other estrogen-responsive tissues. More recently their involvement in the growth and/or function of a number of other tissues, such as the skeleton, the cardiovascular system and the central nervous system, in both males and females, has been recognised. The decreased ovarian production of estrogens which occurs after climacteric has been linked to a number of pathological conditions including osteoporosis, coronary artery disease, depression and Alzheimer's disease (Grese and Dodge, 1996).

There is a large and increasing body of experimental and clinical data supporting the existence of variant estrogen receptor (ER) proteins in both normal and neoplastic estrogen target tissues, including human breast. Therefore, future examination of ER protein in pathogenesis must take into account the co-expression of the wild type ER with

multiple ER variants in any one tissue sample as well as the relative expression of each with respect to others (Murphy et al., 1997)

The mechanism of steroid hormone action has been subjected to extensive study in several leading laboratories. Nevertheless, a lot needs to be known regarding the basic molecular details that underlie hormone action. A special emphasis is given to the mode of action of estrogens in this thesis.

MECHANISM OF ESTROGEN ACTION

Steroid hormones manifest their biological actions by binding to receptive proteins called receptors. The concept of steroid receptor activation developed along with the initial studies on the identification of estrogen receptor (ER) in the hormone target organs (Shyamala and Gorski, 1969; Mohla et al, 1972; Jensen and De Sombre, 1973; Walters, 1985). Sucrose density gradient analysis of the uterine estrogen receptor-^{3}H-estradiol-complex showed the presence of two species of cytosolic estrogen receptors, a 8S form in low salt gradients and a 4S species in high salt gradients. The rapid transformation of the inactive 4S form to the active 5S form was found to occur as a consequence of hormone binding to the receptors. The process of transformation of the inactive estrogen receptor to the active estrogen receptor has been termed as 'estrogen receptor activation' (Grody et al, 1982).

The hormone free ER exists as a heteromeric complex with heat shock proteins and possibly other proteins as well (Csermely, et al, 1998). When the hormone binds to the receptor, the receptor dissociates from the complex and forms a homodimer (5S) which binds to the DNA. This occurs at specific binding sites in the enhancer region of

the estrogen responsive gene called the estrogen response elements (ERE) (Beato, 1989). The ERE is a palindromic pair of hexameric half sites AGGTC AnnnTGATCT (Green and Chambon , 1987). What happens to the ER after this step, is not clearly understood. Thampan (1985, '88) observed that estradiol stimulated nuclear ribonucleoprotein transport in the rat uterus and postulated that the ER, after its stimulation of transcription, formed a constituent of the heterogenous nuclear ribonucleoprotein (hnRNP) complex.

STRUCTURE OF THE ESTROGEN RECEPTOR:

The estrogen receptor gene has been cloned, sequenced and the predicted amino acid sequence determined. The estrogen receptor has 595 amino acids and a molecular mass of 66kDa. Based on the amino acid sequence homology between the human ER (hER) and chicken ER (cER), the ER can be divided into six regions A, B, C, D, E and F (Kumar et al, 1987). The region 'A' is conserved upto 87% between cER and hER and is required for activation of transcription. The regions 'C (100%) and 'E' (94%) are highly conserved. The region 'C' is the DNA binding domain and is hydrophilic containing high amounts of cysteine, lysine and arginine. These residues are required for DNA binding. Deletion of this region results in the loss of the DNA binding capacity of the receptor (Kumar et al., 1986). Attachment of the receptor to the major groove of the DNA is thought to be achieved via a projection from the DNA binding domain called as the 'zinc finger'. These are generated by the co-ordination of zinc ions with four cysteine residues (Green and Chambon, 1987). Steroid receptors have two apparently unrelated zinc fingers encoded by separate exons (Huckaby et al, 1987) with four conserved cysteine residues. The ER molecule has 2 zinc fingers in the 'C region which is analogous to the

xenopus transcription factor TF IIIA (Hartshorne et al, 1986). When hER's region C was replaced with that of the human glucocorticoid receptor (hGR), the hybrid was shown to activate the glucocoriticoid responsive gene (Green and Chambon, 1987), suggesting that the region C determined the target gene specificity. The region E is hydrophobic in nature and is the hormone binding domain (Kumar et al., 1986). A mutant of hER (HE15), which lacks the hormone binding domain, binds to DNA but activates transcription poorly (Kumar et al, 1986, 1987). The regions B, D and F are not well conserved suggesting that their integrity is less important for ER function (Green and Chambon, 1986; Green et al., 1986 and Krust et al., 1986).

The region D has 37 amino acids and functions as a hinge region between the DNA binding and the hormone binding domains. Deletion and insertion experiments revealed no loss of receptor function (Kumar et al, 1988). This region harbours the nuclear localization signal (NLS) which is essential for the transport of ER into the nucleus.

THE CELLULAR LOCALIZATION OF THE ESTROGEN RECEPTOR:

The intracellular localization of the estrogen receptor and other steroid receptors in the cell has been examined in considerable detail. A general agreement has been reached regarding the intracellular localization of the estrogen receptors that the ER is primarily localized in the nucleus. Two other models proposed in this context (a and c, mentioned below) have not withstood the test of time.

a) Two step model: This model proposes that the receptor is cytoplasmic in localization and only upon binding the hormone, it enters the nucleus (Gorski et al., 1968; Jensen et al., 1968).

b) One step model: This model proposes that steroid receptors are present exclusively in the nucleus. Enucleation studies and immunolocalization studies have demonstrated exclusive nuclear localization of ER, progesterone receptor, and thyroid hormone receptor (King and Greene, 1984; McClellan et al., 1984; Welshons et al., 1984 and Perrot et al., 1985). Brink et al (1992) demonstrated that the glucocorticoid receptor is also exclusively nuclear. Raam et al (1988), however, questioned the validity of the methods employed by these workers and suggested that the results they had observed were artifacts of the fixation procedures employed.

c) **Equilibrium affinity model:** The equilibrium model proposed by Sheridian et al (1979) suggested that the hormone free receptors remain in equilibrium partitioned between the nucleus and the cytoplasm according to the free water content of these intracellular components.

NUCLEAR TRANSPORT OF ESTROGEN RECEPTOR:

The ER is synthesized in the cytoplasm and its site of action is in the nucleus. Welshons et al (1984) and King and Greene (1984) proposed that unliganded estrogen receptors are localized exclusively in the target cell nucleus. A potential nuclear localization signal (NLS) of human ER, which is necessary for its transport to the nucleus, was identified in the region of amino acids 256-303. Deletion of this region resulted in the retention of the ER in the cytoplasm. A fusion protein between

β-galactosidase and human ER 256-303 amino acids has been demonstrated to be transported to the nucleus (Picard et al, 1990). Ylikomi et al (1992) reported the identification of three constitutive and one estrogen-inducible nuclear localization signal in the human ER and these were termed as proto-NLSs (pNLSs). It was observed that co-operation of these signals was required for the nuclear accumulation of ERs. These studies suggested that ER translocation from the cytoplasm to the nucleus is NLS-dependent whether it is hormone inducible or not.

A NLS-binding protein which aids in the transport of ER was purified, characterized and its role in ER transport studied by Nirmala and Thampan (1995a and b). The ER NLSBP is a 55kDa protein which strongly binds to a lysine-rich region of ER. It also binds to the cytoskeletal elements, actin and tubulin, and also to the heterologous NLS of SV40 large T antigen.

Based on the observations made by Nirmala and Thampan, the mechanism of transport of ER into the nucleus can be divided into two steps: i) the p55-mediated transport and binding of ER to the nuclear membrane and (ii) An ATP-dependent, 12-14kDa protein(s)-mediated translocation of ER into nucleus. The inherent ATPase function of ER NLSBP generates energy from ATP hydrolysis that is presumably utilised in the nuclear transport of the ER.

HALF LIFE OF ESTROGEN RECEPTOR AND ITS DEGRADATION BY UBIQUITIN PATHWAY:

The concentration of transcriptional regulators like the steroid receptors has to be regulated in the cell. How these receptors are timed and degraded is not known. The half life of the mammalian estrogen receptor in the absence of hormone is approximately 5

days. In the presence of estradiol, the ER levels fall within an hour. The normal levels of **ER** are reached within 4-6 hours that can be blocked by actinomycin D or cycloheximide (Sarff and Gorski, 1971). Thus replenishment of receptors in the cytosol is contributed mainly by *de novo* synthesis of the receptors. Only 10% of the replenished receptors are contributed by recycling of the receptors that have moved out of the nucleus (Horwitz et al, 1983). Thus, the estrogen receptor has to be recognised and degraded selectively. The only known mechanism for selective degradation of proteins is the ubiquitin pathway. Ubiquitination is a post-translational modification wherein the carboxy terminus of ubiquitin is covalently ligated to the e-amino lysine of the protein substrate (Hershko and Ciechenover, 1998). The ubiquitination of proteins selectively targets them for degradation by 26S proteasome (Jentsch and Schlenker, 1995).

Nirmala and Thampan (1995c) showed that the estrogen receptor of the rat uterus is degraded by ubiquitin pathway. They showed that it is estrogen specific and also that estrogen enhanced ER ubiquitination.

ALTERNATE FORMS OF ESTROGEN RECEPTORS:

The non-activated estrogen receptor (naER):

The non-activated estrogen receptor (naER) is different from the classical ER (a) which has been discussed so far and also proteins bearing similar nomenclature in the literature (Sica et al., 1976; Atrache et al., 1985; Redenilh et al., 1987). The naER does not get activated during salt dependent or temperature dependent reactions. It gains access to the DNA after reaching the nucleus only after dimerization with **a** DNA-binding protein, the estrogen receptor activation factor (E-RAF). In short, it is a never activated

receptor unless it dimerizes with E-RAF. The regular receptor (ERα), on the contrary fails to dimerize with the E-RAF (Thampan, 1987).

Physical characteristics of the naER were compared with those of the regular ER. It has a molecular mass of 66kDa, sediments at 4.2S and has a Stokes radius of 36A°. CNBr peptide map analysis revealed that the primary structure of naER was distinctly different from that of the regular ER (Anuradha et al., 1994). The naER was shown to be a tyrosine kinase. Estradiol inhibited the tyrosine kinase activity of naER which could be reversed by tamoxifen, an antiestrogen. naER is also a glycoprotein. Karthikeyan and Thampan (1996) observed that there was an increase in the net yield of naER when the tissue was homogenized in a medium containing Triton X-100. This observation and the assumption that, being a tyrosine kinase, the naER could be grouped into a family of proteins that represents growth factors and oncoproteins, prompted them to examine whether naER is a plasma membrane constituent. Their study generated positive results.

Exposure of purified plasma membrane preparations to estradiol caused dissociation of naER from the membrane into the medium. This receptor movement took place in the presence of diethylstilbestrol while testosterone, progesterone, dexamethasone and tamoxifen did not facilitate the dissociation of the naER from plasma membrane. Tamoxifen also inhibited the estradiol mediated release of naER from plasma membrane. All the properties of naER purified from the plasma membrane matched with those of the naER purified from the cytosol of the goat uterus.

The nuclear estrogen receptor II is the deglycosylated version of naER:

Karthikeyan and Thampan (1995) compared the structural and functional characteristics of the goat uterine nuclear estrogen receptor II with those of the naER

purified from the cytosol. Both the proteins have 66kDa molecular mass and display identical CNBr peptide maps. Both are recognised by antiER IgG. Both the proteins are tyrosine kinases. Unlike the naER, the ER-II is incapable of dimerization with the estrogen receptor activation factor (E-RAF). The naER is a glycoprotein but ER-II is not. ER-II has a higher capacity to bind estradiol but with reduced affinity compared to naER. The sedimentation behavior and Stokes radius of ER-II were different from those of the naER. While the naER displayed a Stokes radius of 36A°, the ER-lI showed a value of 22A° for its Stokes radius. While naER showed a sedimentation value of 4.2S, the ER-II sedimented at 3.8S. The ER-II was shown to have a special affinity to bind to RNA polymerases.

Estrogen Receptor β(ER P):

Kuiper et al (1996) and Mosselman et al (1996) identified a cDNA clone encoding an estrogen receptor different from the regular estrogen receptor. This receptor has been named as ER (3 to differentiate it from the regular ER (ER a). The human and rat ER β clones were identified in cDNA libraries from testis and rat prostate, the tissues not generally considered to be major estrogen target tissues. Similar cDNA for ER p was also found in mouse tissues (Couse et al., 1997).

The rat ER p cDNA encodes a protein of 485 aminoacid residues with a molecular weight of 54200. Rat ER β is highly homologous to rat ER a particularly in the DNA binding domain (>90% amino acid identity) and in the C-terminal ligand binding domain (55% identity). Saturation ligand binding experiments with *in vitro* synthesized ER p protein revealed a single binding component for 17p-estradiol with high affinity (dissociation constant, Kd = 0.6nM) (Kuiper et al., 1997).

Using RT-PCR on RNA from a variety of tissues in the rat, the relative levels of ERp and ERa RNA were compared. Some tissues (such as kidney) contain exclusively ERa while other tissues show a great predominance of ERa (i.e., uterus, pituitary, epididymis). Other tissues show equal or greater levels of ERβ RNA (i.e., ovary, prostate). In brain, ERβ may play a significant role in estrogen action in brain, ovary, prostate and possibly in other tissues (Kuiper, 1997). *In situ* hybridization with cDNA probes to ERP indicate a cell-specific localization in ovary and prostate (Kuiper, 1996). Future studies on various aspects of ERp will throw light on functional significance of ERβ.

PUTATIVE ER mRNAs:

ER like mRNAs distinct from the wild-type ER mRNA have been identified in many known ER positive tissues and cell lines. Some of these have been cloned and characterized from cDNAs representing close to full length transcripts. But in most cases their overall structure has been predicted from more limited sequences derived from reverse transcribed and polymerase chain reaction amplified (RT-PCR) products. Some of the various putative ER cDNAs are D3-ER (61kDa), D5-ER (40kDa), D7-ER (51kDa), D3-4-ER (49kDa), D2-3-7ER (18kDa), D4/7-ER (51kDa), D3-4-ER (34kDa), clone 4-ER (24kDa), exon $(34)^2$ ER (75kDa), ER 69bp (69kDa). The estimated molecular masses in kilodaltons have been given in the brackets. These ER cDNAs have been found to be present in normal and tumorous breast, ovary, uterus, bone, pituitary in humans and rats (Murphy et al., 1997)

PROTEIN-PROTEIN INTERACTIONS INVOLVING THE ESTROGEN RECEPTORS

Steroid receptors are associated with several proteins without which their actions will be incomplete. Some of these proteins are described below:

Estrogen Receptor Activation Factor (E-RAF):

During the seventies, results from various laboratories (Notides and Nielson, 1974; Thrower et al., 1976; Yamamoto, 1974; Yamamoto and Alberts, 1975) suggested that a DNA binding protein which is incapable of binding estradiol possessed the ability to convert the 4S estrogen receptor to the 5S receptor form. Thampan and Clark (1981) gave direct experimental evidence for the existence of this protein in the rat uterine cytosol. This protein was designated as the estrogen receptor activation factor (E-RAF) and was subsequently purified to homogeneity from the goat uterine cytosol (Thampan, 1987). E-RAF is a 66kDa protein and it has DNA-dependent ATPase activity and demonstrates a capacity to introduce structural changes in the DNA. The E-RAF can be resolved into three molecular species, I, HA and IIB by gel filtration. The E-RAF I has a DNA helix stabilizing function and E-RAF IIA and IIB possess DNA helix destabilizing capacity (Thampan, 1989). The E-RAFs share common immunological domains with both ER and naER. E-RAFs I and II are different from naER and ER as revealed by peptide maps (Zafar and Thampan, 1993). The E-RAF is a serine kinase (Anuradha et al., 1994).

Structural characterization of E-RAF was carried out by using an endogenous calcium activated neutral protease (CANP). CANP cleaved E-RAF into two near equal-sized fragments, **a** and P, of molecular masses 32kDa and 30kDa respectively. The

E-RAF a subunit retained the DNA binding domain and naER dimerization domain (Kumar, 1994).

Heat Shock protein 90 (hsp 90):

Heat shock protein 90 is a highly conserved, ubiquitous and abundant protein that is associated with all the steroid receptors (Baulieu, 1987; Pratt. 1990; Pratt et al., 1992). Such an association has been conserved throughout the evolution of eukaryotes from fungi to the highly evolved mammals (Joab et al., 1984). hsp 90 exists as a component in the 9S non-activated steroid receptor complexes in a stoichiometric ratio of 2:1 (2hsp:lSR). hsp90 crosslinks actin filaments (Koyasu et al., 1986; Nishida et al., 1986) and thus the steroid receptors bind to the actin filaments via hsp 90 (Miyata and Yahara, 1991). The non-transformed estrogen receptor complexes can be reconstituted with hsp 90 and the ER (Inano et al., 1990). hsp 90 binding of ER prevents it from binding to the DNA and at the same time maintains the receptor in a conformation required for hormone binding (Bresnick et al., 1989). The direct evidence for this came from the studies of Picard et al (1990) who have shown that mutants having low levels of hsp 90 but normal levels of steroid receptors are not responsive to the steroid as the receptors do not bind the hormone efficiently.

p59:

p59 is associated with all the non-activated steroid receptor complexes and is associated with hsp-90 (Tai et al., 1986; Renoir et al., 1990). In the non-activated steroid receptor complex, two molecules of hsp 90 and one molecule of the steroid receptor are associated with one molecule of p59 (Tai et al., 1993). As p59 is associated with all the

steroid receptors in all the tissues, it must be performing an as yet unidentified but important function.

Heat shock protein 70:

This protein has been shown to be a component of steroid hormone receptor complexes. This protein also associates itself with tumor-supressor proteins like p53 and pRb The functions include protein folding and assembly, mediation of protein translocation across membranes in mitochondria, chloroplasts and endoplasmic reticulum (Rassow, et al., 1997).

Estrogen receptor nuclear localization signal binding protein (p55):

This is a 55kDa protein that is essential for the transport ofER into the nucleus (Nirmala and Thampan, 1995a and b). The details of this protein have been discussed in the section 'nuclear transport of ER'.

p29:

This protein is associated with ER under certain 'activating' conditions. It is qualitatively and quantitatively related to ER but not other steroid hormone receptors. Immunohistochemical assays for p29 show that p29 is atleast as effective as ER in predicting clinical response of patients to hormone therapy (Coffer and King, 1988).

Ubiquitin:

Ubiquitin is a 8.5 kDa protein with 76 amino acids. It is a highly conserved protein in all the eukaryotes right from the yeast to the mammals (Goldstein et al., 1975). Ubiquitin is a marker for protein degradation by 26S proteasome. The tertiary structure determined by X-ray crystallography and 2D NMR revealed that human ubiquitin is highly compact and tightly bonded. The ubiquitin moieties of the ubiquitin-protein

conjugates are believed to be recycled when the target protein is degraded by the ubiquitin-dependent protease. The rigid structure may serve to reduce its susceptibility to degradation (Kumar et al., 1987, Weber et al., 1987). Ubiquitin has two functional sites. A C-terminal site through which ubiquitin is ligated to the e amino group of the lysine residues in the acceptor proteins. A second site is the lys-48 of ubiquitin. This residue can serve as an acceptor site for ubiquitination. Evidence is gathering that ubiquitin has a role to play in nuclear transport of proteins (Johnson and Hochstrasser, 1997).

RECEPTOR HETERODIMERS:

The naER which has no capacity to bind to nuclei on its own, does so by dimerizing with a DNA binding protein, the estrogen receptor activation factor (E-RAF). This heterodimer may exist as a system parallel to the regular ER homodimers in the mammalian uterus. The naER is a tyrosine kinase and the E-RAF is a serine kinase. When the proteins dimerize, their protein kinase function is suppressed. Therefore, these proteins can phosphorylate only when they are separate (Anuradha et al., 1994).

The receptor heterodimers have been identified in other steroid hormone systems like those of thyroid hormone, retinoic acid and vitamin D_3 and also in the viral oncogene products fos and jun which act as transcription factors. The thyroid hormone receptor (TR) isoforms TRα and TRP bind to specific DNA sequences called thyroid hormone responsive elements (TRE) as monomers and dimers. TR binding to TRE can be enhanced by heterodimerization with thyroid hormone receptor auxiliary proteins (TRAP) (Darling et al., 1991). Retinoic acid receptors (RAR) and retinoic-X-receptor (RXR) also heterodimerize with thyroid receptor and enhance the binding of the TR to

TRE (Zhang, et al., 1992). The binding of RAR to its responsive element requires a co-regulator called as RXRβ, that increases the DNA binding capacity and the transcriptional activity of RAR. (Yu et al, 1991). Vitamin D_3 binding to its responsive element requires a nuclear protein called nuclear accessory protein which has a molecular mass of 59-64kDa (Ross, et al., 1992). fos and jun, the products of nuclear proto-oncogenes form a non-covalent heterodimeric association (Sassone-Corsi, et al., 1988).

CHOLESTEROL METABOLISM:

Cholesterol has never ceased to attract the attention of scientists from the most diverse areas of science and medicine, ever since it was first isolated from gall stones in 1784. This is because of its unique structure, complexity of its synthesis, its essential role in membranes of animal cells, being the precursor for steroid hormones and bile acids and of being the cause for many a disease particularly atherosclerosis. The research on cholesterol is of paramount significance. The fact that thirteen noble prizes were awarded to scientists who revealed some of the mysteries shrouding cholesterol is an evidence for this phenomenon (Brown and Goldstein, 1986).

CHOLESTEROL BIOSYNTHESIS:

The biosynthesis of cholesterol is a complex pathway. The synthesis of cholesterol takes place in the cytosol and endoplasmic reticulum of the liver cells. It is interesting to note that all the 27 carbons of cholesterol are derived from the two carbon precursor acetate. The entire pathway can be broadly divided into three distinct processes: (1) conversion of acetate to a C_6 isoprenoid precursor, mevalonate; (2)

conversion of 6 moles of the C_6 mevalonate, via activated C_5 intermediates, to the C_{30} squalene; (3) cyclization of squalene and its transformation to the C_{27} cholesterol. The major intermediate compounds are 3-hydroxy-3-methylglutaryl-CoA, mevalonate, 5-phosphomevalonate, 5-pyrophosphomevalonate, isopentenylpyrophosphate, dimethyl allyl pyrophosphate, geranyl pyrophophate, farnesyl pyrophosphate, presqualene pyrophosphate, squalene, squalene 2, 3-epoxide, lanosterol and 7-dehydrocholesterol. The pathway's major control point is at HMG Co A reductase. This enzyme is regulated by competitive and allosteric mechanisms, phosphorylation/dephosphorylation, and long term control of the rates of enzyme synthesis and degradation (Bloch, 1965; Rilling and Chayet, 1985).

CHOLESTEROL TRANSPORT:

Cholesterol synthesized by the liver is either converted to bile acids for use in digestive processes or esterified by acyl-CoA: cholesterol acyltransferase (ACAT) to form cholesteryl esters which are secreted into the blood stream as part of the lipoprotein complexes. These pathways were delineated in 1950s and 60s through experimental work in many laboratories most notably that of Oncley, (1956), Gofman (1954) and Fredrickson (1974). The five broad classes of plasma lipoprotein are very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), high density lipoprotein (HDL) and chylomicrons. These are globular micelle like particles that consist a non-polar core of triacylglycerols and cholesteryl esters surrounded by an amphiphilic coating of protein, phospholipid and cholesterol.

Chylomicrons:

Chylomicrons transport exogenous (dietary) triacylglycerol and cholesteryl esters from the intestine to the tissues. These have the apoproteins A-I, A-II, B-48, C-I, C-II, C-III, E, have density of less than 0.95g.cm^{-3} and a particle diameter of 8OO-5OOOA^{0}.

Very **low density lipoproteins (VLDL), Intermediate density lipoproteins (IDL), and** Low **density Lipoproteins (LDL):**

These are a group of related particles that transport endogenous triacylglycerols and cholesterol from the liver to the tissues. During circulation, VLDLs, apolipoproteins and triacylglycerols are removed in the capillaries of muscle and adipose tissue, sequentially converting the VLDL to IDL and then to LDL. The VLDL have apoproteins B-100, C-I, C-II, C-III and E. The VLDL has a density of 0.95 to 1.006g/cm^{-3} and a diameter of 300 to 800A°. The IDL has apoproteins B-100, C-III and a density of 1.006-1.010 g/cm^{-3}. The diameter of IDL is in the range of 250-350A^{0}. The LDL has a density of 1.019 to 1.063g/cm^{-3} and a diameter of 180-280A°. LDL is the major cholesterol carrier of the blood stream. This spheroidal particle consists of some 1500 cholesteryl ester molecules surrounded by an amphiphilic coat of 800 phospholipid molecules, 500 cholesterol molecules and one 550kDa molecule of apolipoprotein B-100 (Brown and Goldstein, 1984).

High density lipoprotein (HDL):

HDL has essentially the opposite function of LDL, it removes cholesterol from the tissues. It has apoproteins A-I, A-II, C-I, C-II, C-III, D, and E, a density of 1.063 to 1.210gm/cm^{-3}, and a particle diametre of 50 to 120A°. HDL is assembled in the plasma from components largely obtained through the degradation of other lipoproteins. Circulating HDL probably acquires its cholesterol by extracting it from cell surface

membranes and converts it to cholesteryl esters through the action of lecithin:cholesterol acyltransferase (LCAT), an enzyme that is activated by HDL component apo A-I. HDL therefore functions as a cholesterol scavenger. (Brown and Goldstein, 1987). The levels of plasma HDL are inversely related to the incidence of atherosclerosis and coronary artery disease. The protective effect of HDL is thought to involve the reverse transport of cholesterol from cells of the arterial wall to the liver for disposal. Receptors for HDL have been found which are called as scavenger receptors (Acton et al., 1996).

Cells take up cholesterol through receptor mediated endocytosis of LDL:

Brown and Goldstein (1986) have demonstrated that cells obtain exogenous cholesterol mainly through the receptor mediated endocytosis of LDL particles. The LDL is sequestered by LDL receptor, a cell surface transmembrane glycoprotein which specifically binds both apo B-100 and apo-E. LDL receptors cluster into coated pits which serve to gather the cell surface receptors that are destined for endocytosis while excluding other cell-surface proteins. The coated pits which have clathrin backing, invaginate into plasma membrane to form coated vesicles that subsequently fuse with lysosomes. In lysosomes, the apoB is rapidly degraded to component aminoacids and the cholesteryl esters are hydrolysed by a lysosomal lipase to yield cholesterol which is subsequently incorporated into cell membranes and steroid hormones.

Intracellular Cholesterol Transport:

The intracellular cholesterol transport has caught the attention of many scientists. Many interesting observations about the intracellular transport are coming to light. Caveolae are small invaginations in the plasma membrane and are involved in cholesterol efflux from the cell (Fielding and Fielding, 1995). Caveolae are enriched in cholesterol

binding proteins called caveolins which shuttle between caveolae and Golgi complex. Caveolins transfer cholesterol to apolipoprotein A-I (Conrad et al., 1995). Steroidogenic acute regulatory protein (StAR) transports cholesterol to inner mitochondrial membrane (Clark et al, 1994).

THE PROTEINS THAT REGULATE CHOLESTEROL HOMEOSTASIS

Hydroxymethylglutaryl-CoA (HMGCoA) reductase:

HMGCoA reductase catalyzes the conversion of HMGCoA to mevalonate. It is the rate limiting enzyme in cholesterol biosynthesis and has a molecular mass of 97,072 with 887 amino acids. This protein is divided into two domains. The first domain, the NH_2-terminal (35,000 Daltons) is extremely hydrophobic in character and it traverses the endoplasmic reticulum membrane. The Nth-terminal is located in the lumen of the endoplasmic reticulum. The second domain has a molecular mass of 62,000 Daltons, has water soluble amino acids and projects into the cytoplasm. This domain carries the catalytic site of the enzyme. HMGCoA reductase is synthesized on membrane bound ribosomes (Goldstein and Brown, 1984).

Low Density Lipoprotein Receptor:

This protein was purified by Schneider et al (1982). Full length cDNA for the human LDL receptor was obtained by Russel et al (1983). The mature receptor without the signal peptide (21 amino acids) has 839 amino acids. The LDL receptor can be divided into 5 domains. The first domain consists of NH2-terminal 292 amino acids which is composed of 40 amino acids that is repeated 8 times with some variation. Each of the 40 amino acid repeats contain 6 cysteine residues, which are in register for all of

the repeats. These repeats contain a cluster of negatively charged amino acids. The two ligands of LDL receptor-apoB and apoE contain positively charged lysine and arginine. Orientation of this domain is made outside of the cell membrane. The second domain of the LDL receptor consists of ~350 amino acids that bear a strong homology with the polyprotein precursor epidermal growth factor (EGF). The third domain consists a stretch of 48 amino acids, 18 of which are serine and threonine residues which contain carbohydrate molecules attached in O-glycosidic linkage. This region is located immediately external to the plasma membrane. The fourth domain consists a membrane spanning region of 22 amino acids. The fifth domain is the cytoplasmic tail and has 50 aminoacids. These sequences bind to clathrin and is therefore involved in receptor internalization (Goldstein and Brown, 1984).

Sterol regulatory element binding protein (SREBP):

Animal cells must regulate their biosynthetic pathways so as to produce the required amount of end-products without risking overproduction. Such control is particularly important in cholesterol homeostasis because cholesterol must be supplied for many cellular functions. Excess cholesterol must be avoided because it forms solid crystals that kill cells. Excess cholesterol in bloodstream is also lethal because it deposits in arteries, initiating atherosclerosis (Small and Shipley, 1974). Cholesterol presents a special problem because it is an insoluble lipid that resides almost exclusively in cell membranes How does the cell sense the level of a membrane-embedded lipid and how is that information transmitted to the nucleus to regulate transcription? Answers are emerging from the studies of a family of membrane bound transcription factors called sterol regulatory element binding proteins (SREBPs) that regulate multiple genes of

HMGCoA reductase, HMGCoA synthase, farnesyl diphosphate synthase and squalene synthase by means of a proteolytic cascade (Goldstein and Brown, 1990; Osborne, 1995; Guan, et al., 1995; Ericsson, et al., 1996; Brown and Goldstein, 1997).

The SREBPs also regulate the synthesis of LDL receptor which supplies cholesterol through receptor mediated endocytosis. The SREBPs were found to modulate transcription of genes encoding enzymes of fatty acid synthesis including acteyl CoA carboxylase, fatty acid synthase, stearoyl coA desaturase-1 and lipoprotein lipase (Tontonoz et al., 1993; Kim and Spiegelman, 1996; Lopez et al., 1996; Magana and Osborne, 1996; Shimano, et al., 1996). Thus, SREBPs co-ordinate the synthesis of the two major building blocks of membranes, fatty acids and cholesterol.

The SREBPs were purified from nuclear extracts of cultured HeLa cells (Wang et al, 1993). Three members of the SREBP family have been identified by cDNA cloning (Yokoyama et al., 1993; Hua et al., 1993).

Each SREBP shares a similar tripartite structure consisting : (1) an NH_2-terminal transcription factor domain of ~480 amino acids; (2) a middle hydrophobic region of ~80 amino acids containing two hydrophobic transmembrane segments and (3) COOH-terminal regulatory domain of ~590 amino acids.

In humans, hamsters and mice two members of the SREBP family designated SREBP-1a and SREBP-1c are produced from single gene (human chromosomal location 17pl 1.2). The mRNAs for human SREBP-1a can also undergo alternative splicing at the 3' ends to produce proteins that differ in the last 113 amino acids (Yokoyama et al., 1993; Hua, et al., 1995).

The third member of the SREBP family, designated as SREBP-2 is encoded by a separate gene on chromosome 22ql3 (Hua, et al., 1993, Miserez, et al., 1997).

In order to influence transcription, the amino-terminal domain of SREBPs should be released from the membrane so that it can enter the nucleus. The release is accomplished by a two step proteolytic cascade that is regulated by sterols. In sterol-depleted cells, the cascade is initiated by the protease that clips SREBPs at a site in the middle of their lumenal loop. The cleavage at this site breaks the covalent bond between the two transmembrane domains of SREBPs but both the halves of the protein still remain attached to the membranes. A second protease breaks the site in the amino-terminal which appears to reside in the transmembrane region. This releases the mature amino-terminal domain into the cytosol, from which it rapidly enters the nucleus as a homodimer and binds to the sterol regulatory elements in the promoter regions of various genes of the proteins that are involved in the synthesis and uptake of cholesterol and synthesis of fatty acids. The recognition site in the LDL receptor gene is the sequence '5'-ATCACCCCAC-3' which is designated as sterol regulatory element-1 (SRE-1). The SREBPs are 125kDa proteins and after the cleavage, the 66kDa fragments are released. The cleavage at the luminal loop is tightly regulated by sterols (Brown and Goldstein, 1997).

CHOLESTEROL AND DISEASE:

The defects in the cholesterol metabolism are the cause for many diseases in humans. Some of the diseases are discussed below.

Atherosclerosis:

This is the most common form of arteriosclerosis (hardening of arteries) and is characterized by the presence of atheromas (arterial thickenings) which have a yellow deposit of pure cholesteryl esters. Atherosclerosis is a progressive disease as intracellular lipid deposits in the smooth muscle cells of the inner arterial wall. These lesions eventually become fibrous, calcified plaques that narrow and even block the arteries. The resultant roughening of the arterial wall promotes the formation of blood clots which may also occlude the artery. A blood flow stoppage called as infarction, causes the death of deprived tissues. The atheromas in coronary artery cause 'myocardial infarctions' or 'heart attacks' (Small and Shipley, 1974).

The development of atherosclerosis is strongly correlated with the level of plasma cholesterol. This is particularly evident in individuals with familial hypercholesterolemia (FH). Homozygotes with this inherited disorder have such high levels of cholesterol-rich LDL in their plasma that their plasma levels are three to five fold greater than the average level of ~175 mg/ 100ml. This situation results in the deposition of cholesterol in their skin and tendons as yellow nodules known as xanthomas. However, far greater damage is caused by the rapid formation of atheromas that, in homozygotes, cause death from myocardial infarction at an age as early as 5. Heterozygotes, which comprise ~1 person in 500, are less severely afflicted, they develop symptoms of coronary artery disease after the age of 30. FH was shown to be caused by inherited defects in the gene encoding the LDL receptor. Cells taken from FH homozygotes completely lack functional LDL receptors, whereas those taken from heterozygotes have about one half of the normal

complement. Therefore, homozygotes and, to a lesser extent, heterozygotes are unable to utilise cholesterol in LDL (Brown and Goldstein, 1986).

Leukaemia:

The patients have hypocholesterolemia. The hypocholesterolemia was found to be due to the high LDL receptor activity of the leukaemic cells (Vitols, et al., 1985).

Renal clear cell carcinoma:

The renal clear cell carcinoma or hypernephroma is the most common type of renal malignancy. The neoplastic cells are characterized histologically by a distinctive pale, glassy cytoplasm. It has been determined that the clear appearance of tumor cells results from cellular storage of lipid and glycogen. The lipid most consistently stored in these tumors cells is cholesterol in its ester form. (Gebhard, et al., 1987).

Smith-Lemli-Opitz Syndrome:

This is a developmental disorder in humans with genetic defects in the cholesterol biosynthetic pathway. Δ^7-reductase deficiency has been found as the probable cause for this disorder that occurs in approximately 1 in 20,000 births. Affected individuals are characterized by cranio-facial abnormalities including holoprosencephaly. Congenital heart disease and malformation of limbs. The gene responsible for this disorder was localized to human chromosome 7q321 through cases involving chromosomal translocation. The tissues of affected individuals are characterized by high levels of 7-dehydrocholesterol (Farese and Herz, 1998).

CHOLESTEROL AND ESTROGEN INFLUENCE:

Cholesterol metabolism has been shown to be under estrogenic influence. Estrogen was shown to increase the incorporation of ^{14}C-acetate into cholesterol (Stacey, et al., 1991; Cypriani, et al., 1988b). 3-Hydroxy-3-methylglutaryl-coenzyme A (HMGcoA) reductase has a key role in controlling mitosis through its control of mevanolic acid and cholesterol synthesis as the rate limiting enzyme in their synthetic pathways. HMGCoA reductase levels increased with the estrogen treatment (Abul-Hajj, 1978; Khan and Moulton, 1990) The LDL receptor expression was shown to be enhanced by estrogen (Croce et al., 1996; Colvin, 1996; Grimes, et al., 1996). The regulation of scavenger receptor (HDL receptor) was also shown to be under estrogenic influence (Landschulz, et al., 1996).

Estrogen protects women from cardiovascular disease during the pre-menopausal period. After the menopause, there is a profound increase in cardiovascular diseases which is mainly based on an enhanced development of atherosclerosis caused by estrogen deficiency. Several mechanisms of estrogen mediated protection from cardiovascular disease have been identified including increased HDL, lower LDL, lower VLDL-cholesterol/triglyceride ratio, increased clearance of IDL and LDL via an upregulated LDL receptor, diminished penetration and degradation of LDL in the arterial wall, an inhibition of LDL oxidation by various estrogens and a reversal of inappropriate acetylcholine-mediated vasoconstriction in arteriosclerotic vessels (Knopp, et al., 1994; Kuhl, 1994).

Scope of the thesis

Endogenous regulators of hormone action have been identified in a variety of hormone-receptor systems. The receptor that is under focus in this thesis is an alternative form of estrogen receptor, the non-activated estrogen receptor (naER) which has no capacity to bind to DNA on its own. It dimerizes with a DNA binding protein in the nucleus, the estrogen receptor activation factor (E-RAF). In short, any gene-manipulative function in which naER involves itself should be preceded by its dimerization with the E-RAF Therefore, any factor that prevents this dimerization process can be considered as a potential inhibitor of estrogen action.

The most startling observation of this thesis is that the ubiquitous molecule, cholesterol, is an inhibitor of this category. Finer details of this mystery needs to be unfolded as there is practically no information available in the literature that will identify cholesterol in a potentially negative role. May be that the naER-E-RAF involvement in estrogen action is not a universal phenomenon. It may be restricted to the regulation of certain specific gene functions like those associated with cholesterol homeostasis as there is substantial experimental evidence to show estrogenic involvement in regulating cholesterol synthesis. Under such circumstances, cholesterol will function as an endogenous feed back regulator. Estrogen action through naER -E-RAF heterodimer could (hypothetical) enhance gene expression mechanisms that would in turn, lead to enhanced cholesterol availability within the cell. This high cholesterol level and the subsequent cholesterol binding to the **E-RAF will prevent entry of E-RAF into** the nucleus thereby blocking the gene expression cascade eventually leading to a decreased availability of cholesterol within the cell.

In summary what is being projected in this thesis could be an **as** yet unrecognized reaction that influences cholesterol homeostasis in estrogen-responsive cells. This remains so notwithstanding **the** fact that in this perspective the E-RAF resembles very closely the SREBPs identified by Goldstein and Brown

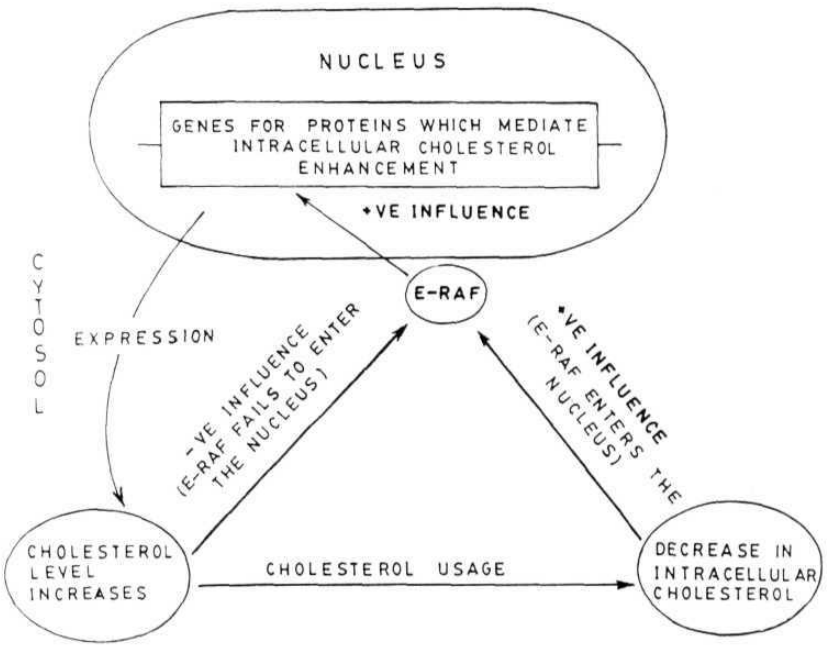


Figure (i) : A Hypothetical presentation to explain E-RAF involvement in cholesterol homeostasis.

CHAPTER - II

MATERIALS & METHODS

[2, 4, 6,7-^{3}H]estradiol-17β (sp. act. 101 Ci/mmol) and [1a, 2a (n)^{3}H] cholesterol (sp. Act. 47 Ci.mmol) were purchased from Amersham. Non-radioactive estradiol-17|3, diethylstilbestrol, p-aminobenzamidine agarose, fluorescein isothiocyanate and phenymethylsulphonyl fluoride were purchased from Sigma, USA. Non-radioactive cholesterol was purchased from E-Merck, Germany DE-52, phosphocellulose and cellulose CF 11 were obtained from Whatman (England); silica gel G was purchased from BDH (England). Sepharose 4B was obtained from Pharmacia. Hydroxylapatite (HAP) was purchased from Bio-Rad. Anti rabbit IgG was obtained from Lupin laboratories, Mumbai. All the solvents used were of HPLC grade purchased from Glaxo and Spectrochem, Mumbai. Synthetic derivatives of cholesterol used in the competitive binding assays were obtained from Aldrich, USA. Routine chemicals of analytical or reagent grade were purchased from local commercial establishments.

Isolation of DNA:

Goat liver DNA was isolated following the method of Marmur (1961). 3gm nuclei were suspended in 25ml of saline EDTA (0.15M NaCl, 0.1M EDTA, pH 8.0) containing 2ml of 25% SDS and were incubated at 60°C for 10 min. The suspension was cooled to room temperature and solid sodium perchlorate was added to the mixture to a final concentration of 1M. The mixture was gently rocked with an equal volume of chloroform-isoamyl alcohol (24:1) for 30 min. After this, the suspension was centrifuged at 7500 rpm for 5 min. The upper aqueous phase was collected and it was layered over 2 volumes of cold ethanol. The nucleic acid was spooled out using a glass rod and it was dissolved in dilute saline citrate (0.015M NaCl, 0.0015M trisodium citrate, pH 7.0). After

getting the DNA dissolved, concentrated saline citrate (1.5M NaCl, 0.15M trisodium citrate) was added to it to give a final concentration of the standard saline citrate (0.15M NaCl, 0.015M trisodium citrate, pH 7.0). This was gently mixed with an equal volume of chloroform-isoamyl alcohol (24:1) for 20 min. and then centrifuged at 7500 rpm for 5 min. The aqueous phase was recovered and the treatment with chloroform-isoamyl alcohol mixture was repeated until no interphase was seen. Finally, the DNA in the aqueous phase was precipitated with two volumes of cold ethanol. The DNA precipitate was used for making DNA Sepharose and DNA cellulose.

CNBr activation of Sepharose 4B and Ligand Immobilization on activated Sepharose 4B:

The Sepharose 4B was activated by using CNBr following the procedure of March et al (1974). One volume of Sepharose 4B was washed with 5 volumes of 1M sodium bicarbonate buffer, pH 9.5 and then gently stirred with one volume of the same buffer. To this suspension, l/20th volume of CNBr solution (2gm CNBr/ml of acetonitrile) was added and it was gently stirred over a magnetic stirrer for 20 min. Then the matrix was washed with 10 volumes of ice cold 0.1M sodium bicarbonate buffer, pH 9.5 followed by equal volumes of cold double distilled water and 0.4M sodium bicarbonate buffer, pH 9.5. Now, the matrix is ready for coupling of the ligands to it. The protein or DNA was dissolved in 0.2M sodium bicarbonate buffer, pH 9.5 and it was gently stirred with activated Sepharose 4B at the rate of 2mg ligand per ml of Sepharose at 4°C for 20 hours. Solid glycine was added to the gel suspension to a final 1M concentration and the mixture was gently stirred for 4 hours at 4°C. The matrix was then washed alternately with 10 volumes each of 0.1M sodium bicarbonate buffer, pH 8.3

containing 0.5M sodium chloride and 0.1M sodium acetate buffer, pH 4.0 also containing 0.5M sodium chloride. The matrix was finally washed with 5 volumes of 10mM Tris-HC1, pH 7.6 and stored in the same buffer with 0.02% sodium azide at 4°C.

Purification of estrogen receptor activation factor (E-RAF):

The method developed by Thampan (1987) was followed. Goat uteri were homogenized (20%) in TEMN buffer (10mM Tris-HCl, pH 7.6 containing 1mM EDTA, 50 mM NaCl and 0.2mM PMSF) using an Ultra Turrax homogenizer. The homogenate was centrifuged at 12,000xg for 15 minutes at 4°C in a refrigerated centrifuge. The soluble cytosolic fraction was prepared from the 12,000xg supernatant following the procedure of van der Hoeven (1981) which involved the precipitation of uterine microsomes using polyethylene glycol. Polyethylene glycol 6000 was added to the 12,000xg supernatant to give a final concentration of 5% and was dissolved by stirring over a magnetic stirrer at 4°C. The resulting solution was centrifuged at 12,000xg for 15 minutes at 4°C. The supernatant formed the cytosol and this was the source for the isolation of the non-activated estrogen receptor (naER), and the estrogen receptor activation factor (E-RAF). The cytosol was chromatographed over a column of p-amino benzamidine agarose and the flow through fraction was collected. This procedure has been adopted in order to reduce the concentration of serine proteases in the cytosol as the enzymes get immobilized on p-amino benzamidine.

The cytosol was stirred with a slurry of Whatman DE-52 suspended in TEMN buffer. The gel was allowed to settle in a beaker kept in ice and the supernatant was collected. This was mixed with a suspension of single stranded DNA Sepharose in

TEMN buffer. The gel was allowed to settle down in a beaker kept in ice and the supernatant was discarded. The gel was washed with 20 volumes of TEMN buffer. The proteins bound to the ss-DNA Sepharose were eluted with 10mM ATP in TEMN buffer. To this eluate, ammonium sulphate was added to yield a final 70% saturation. The precipitate obtained following centrifugation (10,000rpm for 15 minutes at 4^0C) was dissolved in a small volume of 10mM sodium phosphate buffer pH 6.8 containing 1mM EDTA, 12mM monothioglycerol and 0.2mM phenylmethylsulphonyl fluoride (PMSF). It was dialysed overnight against the same buffer. The dialysate was mixed with a 60% suspension of hydroxylapatite in lOmM sodium phosphate buffer. The gel was washed with 100mM sodium phosphate buffer, pH 6.8 and the E-RAF mixture (E-RAF I and E-RAF II) was eluted using three gel volumes of 250mM sodium phosphate buffer. The purified E-RAF appeared as a 66kDa band in silver stained SDS gels.

Isolation of goat uterine hsp 90 and the preparation of hsp 90-Sepharose:

Goat liver hsp 90 was isolated following the procedure outlined by Sullivan et al (1985). The goat liver homogenate (20%) was made in +Mo buffer (lOmM potassium phosphate buffer, pH 7 containing lOmM sodium molybdate, 12mM monothioglycerol and 0.2mM PMSF). The cytosol was prepared by centrifuging the homogenate at 12,000xg at 4^0C for 15 min. The supernatant was stirred with PEG 6000 on a magnetic stirrer to dissolve PEG in the mixture. This was centrifuged at 10,000xg for 15 min. at 4°C. The supernatant obtained was the cytosol. The cytosol was mixed with heparin Sepharose equilibrated with +Mo buffer and the gel was allowed to settle down in a beaker kept in ice. The matrix was washed with 20 volumes of +Mo buffer. The matrix

bound proteins were eluted with 3 volumes of -Mo buffer (same as +Mo buffer but without molybdate). The eluate was mixed with DE-52 equilibrated with -Mo buffer and the gel was allowed to settle down. The supernatant was decanted and the DE-52 was washed with 20 volumes of -Mo buffer. The DE-52 bound proteins were eluted with 3 volumes of 0.3 M NaCl in -Mo buffer. This eluate was diluted with -Mo buffer in order to reduce the concentration of NaCl to 0.050M and was then mixed with hydroxylapatite (HAP) equilibrated with 10mM phosphate buffer. After washing the HAP with -Mo buffer, the HAP bound hsp 90 was eluted with 3 volumes of 250mM phosphate buffer.

The purified hsp 90 was coupled to CNBr activated Sepharose 4B as described by March et al (1974) This matrix was highly useful in the purification of the naER

Purification of non-activated estrogen receptor (naER):

Goat uterine naER was isolated and purified following the method published earlier from our laboratory (Anuradha et al., 1994; Karthikeyan and Thampan, 1996).

The protocol adopted for the purification of E-RAF has been described. The proteins which remained bound to the DE-52 in the E-RAF purification protocol, formed the source for the purification of naER. The DE-52 was washed with 20 gel volumes of TEMN buffer followed by TEM buffer containing 0.3M NaCl. The naER was eluted from the DE-52 using TEM containing 0.5M NaCl. The concentration of NaCl in this fraction was reduced to 50mM by diluting with salt free TEM buffer. Sodium molybdate was added to this eluate to a concentration of 1 OmM. This was then chromatographed on a column of heat shock protein (hsp 90)-Sepharose equilibrated with TEMN buffer containing lOmM sodium molybdate. The hsp 90 Sepharose was washed with 5 volumes

of the equilibration buffer and the naER bound to hsp 90 Sepharose was eluted using molybdate-free TEMN buffer. This eluate was chromatographed on a column of phosphocellulose equilibrated with TEMN buffer The phosphocellulose was washed with 5 column volumes of TEM buffer containing 0.4M NaCl. The naER that was bound to phosphocellulose was eluted with 3 column volumes of TEM buffer containing 0.7M NaCl. The eluate was dialysed against 10mM Tris-HCl containing 12mM monothioglycerol and 0.2mM PMSF. The dialysed protein was concentrated through ultrafiltration. The purified naER appeared as a 66kDa band in silver stained SDS gels.

Extraction and partial purification of the endogenous inhibitor from the goat uterus:

Goat uteri were obtained from a local slaughterhouse. The tissues were homogenized in TEMN buffer using an Ultra Turrax homogenizer. The homogenate was heated in a boiling water bath for 30 minutes following which it was filtered through glass wool. The filtrate was centrifuged at 15,000xg for 15 minutes and the supernatant was mixed with activated charcoal (0.1g/ml) for 1 hour. The charcoal pellet was recovered following centrifugation and was extracted with chloroform-methanol (2:1). The chloroform-methanol extract was dried under a stream of air following which the dried material was dissolved in TEMN buffer containing 10% ethanol. The extract was chromatographed over a column of Whatman DE-52 equilibrated with TEMN buffer. The inhibitor material bound to the DE-52 was eluted using a 50mM to 1M NaCl gradient. The fractions collected were assayed for inhibitor activity following the method described below.

Thin layer chromatographic analysis:

The DE-52 fraction which displayed the inhibitor activity was extracted with chloroform-methanol (2:1). The extracted material was dried under vacuum and was redissolved in the organic solvent used as the mobile phase in the first of the three TLC (silica gel G) systems followed. These were (i) butanol-acetic acid acid-water (70 15:15), (ii)) hexane-ethyl acetate (1:1) and (iii) chloroform-methanol (1:1). The gel area was partitioned and scraped out. Each gel sample was extracted with chloroform-methanol (2:1) following which the extract was dried and assayed for the inhibitory activity. The active fraction from TLC (i) was subjected to analysis in TLC (ii) and that of TLC (ii) was analysed in TLC (iii). The active fraction obtained from TLC (iii) was subjected to GC/MS analysis.

Preparation of DNA cellulose:

Goat liver DNA was extracted following the method of Murmur (1961) and was immobilized on Whatman CF-11 as described by Alberts and Herrick (1971). 3mg goat liver DNA was dissolved in TE buffer (10mM Tris-HCl, pH 7.4, 1mM Na_2EDTA). The Whatman cellulose, CF-11 was added to this preparation at the rate of 1g per 3 ml of DNA. The slurry was stirred till a thick consistency was achieved and it was spread over a glass plate. The plate was left at room temperature to dry overnight. The dried gel was scraped out and lyophilized to remove any residual water. The DNA-cellulose was suspended in 20 volumes of TE buffer for a day at 4°C. After two quick washes, the DNA cellulose was stored at -20^0C in 1ml aliquots.

UV Spectroscopy:

The inhibitor sample was dissolved in 1ml methanol and its absorbance in the UV range was measured against the background of methanol which was used as a blank.

Inhibitor assay:

The extracts of silica gel samples derived from the three TLC systems was collected in assay tubes and dried under a stream of air. Non-activated estrogen receptor (naER), purified from the goat uteri as described in 'methods', was incubated overnight with 20nM ^{3}H-estradiol, at 4°C. The unbound hormone was removed following adsorption to dextran-coated charcoal (1% activated charcoal and 0.1% dextran suspended in 10mM Tris-HCl, pH 7.6 containing 10% glycerol). The ^{3}H-estradiol-naER complex was mixed with an equimolar concentration of E-RAF. The mixture was incubated with DNA cellulose at 30°C for 30 minutes. The reaction was stopped following the addition of 2ml ice cold TEMN buffer to each tube. The samples were centrifuged and the supernatants discarded. The DNA-cellulose pellets were washed twice with ice cold TEMN buffer. The washed pellet was extracted with lml distilled ethanol. The radioactivity associated with the ethanol extract was measured using a Wallac 1409 liquid scintillation counter.

Mass spectroscopy:

Mass spectral analysis was done with VG AUTOSPEC-M mass spectrometer with OPUS V3IX data system. The following conditions were maintained during the analysis: Source temperature: 200°C; Trap current: 200μA; Electron energy: 70eV.

Gas Chromatography/Mass spectral analysis (GC/MS):

GC/MS analysis was carried out on the same instrument using the following parameters. The column used was OV-1 fused silica capillary column of 30mm length, 0.31 mm 1. d and 0.25mm film thickness with an initial temperature set at 100^0C for 5 minutes and a final temperature at 200^0C for 90 minutes The ramp rate of heating was set at 10^0C/min.

Synthesis of Cholesta 4, 6, diene 3 ol:

The method described by Tachibana (1986) was followed. A hexane solution of cholesteryl acetate (2.48g), NBS (N-bromosuccinimide, 1.77 g) and a catalytic amount of benzoyl peroxide was allowed to react under reflux for 40 minutes. The hexane solution was filtered to remove succinimide and the filtrate was evaporated to dryness under reduced pressure below 40^0C. The residue was dissolved in butyl acetate (8ml). Tetrabutylammonium bromide (0,346g) and 2, 4, 6-trimethyl pyridine (1 45g) were added to the butylacetate solution. The mixture was allowed to reflux for 20 minutes. The solution was washed with dilute HC1 and water, dried over sodium sulfate and finally concentrated to a semicrystalline residue under reduced pressure. To the residue methanolic KOH solution (KOH 0.4g, MeOH 10ml) was added and the mixture was allowed to stand overnight at room temperature. The crude cholesta-4, 6-diene-3 ol was collected by filtration and was purified through chromatography on silica gel. **The** synthesized compound was checked for purity through mass spectrometry.

Sucrose density gradient analysis:

A mixture of naER and E-RAF was incubated with 40nM ^{3}H-estradiol overnight at 4^{0}C in the presence or absence of the inhibitor, in a total volume of 250µl. Unbound hormone was removed from the mixture following adsorption to dextran-coated charcoal. The samples were layered over 4ml linear gradients of 5 to 20% sucrose in TEM buffer containing 0.3M NaCl and centrifuged for 16 hours at 55,000 rpm in a SW 65 rotor of a Becktnan ultracentrifuge. The fractions collected were analysed for radioactivity. Human gamma globulin and bovine serum albumin were used as sedimentation markers.

Isolation of nuclei:

The goat uterine nuclei were isolated following the method described by Thampan (1985). Uteri obtained from a local slaughterhouse were immediately brought in ice to the laboratory. The uteri were washed with TCKM buffer (50mM Tris, pH 7.6, 20mM KC1, 1mM $CaCl_2$, 2mM $MgCl_2$, 0.2mM PMSF, 250mM sucrose) and minced finely. A 20% homogenate was made in TCKM buffer using a Polytron homogenizer (PT45-80) at a setting of 4 (1minx4 with an interval of 3 minutes). The homogenate was filtered through a nylon membrane and the filtrate was centrifuged at 800xg at 4°C for 10 minutes. The pellet was not treated with Triton X-100 as was done in the original procedure because it removed the nuclear membrane. Instead, the pellets were straightaway washed twice with TCKM buffer. The washed pellets **were** suspended in 5ml of the same buffer and layered over 30ml pads of TCKM buffer containing 340mM sucrose. Following centrifugation at 800xg for 10 minutes, the final pellet contained purified nuclei.

Nuclear binding of the naER-E-RAF heterodimer:

Goat uterine nuclei were isolated following the method described earlier by Thampan (1985); the nuclei were not treated with Triton X-100. The isolated nuclei were suspended in TCKM buffer (50mM Tris-HCl, pH 7.6 containing 2mM $MgCl_2$. 20mM KC1, 1mM $CaCl_2$, 0.2mM PMSF and 250mM sucrose). The naER-E-RAF mixture was incubated overnight at 0-4°C with 20nM ^{3}H-estradiol and varying concentrations of the inhibitor. The nuclei, suspended in TCKM-sucrose buffer, containing 4mM ATP, were exposed to this incubated mixture at 30^0C for 30 minutes. The incubated samples (250µl) were layered over 10ml pads of TCKM buffer containing 0.34M sucrose and centrifuged at lOOOxg for 10 minutes in a Sorwall RC-5B centrifuge. The nuclear pellet collected at the bottom of the tube was extracted with 1ml ethanol. The radioactivity associated with the ethanol extract was measured using a toluene based and Triton X-100 containing scintillation medium.

Saturation Binding of ^{3}H-cholesterol to E-RAF:

Purified E-RAF was incubated with ^{3}H cholesterol (1 to 20nM) in the presence or absence of a hundred fold excess of unlabeled cholesterol overnight at 4°C in a total volume of 250µl. At the end of the incubation, 250ul of 60% hydroxylapatite (HAP) was added to the mixture and the tubes were left in ice for 30 min. The HAP pellet was washed twice with 2ml of ice cold TEMN buffer and was extracted subsequently with 1 ml ethanol. The radioactivity associated with the ethanol extracts was measured using a toluene based scintillation fluid in a Wallac 1409 liquid scintillation counter. The specific binding activity was estimated as described by Peck and Clark (1977).

Circular dichroism measurement:

Interaction of cholesterol with E-RAF was subjected to CD analysis using a Jasco Spectropolarimeter. Scans were made at a wavelength range of 330 to 240nm (0.5 sensitivity) in order to detect changes in the tertiary structure and also at 250 to 210nm (2.0 sensitivity) in order to identify changes in the secondary structure of the E-RAF.

Purification of Actin :

Actin was purified from the goat skeletal muscle following the procedure of Pardee and Spudich (1982). Acetone powder of the skeletal muscle was made as follows. 250 g of muscle was washed with double distilled water and minced finely The mince was washed extensively with distilled water and extracted for 10 minutes in 500 ml ice cold 150mM potassium phosphate buffer, pH 6.5 containing 100 raM potassium chloride. This was filtered under vacuum. The residue was extracted by stirring successively in (1) 1000 ml of 50 mM sodium bicarbonate for 10 minutes, (2) 500 ml of 1mM EDTA, pH 7 0 for 5 minutes, (3) twice with 500 ml of double distilled water for 5 minutes and (4) 5 times with 250 ml of acetone (at room temperature) for 10 minutes. Each step was followed by filtration under vacuum. The final residue was lyophilized to remove traces of acetone. The acetone powder was obtained and stored at -70°C until further use.

10 g of acetone powder was extracted with 250 ml of TMC buffer (2 mM Tris-HCl, pH 8.0, 0.5 mM β-mercaptoethanol, 0.2 mM $CaCl_2$) containing 0.2 mM ATP for 30 minutes. The mixture was centrifuged at 15000xg for 20 minutes. The supernatant was aspirated very carefully, leaving the turbid layer in the centrifuge tube. To the supernatant potassium chloride, magnesium chloride and ATP were added to final

concentrations of 50 mM, 2 mM and 1 mM respectively. The supernatant was stirred with a glass rod and left undisturbed for 2 hours. The KCl concentration of the supernatant was increased to 0 6 M and the stirring was continued for an additional 90 minutes. The polymerized actin was pelleted by centrifugation at 80000xg for 3 hours. The pellet was homogenized in TMC buffer containing 0.2 mM ATP and dialysed for J days against the same buffer in order to depolymerize actin. The dialysate was centrifuged at 80000xg for 3 hours. The supernatant was collected and chromatographed over a column of DE-52, pre-equilibrated with imidazole buffer (10 mM imidazole pH 8.0, 0.5 β-mercaptoethanol, 0.1 mM $CaCl_2$, 1 mM ATP) containing 100 mM KCl. The column was washed with the same buffer. Elution was carried out with 300 mM KCl in imidazole buffer. The eluate was dialysed against 2 L of TMC buffer containing 50 mM KCl, 2 mM $MgCl_2$ and 1 mM ATP to effect actin polymerization. The dialysate was centrifuged at 80000xg for 3 hours in order to pellet down actin. The pellet was resuspended in TMC buffer containing 0.2 mM ATP and was dialysed against the same buffer for 2 days. The dialysate was centrifuged at 80000xg for 3 hours. The supernatant which contained pure actin was lyophilized. Actin was dissolved in 0.2 M sodium bicarbonate, pH 8.3 containing 0.5 M NaCl and coupled to CNBr activated Sepharose **4B.**

Actin Sepharose Chromatography of E-RAF:

Purified E-RAF was chromatographed over the actin-Sepharose column equilibrated with TEMN buffer and elution was achieved using a 50mM to 1 M NaCl gradient in the same buffer.

Preparation of Bradford's reagent:

10mg coomassie brilliant blue G-250 was dissolved in 5ml of 95% ethanol. To this solution 10ml of phosphoric acid was added and the volume was made up to 100ml using double distilled water and stirred for 10 minutes. Then the whole solution was filtered using a Whatman No. 1 filter paper. This reagent was used within a month of its preparation.

Estimation of Protein:

Quantitative analysis of protein was carried out as described by Bradford et al (1976). To 100ul of protein aliquot, 1ml of Bradford's reagent was added and the mixture was vortexed using a cyclomixer. The intensity of the color formed was measured at 595nm. BSA (10µg) had an absorbance of 0.5 when used as the standard.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE):

SDS-PAGE was carried out as described by Laemmli (1970). The following **solutions** and reagents were used for polymerization and electrophoresis.

i) 30% acrylamide solution: 30g of acrylamide and 0.8g of methylene-bis-acrylamide were dissolved in double distilled water and the volume was made up to 100ml.

ii) Lower Tris (4x): 1.5M Tris-HCl, pH 8.8 buffer containing 0.4% SDS.

iii) Upper Tris (4x): 0.5M Tris-HCl, pH 6.8 buffer containing 0.4% SDS.

iv) Reservoir buffer (10x): 0.1 M Tris buffer containing 0.8M glycine.

v) Gel running buffer (x): 100ml of the reservoir buffer was diluted to 990ml. 10ml of 10% SDS was added to this buffer.

vi) Ammonium per sulfate (APS): 1% APS solution was prepared in double distilled water.

All these buffers were stored in a refrigerator. 10% gels were used in the studies.

A 10% resolving gel was polymerized by adding 2.5ml of double distilled water, 1.5ml of lower Tris buffer and 2ml of acrylamide solution. A 3% snicking gel was layered over the resolving gel. SDS-PAGE was performed at 100V.

Preparation of proteins for denaturing electrophoresis:

The protein samples were precipitated using 100% trichloroacetic acid (TCA). To 1ml of protein sample (10µg), 0.5ml of TCA was added and the Eppendorf tubes containing the protein were left at -20^0C for 1hr. The tubes were centrifuged at 15,000rpm for 15min. The precipitate was washed once with absolute alcohol to remove TCA. The protein was dissolved in 20ul SDS sample buffer (100µl glycerol, 50ul β-mercaptoethanol, 300ul 10% SDS, 125µl upper Tris buffer, 425ul double distilled water and 1mg of bromophenol blue).

Silver staining of SDS gels:

The proteins in the SDS gels were stained with silver nitrate following the procedure of Blum et al., (1987). The gels were transferred to a tray containing a fixative solution (50% methanol, 12% glacial acetic acid, 0.5 ml/1 37% formaldehyde) and were gently rocked for 45 min. Later, the gels were transferred to 50% alcohol and the rocking was continued for 20 min. The alcohol was removed and the gel was treated with sodium thiosulfate (200mg/l) for 1 minute. The gels were washed repeatedly with double distilled

water 3 or 4 times to remove sodium thiosulfate. The gels were rocked with silver nitrate solution (2g $AgNO_3$, 0.5ml/l formaldehyde) for 20 min. The gels were washed well with double distilled water and were developed using a 6% sodium bicarbonate solution containing 0.5ml/l formaldehyde. Finally, the gels were washed well with double distilled water and were stored in 3% acetic acid.

Development of polyclonal antibodies:

Rabbits of an inbred strain were used for raising polyclonal antibodies. The pre-immune sera was collected. About 200µl protein in one ml water was mixed with an equal volume of Freund's adjuvant. The initial dose contained the protein mixed with Freund's complete adjuvant. Three booster doses had the protein mixed (1:1) with incomplete adjuvant and injected at an interval of ten days each. Each time, the animal was injected subcutaneously at 4 or 5 locations including the footpads. Blood was collected from the rabbit following ear puncture one week after the final booster dose. The serum collected was stored in aliquots at -20°C.

FITC-labeling of proteins:

100 µg (100µl) each of naER and E-RAF were separately incubated overnight at 4^0C with 100 µl of FITC solution (1mg FITC in 1ml of 0.15M sodium phosphate buffer, pH 9.5). The Eppendorf tubes were covered with tin foil to avoid light. The unbound FITC was removed using a Sephadex G-25 column equilibrated with TCNM buffer.

Fluorescence microscopic studies of nuclear transport of naER/E-RAF:

The microscopic studies involving FITC labeled naER and E-RAF were carried out using a fluorescence microscope NIKON eclipse E600. Five sets of experiments were carried out.

(1) 0.5µg of FITC labeled naER alone was added to a suspension of freshly isolated uterine nuclei suspended in 2x TCKM buffer containing 4mM ATP.

(2) 0.5µg of FITC labeled naER + unlabeled E-RAF + nuclei.

(3) 0.5µg of FITC labeled E-RAF + nuclei.

(4) 0.5µg of FITC labeled E-RAF + unlabeled naER + nuclei.

(5) 0.5µg of FITC labeled E-RAF + 20nM cholesterol + nuclei.

The magnification of 40x and 100x were used. The photographs were taken using the photographic film ILFORD PAN 400 ASA.

CHAPTER - III

RESULTS

DE-52 chromatography of the inhibitor:

The method adopted for the extraction of the inhibitor material from the goat uterus has been detailed in the experimental section. The material that was adsorbed to the DE-52 matrix was eluted using a 50mM to 1M NaCl gradient in TEM buffer. Individual fractions were assayed for the inhibitor activity as described in 'methods'. The fraction that displayed the capacity to inhibit the DNA binding of E-RAF-^{3}H-E_2-naER complex was recovered for further analysis (Fig. 1). The fractions eluted with the buffer containing 0.5 to 0.65M NaCl showed positive indications for the presence of the inhibitor Control experiments were carried out in order to rule out the possibility that the effect observed was not due to the ionic strength of the elution buffer. A DE-52 column without any adsorbed inhibitor material was subjected to a salt-gradient elution. The fractions collected were subjected to the inhibitor assay. The data presented in figure 1 indicate that the salt concentration in the fractions did not influence the inhibitor activity.

Thin layer chromatography of the inhibitor:

Three different solvent systems were used in the successive thin layer chromatographic isolation of the inhibitor originally eluted from the DE-52 column. These were (i) butanol-acetic acid-water (70:15:15), (ii) hexane-ethyl acetate (1:1) and (iii) chloroform-methanol (1:1). Following chromatography the gel area was fractionated and subsequently extracted with chloroform-methanol (2:1). The extract was collected in assay tubes, dried and subjected to the inhibitor assay. The assay fraction recovered from TLC (ii) was analysed in TLC (iii). There was a clear concentration and enhanced

Figure 1: Whatman DE-52 chromatography of the uterine extract containing the inhibitor. The method adopted in the extraction of the crude inhibitor material has been given in 'methods'. The heat resistant and charcoal-adsorbed material was chromatographed over a column of DE-52 equilibrated with TEMN buffer. Elution was achieved using a 50mM to 1M NaCl gradient in TEM buffer. The fractions collected were assayed for the inhibitory activity (o). Inhibitor-free material eluted from the column using the salt gradient was used as the control (•) in this experiment.

^{3}H-ESTRADIOL BOUND TO DNA CELLULOSE
D.P.M. x 10^{-3}
0
1
2
3
4
2
6
10
14
18
FRACTION NUMBER
1 00
0.75
0.50
0.25
M NaCl (- - - -)

recovery of the inhibitory material noticed in TLC (iii) in comparison to the recovery from the two preceding systems (Fig. 2).

Structural analysis of the active fraction recovered from TLC iii:

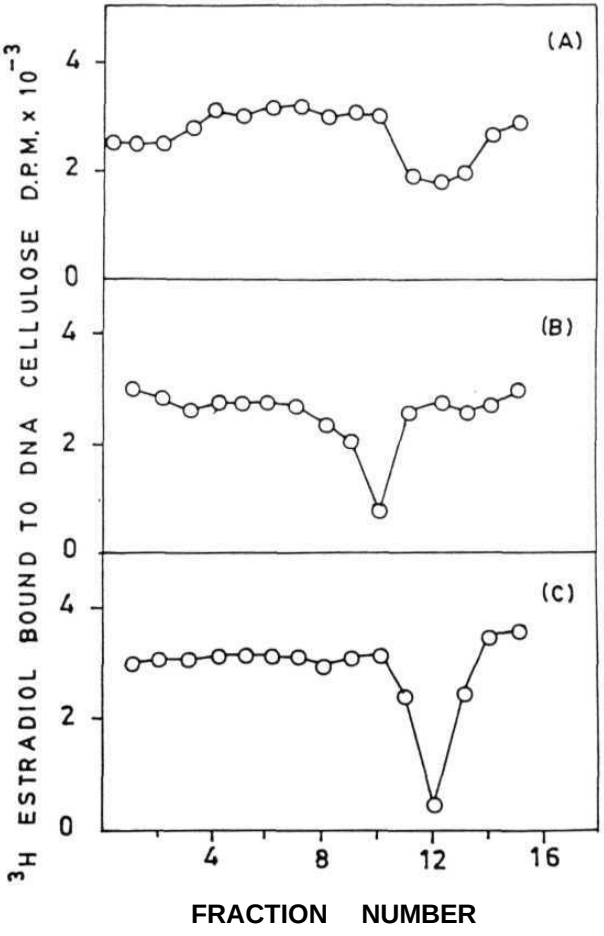
The fraction that contained the inhibitory activity could be identified under UV source due to the high fluorescence displayed by the material. The gel area covered by the fluorescent spot was scraped out and extracted with chloroform.

The inhibitory material partially purified through the TLC systems was subjected to UV spectroscopy. It was shown to have an absorbance maximum at 243nm (Fig. 3A). An inhibitor assay carried out using this partially purified material showed a direct relationship between the inhibitory activity and the concentration of this material in the assay system (Fig. 3B).

Gas chromatographic analysis displayed peaks ranging between 200 and 2400 scans (Fig. 4). The most prominent among them were the peaks with retention times around 37.8 min. (Scan no. 1011) and 53.8 min. (Scan no. 1463).

Mass spectra of the GC peaks of 1463 and 1011 scans indicated that they belonged to molecules with molecular weights 386 and 366 respectively (Fig. 5). A library search carried out for identifying these compounds indicated that while the mass 386 peak was found to represent cholesterol, the mass 366 peak was shown to represent either cholesta-4, 6-diene-3-ol benzoate or cholesta-4, 6-diene-3-ol (Fig. 5). However one would expect the retention time of cholesta-4, 6-diene-3-ol to be close to that of cholesterol. Therefore, the observed retention time for the scan 1011 suggested that it

Figure 2: Thin layer chromatographic analysis of the DE-52 bound inhibitor. The active fraction recovered from the DE-52 column was subjected to TLC using three different solvent systems. It was first subjected to TLC i (A) a solvent system containing butanol-acetic acid-water (70:15:15). The active fraction from this was subjected to TLC ii (B) using hexane-ethyl acetate (1:1) as the solvent system. The active component from this system was subjected to TLC iii (C) with a solvent system containing chloroform-methanol (1:1). The gel area, partitioned into 1cm squares, was extracted with chloroform-methanol (2:1). The extract was dried in assay tubes prior to its exposure to the assay constituents.


(A)
(B)
(C)
4
2
0
4
8
12
16
^{3}H ESTRADIOL BOUND TO DNA CELLULOSE D.P.M. x 10^{-3}
FRACTION NUMBER

could be a non-polar cholestatriene molecule with a possible precursor as cholesta-4, 6-diene-3-ol, because the mass spectra of the latter matched closely with that for scan 1011.

A cluster of peaks appeared between 200 and 800 scans. Of these, three peaks were identified as fatty acids corresponding to molecular weights 228, 256 and 284 (Fig. 6). The mass spectra of these compounds matched well with the mass spectra of myristic acid, palmitic acid and stearic acid in the library search programme. The other peaks in the 200 to 600 scans range were identified as artifacts due to phthalates and some hydrocarbons.

Identification of the 'receptor' protein for the inhibitor:

In order to find out which one of the components identified in the computer search was the true inhibitor, an indirect experimental approach was undertaken. Purified E-RAF and naER were exposed to the inhibitor, isolated through TLC (iii) overnight at 4°C. The proteins were subsequently immunoprecipitated using the corresponding (anti naER or anti E-RAF) polyclonal antibody. The immunoprecipitate was washed extensively with TEMN buffer. The precipitate was extracted with chloroform-methanol (2:1) and the extract was subjected to GC/MS analysis. The gas chromatogram of the E-RAF immunoprecipitae showed some peaks between ~300 and ~500 and a major peak at 1095 scans (Fig. 7A). The 1095 scans peak had a molecular mass of 386 and it was identified as cholesterol upon mass spectral analysis notwithstanding the observation that the retention time of this peak was found to be different from what was shown in figure 5 (Fig. 8). Cholesta-4, 6-diene-3-ol was not present. Traces of both palmitate and stearate were also observed to be bound to the E-RAF. The peaks at 363 and 463 scans were

Figure 3: (A) UV spectrum of the partially purifled inhibitor obtained at the end of TLC (iii). The molecule displayed an absorption maximum at 243nm **(B) DNA cellulose binding assay showing the effect of the partially purifled inhibitor.** Details of the assay method have been given in the methods section. ^{3}H-estradiol-naER complex was incubated with E-RAF and DNA cellulose in the presence of varying concentrations of the inhibitor obtained from TLC (iii). The ^{3}H-E_2 bound to the DNA at the end of the incubation, was measured.

(A)
(B)
ABSORBANCE
0.600
0.400
0.200
0.000
250
300
350
λ
0
0.1
0.2
0.4
INHIBITOR 240nm ABSORPTION UNITS
4.0
2.0
0
^{3}H-ESTRADIOL BOUND TO DNA CELLULOSE D.P.M.x10^{-3}

Figure 4: GC/MS analysis of the inhibitory material obtained at the end of TLC (iii). The peaks selected for further analysis were those which appeared at scans 1463 and 1011 and also the cluster observed between 200 and 800 scans. The peaks observed at 725 and 375 scans were due to phthalate impurity.

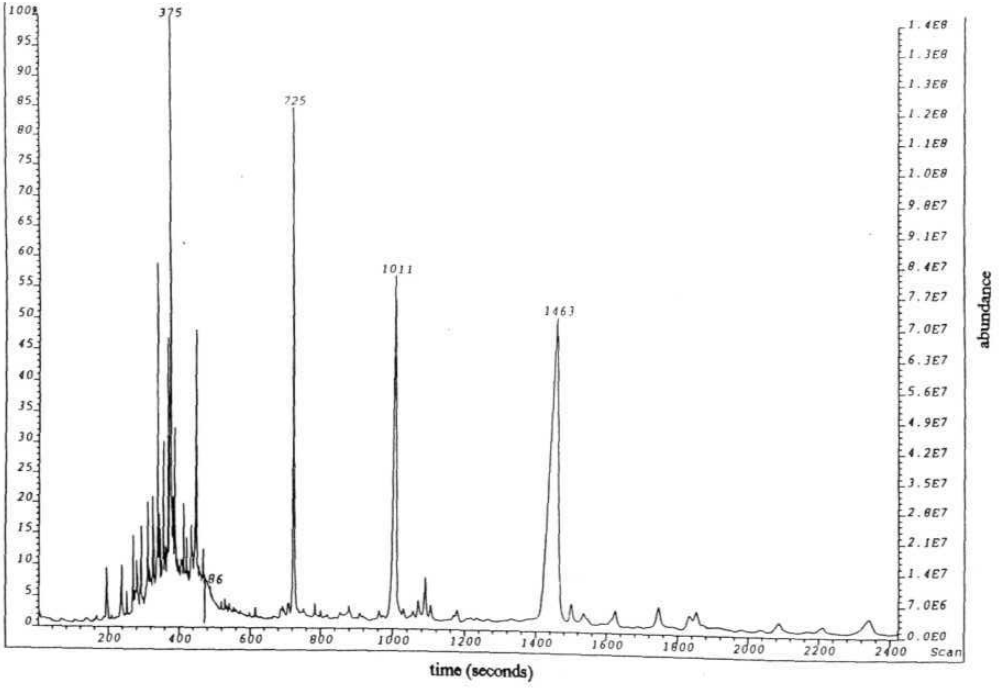

375
725
1011
1463
86
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
Scan
time (seconds)
1.4E8
1.3E8
1.3E8
1.2E8
1.1E8
1.0E8
9.8E7
9.1E7
8.4E7
7.7E7
7.0E7
6.3E7
5.6E7
4.9E7
4.2E7
3.5E7
2.8E7
2.1E7
1.4E7
7.0E6
0.0E0
abundance

Figure 5: Mass spectra of the GC peaks and computer search results of the MS data. The GC 1463 scans peak of figure 4 has been identified as cholesterol while the peak at 1011 scans from GC has been identified as cholesta-4, 6-diene-3-ol or its benzoate derivative.

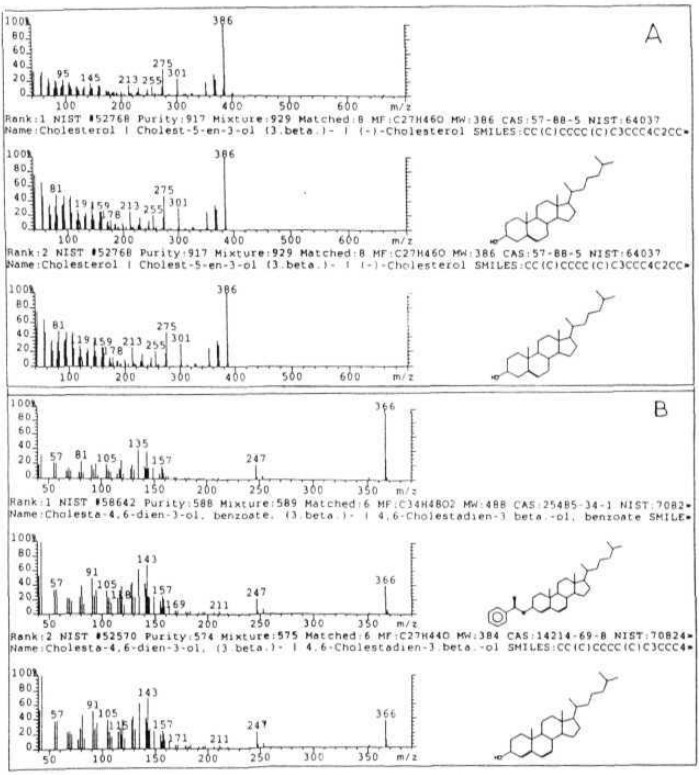

A
386
275
301
95
145
213
255
Rank:1 NIST #52768 Purity:917 Mixture:929 Matched:8 MF:C27H46O MW:386 CAS:57-88-5 NIST:64037
Name:Cholesterol | Cholest-5-en-3-ol (3.beta.)- | (-)-Cholesterol SMILES:CC(C)CCCC(C)C3CCC4C2CC=
Rank:2 NIST #52768 Purity:917 Mixture:929 Matched:8 MF:C27H46O MW:386 CAS:57-88-5 NIST:64037
Name:Cholesterol | Cholest-5-en-3-ol (3.beta.)- | (-)-Cholesterol SMILES:CC(C)CCCC(C)C3CCC4C2CC=
B
366
135
57
81
105
157
247
Rank:1 NIST #58642 Purity:588 Mixture:589 Matched:6 MF:C34H48O2 MW:488 CAS:25485-34-1 NIST:7082
Name:Cholesta-4,6-dien-3-ol, benzoate, (3.beta.)- | 4,6-Cholestadien-3.beta.-ol, benzoate SMILE
143
91
105
366
Rank:2 NIST #52570 Purity:574 Mixture:575 Matched:6 MF:C27H44O MW:384 CAS:14214-69-8 NIST:70824
Name:Cholesta-4,6-dien-3-ol, (3.beta.)- | 4,6-Cholestadien-3.beta.-ol SMILES:CC(C)CCCC(C)C3CCC4
m/z

Figure 6: Mass spectra and the computer search data of the fatty acids located in the peak cluster observed between 200 and 800 scans in the GC trace. (A) pertains to myristic acid, (B) to palmitic acid and (C) to stearic acid.

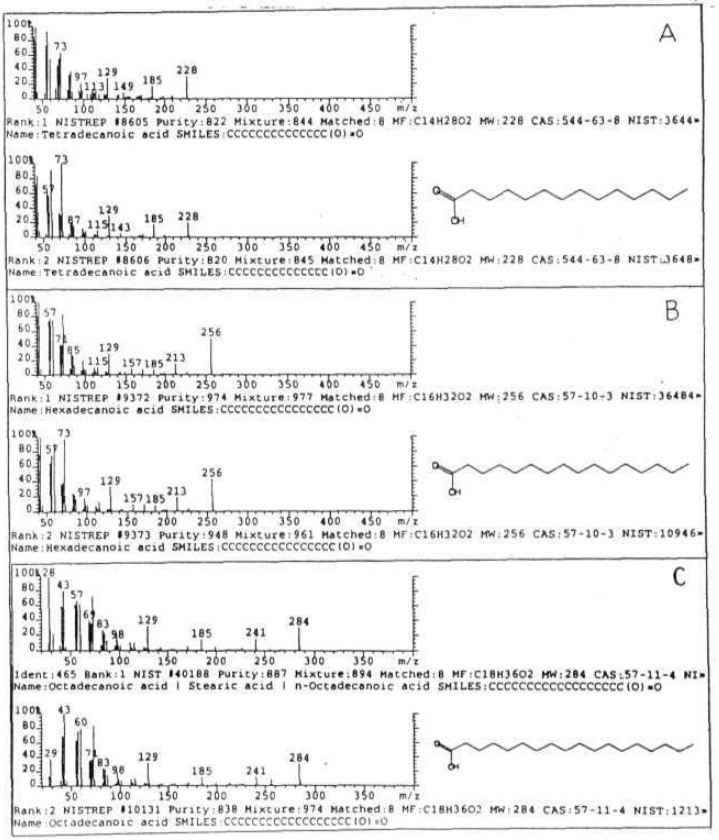

A
Rank:1 NISTREP #8605 Purity:822 Mixture:844 Matched:8 MF:C14H28O2 MW:228 CAS:544-63-8 NIST:3644
Name:Tetradecanoic acid SMILES:CCCCCCCCCCCCCC(O)=O
Rank:2 NISTREP #8606 Purity:820 Mixture:845 Matched:8 MF:C14H28O2 MW:228 CAS:544-63-8 NIST:3648
Name:Tetradecanoic acid SMILES:CCCCCCCCCCCCCC(O)=O
B
Rank:1 NISTREP #9372 Purity:974 Mixture:977 Matched:8 MF:C16H32O2 MW:256 CAS:57-10-3 NIST:36484
Name:Hexadecanoic acid SMILES:CCCCCCCCCCCCCCCC(O)=O
Rank:2 NISTREP #9373 Purity:948 Mixture:961 Matched:8 MF:C16H32O2 MW:256 CAS:57-10-3 NIST:10946
Name:Hexadecanoic acid SMILES:CCCCCCCCCCCCCCCC(O)=O
C
Ident:465 Bank:1 NIST #40188 Purity:887 Mixture:894 Matched:8 MF:C18H36O2 MW:284 CAS:57-11-4 NI
Name:Octadecanoic acid | Stearic acid | n-Octadecanoic acid SMILES:CCCCCCCCCCCCCCCCCC(O)=O
Rank:2 NISTREP #10131 Purity:838 Mixture:974 Matched:8 MF:C18H36O2 MW:284 CAS:57-11-4 NIST:1213
Name:Octadecanoic acid SMILES:CCCCCCCCCCCCCCCCCC(O)=O

Figure 7 : **GC/MS** analysis of the 'inhibitor' associated with the **immunoprecipitates** of E-RAF and naER. Purified E-RAF and naER were immunoprecipitated after they were incubated separately with the partially purified inhibitor. The immunoprecipitated material was analysed by GC/MS. (A) In the E-RAF immunoprecipitate, the peaks that appeared at 1095 scans and the ones that appeared between ~300 to ~500 scans were subjected to mass spectral analysis. (B) In the naER immunoprecipitate, there was no peak that corresponded to the presence of cholesterol as seen in the E-RAF immunoprecipitate.

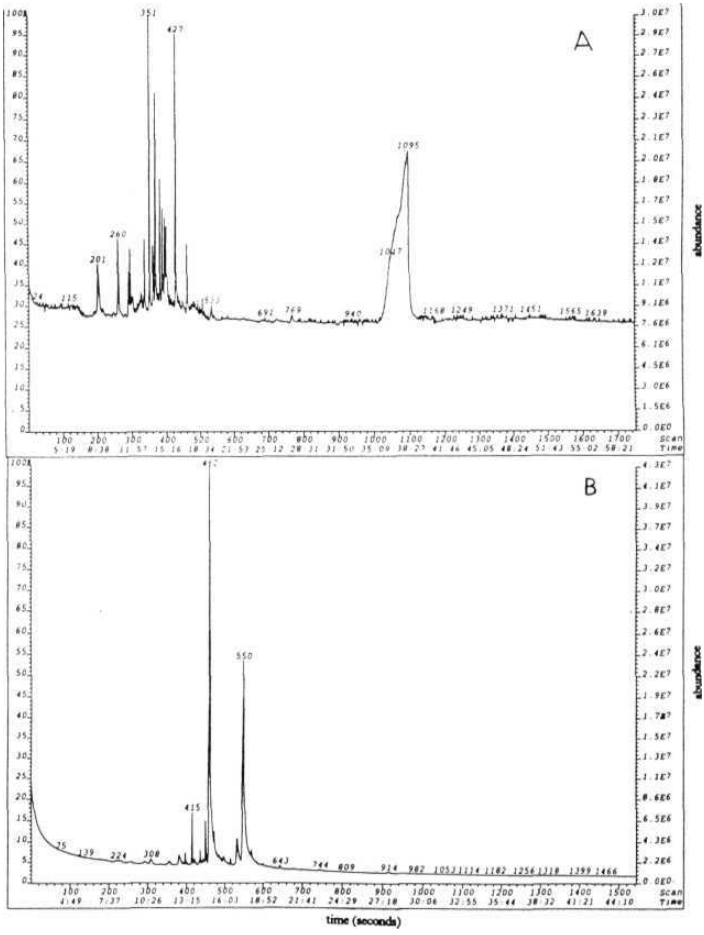

A
B
abundance
Scan
Time
time (seconds)

Figure 8: The computer analysis of the mass spectrum of the 1095 scans peak obtained in GC of the E-RAF immunoprecipitate. The peak observed at 1095 scans was found to be that of cholesterol.

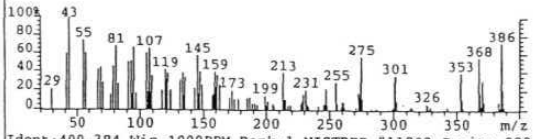
Libraries 1: NISTREP (12593) 2: NIST (62235)

Peaks:	8	7	6	5	4	3	2	1	0
Matches:	2	2	7	12	40	158	787	10485	63335
Total:	2	4	11	23	63	221	1008	11493	74828

Normal Search: 21 of 23 Rank: Purity Mass Weighting: On Presearch Minimum: 20

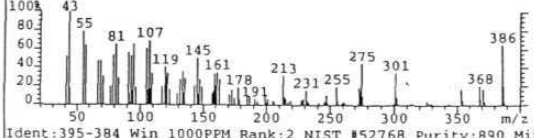
File Text:HCU - U - GC/MS 100 -5M-10/MIN-200-45MIN File:HCU_U Ident:1036_1114-827_900 Win 1000P»

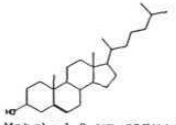
AutoSpec EI+ Function:Magnet TIC:6386422



Ident:400-384 Win 1000PPM Rank:1 NISTREP #11902 Purity:922 Mixture:928 Matched:8 MF:C27H46O MW:»

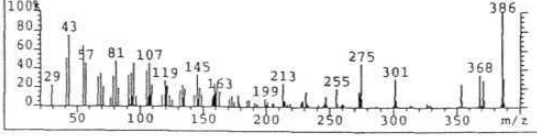
Name:Cholesterol SMILES:CC(C)CCCC(C)C3CCC4C2CC=C1CC(O)CCC1(C)C2CCC34C





Ident:395-384 Win 1000PPM Rank:2 NIST #52768 Purity:890 Mixture:896 Matched:8 MF:C27H46O MW:386»

Name:Cholesterol | Cholest-5-en-3-ol (3.beta.)- | (-)-Cholesterol SMILES:CC(C)CCCC(C)C3CCC4C2CC»



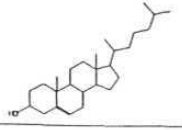
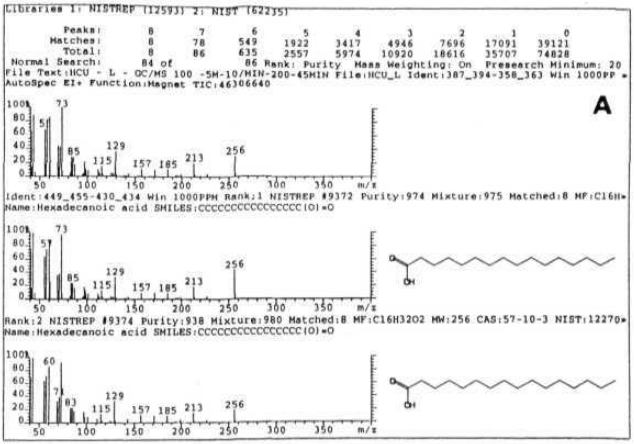
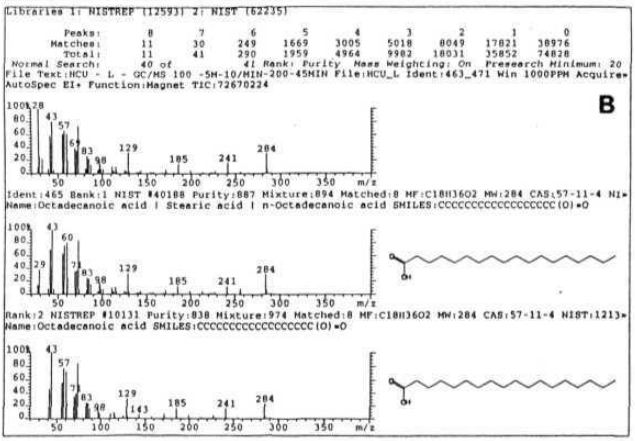


Figure 9: The computer analysis of the mass spectra of the peaks of 363 and 463 scans that appeared in GC of the E-RAF immunoprecipitate. The peaks at 363 and 463 scans corresponded to those of palmitate (A) and stearate (B) respectively.


Libraries 1: NISTREP (12593) 2: NIST (62235)
Peaks: 8 7 6 5 4 3 2 1 0
Matches: 8 78 549 1922 3417 4946 7696 17091 39121
Total: 8 86 635 2557 5974 10920 18616 35707 74828
Normal Search: 84 of 86 Rank: Purity Mass Weighting: On Presearch Minimum: 20
File Text:HCU - L - GC/MS 100 -5H-10/MIN-200-45MIN File:HCU_L Ident:387_394-358_363 Win 1000PP »
AutoSpec EI+ Function:Magnet TIC:46306640
A
Ident:449_455-430_434 Win 1000PPM Rank:1 NISTREP #9372 Purity:974 Mixture:975 Matched:8 MF:C16H»
Name:Hexadecanoic acid SMILES:CCCCCCCCCCCCCCC(O)=O
Rank:2 NISTREP #9374 Purity:938 Mixture:980 Matched:8 MF:C16H32O2 MW:256 CAS:57-10-3 NIST:12270»
Name:Hexadecanoic acid SMILES:CCCCCCCCCCCCCCC(O)=O
m/z


Libraries 1: NISTREP (12593) 2: NIST (62235)
Peaks: 8 7 6 5 4 3 2 1 0
Matches: 11 30 249 1669 3005 5018 8049 17821 38976
Total: 11 41 290 1959 4964 9982 18031 35852 74828
Normal Search: 40 of 41 Rank: Purity Mass Weighting: On Presearch Minimum: 20
File Text:HCU - L - GC/MS 100 -5H-10/MIN-200-45MIN File:HCU_L Ident:463_471 Win 1000PPM Acquire»
AutoSpec EI+ Function:Magnet TIC:72670224
B
Ident:465 Rank:1 NIST #40188 Purity:887 Mixture:894 Matched:8 MF:C18H36O2 MW:284 CAS:57-11-4 NI»
Name:Octadecanoic acid | Stearic acid | n-Octadecanoic acid SMILES:CCCCCCCCCCCCCCCCCC(O)=O
Rank:2 NISTREP #10131 Purity:838 Mixture:974 Matched:8 MF:C18H36O2 MW:284 CAS:57-11-4 NIST:1213»
Name:Octadecanoic acid SMILES:CCCCCCCCCCCCCCCCCC(O)=O
m/z

found to have molecular masses 256 and 284 corresponding to those of palmitate and stearate respectively (Fig.9). Interestingly, no cholesterol and cholesta-4, 6-diene-3-ol were located in the naER immunoprecipitate (Fig.7B).

Direct identification **of the inhibitor as cholesterol :**

In order to find out the true identity of the inhibitor it was essential to carry out assays using the molecules identified in the mass spectra-computer search. Cholesta-4, 6-diene-3-ol was synthesized in our laboratory while the fatty acids and cholesterol were purchased from commercial establishments. DNA-cellulose binding of ^{3}H-E_2 naER in the presence of E-RAF and varying concentrations of these agents was studied. Cholesterol alone was shown to function as the inhibitor. The maximum inhibition was observed in the presence of ~20nM cholesterol (Fig. 10A). Cholesta-4, 6-diene-3-ol was totally ineffective (Fig. 10A). None of the three fatty acids employed could display the inhibitory activity (Fig. 10C). The mass spectrum of cholesta-4, 6-diene-3-ol used in this study is shown in Fig.lOB.

Cholesterol inhibits naER-E-RAF dimerization:

Sucrose density gradient analysis was carried out to find out whether cholesterol influenced the dimer formation between naER and E-RAF. In the absence of cholesterol the complex sedimented at 4.8 to 5.0 S while in the presence of 20nM cholesterol the hormone-binding activity was restricted to the 4.0 to 4.2S peak which represented the free naER (Fig. 11 A).

Figure 10: Analysis of the functional identity of cholesterol and cholesta-4, 6-diene-3-ol. (A) A DNA-cellulose binding assay was carried out using assay systems containing either cholesterol (o) or its metabolite, cholesta-4, 6-diene-3-ol (•). The inhibitory activity is clearly shown here to be associated with cholesterol. (B) is a mass spectrum of the cholesta-4, 6-diene-3-ol synthesized within the laboratory and used in the assay system of (A).

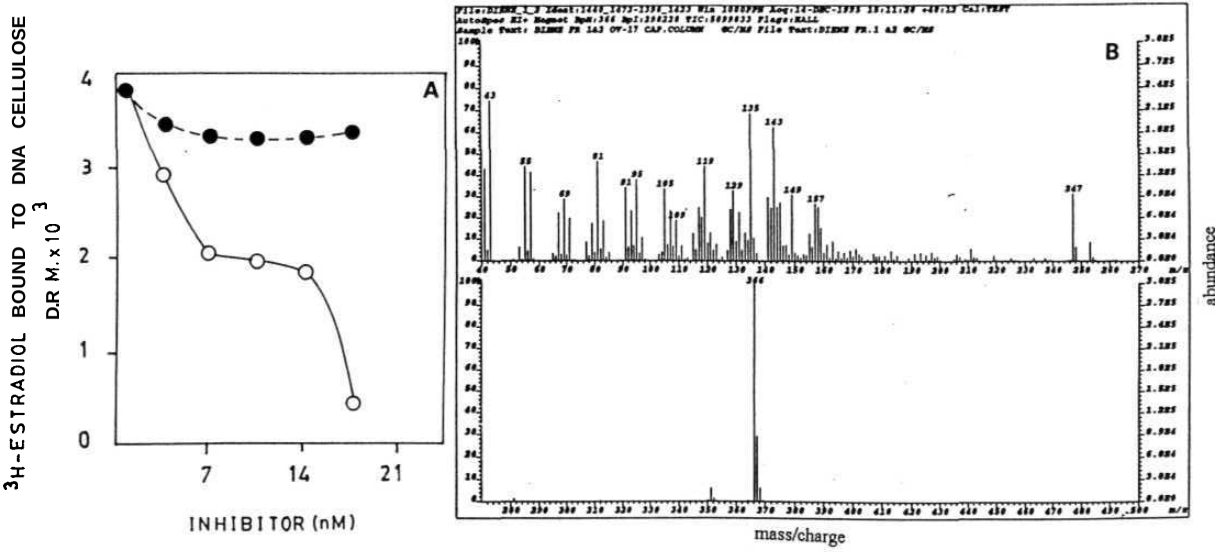
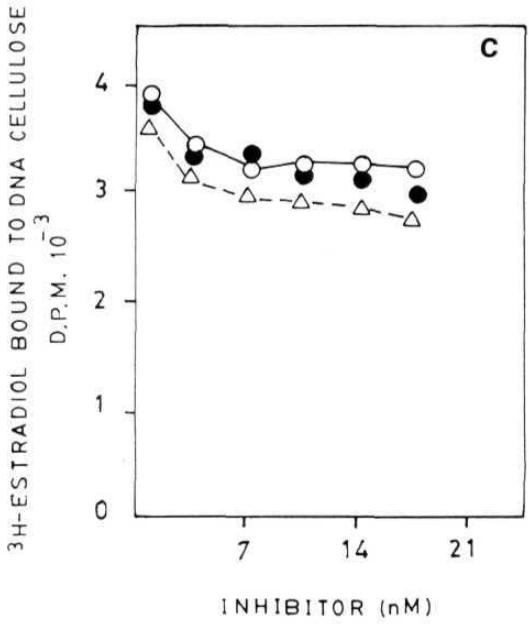

^{3}H-ESTRADIOL BOUND TO DNA CELLULOSE
D.R M. x 10 ^{3}
A
4
3
2
1
0
7
14
21
INHIBITOR (nM)
B
mass/charge
abundance

Figure 10C : The three fatty acids, palmitic (•), stearic (o) and myristic (A) acids failed to inhibit the DNA cellulose binding of ^{3}H-E_2 naER-E-RAF complex.


C
^{3}H-ESTRADIOL BOUND TO DNA CELLULOSE
D.P.M. 10^{-3}
4
3
2
1
0
7
14
21
INHIBITOR (nM)

Cholesterol inhibits the nuclear binding of naER-E-RAF complex:

Effect of cholesterol on the nuclear binding of the ^{3}H-E_2 naER-E-RAF complex was examined. It was observed that cholesterol inhibited the nuclear binding of the ^{3}H-E_2 naER-E-RAF complex in a concentration dependent manner (Fig. 11B). Maximum inhibition in the nuclear binding of the heterodimer was achieved by cholesterol at 20nM concentration.

Saturation binding of ^{3}H-cholesterol to E-RAF:

Cholesterol binds with high affinity ($Kd \sim 1 \times 10^{-10} M$) to E-RAF (Fig. 12B). E-RAF binding sites get saturated with 12nM cholesterol (Fig.12A). The following synthetic derivatives of cholesterol were tested for their capacity to inhibit cholesterol binding to E-RAF. The effect, in terms of their capacity to inhibit cholesterol binding to E-RAF was as follows: (1) Cholesteryl stearate (100%); (2) 5 a cholestan-3-one (100%); (3) (+) Dihydrocholesterol (100%); (4) 5 β–cholestan-3 α–ol (85%); (5) Cholesta-4, 6-dien-3-one (71%); (6) 5 cholesten-3-one (32%); (7) Cholesteryl oleate (32%); (8) (+) 4 Cholesten-3-one (32%), (9) 5-cholesten-3 β ol-7-one (25%); (10) Cholesteryl palmitate (10%). It was also observed that this property ran parallel to the capacity uf the individual compounds to inhibit nuclear binding of the ^{3}H-E_2 naER-E-RAF complex (Fig. 12C). The values in parentheses indicate the % inhibition effected by the test compound (2 μM) on the binding of 20nM ^{3}H-cholesterol to E-RAF.

Figure 11: Functional studies involving cholesterol. (A) Sucrose density gradient analysis of a mixture of ^{3}H-E_2 naER complex and E-RAF incubated either in the presence (o) or absence (•) of 20nM cholesterol. The mixture was layered over a 5 to 20% sucrose density gradient and subjected to centrifugation for 16 hours at 55,000rpm in the SW 65 rotor of a Beckman ultracentrifuge. Fractions collected were analysed for radioactivity. **(B) Effect of cholesterol on the nuclear binding of ^{3}H-E_2-naER-E-RAF complex.** Isolated nuclei were incubated with ^{3}H-E_2-naER and E-RAF in the presence ofvarying concentrations of cholesterol at 30°C for 30 minutes. The incubated samples were layered over 10ml 0.34M sucrose pads and centrifuged at lOOOxg for 10 minutes, as described in 'methods'. The radioactivity associated with the nuclei was measured.

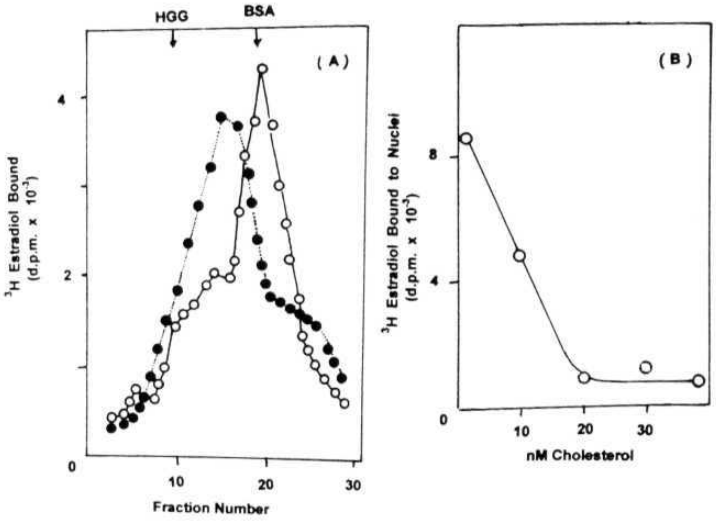
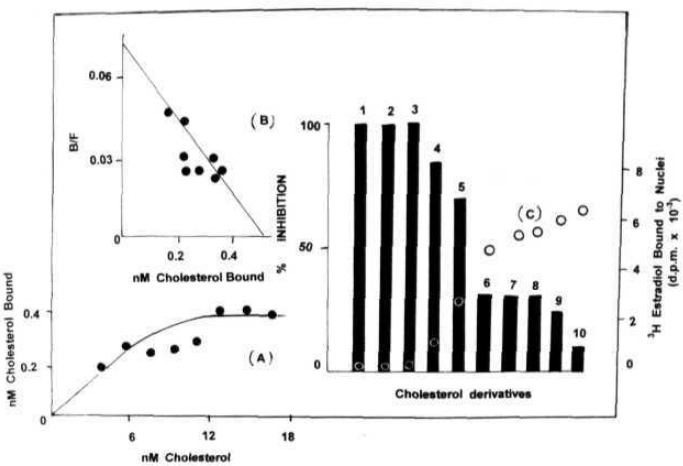

HGG
BSA
(A)
^{3}H Estradiol Bound
(d.p.m. x 10^{-3})
0
2
4
10
20
30
Fraction Number
(B)
^{3}H Estradiol Bound to Nuclei
(d.p.m. x 10^{-3})
0
4
8
10
20
30
nM Cholesterol

Figure 12: Binding of ^{3}H-cholesterol to E-RAF: (A) Saturation binding of ^{3}H-cholesterol to E-RAF, measured using a hydroxylapatite binding assay. (B) Scatchard plot of the binding data presented in (A). (C) Competition studies. A randomly selected derivatives of cholesterol were employed to find out their effectiveness to inhibit ^{3}H-cholesterol binding to E-RAF and also to prevent the nuclear binding of ^{3}H-E_2-naER-E-RAF complex. The histograms show the effect (represented as % inhibition) of the cholesterol derivatives on ^{3}H-cholesterol binding to E-RAF. The compounds were used at 2μM concentration while ^{3}H-cholesterol was employed at 20nM concentrations. The binding was measured using a HAP binding assay. The results of the nuclear binding assay are represented by the circles. Here, the nuclei were incubated with ^{3}H-E_2-naER and E-RAF in the presence of 20nM concentrations of the synthetic compounds The radioactive hormone bound to the nuclei was measured. The synthetic derivatives of cholesterol, selected at random, were:

(1) Cholesteryl stearate; (2) 5a cholestan-3-one; (3) (+) dihydrocholesterol; (4) 5 (3 cholestan-3a-ol; (5) Cholesta 4, 6 dien-3-one; (6) 5 cholesten-3-one; (7) Cholesteryl oleate; (8) (+) 4 cholesten-3-one, (9) 5 cholesten-3P-ol-7-one; (10) Cholesteryl palmitate.


nM Cholesterol Bound
0.4
0.2
0
6
12
18
nM Cholesterol
(A)
B/F
0.06
0.03
0
0.2
0.4
nM Cholesterol Bound
(B)
INHIBITION %
100
50
0
1
2
3
4
5
6
7
8
9
10
Cholesterol derivatives
(C)
^{3}H Estradiol Bound to Nuclei (d.p.m. x 10^{-3})
8
6
4
2
0

CD analysis of cholesterol-E-RAF interaction:

CD measurements were taken to find out whether the high allinity binding of cholesterol introduced any changes in the tertiary (Fig. 13A) or the secondary (Fig. 13B) structure of the E-RAF. A marginal yet noticeable change was observed in both the cases. However, in the tertiary CD the change observed at 280nm was insignificant, apparently indicating that the regions where conformational changes are introduced do not contain tryptophan or tyrosine residues.

E-RAF is an actin binding protein:

It was of interest to observe whether E-RAF binds to actin since that would suggest a possibility that actin mediates the intracellular transport of E-RAF. Purified E-RAF was chromatographed on a column of actin Sepharose. The protein bound to the column was eluted subsequently using a salt gradient and the fractions were assayed for E-RAF in a system containing ^{3}H-E_2-naER and isolated uterine nuclei. The results showed that E-RAF-actin interaction was strong since NaCl concentrations of 0.6-0.7 M was needed in order to dissociate E-RAF from the column (Fig. 14A).

Nuclear entry of FITC labeled E-RAF or naER:

Both naER and E-RAF were labeled with FITC in order to examine their nuclear entry in the presence or absence of the other component of the heterodimer. FITC-labeled naER failed to enter the nucleus on its own (Fig. 15A and F). Following dimerization with the E-RAF the complex remained at the nuclear periphery, incapable of entering the nucleus (Fig. 15B and G). FITC-labeled E-RAF gained an immediate entry into nucleus

(Fig. 15C and H). Following dimerization with naER the complex remained at the nuclear periphery (Fig. 15D and I). Exposure of FITC-labeled E-RAF to 20nM cholesterol prevented the entry of the protein into the nucleus (Fig. 15E and J). The figures (g) and (i) are presented here in order to augment the information quality of figures (G) and (I) respectively. 'g' displays retention of FITC-naER + E-RAF at the nuclear periphery while (i) represents the retention of FITC–E-RAF + naER at the nuclear periphery.

Figure 13: Circular dichroism measurement of cholesterol interaction with E-RAF. E-RAF (~3μg protein in 450 μl) was analysed in a JASCO spectropolarimeter either in the presence (broken line) or in the absence (solid line) of 20nM cholesterol Near UV CD spectra were taken to detect any change that occurs in the E-RAF conformation either at the tertiary structure (A) or at the secondary structure (B) level.

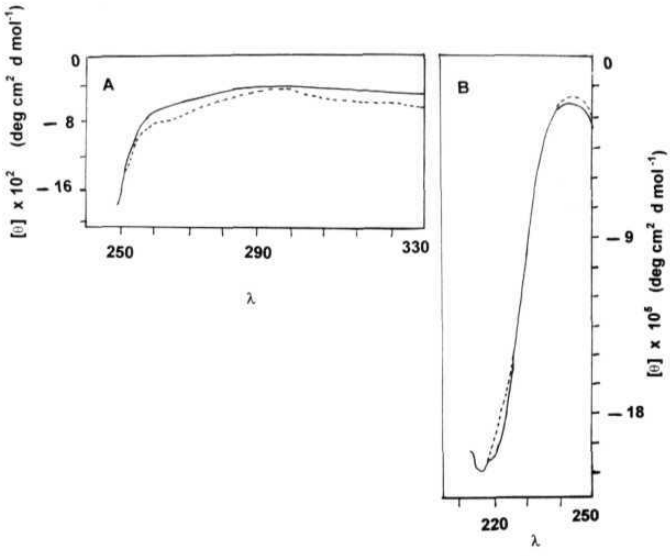

A
B
0
8
16
9
18
250
290
330
220
250
λ
λ
[θ] x 10² (deg cm² d mol⁻¹)
[θ] x 10⁵ (deg cm² d mol⁻¹)

Figure 14: Actin-Sepharose chromatography of purified E-RAF: (A) Purified E-RAF was chromatographed over a column of actin-immobilized on CNBr activated Sepharose 4B. The column was washed with the TEMN buffer. The E-RAF bound to the column was eluted using a salt gradient. Individual fractions were incubated with ^{3}H-E_2-naER and isolated uterine nuclei at 30°C for 30 minutes. The labeled hormone bound to the nuclei was measured following extraction of the nuclear pellet with ethanol and analysing the radioactivity associated with the ethanol extract.

(B) Shows the SDS gel of the E-RAF that was chromatographed on the column and subsequently recovered in the 'peak' fraction.

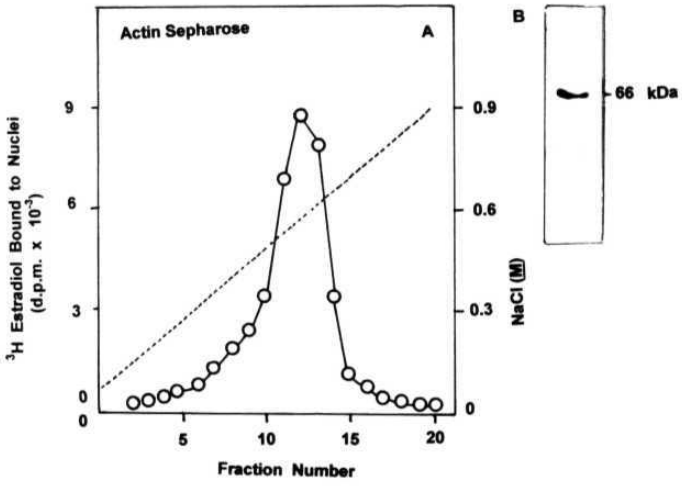
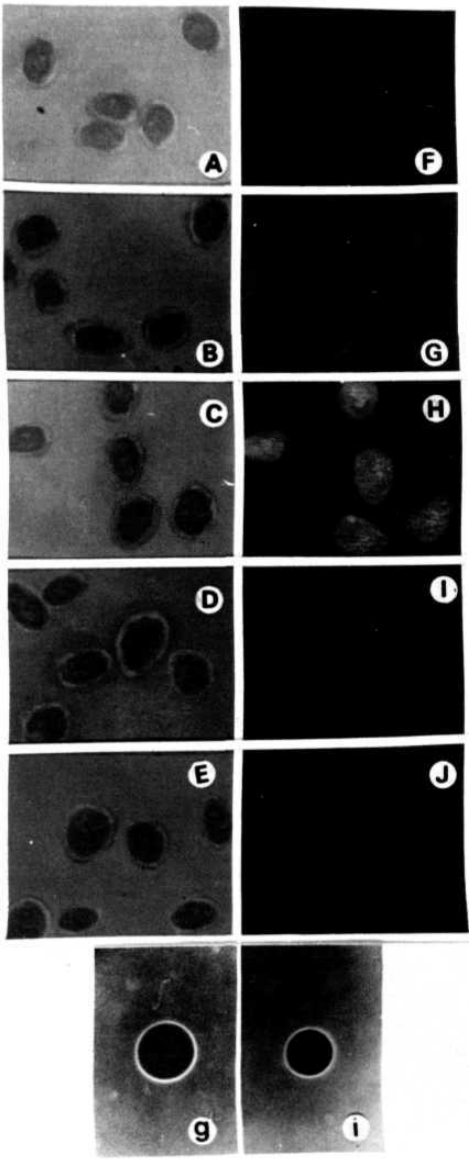

Actin Sepharose
A
B
66 kDa
^{3}H Estradiol Bound to Nuclei
(d.p.m. x 10^{-3})
NaCl (M)
Fraction Number
0
3
6
9
0.3
0.6
0.9
5
10
15
20

Figure 15: Nuclear entry of naER and E-RAF: Both naER and E-RAF were labeled with FITC and incubated with isolated uterine nuclei under appropriate conditions as described in 'methods' A, B, C, D and E are fluorescence micrographs of nuclei, the phase contrast micrographs of which are presented in F, G, H, I and J respectively. (A and F) nuclei incubated with FITC-labeled naER alone. (B and G) nuclei incubated with FITC-labeled naER and unlabeled E-RAF. (C and H) nuclei incubated with FITC-labeled E-RAF alone. (D and I) nuclei incubated with FITC labeled E-RAF and unlabeled naER. (E and J) nuclei incubated with FITC labeled E-RAF and 20nM cholesterol. (g) and (i) have been presented here to augment the effectiveness of (G) and (I) respectively. (g) shows the nuclear (peripheral) binding of FITC-naER + E-RAF and (i) displays the same effect produced by FITC-E-RAF+naER.


A
F
B
G
C
H
D
I
E
J
g
i

CHAPTER - IV

DISCUSSION

Steroid receptor activation is a molecular mechanism that has received wide recognition by investigators working with different steroid receptor systems (Higgins, et al., 1973, Bailly, et al., 1980; Grody et al., 1982). Low molecular weight ligands inhibiting steroid hormone-receptor activation have been known for a long time. During early seventies, Sato and co-workers (Sato et al., 1978, 1980) observed that a heat stable and dialysable component endogenous to the mammalian uterus prevented the estrogen receptor-activation process. The most extensively characterized molecule in this category of inhibitors is the 'modulator' a phosphoglyceride that inhibits the glucocorticoid receptor activation process (Bodine & Litwack, 1988, 1990; Bodine et al., 1994).

The estrogen receptor activation process has been studied in our laboratory, in a totally different perspective. The ER under the present study is not the well-recognized receptor (ER a) that binds as a homodimer to the estrogen responsive element in the target gene. This alternative ER of our interest, the naER, is not a DNA binding protein. It gains access to the DNA only after it dimerizes with the E-RAF, a DNA-binding protein whose binding domain on the estrogen responsive gene still remains undiscovered. The 'receptor activation' in focus here is therefore, the dimerization between the naER and the E-RAF.

The information that the inhibitor is heat stable (Sato et al, 1978) and that the heat treatment step released the inhibitor from the putative cytosolic binding proteins (Bodine and Litwack, 1988), prompted me to heat the uterine homogenate in a boiling water bath during the inhibitor purification. The inhibitor, being a low-molecular weight component, is easily adsorbed to charcoal. This step enables its separation from macromolecular entities. Bodine and Litwack (1990) reported that the low molecular weight modulator of

the GR-complex activation is an anion. This prompted me to use DE-52 chromatography step in the purification protocol. The DE-52 fraction with the inhibitory activity was subjected to thin layer chromatography (TLC) in three different solvent systems. The partially purified inhibitor was subjected to gas chromatography-mass spectrometry (GC/MS).

Gas chromatography is based upon the partition of components between a mobile gas phase and a stationary liquid phase retained as a surface layer on a suitable solid supporting medium. The components of the sample are separated depending on their individual partition coefficients between the stationary and the mobile phase. In the mass spectrometry, the compound under investigation is bombarded with abeam of electrons which produce an ionic molecule or ionic fragments of the original species. The resulting assortment of charged particles is then separated according to their masses. The spectrum produced, called as mass spectrum, is a record of information regarding various masses produced and their relative abundance. In the gas chromatography and mass spectroscopy (GC/MS), both gas chromatography and mass spectroscopy are integrated and the data obtained is analysed by the computer. The computer compares the data obtained with those of the already existing compounds and it comes out with the picture of the most probable candidate molecule.

From the GC/MS analysis of the partially purified inhibitor material it was evident that the inhibitor activity is associated with (a) cholesterol, (b) its metabolite cholesta-4,6 diene-3-ol or (c) one or more of the fatty acids, myristic, palmitic or stearic acids. The assay necessitated the synthesis of the cholesterol metabolite identified in the computer search. There was a noticeable difference in the retention times of cholesterol

as noticed in the gas chromatograms of the partially purified inhibitor and the extract of the material that was bound to the immunoprecipitated E-RAF. The difference could be due to the fact that there were many compounds in the partially purified inhibitor sample in contrast to those bound to the E-RAF immunoprecipitate. The observation that along with cholesterol both palmitic and stearic acids were observed to be associated with the immunoprecipitated E-RAF was indicative of a non-specific association of fatty acids with E-RAF. The fatty acids failed to inhibit the naER-E-RAF dimerization and consequently, the DNA binding of the proteins. Being a basic protein, the E-RAF could form electrostatic interactions with the fatty acids. The interaction apparently did not introduce any structural changes in the E-RAF that could lead to a decrease in its capacity to dimerize with the naER.

Various synthetic derivatives of cholesterol, selected at random, were tested for their capacity to inhibit cholesterol binding to E-RAF as well as interfere with E-RAF-naER dimerization. What was conspicuous was the fact that the compound that could compete for cholesterol binding sites on the E-RAF could also bring about a corresponding inhibition in the nuclear binding of ^{3}H-E_2-naER-E-RAF complex. It was interesting to note that the synthetic cholesta-4,6-diene-3-one could bring about 71% inhibition in cholesterol binding to E-RAF as well as ^{3}H-E_2-naER-E-RAF binding to the nuclei while its close relative, found under natural conditions, choesta-4,6-dien-3-ol failed to bring about this effect. May be that the naER dimerization domain lies very close to the cholesterol binding domain on the E-RAF. The results, however, do serve to highlight the assumption that the binding of cholesterol or its synthetic derivative to E-RAF introduced a conformational change in the protein, rendering it temporarily

incapable of dimerization with naER. This inference is supported by the CD data on the E-RAF-cholesterol interaction.

There has recently been a study that reported the findings regarding the factors that regulate the endogenous E-RAF level within the rat uterus (Kumar and Thampan, 1995). The study involved the use of ELISA in measuring the E-RAF titer in the uterine cell. E-RAF synthesis was followed by quantification of the ^{35}S-methionine-labeled E-RAF that was immunoprecipitated by anti E-RAF IgG. Extreme caution was observed in these studies to prevent the contamination of test samples by ER and naER since both these proteins recognize polyclonal anti E-RAF IgG. It was observed that the E-RAF synthesis in the rat uterus is under the control of both estradiol and progesterone. While non-estrogenic steroids did not influence the E-RAF synthesis, tamoxifen effectively antagonized it. The most striking observation in this study was that of the E-RAF level in relation to different stages of pregnancy in the laboratory rat. The E-RAF titer during pregnancy was found to be closely parallel to the circulating levels of progesterone. It reached a peak towards day 16 of pregnancy. The level declined immediately afterwards reaching an undetectable status a day prior to parturition. Another observation that pointed towards the physiological significance of the E-RAF titre was the total decline in E-RAF produced as a result of *in vivo* exposure of the cycling rat uterus to Mala-N, an oral contraceptive consisting of Norgestrel and ethinyl estradiol.

These observations clearly imply that any agent that blocks the nuclear entry of E-RAF can effectively block estrogen action with reference to a specific gene. It is possible that not all estrogen-regulated genes are controlled by E-RAF/naER or for that matter by the regular ER-homodimer itself. Looking at the relative amounts of naER and

ER within the target cell, it may be speculated that the genes influenced by naER (through E-RAF) and those influenced by ER may follow a 1:10 ratio since that is the value that represents the total recovery of the proteins from a given mass of tissues. E-RAF exists in two functional forms, I and II (Thampan, 1989). While E-RAF II destabilizes the DNA double helix, E-RAF I stabilizes it. Consequently E-RAF II enhances transcription in a reconstituted *in vitro* transcription system while E-RAF 1 inhibits it. Therefore, it will not be out of place to suggest that the functional role of E-RAF is that of a transcription factor, capable of enhancing or blocking gene expression depending on whether which form of E-RAF arrives at the transcription site.

Based on the observations made by other investigators it is possible to speculate that the E-RAF has the status of an orphan receptor. Recent studies point towards an apparent structural and functional relationship between the E-RAF I and E-RAF II on the one hand and the hERR II and hERR I respectively, on the other. hERRI and hERRII were discovered by Evan's group (Giguere et al., 1988). E-RAF I is a transcription inhibitor: a similar function has been attributed to hERR II (Trapp & Holsboer, 1996). If E-RAF is given recognition as an orphan receptor, cholesterol will receive recognition instantaneously as the 'elusive' ligand of an orphan receptor.

lntracellular transport of cholesterol in eukaryotic cells has been the subject of intensive studies in several laboratories. It is known that cholesterol is an essential requirement in plasma membrane formation and also that the major intracellular site of cholesterol synthesis is the endoplasmic reticulum. Therefore, there is a need for an effective mechanism for the transport of cholesterol from the endoplasmic reticulum to the plasma membrane. One possibility that has been suggested is that it is a transport

protein dependent mechanism involving a cholesterol-binding protein (Friedlander et al. al., 1980; Ishibashi and Bloch, 1981; Lange and Matthies, 1984; Pfeifer et al., 1993). Evidence has been presented to show that this transport is both energy and cytoskeletal elements-dependent response. Agents which disrupt the cytoskeletal machinery (Crivello and Jefocate, 1980) and metabolic toxins that inhibit energy release have been shown to block the intracellular transport of cholesterol (Noland et al., 1980, Dempsey et al., 1981). The role of cytoskeletal proteins in cholesterol transport, however remains controversial since recent reports deny any such role in the vesicular traffic of endogenously synthesized cholesterol (Liscum and Underwood, 1995). It has been shown that cholesterol is an unavoidable item required for DNA replication and cell proliferation in mammalian systems (Chen, 1984; and Siperstein, 1984; Tabacik, et al., 1985). Cholesterol synthesis in MCF-7 cells is under estrogenic regulation (Cypriani et al., 1988a and b). Taken together, the experimental data indicate that cholesterol has a functional role to play in estrogen action. The synthetic activities within a cell will be influenced by the structural integrity of the cell which in turn is dependenl on the structural and functional status of the plasma membrane. The negative functional role of cholesterol may be restricted to the expression of a small number of genes mediated by naER-E-RAF heterodimer. That may well be the reason why previous investigators failed to spot any 'antiestrogenic' effect of cholesterol. The reason that is the basis of such an interpretation is the observation that the total mass of ER and naER isolated from a given mass of tissue follows a 10:1 ratio (Thampan, unpublished observation). This may imply that the total number of estrogen-responsive genes that are regulated by the ER and the naER may also follow a 10:1 ratio.

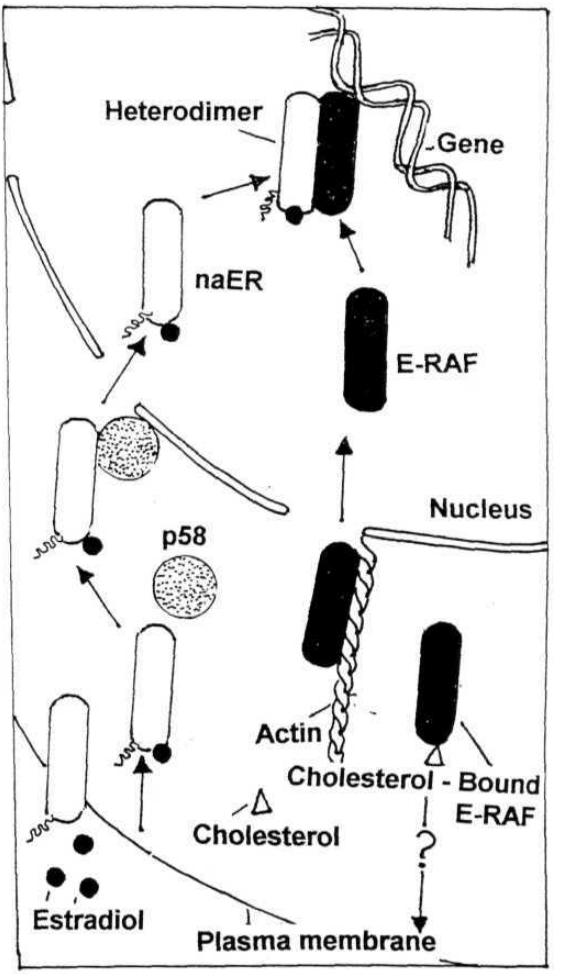
Estradiol-cholesterol interrelationship is exemplified in a different perspective elsewhere. Women are known to be less prone to coronary heart diseases than men during the reproductive age. Advancing age reverses this trend in women when the circulating levels of estradiol recede (Rosenberg et al., 1976). Treatment of post-menopausal women with estradiol reduces the coronary heart diseases risk while opening up the risk of cancer of the reproductive tissues (Sherman et al., 1983). The cholesterol that is under focus here is the extracellular and circulating cholesterol. One possible explanation that could be suggested here is that estradiol may increase the LDL receptors on the plasma membrane which will in turn have a positive effect on the absorption of LDL-cholesterol thereby reducing the circulating level of cholesterol. The internalized cholesterol will immediately be available for plasma membrane synthesis. Recent observations of Lundeen et al. (1997) from experiments carried out on rats provide a reasonable basis for the subject mentioned above. The significance of naER-E-RAF heterodimer in this phase of estrogen action needs to be underscored.

Thus, under the influence of estradiol, the uterine cell is exposed to cholesterol derived from two sources: the extracellular cholesterol internalized through LDL receptors and the intracellular cholesterol which forms the immediate source for the re-building of plasma membrane. E-RAF is an ATPase (Thampan, 1989) and also an actin-binding protein. In view of the various properties associated with the E-RAF, including its affinity for cholesterol binding it is reasonable to speculate that one of the primary functions of E-RAF may be intracellular cholesterol transport, presumably from the endoplasmic reticulum to the plasma membrane. The observation that cholesterol-bound E-RAF fails to enter the nucleus supports this argument since, under these

conditions, the only known function that E-RAF can perform is that of a cholesterol-carrier protein.

Sterol regulatory element binding proteins (SREBPs) as have been described in the 'introduction', are the proteins that regulate cholesterol metabolism by means of a proteolytic pathway. They are a set of 125kDa proteins. During sterol depleted conditions, these proteins undergo cleavage to become 66kDa entities which enter the nucleus as a homodimer and bind to the sterol regulatory elements of various genes of the proteins that are involved in the synthesis and uptake of cholesterol and also in the synthesis of fatty acids (Brown and Goldstein, 1997). The estrogen receptor activation factors (E-RAFs) are also 66kDa proteins. The E-RAFs are also transcriptional factors like SREBPs. It would be interesting to know whether E-RAFs are SREBPs themselves or are atleast similar to them. Future studies on this aspect will throw more light on the cholesterol metabolism and also on cholesterol-estradiol inter-relationships.

Figure 16: A model that highlights the major findings presented in this thesis
Nuclear entry of naER is mediated by a 58kDa protein while that of E-RAF is an independent mechanism (S. Sreeja and R. V. Thampan, unpublished observations). Cholesterol binding prevents the nuclear entry of E-RAF. It is assumed that under these circumstances the E-RAF may function as an intracellular cholesterol transport protein carrying cholesterol to the plasma membrane. This, however, needs to be ascertained experimentally.


Heterodimer
Gene
naER
E-RAF
Nucleus
p58
Actin
Cholesterol - Bound
E-RAF
Cholesterol
?
Estradiol
Plasma membrane

Summary:

The steroid receptor 'activation' process has been well worked out with the regular estrogen receptor (ERα). The steroid receptor 'activation' is the term applied to the process in which the non-activated form of the receptor which cannot bind to the DNA is converted to an activated form which binds to the DNA. The major focus of our laboratory is on an alternative form of estrogen receptor called non-activated estrogen receptor (naER). This receptor never gets activated under the conditions in which the ERa gets activated. The naER gets activated only upon dimerization with a DNA binding and a non-estrogen binding protein called the estrogen receptor activation factor (E-RAF).

During the purification of E-RAF, it was observed that an inhibitor was co-purified along with the E-RAF which prevented the activation of naER. The identification and characterization of this inhibitor is the subject matter of this thesis. A fraction obtained after DE-52 chromatography and thin layer chromatography (TLC) of the cmde inhibitor fraction was found to contain the inhibitory activity. This fraction was analysed through gas chromatography and mass spectrometry (GC/MS) and displayed the presence of 5 major compounds-Cholesterol, cholesta 4, 6 diene-3ol, myristate, palmitate and stearate. Only cholesterol inhibited the activation of naER.

Some functional studies were carried out using cholesterol. It was found that cholesterol bound to E-RAF with high affinity. Cholesterol induced a noticeable change in the secondary and tertiary structures of E-RAF and prevented the dimerization between naER and E-RAF. Cholesterol also prevented the binding of E-RAF to the nucleus. The

naER-E-RAF complex does not enter the nucleus as a dimer while E-RAF enters the nucleus on its own. This clearly indicates that the nuclear entry of naER is through a totally different mechanism, independent of that which governs the E-RAF entry.

It is being mentioned here as a hypothesis that E-RAF-naER complex mav not be a universal mediator of estrogen action. May be that the complex is responsible for the activation of a selected number of genes which may not come under the classical ER influence. Identification of these genes will be a target for studies to be carried out in the future.

Conclusions:

1) The heat stable and dialysable factor that inhibits the 'activation' of the non-activated estrogen receptor (naER) of the goat uterus has been isolated and its structure identified. The term 'activation' is used here to identify the dimerization between the naER and the DNA binding protein, the estrogen receptor activation factor (E-RAF).
2) The major steps involved in the isolation of the inhibitor are: (a) heat treatment of the tissue homogenate in a boiling water bath in order to coagulate the macromolecules, (b) adsorption to activated charcoal, (c) chromatography of the charcoal adsorbed factor on Whatman DE-52, and (d) three successive silica gel thin layer chromatographic steps using three different solvent systems.
3) The analytical procedures used in the characterization of the inhibitor included UV spectroscopy and gas chromatography-mass spectrometry
4) These analytical procedures clearly indicated that the inhibitor under study is unmetabolised cholesterol and also that the target molecule to which cholesterol binds is the E-RAF.
5) Several experiments were carried out in order to gain an insight into the mechanisms by which cholesterol inhibited E-RAF-dependent naER activation. Cholesterol bound to E-RAF with high affinity (Kd=l x 10^{-10}M). Cholesterol-bound E-RAF was incapable of dimerization with naER. Circular dichroism analysis of the cholesterol-E-RAF interaction indicated that it resulted in subtle changes in the 2D as well as 3D structure of the E-RAF.
6) Fluorescence microscopic analysis of the nuclear binding/entry of FITC-labeled naER/E-RAF provided some new revelations. It showed that E-RAF entry into the

nuclei did not require the mediation of another protein. E-RAF-naER heterodimer remained at the nuclear periphery, incapable of entering the nucleus, pointing out to the possibility that the dimerization should take place only after the two proteins reach the nucleus. Cholesterol-bound E-RAF failed to bind to the nuclei even at the peripheral level.

7) This observation finally leads to a speculation. Since cholesterol binding inhibits the E-RAF entry into the nucleus, it indicates that the net result of these reactions will be total inhibition of E-RAF-mediated gene regulatory events (naER is incapable of binding to the DNA on its own). What these genetic events are, need to be identified. It is possible that naER/E-RAF heterodimer is responsible for the activation of only a small set of genes which do not come under the regulatory influence of the classical ER homodimer. If the genes which influence cholesterol homeostasis come under this category (eg. LDL receptor gene), it will be possible to speculate that cholesterol functions here as an internal feedback regulator. When the intracellular level of cholesterol is high, the E-RAF fails to enter the nucleus. Probably, under these conditions, the E-RAF may function as an intracellular cholesterol transport protein. When the intracellular level of cholesterol is low, causing dissociation of cholesterol from E-RAF, the E-RAF regains its freedom to enter the nucleus and initiate gene regulatory events.

8) It needs to be pointed out that in this perspective E-RAF closely resembles the sterol responsive element binding protein (SREBP) identified by Goldstein and Brown. Whether E-RAF binds to the SRE remains to be known.

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