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Estradiol Dependent Anchoring of the Goat Uterine Estrogen Receptor Activation Factor (E-RAF) at the Endoplasmic Reticulum by a 55 kDa Anchor Protein (ap55)

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Abstract The primary intracellular site of localization of the estrogen receptor activation factor (E-RAF) is shown here to be the endoplasmic reticulum where the protein remains anchored through an estrogen dependent mechanism. The retention of E-RAF by the endoplasmic reticulum is facilitated by two proteins: (1) a 55 kDa anchor protein (ap55) which is an integral membrane protein of the endoplasmic reticulum. ap55 is a high affinity estrogen binding protein. A conformational change induced by estrogen binding is thought to favor the anchoring process. (2) The anchoring of E-RAF by ap55 is mediated by yet another protein. This is the 66 kDa transport protein (tp66) which recognizes ap55 on the one hand and E-RAF on the other. The presence of estradiol that saturates the hormone binding sites on ap55 appears to favor the anchoring of tp66–E-RAF complex to ap55. This interaction appears to be weakened by levels of estradiol below 7 nM concentration leading to the dissociation of the tp66–E-RAF complex from ap55. The tp66—E-RAF complex moves towards the nucleus. J. Cell. Biochem. 89: 94–107, 2003.

Key words: estrogen receptor activation factor; endoplasmic reticulum; nuclear transport; membrane anchor protein; microsomal protein binding estrogen

Estrogen receptor activation factors (E-RAFs) are DNA binding proteins that dimerize with an alternative form of estrogen receptor, the non-activated estrogen receptor (naER) [Thampan and Clark, 1981; Thampan, 1987, 1989]. Recent studies reported from our laboratory indicate the apparent involvement of leucine zippers in the dimerization between naER and E-RAF [Jaya et al., 2001]. E-RAF is a 66 kDa protein that lacks estradiol binding function. Though both E-RAF and naER show immunological similarity with the regular estrogen receptor (ERα), they show marked differences in their primary structure as is evident from the data on the CNBr peptide fragment analysis of the two proteins [Zafar and Thampan, 1993; Karthikeyan and Thampan, 1995]. Both naER and E-RAF are protein kinases. While naER is a tyrosine kinase, E-RAF functions as a serine kinase [Anuradha et al., 1994].

E-RAF was previously thought to be a cytosolic protein. It was long assumed that E-RAF is the mediator involved in the transport of naER to the nucleus and vice versa. The recent findings by Thampan et al. [2000] revealed that the heterodimer formed between E-RAF and naER gets 'docked' at the nuclear envelope and also that this dimer formation inhibited the nuclear entry of either protein. The identification of transport proteins involved in the nuclear entry of naER [Sreeja and Thampan, manuscript communicated]

Abbreviations used: ap55, anchor protein 55; BCIP, 5-bromo 4-chloro 3-indoyl phosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; E_2, estradiol 17β; ERα, estrogen receptor α; E-RAF, estrogen receptor activation factor; HAP, hydroxylapatite; hER, human estrogen receptor; naER, non-activated estrogen receptor; NBT, nitroblue tetrazolium; NLS, nuclear localization signal; SDS, sodium dodecyl sulphate; TCA, trichloroaceticacid; tp66, transport protein 66.

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ruled out the possibility of E-RAF involvement in the nuclear transport of naER. This left us with two options to be examined: (1) E-RAF is transported to the nucleus all by itself; (2) E-RAF entry into the nucleus is mediated by other proteins.

Transmembrane proteins in both plasma membrane and endoplasmic reticulum have been shown to function as transcription factors. These proteins undergo proteolytic cleavage and the cleaved intracellular portion then moves to the nucleus where it acts as a transcription factor regulating transcription of target genes [Wang et al., 1994; Hua et al., 1996; Ni et al., 2001]. The sterol responsive element binding proteins (SREBPs) identified by Goldstein and Brown team are transmembrane proteins that undergo proteolytic cleavage at the endoplasmic reticulum and the released protein rapidly enters the nucleus and influences transcription of genes involved in cholesterol homeostasis. E-RAF showed functional similarity to SREBPs in having cholesterol binding property [Thampan et al., 2000] and also in transcriptional activation. This information prompted us to examine the endoplasmic reticulum as the possible intracellular site of localization of E-RAF.

During the course of the present study it was observed that E-RAF was retained at the endoplasmic reticulum in an estrogen dependent manner. Since E-RAF does not have any estrogen binding function, it seemed likely that an estrogen binding protein in the endoplasmic reticulum could be involved in this estrogen mediated retention/release of E-RAF. There have been earlier reports of estrogen binding sites associated with the endoplasmic reticulum [Parikh et al., 1980; Monje and Boland, 1999]. Evans and Muldoon [1991] showed that endoplasmic reticulum possesses estrogen-binding sites with biochemical properties that differ from those of the classically described cytosolic (loosely associated nuclear) estrogen receptor. Muldoon et al. [1988] reported the existence of high affinity estrogen binding sites associated with the microsomal fractions. Though these sites showed many properties in common with the cytosolic receptors, they failed to get activated to a DNA-binding form under conditions which permit such activation of cytosolic receptors. Another feature that differentiated it from the regular receptor was its affinity for binding progesterone.

In view of the above reports, experiments were designed to probe into the possible existence of an estrogen binding protein that mediates the retention/release of E-RAF and the results obtained were supportive of this hypothesis. Though the major aim was to identify the protein retaining E-RAF in the endoplasmic reticulum, we were fortunate to stumble across two proteins in the process, one having an anchoring function and the other having a nuclear transport function. These observations came as the long awaited answer to the questions related to the intracellular location as well as the nuclear entry of E-RAF.

MATERIALS AND METHODS

2,4,6,7-[^{3}H] estradiol-17β (specific activity 101 Ci/mmol) was purchased from Amersham, England. Non-radioactive estradiol-17β, progesterone, dexamethasone, tamoxifen, *p*-amino benzamidine agarose, phenyl methyl sulfonyl fluoride (PMSF), 5-bromo 4-chloro 3-indoyl phosphate (BCIP), nitroblue tetrazolium (NBT), heatshock protein 90 (hsp90)-Sepharose, and concanavalin A-agarose were purchased from Sigma Chemicals Co. St. Louis, MO, USA DE-52 and phosphocellulose were obtained from Whatman, International Ltd., Maidstone, England. Sepharose 4B and CM Sepharose were obtained from Amersham-Pharmacia Biotech UK Ltd., Buckinghamshire, England. Hydroxylapatite (Biogel-HTP) was purchased from Bio-Rad, Chennai, India. Alkaline phosphatase (ALP)-coupled anti-rabbit IgG was obtained from Genei, Bangalore, India. Routine chemicals used in the study were of reagent grade, obtained from local commercial establishments.

Buffers

Four major buffer systems were used in these studies.

1. TEM buffer: 10 mM Tris-HCl, pH 7.6, 1 mM ethylene diamine tetraaceticacid (EDTA), 12 mM monothioglycerol, and 0.2 mM PMSF.
2. TM buffer: 10 mM Tris-HCl, pH 7.6, 12 mM monothioglycerol, and 0.2 mM PMSF.
3. TMKC-sucrose buffer: 50 mM Tris-HCl, pH 7.6, 2 mM $MgCl_2$, 20 mM KCl, 1 mM $CaCl_2$, and 250 mM sucrose. This buffer was used during the isolation of goat uterine nuclei and microsomes. The buffer was used as a 2 × preparation for the initial suspension of isolated nuclei.

4. TCMMN buffer: 10 mM Tris-HCl, pH 7.6, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.2 mM PMSF. This buffer was used for con A-agarose chromatography.
5. Phosphate buffer: 10 mM sodium phosphate buffer, pH 6.8 containing 0.1% triton X 100. The buffer was used in the purification of ap55 through chromatography on CM Sepharose.

Preparation of Goat Uterine Cytosol

Goat uteri were obtained from a local slaughter house and transported in ice to the laboratory for experimentation. The uteri were homogenized in TMKC sucrose buffer using an Ultra Turrax (Germany) homogenizer. The homogenate was filtered using a nylon mesh and centrifuged at 800*g* for 10 min in a Sorvall RC 5B high speed refrigerated centrifuge. The crude nuclear pellet thus obtained was discarded and the supernatant was centrifuged at 15,000*g* for 15 min. Precipitation of microsomes in the high speed supernatant fraction was achieved as described by van der Hoeven [1981]. Polyethylene glycol-6000 (PEG) was added, under constant stirring, to the post-mitochondrial supernatant to give a final 5% concentration of PEG. The mixture was centrifuged at 20,000*g* for 20 min (4°C). The pelleted microsomes and the supernatant (cytosol) were used in the experimental studies.

Isolation of Microsomes and Endoplasmic Reticulum

Goat uterine microsomes were isolated following the procedure described above and the endoplasmic reticulum was isolated following the procedure of Dallner [1997]. Triton X 100 was added to the buffer in which the microsomes or the endoplasmic reticula were suspended, to a final concentration of 0.1%, 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. The E-RAF appeared as a homogenous 66-kDa band in silver stained sodium dodecyl sulphate (SDS) gels.

Purification of naER

The naER from the goat uterine cytosol was purified following the method described by Anuradha et al. [1994]. The naER appeared as a homogeneous 66-kDa band in silver-stained SDS gels.

Examination of the Role of Estradiol in the Retention of E-RAF by the Microsomes

Goat uteri were homogenized in the TMKC Sucrose buffer both in the presence and absence of exogenous estradiol. Estradiol was added to the homogenizing medium to a final concentration of 0.36 μM. E-RAF was purified from the cytosol and also from the detergent (0.1% Triton X-100) extracts of microsomes. The E-RAF thus isolated was subjected to SDS–PAGE.

E-RAF Purification Using an Affinity Chromatography Method

This has been carried out using a procedure published recently by Jaya et al. [2001]. Purified naER, immobilized on Sepharose 4B, was used as an affinity matrix for the immobilization of E-RAF. The detergent extract of the endoplasmic reticulum was centrifuged at 105,000*g* for 1 h and the resulting supernatant was chromatographed over a column of naER Sepharose. The column was washed initially with TEM buffer containing 1 M NaCl and subsequently with salt free TEM buffer. Elution was achieved using TEM buffer containing 4 mM leucine as described by Jaya et al. [2001]. The basis of the method was the observation that free leucine could dissociate protein dimers which dimerize due to the involvement of leucine zippers while all other naER-bound proteins will be removed during washing of the column with buffer containing 1 M NaCl. The fractions collected were assayed for absorbance at 280 nm. The peak absorbance fraction contained a highly homogenous preparation of E-RAF.

SDS–PAGE and Western Blot Analysis

Denaturing gel electrophoresis in the presence of SDS was performed following Laemmli [1970] method. The gels were stained with silver nitrate as described by Blum et al. [1987]. Western blot analysis was performed as described by Towbin et al. [1979].

Isolation of E-RAF-Associated Proteins From the Detergent Extracts of Microsomes

Goat uterine microsomes were isolated and suspended in TMKC-sucrose buffer containing 0.1% Triton X-100 and 360 nM estradiol. The suspension was subjected to ultracentrifugation. The supernatant was recovered and subjected to chromatography over a 25 ml column of DEAE cellulose (Whatman DE-52). The column was equilibrated and washed well with TEM buffer containing 36 nM estradiol and elution of the DE-52 bound proteins was achieved using TEM buffer containing 1 M NaCl and 36 nM estradiol. The aim was to isolate a protein that mediates retention of E-RAF by the endoplasmic reticulum in an estradiol dependent manner. The DE-52 bound proteins were chromatographed over an affinity matrix of E-RAF Sepharose equilibrated with buffer containing 1 M NaCl and 36 nM estradiol. The column was washed well with the same buffer and elution was carried out in a triphasic manner. Phase I: TEM buffer containing 36 nM estradiol but deficient in NaCl. Phase II: TEM buffer free of NaCl and estradiol. Phase III: TEM buffer containing 36 nM progesterone, but without NaCl and estradiol.

Transport Assay for the Nuclear Entry of E-RAF

Purified naER (1 μg) was incubated overnight at 4°C with 20 nM [^{3}H]-estradiol. Unbound hormone was separated from the [^{3}H] E_2–naER complex after adsorption of the free hormone to dextran coated charcoal (1% charcoal and 0.1% dextran in 50 mM Tris-HCl, pH 7.6, containing 10% glycerol). The [^{3}H] E_2–naER complex was then incubated with isolated goat uterine nuclei suspended in 2 × assay buffer. E-RAF (0.6 μg) and the protein that influences E-RAF transport into the nuclei (tp66) were added to this mixture and the incubation was carried out at 30°C for 30 min. The incubated tubes were transferred to an ice bath and 2 ml of ice cold assay medium was added to each tube. The nuclei were sedimented after centrifugation at 800*g* for 10 min, washed once again with 2 ml of ice cold assay buffer, and finally extracted with 1 ml ethanol. The radioactivity associated with the ethanol extract was measured using a Wallac liquid scintillation counter and a Triton X-100 containing scintillation mixture. The nuclear transport activity was expressed as [^{3}H]-estradiol bound to nuclei, in counts per minute. In designing this procedure the basic understanding was that E-RAF could be sandwiched between ^{3}H-E_2 naER and tp66. The recognition of a nuclear pore complex protein by tp66 facilitated the "docking" of the complex of three proteins at the nuclear periphery. Measurement of the ^{3}H-E_2 bound to the nuclei gave an indirect indication for the quantity of E-RAF transported into the nuclei, in spite of the fact that the naER–E-RAF complex is bound only to the periphery of the nuclei.

Concanavalin A (con A) Agarose Chromatography

The purified proteins were chromatographed over a 5 ml column of con A-agarose equilibrated with TCMMN buffer. The flowthrough volume from this column was collected in 1 ml fractions. After washing the column with TCMMN buffer, elution was carried out using TCMMN buffer containing 0.3 M methyl-α-D glucopyranoside. Fractions collected were analyzed for their absorbance at 280 nm and the peak fractions were subjected to SDS–PAGE.

Hydroxylapatite Binding Assay for Estradiol Binding to ap55

HAP binding assays were carried out in order to examine the saturation binding of estradiol to ap55. The purified protein was incubated with 0–20 nM [^{3}H]-estradiol in the presence and absence of 100× diethylstilbestrol overnight at 4°C in order to obtain the non-specific and total binding data respectively. The specific binding activity was calculated by deducting the non-specific binding data from the total binding data. The radioactivity associated with the protein was measured through a HAP adsorption assay described by Clark and Peck [1979].

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.

naER Sepharose

naER purified to homogeneity, was coupled to CNBr activated Sepharose 4B.

ap55 Sepharose

The method employed in the purification of ap55 has been detailed in the text. Purified ap55 was coupled to CNBr activated Sepharose 4B.

tp66 Sepharose

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

RESULTS

Subcellular Distribution of E-RAF

In order to study the subcellular distribution of E-RAF, cytosol and microsomes were isolated from goat uterine homogenates both in the presence and absence of exogenous estradiol. The two homogenates (one with externally added estradiol while the other functioned as the control) were processed independently and the E-RAF was purified from each homogenate using the protocol developed by Thampan [1987]. SDS–PAGE analysis of the four fractions revealed that there was a biphasic distribution of E-RAF in the cytosol and the microsomes. When the cytosol and microsomal fractions were prepared from homogenates that contained externally added estradiol, it was observed that the E-RAF was almost exclusively associated with the microsomal fraction. In the absence of added estradiol in the homogenate the major part of E-RAF appeared in the cytosol and a corresponding decrease was noticed in the recovery of E-RAF from the microsomal fraction (Fig. 1A).

Three different hormonal agents viz estradiol, tamoxifen, and dexamethasone were used in order to study the effect of these agents on the distribution of E-RAF during tissue homogenization. The hormones were added to a final concentration of 36 nM in the homogenizing media and microsomes were isolated from the three homogenates from which E-RAF was purified. Out of the three hormonal agents used only estradiol was found to favor the retention of E-RAF within the microsomes, while tamoxifen and dexamethasone remained ineffective (Fig. 1B).

The effect of exposure to varying concentrations of estradiol on the retention or release of E-RAF from the microsomes was analyzed. Microsomes were isolated from uterine homogenates made in the presence of 0.36 μM estradiol and aliquots of microsomal fractions, suspended in TMKC sucrose, were incubated in media containing 0–9 nM estradiol at 30°C for 30 min. The incubated samples were centrifuged at 15,000 rpm for 20 min and the supernatants collected were analyzed for the presence of E-RAF. Fifty microlitre aliquots from each of the nine samples were subjected to Western blot analysis using anti E-RAF IgG. The control (no estradiol in the medium) showed a heavy presence of E-RAF that was released from the microsomes into the medium. A gradual decrease was observed in the dissociation of E-RAF from the microsomes into the media as the concentration of estradiol that was added to the media increased (Fig. 1C). An increase in the concentration of estradiol, from 6.3 to 7.2 nM, resulted in a marked decrease in the dissociation of E-RAF from the microsomes.

Localization of E-RAF at the Endoplasmic Reticulum

The possibility that the E-RAF is primarily localized in the microsomal fraction was evident from the previous study. Endoplasmic reticulum was isolated and E-RAF was purified through affinity chromatography on naER Sepharose as described in 'Materials and Methods'. The basis of the experiment was the observation that E-RAF alone dimerized with naER, involving leucine zippers, and that the dissociation of the dimer into its units could be brought about by exposing the dimers to 4 mM leucine. The absorbance (280 nm) pattern and the subsequent SDS gel analysis of the peak absorbance fraction revealed the presence of E-RAF in the endoplasmic reticulum (Fig. 2).

Detection of Proteins That Associate With E-RAF in an Estrogen Dependent Manner

Proteins of the endoplasmic reticulum that interacted with E-RAF were isolated as described in Materials and Methods. The three step elution profile showed a similar pattern. The peak fractions derived from the three different elutions were subjected to SDS–PAGE analysis. The silver stained gel displayed two bands with molecular mass of 66 and 55 kDa in all the three samples eluted from the column (Fig. 3).

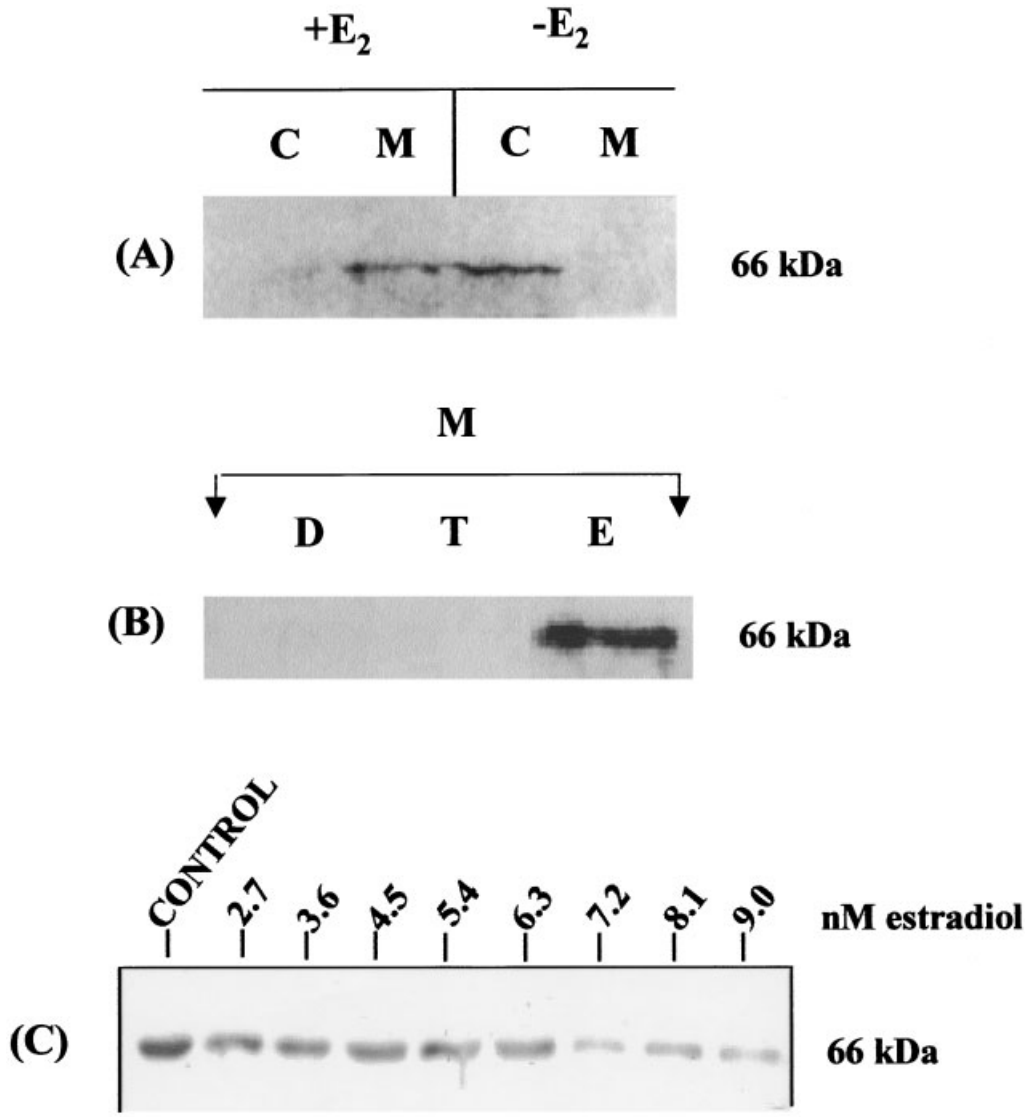

Fig. 1. Subcellular distribution of E-RAF in the goat uterus. **A**: Microsomes and cytosol were isolated from uterine homogenates prepared both in the presence and absence of externally added estradiol (360 nM). The figure shows SDS–PAGE analysis of the E-RAF, purified from microsomes (M) and cytosol (C) isolated either in the presence of estradiol ($+E_2$) or in its absence ($-E_2$). **B**: Hormonal specificity associated with the retention of E-RAF by the microsomes. The microsomes were isolated from homogenates made in the presence of 360 nM concentration of one of the three hormonal agents mentioned below: E-RAF was purified from the detergent (0.1% Triton X 100) extracts of microsomes isolated from these homogenates. E, estradiol; D, dexamethasone; T, tamoxifen. **C**: Western blot analysis of E-RAF released from the microsomes following exposure to varying concentrations of estradiol. Microsomes were isolated from uterine homogenates containing externally added estradiol (360 nM). One millilitre aliquots of resuspended microsomes were added to the media containing varying concentrations of estradiol (0–9 nM). The samples were incubated at 30°C for 30 min following which they were subjected to centrifugation at 15,000 rpm for 20 min. The proteins, precipitated with TCA, were subjected to SDS–PAGE and, following their transfer to a nitrocellulose membrane, were exposed first to anti E-RAF IgG and subsequently to alkaline phosphatase-coupled anti rabbit IgG. The blots were stained with BCIP/NBT.

Role of Estradiol in the Nuclear Binding of E-RAF–[^{3}H]-E_2 naER Complex in the Presence of the 66 and 55 kDa Proteins

The effect of exposure of the medium containing E-RAF, ^{3}H-E_2 naER, isolated nuclei, and the microsomal proteins to increasing concentrations of estradiol on the nuclear transport of E-RAF was examined. The 66 kDa protein has been designed as the transport protein (tp66) and the 55 kDa protein as the anchor protein (ap55) following the experiments, results of which have been presented at a later stage in this text. Nuclear transport assay of E-RAF was

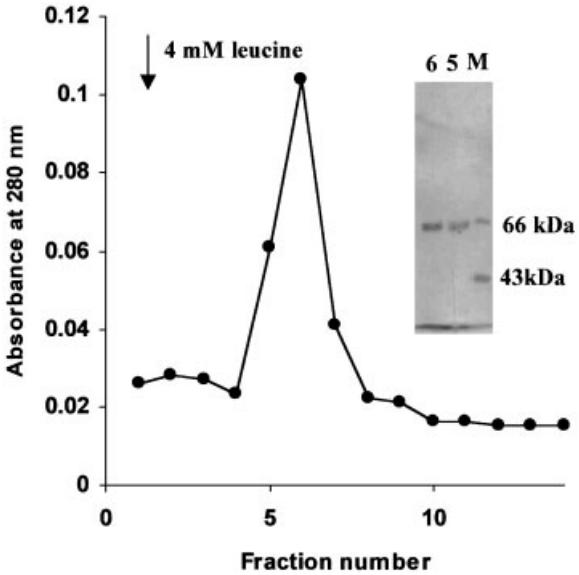

Fig. 2. Localization of E-RAF in the endoplasmic reticulum. Membranes of the isolated endoplasmic reticulum were detergent treated following suspension of the membranes in TEM buffer containing 0.1% Triton X 100. The suspension was centrifuged at 105,000*g* for 1 h and the supernatant was collected. E-RAF was purified from the supernatant through affinity chromatography over a 5 ml column of naER Sepharose. The column was washed first with TEM buffer containing 1 M NaCl and subsequently with salt free TEM buffer. Elution was achieved using TEM buffer containing 4 mM leucine. The fractions collected were analyzed for absorbance at 280 nm. The inset shows a silver stained SDS gel of the peak fractions (5 and 6) along with the molecular weight markers (M).

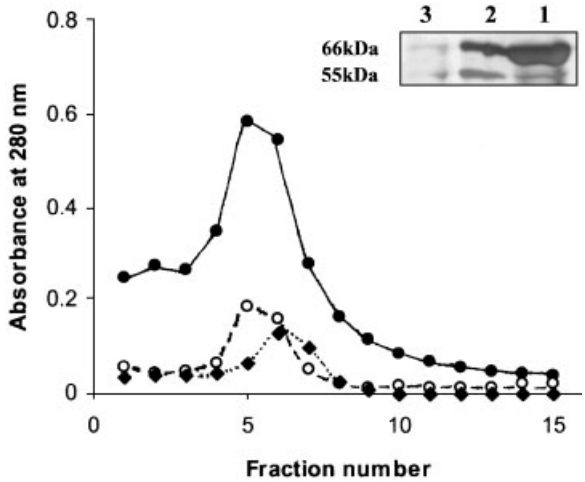

Fig. 3. Isolation of p55–p66 complex through affinity chromatography on E-RAF Sepharose. **A**: Detergent extracts of microsomes (prepared in the presence of 360 nM estradiol) were chromatographed over a column of DE-52 equilibrated with TEM buffer containing 36 nM estradiol. The column was washed with TEM buffer containing 36 nM estradiol and elution was achieved using TEM buffer containing 1 M NaCl and 36 nM estradiol. The eluate from the DE-52 column was chromatographed over a 5 ml column of E-RAF Sepharose equilibrated with buffer containing 1 M NaCl and 36 nM estradiol. The column was washed with the same buffer and elution was achieved in three separate steps. (●) Elution in the absence of NaCl using buffer containing 36 nM estradiol. (○) Elution in the absence of estradiol and NaCl. (▲) Elution using buffer containing 36 nM progesterone, in the absence of estradiol and NaCl. **B**: SDS gel profiles of the fractions which showed peak absorbance at 280 nm. The gel was stained with silver nitrate.

carried out in the presence of increasing concentrations of estradiol (0–36 nM) while keeping the ap55–tp66 concentration constant at 0.4 μg per assay medium. Increase in estradiol concentration beyond 7 nM created an inhibitory effect on the nuclear entry of E-RAF (Fig. 4A). This result may be examined in the light of the data presented in Figure 1C. Estradiol alone showed an inhibitory effect on the nuclear transport of E-RAF while the other hormonal agents which included dexamethasone, tamoxifen, and testosterone remained ineffective (data not shown).

Estradiol Binding to ap55

The purified ap55 was subjected to estradiol binding analysis. The saturation binding data of ap55 showed a sigmoid pattern. The saturation in hormone binding was achieved in the presence of 16–18 nM estradiol (Fig. 4B). The Kd value has not yet been ascertained.

Purification and Separation of the 55 kDa (ap55) and 66 kDa (tp66) Proteins

Attempts were made in order to separate the two proteins from each other to facilitate their characterization.

Purification of the 66 kDa protein (tp66) was achieved in three steps.

(1) DE52 chromatography: The microsomal detergent extracts were first chromatographed over a 5 ml column of DE-52. The column was washed extensively with TM buffer and elution was achieved using a linear gradient of 0–2 M NaCl in the same buffer (Fig. 5A). Preliminary attempts to chromatograph the tp66–ap55 mixture on a column of E-RAF Sepharose showed that while ap55 appeared in the E-RAF Sepharose flowthrough fraction, tp66 was retained by the column, to be eluted subsequently with a high ionic strength buffer. This

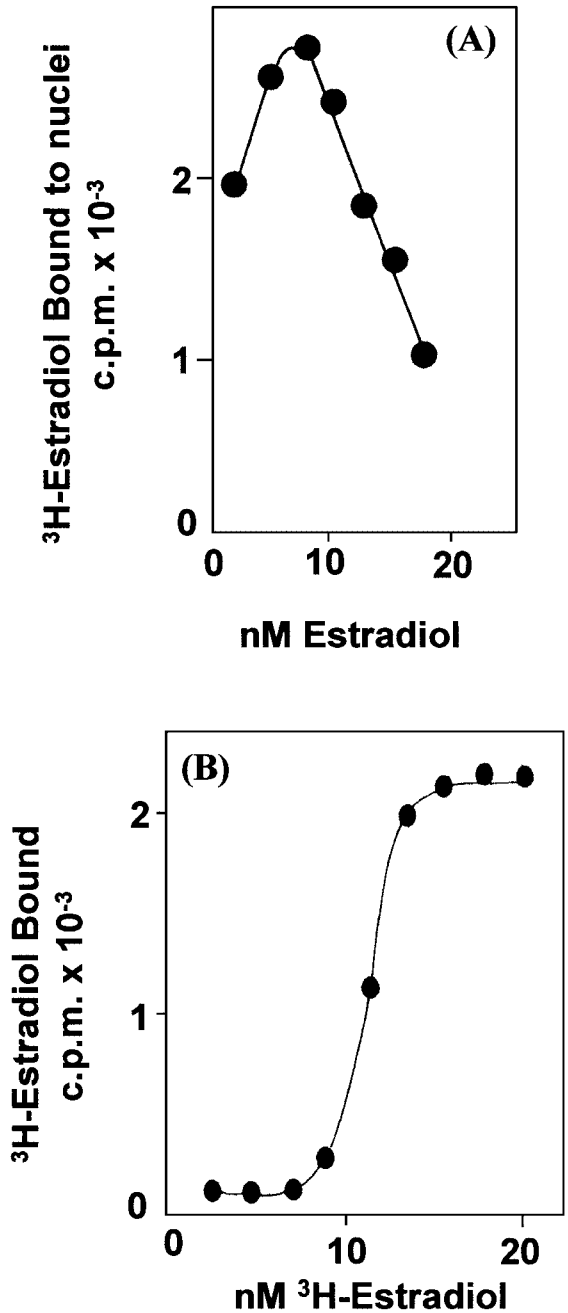

Fig. 4. **A**: Estrogenic influence on the nuclear entry of E-RAF. Effect of exposure of the ap55–tp66–E-RAF complex to varying concentrations of estradiol (0–36 nM) on the nuclear binding of ^{3}H-E_2 naER–E-RAF–tp66 complex was analyzed. Isolated nuclei were incubated with ^{3}H-E_2 naER (1 μg), E-RAF (0.6 μg), ap55–tp66 complex (0.4 μg), and varying concentrations of unlabeled estradiol at 30°C for 30 min. ^{3}H-E_2 naER bound to the nuclei was measured as described in the methods section. **B**: Binding of ^{3}H-estradiol to ap55. Purified ap55 was incubated overnight with 0–20 nM [^{3}H]-estradiol at 4°C (in the presence and absence of 100× diethylsilbesterol) and saturation binding analysis was carried out using a HAP adsorption assay method. The radioactivity associated with the HAP pellet was measured.

observation indicated that p66 was in a position to interact with both ap55 and E-RAF. Previous reports published from this laboratory demonstrated the existence of a 55 kDa protein that transports the regular estrogen receptor to the goat uterine nucleus [Nirmala and Thampan, 1995; Sai padma and Thampan, 2000] and a 62 kDa protein that transports the naER to the nucleus [Sreeja and Thampan, manuscript communicated]. It was decided to check the possibility whether the 66 kDa protein identified in the present study is the much sought after transport protein that transports E-RAF to the nucleus.

(2) Nuclear transport assay for E-RAF: The fractions collected from the DE-52 column were subjected to a nuclear transport assay. The basis of this assay is the information that the heterodimer formed between E-RAF and naER 'docks' at the nuclear periphery wherein the protein that recognizes the 'docking' protein is the E-RAF. A measure of the radioactivity (^{3}H-estradiol bound to naER) associated with the isolated nuclei provides an indirect account of the E-RAF that is retained at the nuclear periphery. The ninth fraction recovered from the DE-52 column, in the present study, displayed a peak activity (Fig. 5A). This active fraction was subjected to additional purification.

(3) E-RAF sepharose chromatography: The fractions which belonged to the activity peak were pooled and chromatographed over a 5 ml column of E-RAF-Sepharose. The column was washed well with TEM buffer and elution was achieved using a linear gradient of 0–1 M NaCl (Fig. 5B). SDS–PAGE analysis of the fraction showing peak absorbance revealed a single band of 66 kDa. Since a nuclear transport function has been attributed to this 66 kDa protein, it will henceforth be represented as tp66 (transport protein 66) (Fig. 5C).

Glycoprotein Nature of the 55 kDa Protein

The tp66–ap55 complex that was recovered from the E-RAF Sepharose matrix was chromatographed over a 5 ml column of con A-agarose equilibrated with TCMMN buffer. The flow-through fraction as well as the fractions eluted with buffer containing 0.3 M methyl-α-D

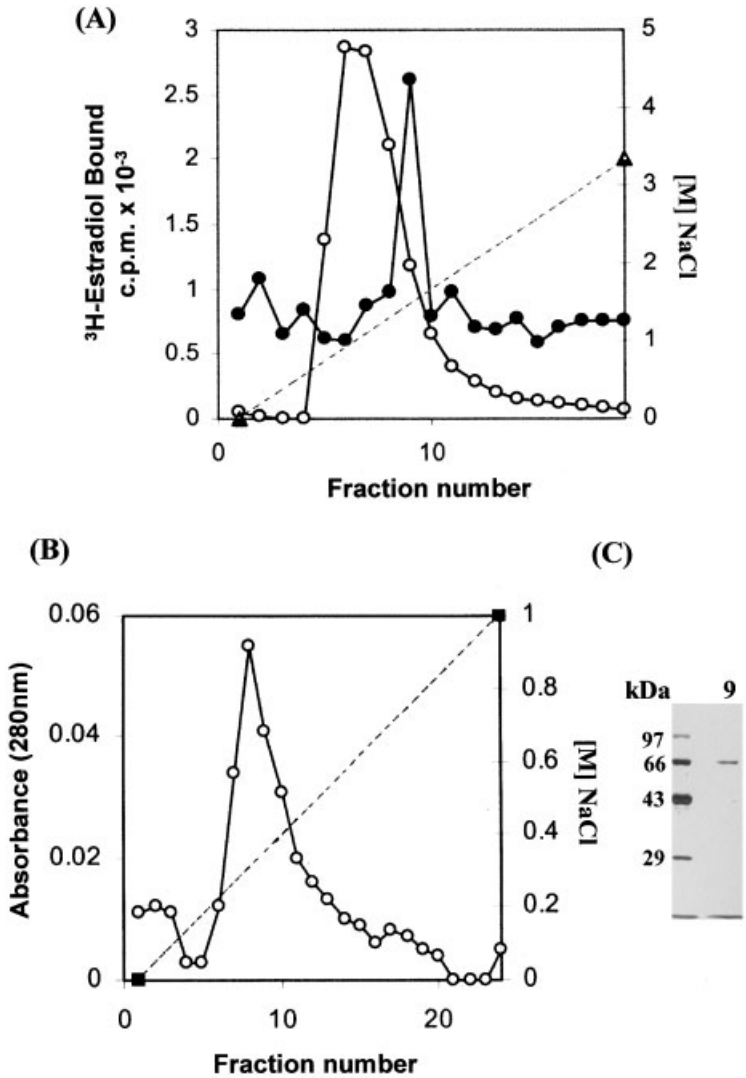

Fig. 5. Purification of tp66. **A**: Detergent extracts of microsomes made in TMKC sucrose buffer containing 0.1% Triton X 100 and 36 nM estradiol were chromatographed over a 5 ml column of DE-52 and the column was washed with TEM buffer (without estradiol). Elution was achieved using a 0–2 M NaCl gradient in TEM buffer without estradiol. One millilitre fractions were collected. Absorbance at 280 nm was measured (○). The fractions from DE-52 were subjected to nuclear transport assay and the radioactivity associated with the nuclei was measured (●). **B**: The fractions which represented the peak activity were 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 0–1 M NaCl in TEM buffer. The fractions were examined for absorbance at 280 nm. **C**: The peak fractions from E-RAF Sepharose were subjected to SDS–PAGE. M, protein molecular weight marker; 9, peak fraction from E-RAF Sepharose.

glucopyranoside were collected (Fig. 6A). Both these fractions were rechromatographed independently over a column of E-RAF Sepharose and the resultant flowthrough and E-RAF-bound fractions were collected. It was observed earlier that the complex formed between the 66 and 55 kDa proteins interacted with E-RAF Sepharose only in the presence of estradiol. Under such conditions the proteins in the flowthrough as well as the fractions bound to conA-agarose should ideally fail to interact with E-RAF when rechromatographed in the absence of estradiol. The flowthrough fractions from the E-RAF Sepharose were therefore pooled separately, concentrated, and subjected to SDS–PAGE analysis. The silver stained gel of the E-RAF Sepharose flowthrough fractions (con A-agarose bound proteins) revealed the presence of a single purified band of 55 kDa. Since this protein is attributed with an anchoring function, it will be identified as the anchoring protein 55 (ap55). The con A-agarose flowthrough fractions revealed a tp66–ap55 complex in the gel (Fig. 6B).

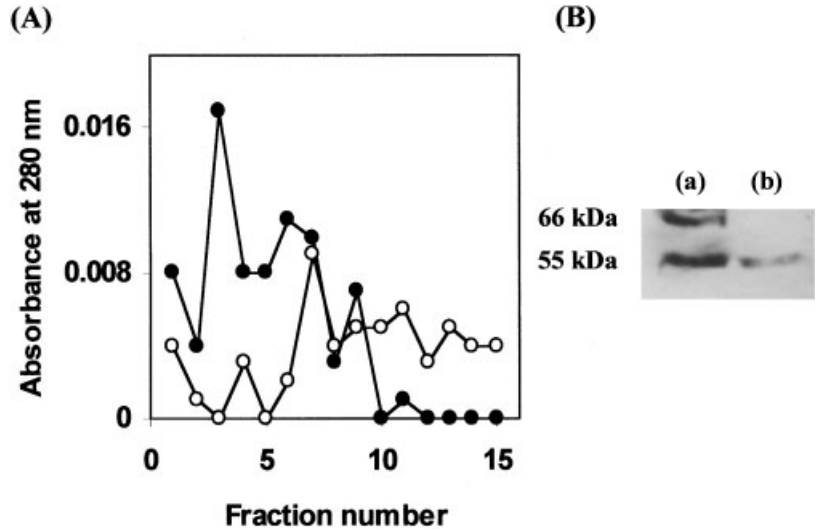

Fig. 6. Identification of ap55 as a glycoprotein. **A**: tp66–ap55 complex obtained from the E-RAF Sepharose column was chromatographed over a column of con A-agarose equilibrated with TCMMN buffer. The flowthrough fractions were collected (●). The column was washed with TCMMN buffer and elution was carried out using the same buffer containing 0.3 M methyl-α-D glucopyranoside (○). The flowthrough as well as con A-agarose bound fractions were rechromatographed over a column of E-RAF Sepharose independently and the corresponding flowthrough fractions were collected. **B**: SDS–PAGE analysis of (a) conA-agarose flowthrough fraction and (b) conA-agarose eluate using methyl-α-D glucopyranoside.

Purification of ap55

The detergent extracts of microsomes were chromatographed over a 5 ml column of DE-52. The flowthrough fractions collected were subjected to chromatography over a 5 ml column of CM Sepharose. 0.1% Triton X 100 was added to the buffers used in the purification of ap55 in order to prevent aggregation of membrane proteins. The column was washed with 10 mM phosphate buffer containing 0.1% Triton X 100. Elution was achieved using a linear gradient of 0–1 M NaCl in the same buffer (Fig. 7A). SDS–PAGE analysis of the fractions revealed the presence of a homogenous 55 kDa protein (Fig. 7B).

Retention of tp66 by ap55 is an Estrogen Dependent Process

The experiment was designed to identify the protein that interacts with ap55 in an estrogen dependent manner. Purified ap55 was coupled to CNBr-activated Sepharose in order to produce an affinity column matrix. The microsomes were isolated from tissue homogenates made in TMKC-sucrose buffer containing 360 nM estradiol. The isolated microsomes were then suspended in an estradiol free medium and incubated at 30°C for 30 min. The suspension was subjected to ultracentrifugation at 100,000*g* for 1 h and the supernatant was collected. It was then chromatographed over a 25 ml column of DE-52. The flowthrough fraction (containing free ap55) was discarded

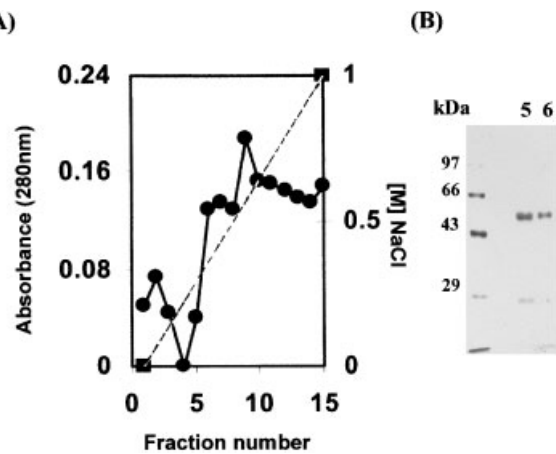

Fig. 7. Purification of ap55. Microsomes were isolated from homogenates containing 360 nM estradiol. Detergent extracts of microsomes (0.1% Triton X 100 in TMKC sucrose buffer) were chromatographed over a 5 ml column of DE-52 equilibrated with TEM buffer and the flowthrough fractions were collected. **A**: The flowthrough fractions were then chromatographed over a 5 ml column of CM Sepharose. The column was washed with 10 mM sodium phosphate buffer, pH 6.8 containing 0.1% Triton X 100. Elution was achieved using a 0–1 M NaCl gradient in the same buffer. One millilitre fractions were collected and analyzed for absorbance at 280 nm. **B**: SDS–PAGE analysis of the eluted fractions. The number indicates the fraction number.

and the column was washed with TEM buffer. Elution of the DE-52 bound proteins was achieved using TEM buffer containing 1 M NaCl. Estradiol was added to this eluate to a concentration of 36 nM and this fraction was chromatographed over a 5 ml column of ap55 Sepharose, equilibrated with TEM buffer containing 1 M NaCl and 36 nM estradiol. The column was washed first with the same buffer and subsequently with salt free buffer containing 36 nM estradiol. Elution was achieved using a decreasing estradiol gradient (36 to 0 nM) (Fig. 8A). The peak fraction (absorbance at 280 nm) was subjected to SDS–PAGE, which revealed a single homogenous band of 66 kDa (Fig. 8B). The high ionic strength buffer that was used in washing the column was effective in dissociating E-RAF from tp66 while the latter remained bound to the immobilized ap55 in the presence of estradiol. Subsequent elution of the column with estradiol-free buffer resulted in the dissociation of tp66 from ap55.

The estradiol mediated interaction between ap55 and tp66 was further confirmed through the chromatography of partially purified ap55 (DE-52 flowthrough fraction) over a column of tp66 Sepharose in the presence of 36 nM estradiol. The column was washed first with

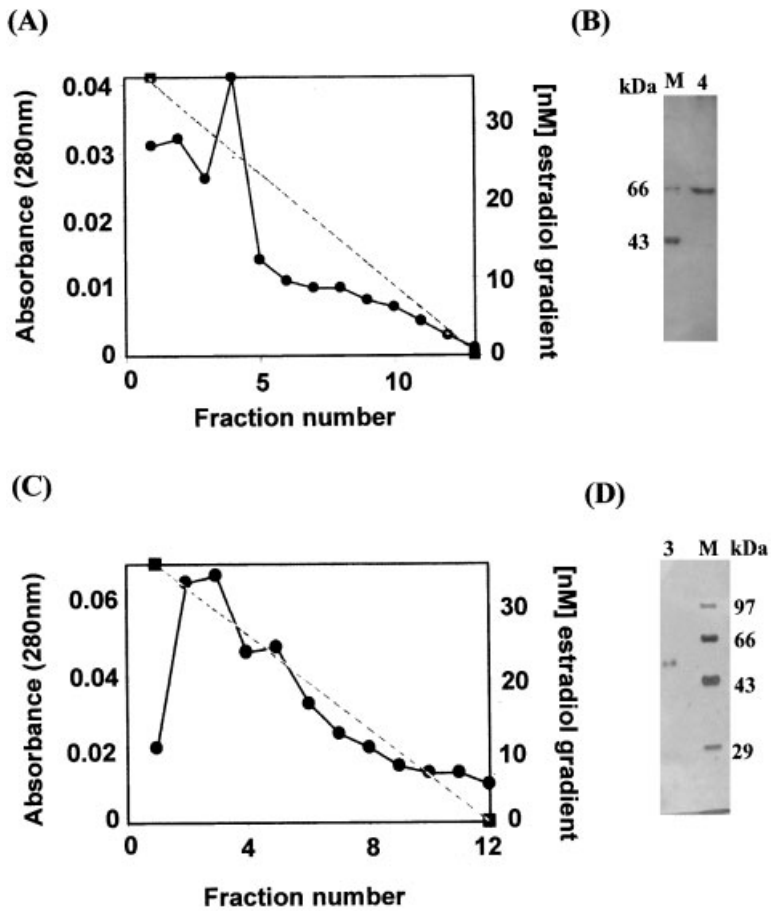

Fig. 8. Estradiol dependent retention of tp66 by p55. Microsomes, isolated from tissue homogenates containing 360 nM estradiol, were incubated at 30°C for 30 min in a medium containing TMKC-sucrose buffer without estradiol. Following ultracentrifugation (100,000*g* for 1 h) the supernatant was collected and chromatographed over a 25 ml column of DE-52. The flowthrough fraction was discarded. The column was then washed with TEM buffer and elution was carried out using TEM buffer containing 1.5 M NaCl. **A**: The eluate was incubated with 36 nM estradiol following dilution of the salt concentration to 150 mM and was re-chromatographed over a column of ap55 Sepharose equilibrated with TEM buffer containing 36 nM estradiol. The column was washed with the same buffer and elution was achieved using a decreasing gradient of 36 to 0 nM estradiol (●). **B**: SDS–PAGE analysis: 4, fraction showing peak absorbance; M, molecular weight marker. **C**: Partially purified ap55 (DE-52 flowthrough fraction of detergent extracts of microsomes) was chromatographed over a column of tp66 Sepharose in the presence of 36 nM estradiol. The column was washed with TEM buffer containing 36 nM estradiol and elution was achieved using a decreasing gradient of 36–0 nM estradiol. **D**: SDS–PAGE analysis: 3, fraction showing peak absorbance; M, molecular weight marker.

buffer containing 1 M NaCl and 36 nM estradiol and subsequently with salt free buffer containing 36 nM estradiol. Elution was achieved using a decreasing gradient (36 to 0 nM) of estradiol (Fig. 8C). SDS–PAGE of the peak fraction (absorbance at 280 nm) showed the presence of a homogenous 55 kDa protein.

ap55 Inhibits the Nuclear Entry of E-RAF in the Presence of Estradiol

The effect of increasing concentrations of ap55 on the nuclear transport of E-RAF was examined both in the presence and absence of exogenous estradiol. Nuclear transport assay of E-RAF was carried out in the presence of increasing concentrations of ap55 (0–0.5 μg) while keeping the tp66 concentration constant at 0.4 μg per assay medium. In the presence of 36 nM estradiol a further increase in the concentration of ap55 created an inhibitory effect on the nuclear entry of E-RAF while ap55 failed to inhibit the nuclear transport of E-RAF in the absence of estradiol (Fig. 9).

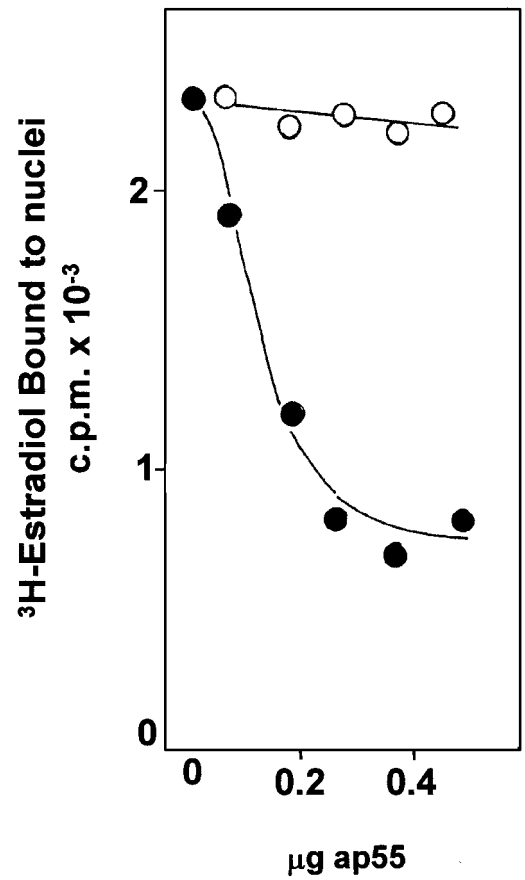

Fig. 9. Inhibition of the nuclear entry of E-RAF by ap55. Effect of exposure of the tp66–E-RAF complex to increasing concentrations of ap55 (0–0.5 μg) on the tp66 mediated nuclear transport of E-RAF was analyzed. Isolated nuclei were incubated with ^{3}H-E_2 naER (1μg), E-RAF (0.6 μg), tp66 (0.4 μg), and increasing concentrations of ap55 both in the presence (●) and absence (○) of 36 nM estradiol at 30°C for 30 min. ^{3}H-E_2 naER–E-RAF complex bound to the nuclei was measured as described in the experimental section.

DISCUSSION

Experiments aimed at identifying the anchoring mechanism by which E-RAF is retained by the endoplasmic reticulum brought to the surface two proteins, of molecular mass 55 and 66 kDa. This retention by the endoplasmic reticulum was found to be an estrogen dependent phenomenon. The visible explanation for this process is that estrogen binds to either one of the two proteins and the conformational change induced in the protein by the hormone is responsible for the retention/release of E-RAF. E-RAF does not bind estradiol. The only candidate with estrogen-binding function seemed to be the anchor protein ap55 and the assumption was found to be correct since ap55 showed high affinity for binding estradiol 17β.

Attempts to characterize the anchor protein function also served to highlight the functional role of the 66 kDa protein, tp66. The protein displayed a nuclear transport function. It may be observed that there were three levels of dissociation of the detergent solubilized proteins from E-RAF following chromatography on a column of E-RAF Sepharose in the experiment presented in Figure 3. The column was initially equilibrated with buffer containing 36 nM estradiol and 1 M NaCl. Elution with salt free buffer containing 36 nM estradiol resulted in the appearance of a major absorption peak representing proteins that dissociated from the column as a result of the breakage of salt bridges. Further elution using estradiol-free buffer apparently introduced conformational changes in the ap55 which, in turn, led to the dissociation of either ap55 alone or the ap55–tp66 complex from the E-RAF Sepharose. Elution with progesterone apparently had its influence at the level of E-RAF. Progesterone binding to E-RAF and the consequent changes in E-RAF conformation caused the elution of the remaining amount of tp66–ap55 complex from the E-RAF Sepharose matrix.

Separation of ap55 from tp66 proved to be a difficult task, since both came as a complex

during chromatography on a variety of conventional matrices. Finally, DE-52 proved to be the matrix of choice in separating tp66 from ap55. Though the role of tp66 at that time was far from being known, it seemed likely that the protein was associated with a transport function. Since ap55 was found to be an integral membrane protein, it was necessary to use detergents for the solubilization of the protein from the membrane. It was of interest to find out whether ap55 was glycoprotein in nature. ConA-agarose chromatography was considered as an option and it confirmed the glycoprotein nature of ap55. The presence of tp66–ap55 complex in the conA-agarose flowthrough fraction could be explained by the fact that binding efficiency of a particular protein to an affinity matrix depends on the availability of unoccupied binding sites. The available binding sites on the column could have been occupied by the ap55 that appeared as a single band in the eluate while the rest of the unbound ap55 appeared in the flowthrough fraction, in the form of a complex with tp66.

The tight association between the tp66 and ap55 that withstood the washes of E-RAF Sepharose affinity matrix with high ionic strength buffer helped to shed light on the basic nature of the anchor protein, ap55. Hence theDE-52 flowthrough fraction proved to be a favorable source for the recovery of ap55. The use of the cation exchanger CM Sepharose helped in attaining the desired purity of ap55.

Since E-RAF and tp66 possessed the same molecular mass it was necessary to distinguish between the two proteins and also to find out which among the two interacted with ap55 in an estrogen dependent manner. A DE-52 chromatography step was introduced for this purpose. Since E-RAF is a basic protein, it fails to bind to DE-52 and appears in the flowthrough fraction. tp66, on the other hand remains bound to DE-52 and using the ap55 Sepharose affinity chromatography, tp66 was identified as the protein interacting with ap55 in an estrogen dependent manner.

Thus, there are at least 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. The ap55 is being represented here as an integral protein of the endoplasmic reticulum, the conformation of which is controlled by the binding of estradiol. In the absence of estradiol or 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 and the complex in turn, moves into the nucleus. When exposed to an estradiol concentration beyond 7 nM, ap55 retains the tp66–E-RAF complex, thereby inhibiting the nuclear entry of E-RAF.

The data presented in the companion paper [Govind et al., 2003] probes deeper into the functional characteristics of the protein tp66. Indirect evidence demonstrates it to be a nuclear localization signal binding protein and also with a capacity to interact with a specific protein of the nuclear pore complex. Two factors seem to govern the E-RAF transport: (1) the release of tp66 from the fold of ap55; (2) the existence of E-RAF in a conformation that favors its interaction with tp66. Any change in these two events was found to exert a negative influence on the nuclear entry of E-RAF. Even though earlier reports from our laboratory have indicated a functional role for E-RAF in estrogen action in view of its exclusive property to dimerize with the naER [Jaya et al., 2001], this and the companion paper provide an insight for the first time for a true functional role for E-RAF in the biology of estrogen action. It is anticipated that additional studies related to the structural and molecular biology of E-RAF will provide an understanding of estrogen action in a newer and wider perspective.

Though the estrogenic and non-genomic effects mediated by estrogen receptor localized in the plasma membrane is gaining attention, very little is known about the estrogen binding sites associated with the endoplasmic reticulum. The 'receptor' associated with it and the functional significance of these sites remain elusive. The purification of an estrogen binding entity from the endoplasmic reticulum could be examined in the light of earlier reports on the identification of estrogen binding sites associated with the endoplasmic reticulum [Parikh et al., 1980; Evans and Muldoon, 1991; Monje and Boland, 1999]. Whether the estrogen binding anchor protein presented in this report is a variant form of the estrogen receptor could be identified only after the aminoacid sequence of this protein is made known.

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