4.0. GENE REGULATORY FUNCTIONS OF ESTROGEN RECEPTOR ACTIVATION FACTOR (E-RAF) UNDER PROGESTERONE INFLUENCE

Estrogen receptor activation factor (E-RAF) is a high affinity progesterone binding protein (Govind et al., 2003a, 2000b). The studies reported by these authors indicated that progesterone binding to E-RAF resulted in the dissociation of tp66 (transport protein 66) from E-RAF, causing the inhibition of nuclear entry of E-RAF. Earlier studies reported from this laboratory (Premkumar and Thampan, 1995) had shown that E-RAF expression in the rat uterus was influenced by both estradiol and progesterone. In addition, during pregnancy, the E-RAF titer in the rat uterus was found to increase steadily during the first half, reaching the peak during mid pregnancy. The E-RAF level in the pregnant uterus declined subsequently, reaching an insignificant level immediately before parturition. A careful analysis of these results indicates a full progestestational control of E-RAF gene expression in the rat uterus during pregnancy. At the same time it was also indicative of E-RAF mediation in progesterone action during pregnancy.

The studies presented in this chapter were carried out to examine this possibility; whether E-RAF mediated gene expression is also regulated by progesterone.

4.1. Confocal microscopic studies on the intracellular movement of E-RAF in goat uterine endometrial cells under progesterone influence

In order to study the intracellular movement of E-RAF in the presence of varying levels of progesterone, a primary culture of goat endometrial cells was exposed to different concentrations of progesterone (0, 4, 16, 24, 32 nM) for a duration of 48 hours. Subsequent to this, the cells were fixed, permeabilized and exposed to a
polyclonal antibody against goat uterine E-RAF. The protein was visualized using FITC labeled anti rabbit IgG. Confocal microscopy of the cells clearly showed that the nuclear entry of E-RAF was influenced by a highly critical concentration of progesterone in the medium. When exposed to low progesterone concentrations (4 nM) the cells displayed the presence of E-RAF mainly in the cytoplasmic area around the nucleus whereas, exposure of the cells to progesterone at a concentration of 16 nM was found to result in a total nuclear entry of E-RAF (Fig. 4.1.1). E-RAF was seen exclusively localized in the cytosol in the presence of higher progesterone concentrations (24, 32 nM) (Fig. 4.1.2).

4.2.1. Identification of the transport protein that mediates the nuclear entry of E-RAF- progesterone complex
Since exposure of E-RAF to progesterone makes the protein inaccessible for tp66 dependent nuclear transport, it was evident that yet another protein should be involved in the nuclear entry of progesterone- E-RAF complex. The experiment presented here was carried out to examine this possibility. Isolated nuclei of goat uteri (the nuclei that were exposed to 0.1% Triton X 100, during isolation, as well as the nuclei which were not exposed to the detergent) were incubated with \(^3\)H-progesterone-E-RAF complex at 30 °C for 30 minutes. The \(^3\)H-progesterone that was bound to the nuclei was measured as described earlier. It was observed that the detergent treated nuclei failed to bind progesterone-E-RAF complex while the control nuclei retained high level of the protein-hormone complex. The results were clearly indicative of the presence of a nuclear periphery associated factor that mediated the nuclear binding of progesterone-E-RAF complex (Fig. 4.2.1).

4.2.2. Presence of ATP is essential for the nuclear transport of E-RAF progesterone complex
Isolated nuclei were suspended in 2 X TMKC sucrose buffer, either in the presence or absence of 8 mM Mg\(^{2+}\) ATP. The nuclei were incubated with \(^3\)H
progesterone – E-RAF complex (E-RAF, incubated overnight with 20 nM \(^3\)H progesterone and subjected to exposure to dextran coated charcoal, to remove free progesterone) at 30 °C for 30 minutes. The incubated nuclei were washed with TMKC- sucrose buffer and extracted with ethanol. Radioactivity associated with the ethanol extract was measured. The results clearly indicated that ATP was essential to be present in the transport assay medium for the nuclear transport of E-RAF- progesterone complex to take place (Fig. 4.2.2).

### 4.3. Isolation of the transport protein

An affinity column, E-RAF Sepharose, was made by immobilizing purified E-RAF on CNBr activated Sepharose 4 B. The column was equilibrated with 50 mM Tris-HCl, pH 7.6 containing 0.2 mM PMSF and 20 nM progesterone. A detergent extract of goat uterine nuclei was made by suspending the nuclei in 50 mM Tris–HCl, pH 7.6 containing 250 mM sucrose, 20 mM KCl, 2 mM MgCl\(_2\), 1 mM CaCl\(_2\), 0.2 mM PMSF, 20 nM progesterone and 0.1 % Triton X 100. Following centrifugation at 800 x g for 10 minutes the supernatant was collected and chromatographed over a column of E-RAF Sepharose equilibrated with buffer containing 20 nM progesterone. The column was washed with this buffer and elution was achieved using 0-1 M NaCl gradient in the same buffer. The fractions (1 ml) collected were analyzed for absorbance at 280 nm and the ability to transport (bind) \(^3\)H-progesterone complex to the nuclei (Fig.4.3.1).

#### 4.3.1. Nuclear transport assay mediated by the E-RAF Sepharose bound protein

Goat uterine nuclei were isolated as reported by Thampan (1985). The nuclei were suspended in 2 x TMKC- Sucrose buffer (50 mM Tris- HCl pH 7.6 containing 500 mM sucrose, 40 mM KCl, 8 mM MgCl\(_2\), 2 mM CaCl\(_2\), 0.4 mM PMSF and 8 mM ATP). Purified E-RAF (100 \(\mu\)l) was incubated overnight at 4 °C
with 20 nM $^3$H progesterone. The free hormone was separated from the bound following exposure to an equal volume (100 μl) of dextran coated charcoal (DCC) suspension (1% charcoal and 0.1% dextran) in 10 mM Tris- HCl pH 7.6 containing 10 % glycerol. 150 μl of the supernatant was mixed with 100 μl of the E-RAF Sepharose column eluate and incubated at 30 °C for 30 minutes after the addition of 250 μl of the nuclear suspension. The incubated nuclei were cooled in ice and 2 ml of ice-cold TMKC- sucrose buffer (containing 250 mM sucrose) was added to the assay tubes. The tubes were centrifuged at 800 x g for 10 minutes. The nuclear pellets were extracted with 1 ml ethanol and the ethanol extracts were examined for radioactivity using a Wallac liquid scintillation counter (Fig.4.3.1).

4.3.2. Identification of dp55
The affinity chromatography presented evidence for a protein that facilitated progesterone E-RAF complex binding to isolated nuclei (Fig 4.2 a). SDS- PAGE analysis of the peak fraction displayed a 55 kDa protein, which is hereby identified as dp55 (docking protein 55) (Fig 4.3.2).

4.4. Confirmation of dp55 dependent transport of progesterone-E-RAF
To observe whether progesterone has a facilitatory role to play in the transport, FITC-E-RAF was incubated with varying (nM) concentrations of progesterone (0, 3, 5, 7,10,13,16,18,20,23 nM) before exposing the nuclei to the mixture. The nuclei used in this study were not exposed to the detergent. The dp55 therefore was associated with the nuclear periphery. Under the above experimental conditions the nuclear entry of FITC-E-RAF was observed only in the presence of 23 nM progesterone (Fig. 4.4).
4.5. Collagenase gene expression in goat endometrial cells as a measure of E-RAF mediated gene regulatory event

Earlier studies reported from our laboratory had indicated an apparent correlation between the presence of E-RAF and collagenase gene expression in the rat uterus during various hormonal exposures. It raised a question whether E-RAF had a direct role to play in collagenase gene expression in the uterine nucleus. It was felt that the endometrial cell culture should provide the appropriate experimental tool to analyse this possibility. As was done in earlier studies, endometrial cells were exposed for 48 hours to varying concentrations of progesterone. At the end of the hormonal exposure period the culture medium was collected and assayed for collagenase activity using the hydroxyproline release measurement method, given in the experimental section. Here the results displayed a biphasic pattern. An initial highly marginal increase in the enzyme activity was noticed when the cells were exposed to progesterone concentrations below 10 nM. Increase in progesterone concentration above 10 nM in the medium provided a clear enhancement in collagenase activity, reaching a peak in the medium containing 18 nM progesterone (Fig. 4.5).
Goat endometrial cells were exposed to varying concentrations of progesterone for 48 hours following which the cells were fixed, permeabilized and exposed to anti E-RAF IgG, followed by FITC-labeled anti rabbit IgG (A). Nuclei were stained with propidium iodide (B). The intracellular movement of E-RAF was visualized by merging the images (C). The results clearly indicate an enhanced nuclear localization of E-RAF in the presence of 16nM progesterone.
Figure 4.1.1

A

B

C

0 nM

4 nM

16 nM
Figure 4.1.2

24 nM

32 nM
Figure 4.2. Identification of a nuclear periphery associated factor involved in the nuclear transport of E-RAF progesterone complex

(1) Goat uterine nuclei were isolated as explained in the text. One half of the nuclear preparation was kept as the control D (-) while the remaining half was subjected to exposure to 0.1% Triton X 100 in TMKC- sucrose buffer (D+). The two nuclear preparations were incubated with E-RAF- $^3$H- progesterone complex at $30^\circ$C for 30 minutes following which the labeled hormone that was bound to the nuclei was measured.

(2) The nuclei which were not exposed to the detergent were grouped into two. One batch was suspended in 2 X TMKC- sucrose containing 8 mM ATP (+ATP) while the other batch was suspended in ATP free buffer (-ATP). The two batches of nuclei were incubated with E-RAF- $^3$H- progesterone complex at $30^\circ$C for 30 minutes following which the labeled hormone that was bound to the nuclei was measured.
Figure 4.2

(1)

(2)
Figure 4.3.1 Isolation of the nuclear transport protein that transports progesterone – E-RAF complex.

Detergent extracts of goat uterine nuclei made in TMKC sucrose buffer containing 20 nM progesterone and 0.1 % Triton X 100 was chromatographed over a column of E-RAF sepharose equilibrated with TEM buffer containing progesterone. The column was washed with this buffer and elution was achieved using 0-1 M NaCl gradient in the same buffer. The 1 ml fractions collected were analyzed for absorbance at 280 nm (solid circle). The fractions from E-RAF sepharose column were subjected to nuclear transport assay and the radioactivity associated with the nuclei (open square) was measured.

Figure 4.3.2 SDS- PAGE analysis of the peak fraction presented in Figure 4.3.1

M- Marker, S- Sample.
Figure 4.3

![Graph showing fraction number on the x-axis and H-progesterone bound to nuclear c.p.m. x 10^3 on the y-axis. The graph also shows absorbance at 280 nm and M and S markers. The markers indicate protein bands at 66 kDa and 43 kDa.](image-url)
Figure 4.4. Role of progesterone in the nuclear entry of FITC-labeled E-RAF

Purified E-RAF was labeled with FITC as described in the text. FITC – labeled E-RAF was incubated with varying concentrations of unlabeled progesterone (0, 3, 5, 7, 10, 13, 16, 18, 20 and 23 nM) overnight at 4°C. 20 µl of the incubated material was mixed with 20 µl of isolated goat uterine nuclei, suspended in 2 x TMKV-sucrose buffer containing 8 mM Mg²⁺ ATP on a microscopic slide. The nuclei were examined under a fluorescence microscope. (A) The phase contrast micrograph of isolated nuclei and (B) the same nuclei incubated with FITC-E-RAF that was exposed to 23 nM progesterone. FITC-E-RAF that was exposed to lower concentrations of progesterone did not display any clear nuclear entry signal.
Figure 4.4

A

B
Figure 4.5. Collagenase gene expression in goat endometrial cells as a measure of E-RAF mediated gene regulatory event

Endometrial cells were exposed for 48 hours to varying concentrations of progesterone. At the end of the hormonal exposure period the culture medium was collected and assayed for collagenase activity using the hydroxyproline release measurement method, given in the experimental section. An initial highly marginal increase in the enzyme activity was noticed when the cells were exposed to progesterone concentrations below 10 nM. Increase in progesterone concentration above 10 nM in the medium provided a clear enhancement in collagenase activity, reaching a peak in the medium containing 18 nM progesterone.
Figure 4.5

![Graph showing the relationship between hydroxyproline released and progesterone concentration. The graph displays a peak at around 15 nM progesterone with a corresponding hydroxyproline release of approximately 400 μg.]
Figure 4.6. E-RAF transport into the nucleus: An integrated model.

This shows that there are two independent channels in E-RAF action: the estrogen mediated and the progesterone mediated. In the former case, tp66 mediates the nuclear transport or free E-RAF. In the latter case dp55 mediates the nuclear movement of progesterone-E-RAF complex. Whether the nuclear pore complex protein that docks the two proteins is the same is the same (npcp 38) or different is not known at this stage.
NUCLEAR ENTRY OF E-RAF: TWO PATHWAYS

- **NPC**
- **E-RAF**
- **Cytosol**
- **E-RAF with altered conformation**
- **Endoplasmic reticulum**
- **Progesterone**
- **Nuclear membrane**
- **Nucleus**

**Pathway 1:**
- Estradiol
- E-RAF
- Cytosol
- E-RAF with altered conformation
- Progesterone
- Nuclear membrane
- Nucleus

**Pathway 2:**
- Ap55
- Tp66
- E-RAF
- Cytosol
- NPC
- Nucleus
4.6. DISCUSSION

The studies presented in this experimental chapter clearly indicate that progesterone bound E-RAF has a definitive role to play in the regulation of gene expression in the uterine nucleus. Earlier studies reported from this laboratory (Govind et al. 2003a, 2003b) demonstrated an inhibitory role for progesterone in the estrogen dependent nuclear entry of E-RAF since progesterone bound E-RAF dissociated from tp66.

The confocal microscopic studies presented in this section gave the first indication that progesterone bound E-RAF did enter the nucleus in the endometrial cells in culture. Nuclear localization of E-RAF was seen in cells exposed to 16 nM progesterone. In the presence of 4 nM progesterone E-RAF was evenly distributed outside the nucleus, possibly associated with the endoplasmic reticulum. Progesterone concentrations of 24 and 32 nM again presented a cytoplasmic localization, but with a difference. The fluorescence distribution was not like that found with cells exposed to 4 nM progesterone. In the presence of higher concentrations of progesterone the E-RAF appeared to be localized in selected spots in the cytoplasm, which could possibly represent the mitochondria. This inference is based on a recent observation made in our laboratory (Vidya, S.K. and Thampan, R.V. unpublished observation) that E-RAF has specific acceptor proteins in the mitochondria. The mitochondrial localization of progesterone bound E-RAF and its possible physiological significance will be examined by these investigators in greater detail.

The identification of dp55 as the protein that facilitates the nuclear entry of progesterone-E-RAF complex underlines the independent nature of the two phases in E-RAF action: (a) the estrogen influenced and (b) the progestogen
influenced (Figure 4.6). E-RAF nuclear transport, as it may be noticed, does not require two ‘importin’ proteins, representing ‘importin’ α and β. In the classical case while α recognizes the protein to be transported, β identifies the nuclear pore complex where it gets ‘docked’. The importin α is an actin/ tubulin binding protein (Nirmala and Thampan, 1995 a,b). In the present case, E-RAF retains the actin- binding property, thereby displaying a crucial feature associated with importin α in conventional case. In brief, E-RAF nuclear entry is regulated by two ‘importin β’(s), the tp66 and dp55, tp66 during the ‘estrogenic’ phase of E-RAF action and dp55 during the ‘progestational’ phase.

There is a distinct possibility that the collagenase gene expression observed in endometrial cells in culture under progesterone influence is E-RAF mediated. The confocal microscopic studies had shown a preferential nuclear localization of E-RAF when the cells were exposed to 16 nM estradiol. It may be noticed that the peak in collagenase was noticed when the cells were under the influence of progesterone within this concentration range. Additional increase in hormone concentration in the medium did not result in a fresh increase in collagenase gene expression and the subsequent release of the enzyme into the medium. It is known that c-fos and c-jun that enhance collagenase gene expression recognizes the AP-1 sequence in the gene. Whether E-RAF is an AP-1 sequence binding protein remains to be known. Studies in this direction are underway and will be accomplished by other investigator groups in our laboratory.

The nuclear entry of FITC-E-RAF observed in the presence of progesterone deserves a comment. The concentration of progesterone, 23 nM that brought about the nuclear entry of FITC-E-RAF in cell free system is higher than the concentration, 16 nM, that facilitated the nuclear entry of E-RAF in the primary
culture of endometrial cells. It is possible that the factors that regulated the protein movement in living cells are not fully reproduced under cell-free conditions. Further the harsh treatments to which the E-RAF has been exposed to during E-RAF labeling (eg. pH 9.5) could have brought about subtle changes in the protein behaviour. Consequently, the E-RAF conformation brought about by 16 nM progesterone, that facilitated its interaction with dp55 and the subsequent nuclear entry in the living cell could be reproduced only in the presence of 23 nM progesterone under cell-free conditions.

When E-RAF was discovered during the early eighties, by Thampan and Clark (1981), it was only considered to function as a DNA binding protein that heterodimerized with an estrogen receptor that did not have a DNA binding function. A remarkable change has been seen in the perception of this protein as a gene regulatory factor during the past two decades. The recent observations made at our laboratory, pertaining to the mitochondrial localization of E-RAF, are bound to lead to highly novel discoveries on the biological functions of this protein. Whether the gene sequences recognized by free E-RAF and the progesterone bound E-RAF are different remains to be known eventhough it is a possibility that cannot be overlooked. What the present study has succeeded in highlighting is the reality that there are two distinct channels of gene regulation under e-RAF influence, one under estrogentic influence and the other under progestational influence. The two channels are independent since progesterone binding to E-RAF serves to terminate the ‘estrogenic’ phase, by dissociating E-RAF from tp66. Possibly, as future investigations may reveal, there are molecular events both in the nuclei and the mitochondria that are under E-RAF influence during the progestational phase.