Journal of Cellular Biochemistry 89:108–119 (2003)

Proteins That Mediate the Nuclear Entry of the Goat Uterine Estrogen Receptor Activation Factor (E-RAF): Identification of a Molecular Basis for the Inhibitory Effect of Progesterone on Estrogen Action

Anitha P. Govind, S. Sreeja, and Raghava Varman Thampan*

Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram-695014, Kerala, India

Abstract A 66 kDa transport protein, tp66, has been identified as the protein that mediates the nuclear transport of the estrogen receptor activation factor (E-RAF). Indirect evidence shows that tp66 influences the transport of E-RAF mainly by recognizing the nuclear localization signals (NLS) on the latter. A 38 kDa nuclear pore complex protein (npcp38) has been identified to which tp66–E-RAF complex gets 'docked' prior to the nuclear entry of E-RAF. Progesterone binding to E-RAF serves to dissociate E-RAF from the tp66 thereby inhibiting the nuclear entry of E-RAF. The demonstration of the high affinity progesterone binding property of E-RAF adds credibility to the above findings. A change in conformation of E-RAF being brought about by progesterone binding is evident from the results of the circular dichroism (CD) analysis. This appears to be the fundamental reason behind the dissociation of the tp66–E-RAF complex under progesterone influence and provides a molecular basis for the estrogen 'antagonistic' action of progesterone. A nuclear run-on transcription assay clearly demonstrates the transcription-activation function of E-RAF II, also reaffirming the functional role of tp66 in the nuclear entry of E-RAF. J. Cell. Biochem. 89: 108–119, 2003.

Key words: estrogen receptor activation factor; progesterone binding protein; nuclear transport; nuclear pore complex protein; nuclear localization signals

Estrogen receptor activation factors (E-RAFs) are DNA binding proteins with Mg^{2+} ATPase activity and having the capacity to dimerize with an alternative form of estrogen receptor, the non-activated estrogen receptor (naER) [Thampan and Clark, 1981; Thampan, 1987, 1989]. Two structurally and functionally distinct E-RAF forms exist, I and II. E-RAF I stabilizes the DNA double helical structure while E-RAF II destabilizes it. E-RAF II enhances transcription in an in vitro system while E-RAF I inhibits transcription. Both I and II are 66 kDa proteins; due to differences in their molecular shapes, they display distinct sedimentation values [Thampan, 1987, 1989]. The functional studies presented in this report were carried out exclusively on goat uterine E-RAF II.

The nucleocytoplasmic transport of macromolecules proceeds through the nuclear pore complexes (NPCs) that perforate the double membrane of the nuclear envelope [Feldherr et al., 1984]. The NPC is a macromolecular structure composed of proteins referred to as nucleoporins. The transport of macromolecules through NPCs is generally energy dependent and requires a number of soluble nuclear transport factors. The nuclear transport of proteins involves the mediation of a group of transport proteins called importins [Gorlich et al., 1994; Moroianu et al., 1995]. Importin

Abbreviations used: ap55, anchor protein 55; CD, circular dichroism; E_2, estradiol 17β; ERα, estrogen receptor α; E-RAF, estrogen receptor activation factor; FITC, fluorescein isothiocyanate; HAP, hydroxylapatite; hER, human estrogen receptor; naER, non-activated estrogen receptor; NLS, nuclear localization signal; NPC, nuclear pore complex; npcp38, nuclear pore complex protein 38; SDS, sodium dodecyl sulphate; TCA, trichloroaceticacid; tp66, transport protein 66.

Grant sponsor: Department of Biotechnology, Government of India; Grant sponsor: Indian Council of Medical Research (ICMR) Senior Research Fellowship.

*Correspondence to: Raghava Varman Thampan, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram-695014, Kerala, India. E-mail: raghavavarman@yahoo.com

Received 11 December 2002; Accepted 13 December 2002

DOI 10.1002/jcb.10482

α is known to recognize the nuclear localization signals (NLS) on the nuclear proteins: in other words, this protein functions as the NLS receptor. The importin α is further recognized by the importin β which in turn 'docks' at the NPC.

Studies carried out in our laboratory on the nuclear transport of goat uterine estrogen receptor α has clearly identified the involvement of two proteins: a 55 kDa protein (p55) that binds to the NLS on the ER and a 12 kDa protein (p12) that recognizes p55 on the one hand and a NPC protein on the other [Nirmala and Thampan, 1995; Sai Padma and Thampan, 2000; Sai Padma et al., 2000]. In the alternative estrogen receptor system that involves the naER, the NLS binding protein that mediates the transport of naER to the nucleus is a 58 kDa protein, p58. At the NPC, the naER–p58 complex binds to a 62 kDa protein, p62. It is further observed that p62 gets 'docked' at a 66 kDa nuclear pore complex protein, npcp66 [Sreeja and Thampan, manuscript communicated].

The transport of E-RAF to the nucleus has been shown to be carried out by a 66 kDa transport protein, tp66 [Govind, Sreeja, and Thampan, companion paper]. Further characterization of tp66 forms the major focus of this paper. An attempt has been made to identify whether tp66 functions as the NLS receptor. It has also been possible to identify the NPC protein that 'docks' tp66 at the NPC. In the process of identifying the proteins involved in the nuclear transport of E-RAF, a novel regulatory mechanism by which the female sex hormones regulate the nuclear entry of E-RAF has come to light.

MATERIALS AND METHODS

2,4,6,7-[^{3}H] estradiol-17β (specific activity 101 Ci/mmol), 1,2,6,7-[3H] progesterone (specific activity 110 Ci/mmol), Sepharose 4B, NP-40, diethyl pyrocarbonate (DEPC), and CM Sepharose were purchased from Amersham-Pharmacia Biotech UK Ltd., Buckinghamshire, England. α-^{32}P uridine triphosphate (specific activity 3,000Ci/mmol) was obtained from BRIT (Board of Radiation and Isotope Technology), Bhabha Atomic Research Centre, Mumbai, India. Non radioactive estradiol-17β, progesterone, *p*-amino benzamidine agarose, phenyl methyl sulphonyl fluoride (PMSF), ATP, GTP, CTP, UTP, hsp 90 Sepharose, and fluorescein isothiocyanate isomer I (FITC) were purchased from Sigma Chemicals Co., St. Louis, MO, USA. DE-52 and phosphocellulose were obtained from Whatman International Ltd., Maidstone, England. Hydroxyl apatite (Biogel-HTP) was purchased from Bio-Rad, Chennai, India and RNasin from Genei, Bangalore. Routine chemicals used in the study were of reagent grade, obtained from local commercial establishments.

Isolation of Microsomes and Endoplasmic Reticulum

Goat uterine microsomes were isolated following the procedure of van der Hoeven [1981] and the endoplasmic reticulum was isolated following the procedure of Dallner [1997]. Triton X 100 was added to the buffer with suspended microsomes or endoplasmic reticulum to a final 0.1% concentration in order to solubilize the membrane proteins.

Isolation of Goat Uterine Nuclei

Goat uterine nuclei were isolated as described by Thampan [1985].

Purification of E-RAF

The method developed by Thampan [1987] was followed.

Purification of naER

The method described by Anuradha et al. [1994] was followed.

Purification of tp66

The method has been described in the companion paper.

Purification of ap55

The procedure has been detailed in the companion paper.

Transport Assay for the Nuclear Entry of E-RAF

Details of the procedure have been given in the companion paper. In brief, intact nuclei or detergent treated nuclei (treated with 0.1% Triton X 100) were incubated at 30°C for 30 min with [^{3}H] E_2-naER (1 μg) and E-RAF (1.2 μg). The labeled estradiol that was bound to isolated nuclei was extracted using ethyl alcohol and measured in a scintillation counter. The nuclear transport activity was expressed as [^{3}H]-estradiol bound to nuclei, in counts per minute.

Preparation of Affinity Matrices

The following matrices were used during various stages of experimentation. Covalent coupling of peptides and proteins to cyanogen bromide activated Sepharose 4B was achieved following the method described by March et al. [1974].

E-RAF Sepharose

E-RAF was purified to homogeneity and coupled to CNBr-activated Sepharose 4B.

tp66 Sepharose

Purification of tp66 was achieved following the method described in the text. Purified goat uterine tp66 was coupled to CNBr-activated Sepharose 4B.

NLS-Sepharose

The hER NLS sequence published by Chambon's group [Ylikomi et al., 1991] was used in the synthesis of the NLS peptide following the protocol developed by Renil et al. [1994]. The synthetic peptide was coupled to CNBr-activated Sepharose 4B.

Wheat Germ Agglutinin (WGA) Sepharose

WGA was purified from unprocessed wheat germ following the procedure of Nagata and Burger [1974]. Electrophoretically pure WGA was coupled to CNBr-activated Sepharose 4B.

Hydroxylapatite Adsorption Assay for Progesterone Binding to E-RAF

E-RAF, purified to homogeneity, was incubated overnight (4°C) with [^{3}H]-progesterone (0–20 nM) $\pm$ 100 $\times$ unlabeled progesterone and the radioactivity associated with the protein was determined using a HAP adsorption assay [Clark and Peck, 1979].

Circular Dichroism Measurement

Interaction of progesterone with E-RAF was subjected to circular dichroism (CD) analysis using a Jasco Spectropolarimeter. Scans were made at the far-UV wavelength range of 250–210 nm (2.0 sensitivity), in order to identify changes in the secondary structure of E-RAF.

Isolation of Goat Uterine Nuclear Envelope and the NPC

The method designed by Davis and Blobel [1986] was followed.

Fluorescence Microscopic Studies

Purified E-RAF was labeled with FITC as described by Chard [1987]. FITC-labeled E-RAF was incubated with detergent treated nuclei in a nuclear transport assay mixture containing 10% glycerol, at room temperature. The E-RAF movement was monitored under a Nikon-ε-600 fluorescence microscope.

Nuclear Run-On Assay

Nuclei were prepared following a slight modification of the method published by Thampan [1985]. The isolated nuclei were suspended in buffer containing 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 20 mM KCl, 0.25 M sucrose, and Nonidet P-40 (0.5% final concentration). The nuclei were gently pipetted up and down in a tube kept on ice for 5–15 min. It was then layered over a cushion of 0.34 M sucrose in TMK buffer and centrifuged at 800 $\times g$ for 10 min. The nuclear pellet obtained was washed twice in run-on buffer (20 mMTris HCl, pH 8, 5 mM $MgCl_2$, 150 mM KCl). Aliquots of 5×10^7 nuclei in run-on buffer were incubated with 10 μCi of [^{32}P]-UTP in the reaction buffer containing 0.6 mM each of ATP, GTP, CTP, 0.06 mM UTP, RNasin ribonuclease inhibitor (50 U) along with a combination of proteins of interest in a total volume of 200 μl for 30 min at 30°C. One milliliter TRIzol reagent was added to the reaction vial and total RNA was isolated following the manufacturer's protocol. Denatured RNA was subjected to electrophoresis on a 1.2% formaldehyde gel containing 1 $\times$ 3-[N-morpholino]-propanesulfonic acid (MOPS) and 2.2 M formaldehyde [Sambrook et al., 1989]. The gels were dried and autoradiography was performed using a phosphor imager (molecular imager FX, Bio-Rad Laboratories, Hercules, CA, USA).

RESULTS

Progesterone Mediated Dissociation of E-RAF–tp66 Complex

Microsomes, isolated from uterine homogenates in the presence of 360 nM estradiol, were suspended in an estradiol free medium. Following incubation for 30 min at 30°C, the suspension was subjected to ultra centrifugation at 105,000 $\times g$ for 30 min. The supernatant thus obtained was chromatographed over a 25 ml column of DE-52. The flowthrough fraction was collected. The column was washed well with TEM buffer and elution was achieved using

TEM buffer containing 1.5 M NaCl. The salt concentration in the eluate was diluted to 150 mM following which the sample was chromatographed over a 5 ml column of E-RAF Sepharose. The column was washed with TEM buffer and elution was achieved using a 0–36 nM progesterone gradient (Fig. 1A). The peak fraction (absorbance at 280 nm) revealed the presence of a highly homogenous 66 kDa protein in the SDS gel (Fig. 1B). The nuclear transport assay revealed that the 66 kDa protein is tp66. The DE-52 flowthrough fraction was subjected to chromatography over a 5 ml column of tp66 Sepharose (E-RAF appears in the DE-52 flowthrough fraction). Elution was achieved using a 0–40 nm progesterone gradient (Fig. 1C). SDS gel analysis revealed that the peak absorbance fraction contained a 66 kDa

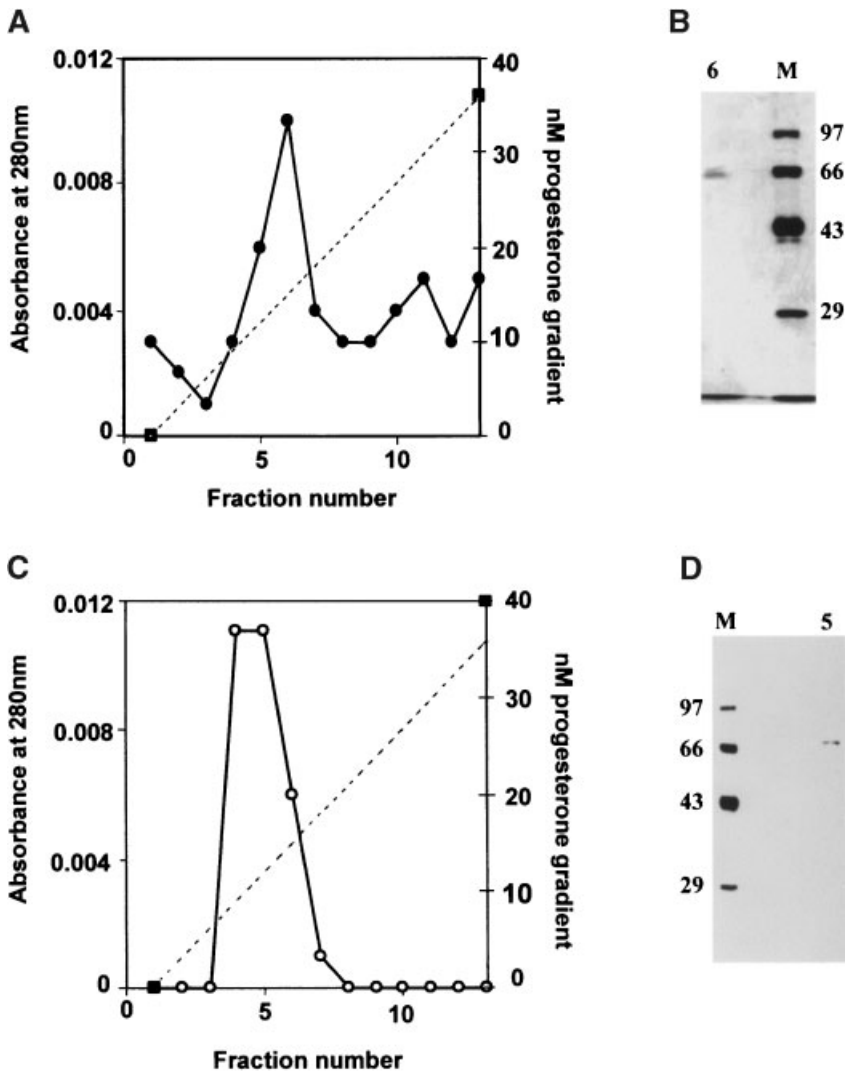

Fig. 1. Progesterone mediated dissociation of E-RAF–tp66 complex. Microsomes isolated in the presence of 360 nM estradiol were suspended in an estradiol-free medium and centrifuged at 100,000 × *g* for 1 h. The supernatant obtained after ultracentrifugation was chromatographed over a 25 ml column of DE-52. The flowthrough fraction was collected. The column was washed well with TEM buffer and elution was achieved using TEM buffer containing 1.5 M NaCl. **A**: The salt concentration of the DE-52 eluate was diluted to 150 mM following which the fraction was chromatographed over a 5 ml column of E-RAF Sepharose. The column was washed with TEM buffer and elution was achieved using a linear gradient of 0–36 nM progesterone. Absorbance of the fractions at 280 nm was recorded (●). **B**: SDS–PAGE analysis of the peak fraction: 6, peak fraction number; M, Molecular weight marker. **C**: The DE-52 flow through fraction obtained was chromatographed over a column of tp66 Sepharose and elution was achieved using 0–36 nM progesterone gradient. Absorbance of the fractions at 280 nm was measured (○). **D**: SDS–PAGE analysis of the purified estrogen receptor activation factor (E-RAF) recovered from the tp66 Sepharose column.

protein that was subsequently confirmed to be the E-RAF (Fig. 1D).

Binding of Progesterone to E-RAF

E-RAF was shown to bind progesterone with high affinity as displayed by the HAP binding assay. The saturation binding was achieved as a biphasic process reaching the saturation limits in the presence of 16 nM ^{3}H-progesterone (Fig. 2A).

Progesterone Inhibits Nuclear Entry of E-RAF

The effect of increasing concentrations of progesterone on the nuclear transport of E-RAF was studied. Nuclear transport assay was carried out using detergent treated nuclei in the presence of increasing concentrations of progesterone (0–36 nM) keeping the tp66 concentration constant at 0.8 μg per assay medium. An inhibitory effect on the nuclear entry of E-RAF was evident in response to the exposure of E-RAF to increasing concentrations of progesterone (Fig. 2B).

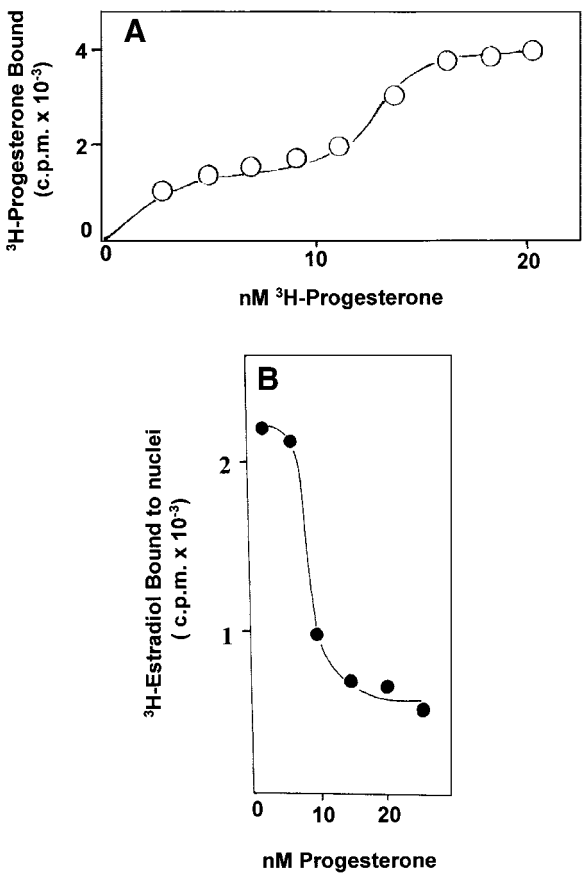

Fig. 2. **A**: Binding of ^{3}H-progesterone to E-RAF. Purified E-RAF was incubated overnight with 0–20 nM [^{3}H]-progesterone in the presence or absence of 100 × unlabelled progesterone at 4°C and saturation binding analysis was carried out using a HAP adsorption assay. The radioactivity associated with the HAP was measured. **B**: Effect of progesterone on the tp66 mediated transport of E-RAF. [^{3}H] E_2-naER (1 μg) was incubated with E-RAF (1.2 μg), tp66 (0.8 μg), and detergent treated nuclei in the presence of increasing concentrations (0–36 nM) of progesterone at 30°C for 30 min and the transport assay was performed as described in the methods section. The [^{3}H] E_2-naER–E-RAF complex that was bound to the nuclei was measured.

CD Analysis of E-RAF Following Progesterone Binding

CD measurements were taken to find out whether progesterone introduced any changes in the secondary structure of the E-RAF (Fig. 3). The major structural feature associated with the E-RAF as displayed by the CD plot, appeared to be in the form of β sheets and α helix since the prominent CD appeared between 220 and 210 nm. The CD data indicated that progesterone binding did introduce significant conformational changes in the E-RAF molecule.

Estradiol Mediated Inhibition of the Nuclear Entry of E-RAF

Crude nuclei with associated nuclear envelope and endoplasmic reticulum were used for studying the transport of E-RAF in the presence of increasing concentrations of estradiol. Crude nuclei were used to ensure the presence of a system with endogenous tp66 and ap55. An inhibition of nuclear entry could be observed in the presence of a concentration of estradiol beyond 7 nM. Previous results (shown in the

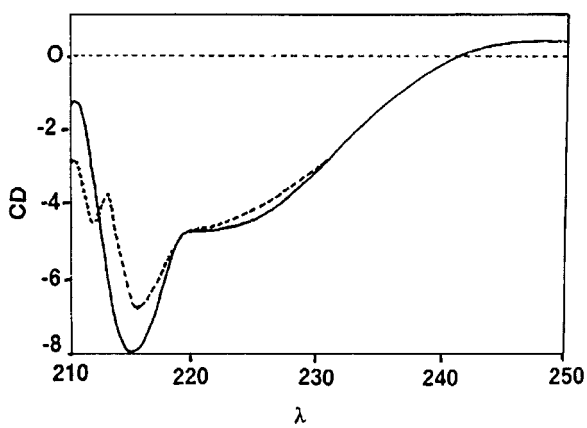

Fig. 3. Circular dicroism (CD) measurements of progesterone interaction with E-RAF. CD of E-RAF (~80 μg protein per 450 μl) was analyzed both in the presence (----) and absence (—) of 20 nM progesterone using a JASCO spectropolarimeter. Far UV CD spectra were taken to detect any changes that occur in the E-RAF conformation at the secondary structure level.

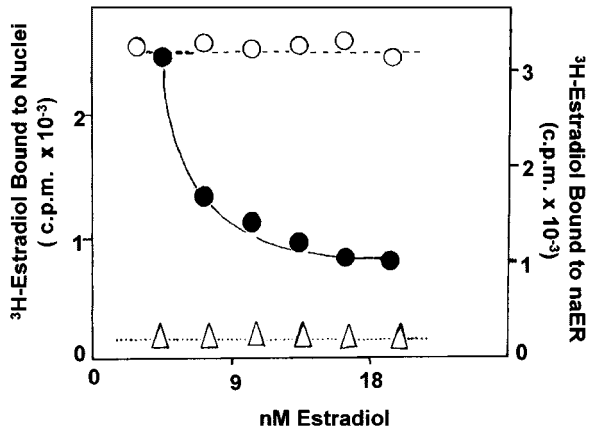

Fig. 4. Effect of estradiol on the nuclear entry of E-RAF. Crude nuclei were incubated with [^{3}H] E_2-naER (1 μg) in the presence of increasing concentrations (0–20 nM) of estradiol. The [^{3}H] E_2-naER–E-RAF complex that was bound to the nuclei was measured (●). As a control (○), ^{3}H-E_2 naER was exposed to varying concentrations (0–20 nM) of estradiol, in the absence of nuclei, in order to confirm that the observed decrease in ^{3}H-E_2 naER bound to the nuclei was not due to the dissociation of ^{3}H-E_2 from the naER under the existing experimental conditions. Another control experiment (△) was carried out using detergent treated nuclei, under the same experimental conditions as those employed in the experiments with the crude nuclei. The results clearly indicate that what was observed with the crude nuclear system was due to the factors associated with the outer nuclear membrane that contributed ap55, tp66, and E-RAF to the system.

companion paper) have indicated that this is a concentration that favors the anchoring of tp66–E-RAF complex to ap55 located in the endoplasmic reticulum thereby inhibiting the nuclear entry of E-RAF (Fig. 4).

tp66 Functions as the Transport Protein for E-RAF

The transport function of tp66 was confirmed by studying the effect of increasing concentrations of tp66 on the nuclear entry of E-RAF. An enhanced nuclear transport of E-RAF could be observed along with a graded increase in the tp66 concentration (Fig. 5A).

tp66 is a NLS-Binding Protein

The involvement of tp66 in the nuclear transport of E-RAF was further confirmed when purified tp66 showed capacity to bind to a column of NLS-Sepharose in which hER NLS peptide was coupled to CNBr activated Sepharose. The elution from the NLS-Sepharose could be achieved using an increasing NaCl gradient (Fig. 5B).

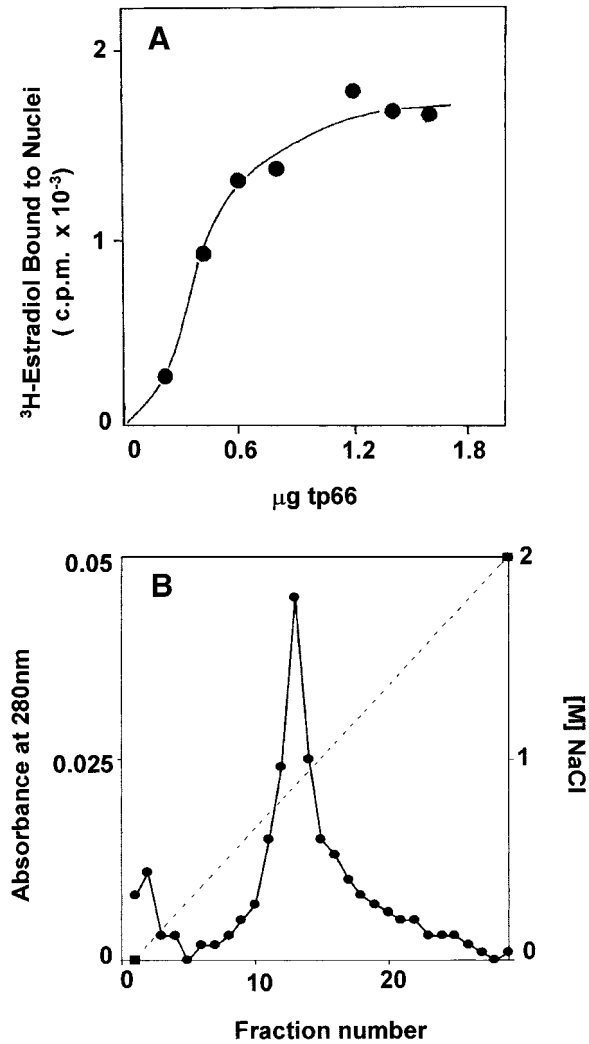

Fig. 5. **A**: tp66 dependent binding of E-RAF–[^{3}H] E_2-naER complex to nuclei. Isolated nuclei (detergent treated) were incubated with E-RAF (1.2 μg) and [^{3}H] E_2-naER (1 μg) in the presence of varying concentrations (0–1.6 μg) of tp66. The [^{3}H] E_2-naER–E-RAF complex that was bound to the nuclei was measured (●). **B**: Binding of tp66 to hER NLS peptide-Sepharose. Purified tp66 was chromatographed over a column of hER NLS-Sepharose, the column was washed with TEM buffer and elution was achieved using a linear gradient of 0–2 M NaCl. One-microliter fractions were collected and the absorbance of the fractions at 280 nm was measured (●).

A 38 kDa Protein 'Docks' tp66 at the NPC

Isolated NPCs were exposed to a medium containing urea to solubilize the proteins [Davis and Blobel, 1986] and the solubilized proteins were chromatographed over a 5 ml column of tp66 Sepharose. The column was washed well with TEM buffer and elution was achieved using a three-column volume of TEM buffer

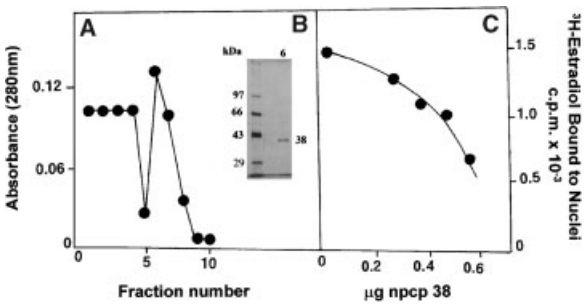

Fig. 6. Identification of the nuclear pore complex protein (npcp38) that 'docks' at tp66. Solubilized NPC proteins were chromatographed over a column of tp66 Sepharose and the proteins bound to the column were eluted using 1 M NaCl. The salt concentration of the final eluate was diluted to 0.05 M and the material was rechromatographed over a 5 ml column of WGA Sepharose in the presence of 0.1% Triton X 100. The column was washed well with TEM buffer containing 0.1% Triton X 100 and elution of the bound proteins was achieved using the same buffer containing 0.5 M *N*-acetyl glucosamine. **A**: Absorbance at 280 nm. **B**: SDS–PAGE analysis of the peak fraction. **C**: Competition assay carried out in order to confirm that npcp38 is the tp66 'docking' protein. Detergent treated nuclei were incubated with [^{3}H] E_2-naER (1 μg), E-RAF (1.2 μg), tp66 (0.8 μg), and varying concentrations of npcp38. The [^{3}H] E_2-naER–E-RAF complex that was bound to the nuclei was measured.

containing 1M NaCl. The concentration of NaCl in the eluted fraction was diluted to 0.05 M using TEM buffer containing 0.1% Triton X 100 and the diluted material was chromatographed over a 5 ml column of WGA Sepharose. The column was washed well with TEM buffer containing 0.1% Triton X 100 and elution was achieved using the same buffer containing 0.5 M N-acetyl glucosamine (Fig. 6A). The SDS–PAGE analysis of the peak fractions revealed the presence of a highly homogenous 38 kDa protein (Fig. 6B). This protein will be henceforth identified as NPC associated protein 38 (npcp38).

A competition assay was carried out in order to ascertain the functional role of npcp38 in 'docking' tp66 at the pore complex. Detergent treated nuclei were used in the study to examine the E-RAF transport by tp66 following exposure to increasing concentrations of npcp38. The basis behind the experiment was that the free npcp38 in the assay medium would interact with tp66, prevent it from docking at the available npcp38 of the NPC, and thereby inhibit the nuclear entry of E-RAF. Increasing concentrations of free npcp38 showed a dose dependent inhibitory effect on the tp66 mediated nuclear transport of E-RAF (Fig. 6C).

Fluorescence Assay for E-RAF Transport Into Isolated Nuclei

Nuclear transport of FITC labeled E-RAF was carried out in order to confirm the nuclear entry of E-RAF that was indicated in the previous experiments. Detergent treated nuclei, suspended in the assay buffer were incubated with FITC-E-RAF (1 μg). No binding of FITC-E-RAF to nuclei was observed under these conditions (Fig. 7B). Addition of tp66 (0.8 μg) to this medium resulted in an immediate entry of FITC-E-RAF into the nuclei (Fig. 7C,F). Presence of 20 nM progesterone in the assay medium resulted in a total inhibition of the nuclear entry of FITC-E-RAF (Fig. 7D). The ATP dependence on the nuclear transport of E-RAF was evident in that no nuclear entry could be observed in the absence of ATP added to the medium (Fig. 7G). Also, at 10 μg/ml concentration, quercetin partially blocked the nuclear entry of FITC-E-RAF (Fig. 7H). The anchor protein ap55 showed its inhibitory effect on the nuclear entry of E-RAF only when exposed to estradiol (Fig. 7K) whereas in the absence of the hormone, the protein failed to inhibit the E-RAF transport into isolated nuclei (Fig. 7J).

E-RAF II Influences Transcription of mRNA

A nuclear run-on assay system that enables the rate of ongoing transcription of genes by measuring the incorporation of radioactive rUTP into nascent transcripts was carried out in order to study the transcription stimulation function of E-RAF II. Nuclear run-on assays were carried out both in the presence and absence of E-RAF and other agents. In the control, a basal level of transcription could be observed. In the presence of the transport protein tp66, E-RAF showed marked influence on the transcription process. Addition of progesterone to the system down regulated the transcription to the basal level and so did the exposure of the nuclei to ap55 in the presence of estradiol. In the absence of estradiol, ap55 did not show any inhibitory effect whatsoever. A transcriptional activation effect of E-RAF on the synthesis of certain high molecular weight species of mRNA in the goat uterine nuclei is evident from the results of the run-on assay experiment (Fig. 8).

DISCUSSION

Elution of the ap55–tp66 complex that was bound to the E-RAF-Sepharose matrix by

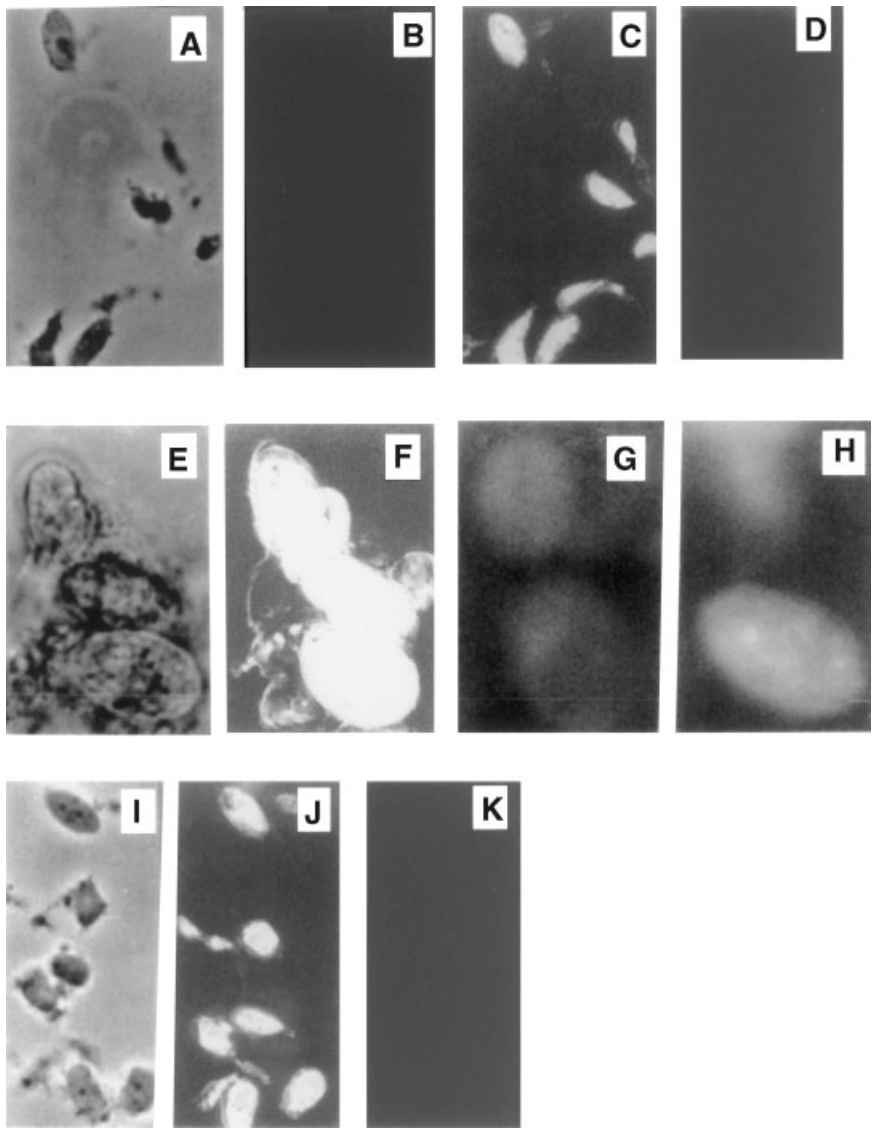

Fig. 7. Fluorescence assay for the E-RAF transport into isolated nuclei. **A**: Detergent treated nuclei suspended in a typical nuclear transport medium containing glycerol (phase contrast 40×). **B**: Fluorescence micrograph of detergent-treated nuclei in nuclear transport medium containing FITC-E-RAF, in the absence of tp66. **C**: Fluorescence micrograph of the system of (B) with added tp66 (40×). **D**: System of (C) with prior exposure to 20 nM progesterone. **E**: Detergent treated nuclei in a nuclear transport assay medium (phase contrast 100×). **F**: Fluorescence micrograph of nuclei shown in (E) in the presence of FITC-E-RAF and tp66 (100×). **G**: Fluorescent micrograph of nuclei exposed to FITC-E-RAF and tp66 in the absence of ATP (100×). **H**: Fluorescence micrograph of nuclei exposed to FITC-E-RAF and tp66 in a medium containing ATP and 10 μg/ml quercetin (100×). **I**: Detergent treated nuclei in a transport medium containing FITC-E-RAF, tp66, and ap55 (phase contrast). **J**: Fluorescent micrograph of the system presented in (I). **K**: Fluorescent micrograph of a system containing detergent-treated nuclei, FITC-E-RAF, tp66, and ap55 in the presence of 20 nM estradiol.

progesterone (discussed in the companion paper) pointed towards an apparent progesterone influence on the interaction between E-RAF and the ap55–tp66 complex. The interaction between E-RAF and tp66 was shown to be weakened in the presence of progesterone. The elution of tp66 from E-RAF Sepharose and vice versa in the presence of progesterone suggested that one of the two proteins has a progesterone binding function. At that stage of the experiment, it was not possible to predict the level at which progesterone brought about this dissociation. The possible involvement of a progesterone-regulated mechanism was further reinforced, when E-RAF showed a high affinity progesterone binding property. The data shows

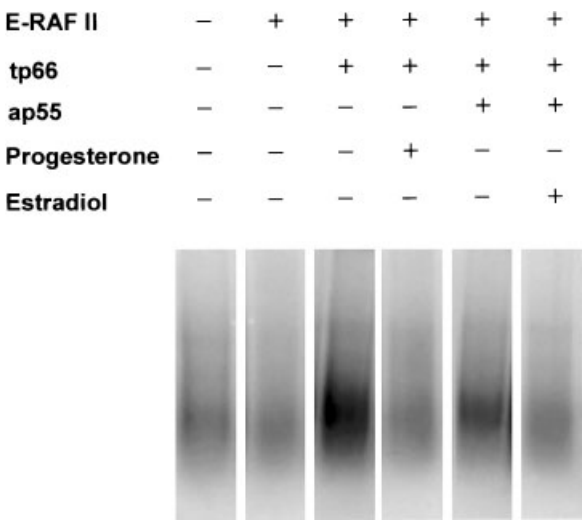

Fig. 8. Transcriptional activity of E-RAF measured in a nuclear run-on assay system. ^{32}P-UTP incorporated into RNA was measured under the following conditions: nuclei (5×10^{-7}) were incubated with (1) control (none); (2) 0.6 μg E-RAF alone; (3) 0.6 μg E-RAF+0.6 μg tp66; (4) 0.6 μg E-RAF+0.6 μg tp66+20 nM progesterone; (5) 0.6 μg E-RAF+0.6 μg tp66+0.6 μg ap55; (6) 0.6 μg E-RAF+0.6 μg tp66+0.6 μg ap55+20 nM estradiol. The total RNA was isolated from each system and subjected to electrophoresis in a 1.2% agarose-formaldehyde denaturing gel. The autoradiographic image was obtained using a Bio-Rad phosphor imager FX.

that it has more than one set of binding sites for progesterone and the saturation of one site appears to increase the affinity for the second site, in the process giving rise to a biphasic saturation plot.

CD analysis served to confirm the assumption that progesterone induces conformational changes in the E-RAF, which in turn leads to the dissociation of E-RAF from tp66. This prevents the nuclear entry of E-RAF since it requires the assistance of the transport protein tp66 as is evident from the inhibitory effect displayed by progesterone on the nuclear entry of E-RAF.

The whole scenario of the nuclear transport of E-RAF could be understood only when the inhibitory role played by the anchor protein ap55 is considered. The membrane anchor protein identified in the companion paper inhibits nuclear entry of E-RAF in the presence of estradiol. The nuclear transport assay carried out using crude nuclei confirmed the above findings. The outer nuclear membrane along with the network of endoplasmic reticulum associated with it remains intact in the crude nuclei providing a ready source for ap55 and tp66 in the assay medium. Inhibition of E-RAF transport that occurred in response to an increase in the concentration of estradiol in the medium could be explained under the light of the previous findings that ap55 present in the endoplasmic reticulum undergoes a conformational change in the presence of estradiol, holding tp66–E-RAF complex to the endoplasmic reticulum and thereby inhibiting the nuclear entry of E-RAF.

The effect of tp66 on E-RAF transport is being confirmed by the transport assay. Indirect evidence shows that tp66 recognizes NLS on the E-RAF. In these experiments, the NLS peptides were synthesized based on the published data on hER NLS sequence [Ylikomi et al., 1991]. The NLS sequence of E-RAF is not yet known. The transport of E-RAF to nuclei is an energy dependent process as is evident from the FITC experiments wherein the nuclear entry of FITC-E-RAF was shown to be inhibited by the absence of ATP in the medium. The NPC is extremely large and is currently estimated to contain between 40 and 100 distinct proteins out of which only a few have been fully characterized [reviewed in Corbett and Silver, 1997]. A 39 kDa protein has been reported to be present in the NPC, which apparently has a molecular mass closest to that of npcp38. Seh1p is a 39 kDa protein that forms the subcomplex of the much larger nucleoporin Nup84 in yeast [Siniossoglou et al., 1996; reviewed in Doye and Hurt, 1997]. Further characterization of npcp38 will contribute to the information pool generated by the wide array of npcps that are being explored.

The transcriptional activation function of E-RAF II is evident from the nuclear run-on assay. At this stage, the identity of mRNAs being regulated by E-RAF cannot be pointed out. It could only be said that there is a definite enhancement in the transcription of certain mRNA species. Sequence analysis of the enhanced RNA species would help to bring into focus the possible target genes that are being regulated by the E-RAF.

We have thus identified two proteins that influence the distribution of E-RAF in the sub cellular compartments. ap55, the anchor protein involved in the retention of tp66–E-RAF complex in the endoplasmic reticulum and tp66, involved in the transport of E-RAF from the cytosol to the nucleus. Both these events seemed to be under the regulation of two

well-characterized steroid hormones having opposite effects, estradiol and progesterone. Either of the hormones at physiological concentrations produced results, the net effect being the inhibition of the nuclear entry of E-RAF. An understanding of its functional significance may provide insight into the physiological changes that are being brought about by the delicate balance between estradiol and progesterone in the female reproductive cycle. The fact that E-RAFs are potential transcription factors points towards the existence of a fine tuned transcriptional regulation of selective genes under the influence of estradiol.

The functional significance associated with the inhibition of the nuclear entry of E-RAF by estradiol at 7 nM concentrations or above is not difficult to understand. The nuclear entry of naER following its internalization from the plasma membrane takes place only upon exposure of naER to estradiol [Karthikeyan and Thampan, 1996]. It is possible that the internalization of naER from the plasma membrane brings about organizational changes in the plasma membrane structure, leading to the cellular entry of free estradiol from the extra cellular space. This enhanced intracellular concentration of estradiol could lead to the retention of E-RAF–tp66 complex at the ap55 anchored to the endoplasmic reticulum. This is possibly an internal regulatory mechanism that could prevent a dimerization between the E-RAF and naER outside the nucleus. The dimerized complex fails to enter the nucleus since the transport mechanisms of the two proteins are distinct and different. This internal regulatory mechanism is, therefore, necessary in order to ensure that the dimerization between the two proteins, involving "leucine zippers" [Jaya et al., 2001], takes place only after the E-RAF and the naER enter the nucleus independently. We had proposed a model earlier to explain this phenomenon [Govind and Thampan, 2001], underlying the need for the naER–E-RAF heterodimer to reach the transcription initiation point. In view of the affinity of naER to bind to the RNA polymerase and the DNA binding nature of the E-RAF, it may be proposed that the heterodimer could reach the transcription initiation site on the estrogen responsive gene, where naER binds to the RNA polymerase while the E-RAF binds to the gene. The dissociation of naER from E-RAF could then be brought about by the naER transforming factor, a 62 kDa protein that deglycosylates the naER and transforms it to nuclear estrogen receptor II [Jaya and Thampan, 2000].

What has been observed in these studies provide an unexpected clue regarding the molecular basis for the 'estrogen antagonistic' action of progesterone. It is known from the information available in the literature that estradiol action fails to manifest so long as the system is dominated by progesterone [Premkumar and Thampan, 1995]. This is a phenomenon observed under regular estrus cycle and also during the pregnancy. If E-RAF is an essential factor required in the estradiol-regulated transcriptional mechanisms, then prevention of its nuclear entry will lead to cessation of estrogen action. The results presented here clearly show that progesterone binding to E-RAF leads to the dissociation of tp66 from E-RAF as a result of which the E-RAF fails to enter the nucleus. Decline in the intracellular concentrations of progesterone will automatically lead to the return of the native conformation of E-RAF, which is essential for its interaction with tp66 and the consequent nuclear entry.

The new information is being summarized in the model presented in Figure 9. The protein ap55 is being represented here as an integral membrane protein of the endoplasmic reticulum; the conformation of ap55 appears to be controlled by the binding of estradiol to the cytosolic domain of ap55. In the presence of concentrations of estradiol below 7 nM, the conformation of ap55 favors the release of tp66–E-RAF complex from its fold. The tp66–E-RAF complex, once released, moves into the nucleus, possibly through a cytoskeletal protein-dependent mechanism.

Progesterone binding to E-RAF introduces conformational changes in the protein, initiating the dissociation of tp66 from E-RAF. In the absence of tp66, E-RAF fails to reach the nucleus. This appears to be a molecular basis for the estrogen 'antagonistic' action of progesterone.

ACKNOWLEDGMENTS

Financial assistance received from a programme support of the Department of Biotechnology, Government of India is gratefully acknowledged. APG is a recipient of the Indian

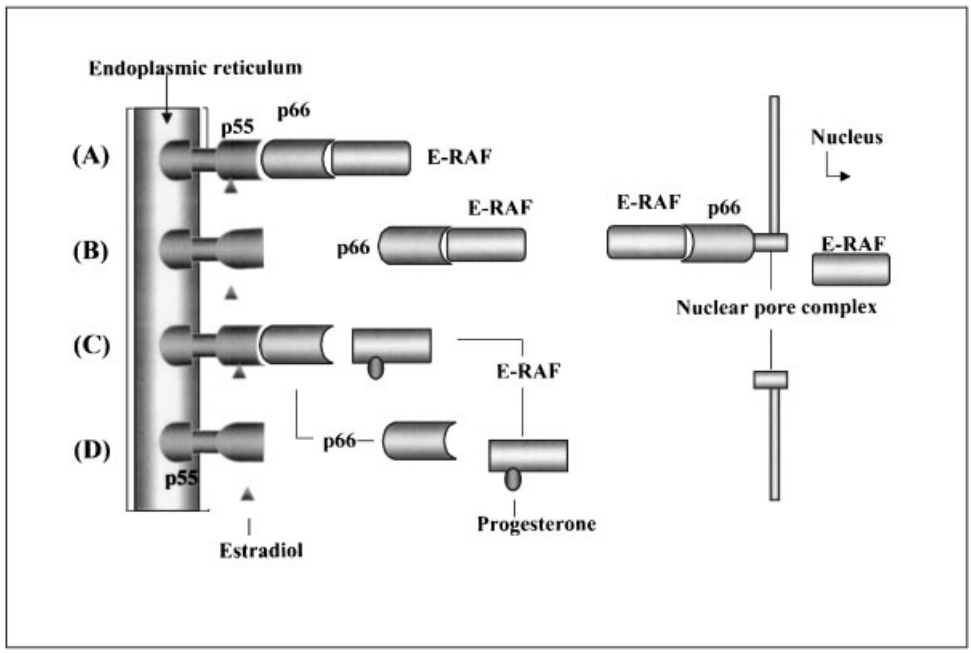

Fig. 9. A model presented to explain the influence of various proteins and hormonal agents on the nuclear entry of E-RAF. **A**: E-RAF remains anchored to the endoplasmic reticulum through its interaction with ap55 and tp66 in the presence of estradiol that saturates the estradiol binding sites on the ap55. **B**: Low levels of estradiol results in the release of the E-RAF–tp66 complex from ap55 initiating the transport of E-RAF into the nucleus. **C**: Progesterone binding to E-RAF brings about the dissociation of E-RAF from tp66. The presence of estradiol favors anchoring of tp66 to ap55, thereby inhibiting nuclear entry of E-RAF. **D**: Presence of progesterone causes dissociation E-RAF from tp66 while low levels of estradiol under the same condition results in the dissociation of tp66 from ap55. Disrupted interaction between tp66 and E-RAF results in the inhibition of the nuclear entry of the latter. This is the molecular mechanism proposed in this text, in order to explain the estrogen 'antagonistic' action of progesterone.

Council of Medical Research (ICMR) Senior Research fellowship.

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