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Protein–Protein Interactions That Precede the Nuclear Entry of Goat Uterine Estrogen Receptor Under Cell-Free Conditions

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Abstract Mechanisms associated with a regulated nuclear entry of the goat uterine estrogen receptor (gER), under the influence of estradiol, have been examined using a cell-free system. The gER transport into the nucleus is mediated by a 55-kDa cytosolic protein, p55. Experimental evidence has been provided to demonstrate that p55 binds to the nuclear localization signals (NLS) on the gER. Under hormone-free conditions, a 28-kDa protein, p28, binds to the NLS and prevents the p55 interaction with the NLS. This inhibition is reversed by a 73-kDa protein, p73. It appears that p73 associates with the hormone binding domain (HBD) of the gER under hormone-free conditions. Estradiol binding to the HBD facilitates p73 interaction with p28. This leads to the dissociation of p28 from the NLS, which, in turn, facilitates the binding of p55 to the NLS on the gER. Both p28 and p55 cross-react with monoclonal antibodies against hsp-25 and hsp-70, indicating a possibility that p28 and p55 belong to a superfamily of molecular chaperones. J. Cell. Biochem. 78:650–665, 2000.

Key words: estrogen receptor; goat uterus; nuclear localization signals (NLS); NLS binding proteins; nuclear transport

It is now well recognized that proteins primarily localized in the nuclei contain a set of amino acid sequences known as nuclear localization signals (NLSs) [Picard and Yamamoto, 1987; Gorlich and Mattaj, 1996]. Unoccupied estrogen receptors (ERs) are predominantly nuclear in localization [King and Greene, 1984; Welshons et al., 1984]. A potential NLS was identified in the human ER (hER) in the region of amino acids 256–303 by Ylikomi et al. [1992], who reported the identification of three constitutive and one estrogen-inducible nuclear localization signals in the hER, termed the proto NLSs (pNLSs). Exposure of cells transfected with wild-type ER expression vectors to estradiol resulted in a reproducible and significant increase in the nuclear accumulation of hER. It was assumed that this nuclear accumulation of hER was contributed by the hormone-inducible pNLS in the ER- hormone binding domain (HBD) [Ylikomi et al., 1992]. The first report regarding a transport protein that facilitated the nuclear entry of ER was made by Nirmala and Thampan [1995a, 1995b], who observed that in the goat uterus, ER transport to nuclei was mediated by a 55-kDa protein, p55. They provided indirect evidence to show that the p55 recognized the NLS on the goat uterine estrogen receptor (gER). With the data presented in this communication, it is possible to suggest that p55 is equivalent to the importin α reported by previous investigators [Gorlich and Mattaj, 1996].

Although the targeting role of NLS has been known for some time, recent results indicate that NLS-dependent nuclear protein import is precisely regulated. Phosphorylation appears to be the major mechanism controlling the nuclear transport of a number of proteins including transcription factors such as NF-κB, C-rel, dorsal, and sw15 from yeast [Baeuerle and Baltimore, 1988; Gilmore, 1990; Ghosh and Baltimore, 1990; Moll et al., 1991]. Cytoplasmic retention factors, intra- and intermolecular NLS masking, and NLS masking through phosphorylation are some of the mechanisms by which nuclear transport has been shown to be regulated.

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The present report identifies p55 as the gER NLS-binding protein. The studies carried out using cell-free systems indicate that the p55–gER interaction is being regulated by two other proteins, the availability of which at the ER-NLS site is influenced by estradiol. This is possibly the first report showing that the nuclear entry of ER is under estrogenic control.

MATERIALS AND METHODS

2,4,6,7-[^{3}H] estradiol-17β was purchased from Amersham, Buckinghamshire, England. Monoclonal anti-heat-shock protein (hsp)-25 (clone 1AP-28), monoclonal anti hsp-70 (clone BRM-22), anti-hsp 90 immunoglobulin G (IgG), anti-actin IgG, 5-bromo 4-chloro,3-indolyl phosphate (BCIP), nitroblue tetrazolium (NBT), fluorescein isothiocyanate (FITC), isomer I, estradiol-17β, Nonidet p-40, and 4-chloro 1 naphthol were obtained from Sigma Chemical Co., St. Louis, MO. Sepharose 4B and Sephadex G 100 were purchased from Pharmacia, Uppsala, Sweden. Horseradish peroxidase–coupled anti-rabbit IgG and alkaline phosphatase–coupled anti-mouse IgG were obtained from Genei, Bangalore, India. Phenyl methyl sulphonyl fluoride (PMSF) and silver nitrate were purchased from E-Merck, Darmstadt, Germany. Routine chemicals used in this study were of analytical or reagent grade and were obtained from local commercial establishments.

Two major buffer systems were used in these studies:

1. TEM buffer: 50 mM Tris-HCl, pH 7.6 containing 1 mM ethylene diamine tetraacetic acid, 12 mM monothioglycerol, and 0.2 mM PMSF. TEMN buffer contained, in addition, 50 mM NaCl.
2. TMKC-sucrose buffer: 50 mM Tris-HCl, pH 7.6 containing 5 mM $MgCl_2$, 25 mM KCl, 3 mM $CaCl_2$, 0.2 mM PMSF, and 250 mM sucrose. This buffer was used during isolation of goat uterine nuclei. The buffer was used as a 2x preparation for the initial suspension of isolated nuclei.

Preparation of Goat Uterine Cytosol

Goat uteri were obtained from a local slaughterhouse, transported in ice to the laboratory, and stored frozen at −75°C until used. The frozen uteri were thawed, minced finely, and homogenized in TEMN buffer using an Ultra Turrax (Germany) homogenizer. The homogenate was centrifuged at 15,000 *g* for 15 min in a Sorvall RC 5B high-speed refrigerated centrifuge. 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 postmitochondrial supernatant to give a final 5% concentration of PEG. The mixture was stirred in the cold for 30 min to dissolve the PEG and was centrifuged at 20,000 *g* for 20 min (4°C). The pelleted microsomal fraction was discarded and the supernatant (cytosol) was used in the experiments.

Isolation of Goat Uterine Nuclei

Goat uterine nuclei were isolated as described by Thampan [1985]. The final nuclear pellet was suspended in 2x nuclear transport assay buffer (50 mM Tris-HCl, pH 7.6, 6 mM $CaCl_2$, 10 mM $MgCl_2$, 50 mM KCl, 500 mM sucrose, and 8 mM ATP).

Purification of gER

The method developed by Zafar and Thampan [1993] was followed. The gER appeared as a homogeneous 66-kDa band in silver-stained sodium dodecyl sulfate (SDS) gels.

Nuclear Transport Assay

The purified gER was concentrated through ultrafiltration. One hundred microliter aliquots of the preparation was incubated overnight at 4°C with 20 nM ^{3}H-estradiol in a final volume of 150 μl. Unbound hormone was separated from the ^{3}H-E_2-ER complex after adsorption of the free hormone to dextran coated charcoal (1% charcoal and 0.1 % dextran in 50mM Tris-HCl, pH 7.6 containing 10% glycerol). The ^{3}H-E_2-ER complexes (2 μg in 200 μl buffer) were mixed with goat uterine nuclei suspended in 2x assay buffer, as already described herein. The proteins that influence gER transport into the nuclei 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. The nuclei were washed once again with 2 ml of ice cold assay buffer and were finally extracted with 1 ml ethanol. The ethanol extract was collected in scintillation vials. Radioactivity in these extracts was mea-

sured 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 disintegrations per minute.

Preparation of Affinity Matrices

The following matrices were used during various levels 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].

1. Actin-Sepharose: Actin was purified from goat skeletal muscle following the procedure of Pardee and Spudich [1982]. The purified actin was coupled to CNBr-activated Sepharose 4B.
2. gER-Sepharose: gER, purified to homogeneity, was coupled to CNBr-activated Sepharose.
3. hER-NLS peptide Sepharose: The hER NLS sequence published by Chambon's group [Ylikomi et al., 1992] was used in the synthesis of hER NLS peptide. The hER amino acids 256–303 (RKRDRGGRMLKHKRQRDDGEGRGEVGSAGDMR AANL WPSPLMIKRSKK) was synthesized on hydroxymethyl tetraethyleneglycol diacrylate crosslinked polystyrene support using standard Fmoc solid-phase peptide synthesis protocol [Renil et al., 1994].The coupling reactions were carried out using dicyclohexyl carbodiimide coupling procedure and threefold excess of Fmoc protected amino acid derivatives. The stepwise deprotection was carried out using 20% piperidine in N,N′ dimethyl formamide. Finally, after the synthesis, the peptide was cleaved from the support after exposure to trifluoroacetic acid:thioanisol: m-cresol (10:1:1) mixture at 40°C for 6 h. The peptide was purified by reprecipitation with diethylether and was coupled to CNBr-activated Sepharose B.

Control hER-NLS peptide Sepharose: The control peptide consisted of the same amino acid sequence as that of the hER NLS peptide, except that the pNLS sequences were substituted by poly alanine (AAAAAGGRM LAAAAAADDGEGRGEVGSA GDM RAA NL WPSPLM IAAAAA). The procedure adopted by Renil et al. [1994] was followed for the synthesis of this peptide.

hER–HBD–Sepharose. The hER HBD peptide (amino acids 302–320 (AAAKKNSLALSLTA DQMVSALL) was synthesized in the laboratory [Renil et al., 1994] and coupled to CNBr activated Sepharose 4B. One milligram of the synthetic peptide was coupled to 1 ml packed Sepharose.

p55 Sepharose. Goat uterine p55 was purified following the method developed by Nirmala and Thampan [1995 a and b] and coupled to CNBr activated Sepharose (5 mg protein per 7 ml packed Sepharose).

p12 Sepharose. Purification of p12 was achieved following the method described by Sai Padma and Thampan [2000]. p12 is the nuclear pore complex-associated protein that binds p55. Detergent extracts of isolated nuclei, made in TMKC-sucrose buffer containing 0.05% Triton X-100 was lyophilized. The lyophilized material was dissolved in a small volume of TEMN buffer containing 0.3 M NaCl and was chromatographed over a column (1.5 × 75 cm) of Sephadex G-100 equilibrated with the same buffer. Fractions collected were incubated with nuclear estrogen transport assay systems containing detergent-treated nuclei. The fractions that displayed peak activity (stimulation of ^{3}H-E_2-ER entry into detergent-treated nuclei) were pooled, concentrated, and subjected to rechromatography on Sephadex G-100. The ensuing fractions that displayed activity were pooled, concentrated, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). A single homogeneous band of 12 kDa was observed after silver staining.

SDS PAGE and Western Blot Analysis

Denaturing electrophoresis in the presence of SDS was performed following Laemmli's [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].

Fluorescence Microscopic Studies

gER was labeled with FITC as described by Chard [1987]. FITC-labeled gER was incubated with isolated nuclei in a nuclear transport assay mixture, and the gER movement was monitored under a Nikon (Japan) fluorescence microscope.

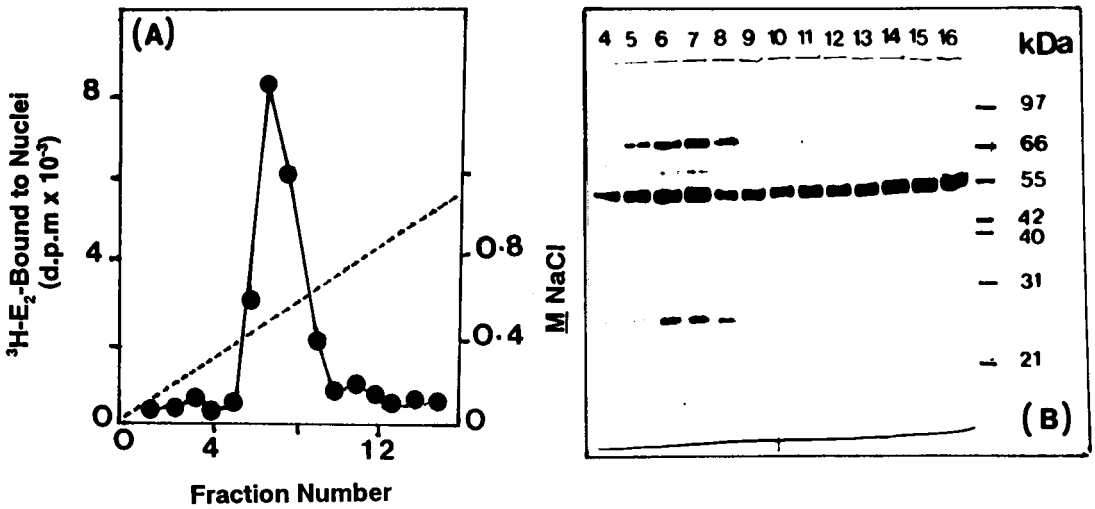

Fig. 1. Isolation of gER-bound proteins. **A**: Goat uterine cytosol was chromatographed over a column of gER-Sepharose (10 ml) equilibrated with TEMN buffer. The column was washed with the buffer, after which the proteins bound to the column were eluted using a linear salt gradient in TEMN buffer. Fractions collected were subjected to the nuclear transport assay. The ^{3}H-E$_2$-gER complexes bound to the nuclei were measured. **B**: Proteins in fractions 4 to 16 were precipitated with TCA, and the precipitated protein fraction was subjected to SDS-PAGE. The numbers displayed on the lanes correspond to the fraction numbers in (A). The gels were stained with silver nitrate.

RESULTS

Identification of gER-Binding Proteins of the Goat Uterine Cytosol

It was assumed that factors that regulate the nuclear entry of gER might bind to the NLS, mask it, and make it unavailable for interaction with the transport protein, p55. The matrix of obvious choice for the isolation of these regulatory factors was gER Sepharose, because it was thought that the regulatory proteins could be isolated after their binding to the NLS sequences on the immobilized gER.

Goat uterine cytosol was chromatographed over a column of gER Sepharose, preequilibrated with TEMN buffer. The unadsorbed fractions from the column were recycled on to the column to optimize the binding of proteins to gER-Sepharose. The column was washed extensively with TEMN buffer, and elution of gER-bound proteins was achieved using a linear gradient of 0–1 M NaCl in TEMN buffer. Fractions collected were subjected to the nuclear transport assay. The protein isolation steps were carried out at 4°C.

The active fractions that displayed the gER transport function were eluted from the gER-Sepharose as a single peak of activity, using buffer containing 0.5–0.6 M NaCl (Fig.1A). Fractions that cut across the activity peak were collected and the proteins precipitated with trichloroacetic acid (TCA) were subjected to SDS-PAGE. The silver stained gel displayed three major protein bands, of molecular mass 73, 55 and 28 kDa (Fig.1B).

It was striking to note that the nuclear transport activity was detected only in those fractions that contained a 73-kDa protein, p73 in addition to p55. Many fractions that contained p55 did not display the transport function. p73 was not present in these fractions. Instead, these fractions displayed another prominent protein band at 28 kDa. It was clear that this protein, p28, made all the difference by inhibiting the p55-dependent gER transport process. It was also evident that this inhibitory effect was neutralized by p73. Subsequent experiments were designed and carried out to confirm these observations made in the first experiment. Another protein band, with a molecular mass of 60 kDa, seen in the gel, did not appear consistently in all the experiments. Therefore, we did not pay attention to this protein in the subsequent studies. The possibility exists that this protein is a proteolyzed derivative of p73.

Chromatographic Isolation of p73, p55, and p28

Goat uterine cytosol was chromatographed over a 10-ml column of gER-Sepharose. The

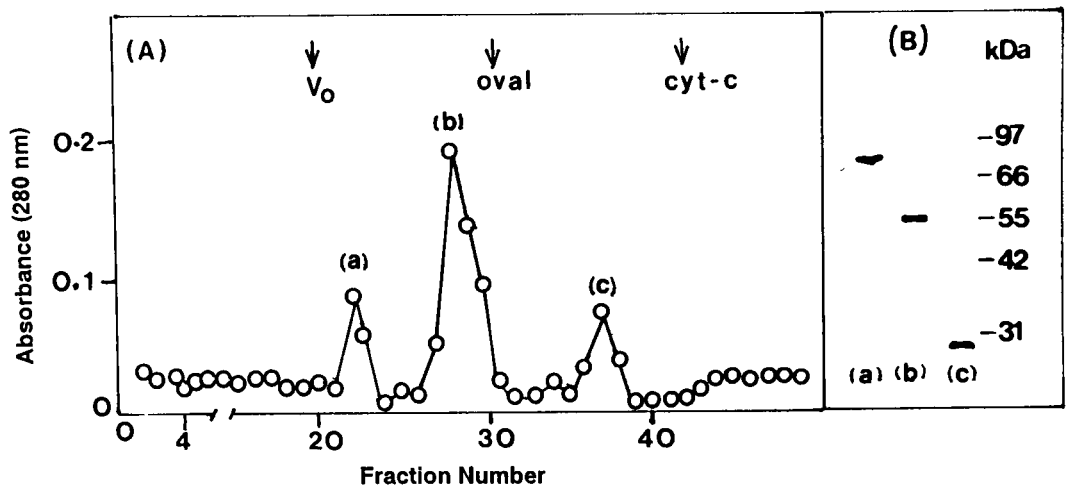

Fig. 2. Gel filtration analysis of gER-Sepharose-bound proteins on Sephadex G100. **A**: Cytosolic proteins bound to gER-Sepharose were eluted from the column using 1M NaCl in TEM buffer and precipitated with $(NH_4)_2SO_4$ at 0.7 saturation. The precipitate was dissolved in a small volume of TEM buffer and dialyzed overnight against the same. The concentrated protein fraction was chromatographed over a column of Sephadex G100 equilibrated with TEM buffer containing 0.3 M NaCl. Elution was achieved using this buffer. The fractions collected were analyzed for their absorbance at 280 nm. **B**: SDS-PAGE of proteins belonging to the fractions representing the three different absorbance peaks of the gel filtration column. The proteins were precipitated with TCA, washed with alcohol, and subjected to SDS-PAGE. The gels were stained with silver nitrate.

column was washed extensively with the buffer and gER-bound proteins were eluted with TEM buffer containing 1 M NaCl. The eluted proteins were precipitated with $(NH_4)_2SO_4$ at 0.7 saturation. The proteins, redissolved in a small volume of the buffer, were dialyzed overnight against the same and concentrated through ultrafiltration. The concentrated protein preparation was chromatographed over a column of Sephadex G100 equilibrated with TEMN buffer containing 0.3 M NaCl. Fractions collected were examined for their absorbance at 280 nm. Three clear peaks were observed, apparently representing the p73, p55, and p28 (Fig. 2A). Subsequent SDS gel analysis confirmed this assumption. The three proteins, p73, p55, and p28 appeared as highly pure and homogeneous bands in silver-stained SDS gels (Fig. 2B). The protein preparations were dialyzed extensively against 50 mM Tris HCl, pH 7.6 and subsequently concentrated through ultrafiltration.

p28 Inhibits p55-Mediated Nuclear Entry of gER

^3H-E_2-gER (0.5 μg protein) was incubated with isolated nuclei and p55 (0.5 μg) in the presence of varying concentrations of p28 (0–0.1 μg) The incubation was carried out at 30°C for 30 min. ^3H-E_2-gER that was bound to the nuclei was quantitated. It was observed that the transport function was inhibited by the addition of p28 to the medium (Fig. 3A).

p73 Reverses the p28-Mediated Inhibition of gER Transport

The possibility that p73 can reverse the p28 mediated inhibition of gER transport was examined using as assay system containing p55 (0.5 μg), p28 (0.1 μg), and increasing concentrations of p73 (0–0.1 μg) in addition to ^3H-E_2-gER and isolated nuclei. The incubation was carried out at 30°C for 30 min. It was observed that p73 reversed the p28-mediated inhibition of the gER transport (Fig. 3B). In the absence of p73, the transport was completely inhibited by p28.

Fluorescence Assay of the p55-Mediated FITC-gER Transport Into the Nuclei

The basic nuclear transport assay that measures ^3H-E_2-gER binding to the nucleus does not distinguish between gER binding to the nuclear membrane and the actual nuclear entry of the gER. To confirm the nuclear entry of the protein, it was essential to label the gER with FITC and examine the transport process under a fluorescence microscope. The nucleus (Fig. 4A) did not display any gER transport in

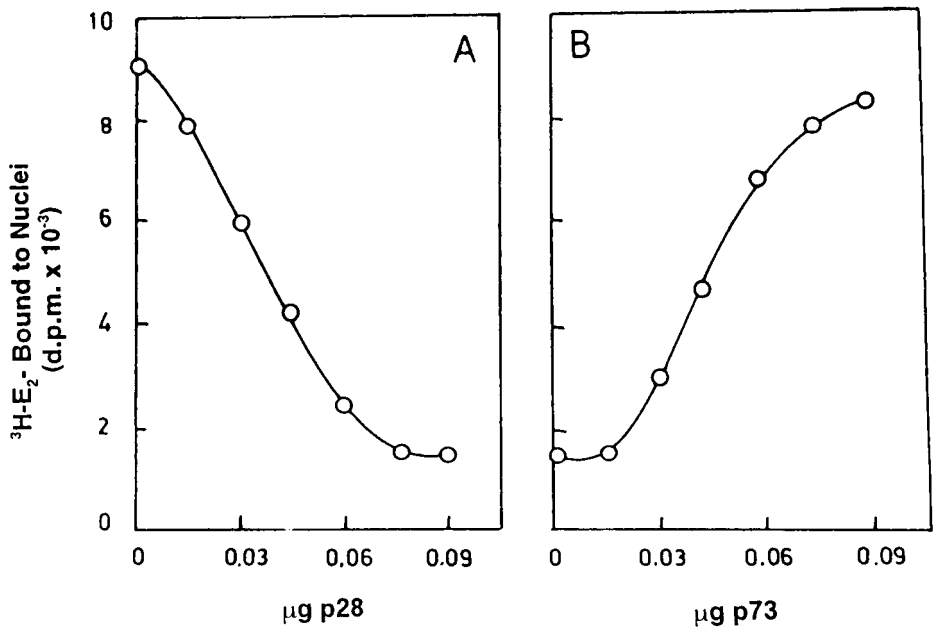

Fig. 3. Effect of p28 and p73 on the p55-mediated nuclear transport of gER. **A**: ^{3}H-E$_2$-gER complexes were incubated with p55 (0.5 μg), isolated nuclei, and p28 (0–0.1 μg) at 30°C for 30 min and the transport assay was performed as described in Materials and Methods. **B**: Effect of p73 on the p28-influenced inhibition of ^{3}H-E$_2$-gER transport: ^{3}H-E$_2$-gER complexes were incubated with p55 (0.5 μg), p 28 (0.1 μg), nuclei, and increasing concentrations (0– 0.1 μg) of p73 at 30°C for 30 min. The nuclear transport assay was carried out as described in Materials and Methods.

the absence of p55 (Fig. 4B). The transport was completed within 10 min of exposure to p55 (Fig. 4C). Addition of p28 to an assay system containing FITC-gER, p55, and nuclei inhibited the transport process (Fig. 4D). The inhibition was reversed after the addition of p73 to this system (Fig. 4 E,F).

Competition Between p55 and p28 for gER Binding Sites

It was of interest to find out whether p28 competed for the p55 binding sties on the gER. ^{3}H-E$_2$-gER (0.5 μg) was incubated with goat uterine nuclei and varying molar concentrations of p55. Two different concentrations (0.03 μg and 0.06 μg) of p28 were added to the reaction media. The transport assay was carried out as described previously, and the results were presented in the form of a Lineweaver-Burke plot (Fig. 5). The results are clearly indicative of the possibility that the p55 and p28 competed for the same site on the gER.

Direct Evidence of the Binding of p55 and p28 to the NLS on the ER

The hER NLS machinery consists of three constitutive NLSs (pNLSs) and one hormone-inducible NLS in the HBD, within the structural framework of amino acids 256–303. The three pNLSs are constitutive in the sense that they promote ER transport even in the absence of the hormone. The hormone-inducible pNLS in the HBD of hER on its own is not efficient for ensuring efficient nuclear accumulation, but cooperates with the constitutive pNLSs. These inducible pNLS apparently contribute to the nuclear accumulation of the wild type-hER in the presence of the hormone [Ylikomi et al., 1992].

The data on the hER were used in the present study because similar structural data on the gER are not yet available. Based on the published results on the hER, three types of NLS peptides were synthesized in the laboratory:

1. Peptide consisting of all three pNLSs and the intervening sequences (amino acids 256–303).
2. Peptide with the same organization as mentioned herein, except that the pNLS sequences were replaced by poly alanine.
3. Peptide representing amino acids 302–320 of the hER. This formed the HBD of the hER.

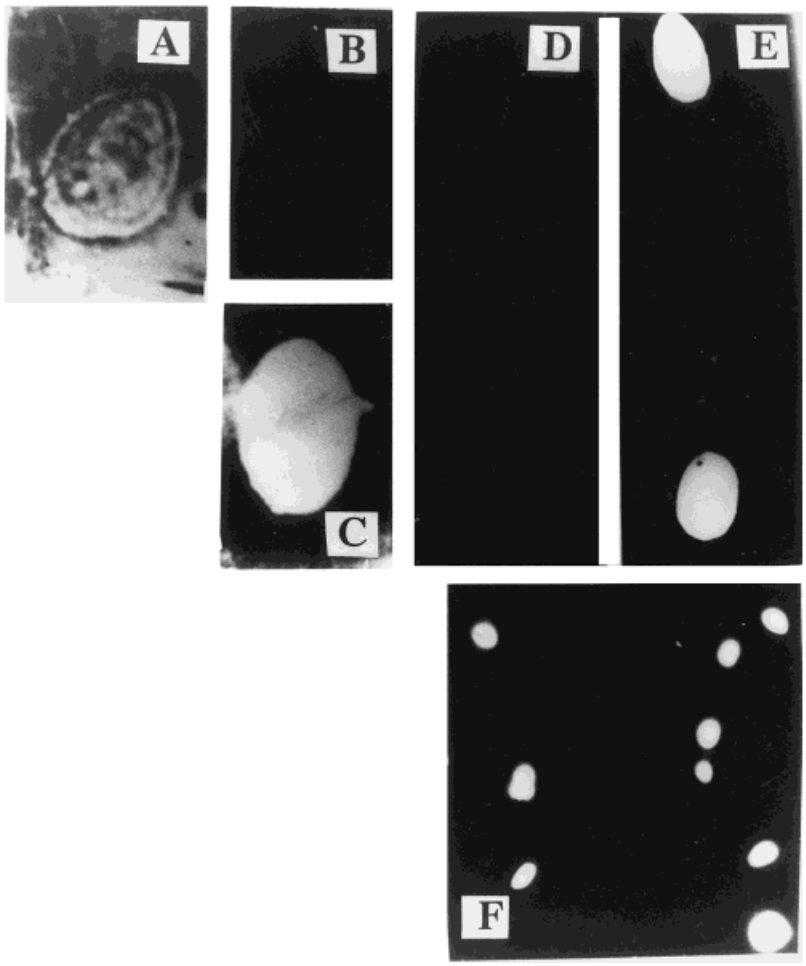

Fig. 4. Fluorescence assay for the gER transport into nuclei. Goat uterine nuclei were suspended in the nuclear transport assay buffer containing ATP and incubated with FITC-labeled gER at room temperature for 30 min. **A**: Phase-contrast micrograph of an isolated nucleus exposed to the transport medium without p55 (×100). **B**: Fluorescence micrograph of (A). **C**: Fluorescence micrograph after the addition of 0.5 μg p55 to the assay medium containing nuclei and FITC-gER. **D**: Effect of addition of p28 (0.1 μg) to a nuclear transport assay medium containing nuclei, FITC-gER, and p55 (0.5 μg). **E:** The medium in (D) after the addition of 0.1 μg p73 (×100). **F:** Results of (E) shown in a larger field (×40).

Incidentally, pNLS-1 overlaps with the HBD of the hER. Affinity columns were made using the three synthetic peptides already mentioned and were identified as NLS-peptide Sepharose, NLS (control) peptide Sepharose, and HBD peptide Sepharose, respectively.

Goat uterine cytosol was chromatographed over a column of ER-NLS peptide Sepharose. The flow-through fractions from this column were recycled on the column for maximal binding. The column was washed extensively with TEMN buffer. Elution of NLS-bound protein was achieved using a linear NaCl gradient (0–1 M) in TEM. The proteins eluted from the column appeared as a clear 280-nm absorbance peak, eluted with NaCl between 0.5 M and 0.6 M concentrations (Fig. 6A). The peak fractions were subjected to SDS-PAGE. The fractions displayed only two proteins, of molecular mass 55 kDa and 28 kDa (Fig. 6B). The results indicated that among the total proteins of the goat uterine cytosol, only p55 and p28 displayed the ability to interact with hER-NLS. The (control) NLS peptide Sepharose column failed to bind any of these proteins, confirming that p55 and p28 bound to the NLS on the ER (data not shown).

p73 Binds to the HBD on the ER

Results of the previous experiment showed that p73 did not bind to the NLS peptide Sepharose. This was also indicative of a possi-

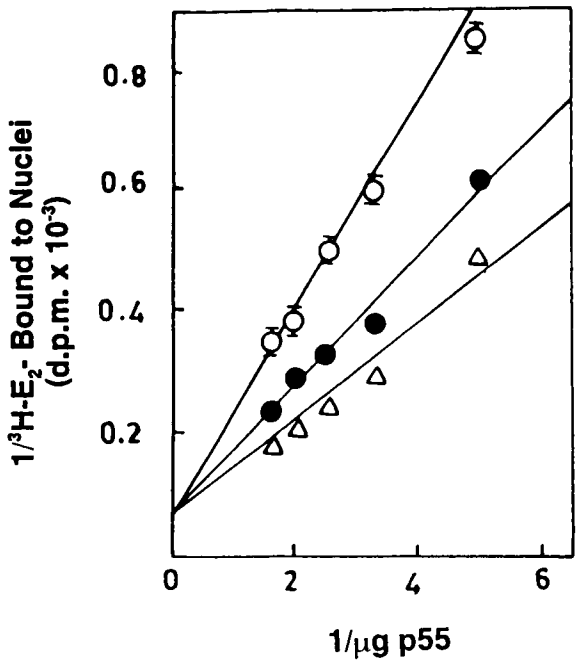

Fig. 5. Competition between p55 and p28 for binding sites on the gER: Nuclei were incubated with ^{3}H-E$_2$-gER and varying molar concentrations of p55 either in the presence or absence of p28 at 30°C for 30 min. The ^{3}H-E$_2$-gER bound to the nuclei was measured and the results are presented in the form of a Lineweaver-Burke plot. Open triangle: no p28; filled circle: 0.03 μg p28; open circle: 0.06 μg p28.

bility that the interaction of p73 with the ER was direct and not mediated by p55 or p28. To find out whether p73 bound to the HBD of the ER, hER-HBD peptide was synthesized and coupled to Sepharose 4B.

Goat uterine cytosol was chromatographed over a column of hER-HBD peptide Sepharose. The column was washed extensively and the peptide bound proteins were eluted using a salt gradient. The fractions were collected and their absorbance at 280 nm measured. The eluted proteins appeared as a single peak of absorbance at 280 nm (Fig. 7A). The fractions were subjected to SDS-PAGE. The silver-stained gel displayed three proteins of molecular mass 73 kDa, 55 kDa, and 28 kDa (Fig. 7B).

Estradiol Releases p73 From the HBD of gER

It was of interest to examine the possibility whether E_2 binding to HBD will bring about the release of p73 from the HBD. Previous studies showed the presence of all three proteins in the protein fraction eluted from the HBD Sepharose. The reason for this may lie in the possibility that the pNLS-1 overlaps the HBD. Therefore, the p55 and p28 that were recovered in the fractions could have remained bound to the residual pNLS, 1 before elution.

In the first experiment, goat uterine cytosol was chromatographed over a column of gER Sepharose, and the column was washed extensively with TEMN buffer. Elution was achieved using TEMN buffer containing 20 nM estradiol-17β. The fractions collected were examined for their absorbance at 280 nm. It was observed that the proteins dissociated from the column as a single major peak immediately after the start of the elution (Fig. 8A). The peak fractions were subjected to SDS-PAGE. Silver-stained gels displayed all of the three proteins: p73, p55, and p28 (Fig. 8B).

The foregoing result deserved a close examination. This time the three proteins, purified to homogeneity, were chromatographed independently over gER Sepharose. Elution was achieved with the buffer containing 20 nM estradiol. The eluted fractions were subjected to SDS-PAGE followed by silver staining. p73 alone was recovered in the E_2-eluted fractions; p55 and p28 remained bound to the gER Sepharose column under these conditions (Fig. 9A). This was a clear indication of the possibility that the appearance of p55 and p28 in the eluate was caused by p73 influence.

The hormone specificity of this release required scrutiny. After chromatography of p73 over gER, Sepharose elution was carried out using buffer containing one of the agents (20 nM) mentioned below: diethylstilbestrol (DES), estradiol-17β, testosterone, dihydrotestosterone, progesterone, and dexamethasone. Other than DES and estradiol, no agent succeeded in dissociating p73 from gER Sepharose (Fig. 9B).

Immunological Similarity Among p55, p28 and Heatshock Proteins 70 and 25

Because the proteins p73, p55, and p28 displayed functions that resembled those of molecular chaperones, it was of interest to test the immunological similarity, if any, between these proteins on the one hand and hsp-70 and hsp-25 on the other. A protein mixture containing p73, p55, and p28 was subjected to SDS-PAGE. The proteins were transferred to nitrocellulose membranes (in duplicates) and were exposed to one of the two monoclonal antibodies anti hsp-70 IgG (clone BRM-22) or anti

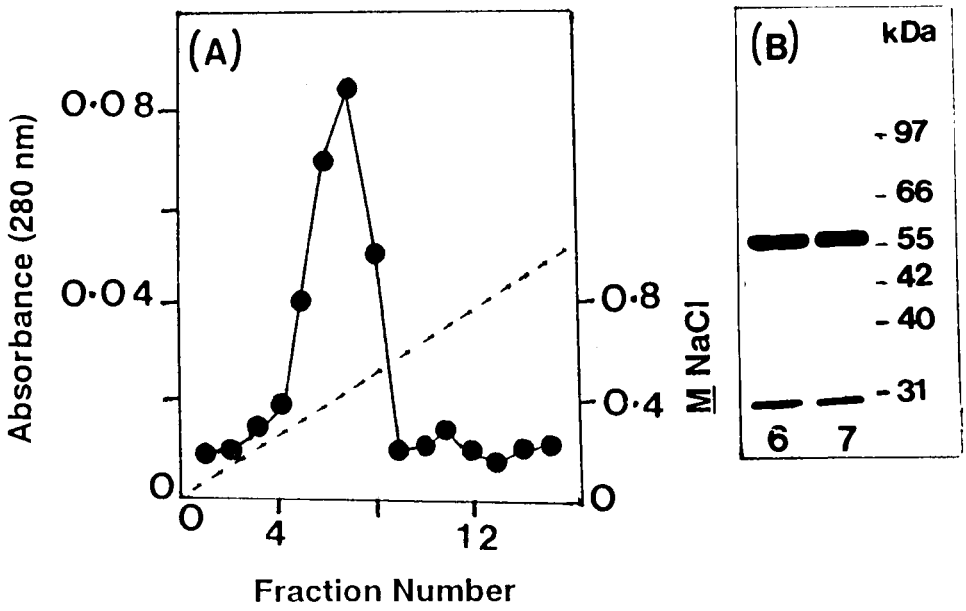

Fig. 6. hER-NLS peptide Sepharose chromatography of goat uterine cytosolic proteins. **A**: Goat uterine cytosol was chromatographed on an hER-NLS peptide Sepharose column. The peptides represented the pNLS1, pNLS2, and pNLS3 of hER with the amino acids 256 to 303 of the hER. The column was washed with the equilibration (TEMN) buffer, and elution was achieved using a linear salt gradient. The fractions collected were examined for their absorbance at 280 nm (closed circles). A similar experiment was carried out using an NLS (control) peptide Sepharose column. No proteins were found associated with this column (results not shown). **B**: The fractions belonging to the absorbance peak were subjected to SDS-PAGE in 10% gels. The gels were stained with silver nitrate. The numbers shown on the lanes correspond to the fractions representing the peak in (A).

hsp-25 IgG (clone-IAP 28). The blots were re-exposed to alkaline phosphatase–coupled anti-mouse IgG and finally stained with BCIP/NBT. Both anti hsp-70 and anti hsp-25 IgGs cross-reacted with p55 as well as p28. p73 was not recognized by these antibodies (Fig. 10). Anti hsp-90 IgG was used as a control in these experiments. This antibody failed to cross-react with the three proteins mentioned herein (Fig. 10).

Is p28 a Proteolytic Derivative of p55?

Because both p55 and p28 bound to ER-Sepharose as well as NLS-peptide Sepharose and were recognized by monoclonal antibodies against hsp 70 and hsp 25, it may be argued that p28 is a product of proteolysis of p55. This remains so in the absence of data on the amino acid sequence of the two proteins. Two additional experiments were carried out to examine this possibility.

1. Actin-Sepharose chromatography: Earlier studies [Nirmala and Thampan, 1995a, 1995b] had shown that p55 displayed high affinity for binding to actin. It was therefore decided to examine whether p28 also possessed this property. A 1:1 mixture of p55 and p28 was chromatographed over a column of actin Sepharose. Elution was achieved using a 0–1 M NaCl gradient. The fractions collected were analyzed for their effect on the nuclear transport of gER. A single peak of activity was eluted with 0.6 M NaCl (Fig.11 A). SDS-PAGE of the peak fractions revealed the presence of p55 alone (Fig. 11B).
2. p12-Sepharose chromatography: A 12-kDa protein, p12, of the nuclear membrane/pore complex is involved in the p55-mediated gER transport into the nucleus (see Materials and Methods). A mixture of p55 and p28 was chromatographed over a column of p12-Sepharose, after which elution of the p12-bound proteins was achieved using an NaCl gradient. p55 alone was found to bind to p12 immobilized on Sepharose (Fig. 11A,B).

Free NLS Peptide Competes With gER for Binding to p55:

The nuclear transport assay mixture containing ^{3}H-E_2-ER complexes (0.5 μg ER), p55

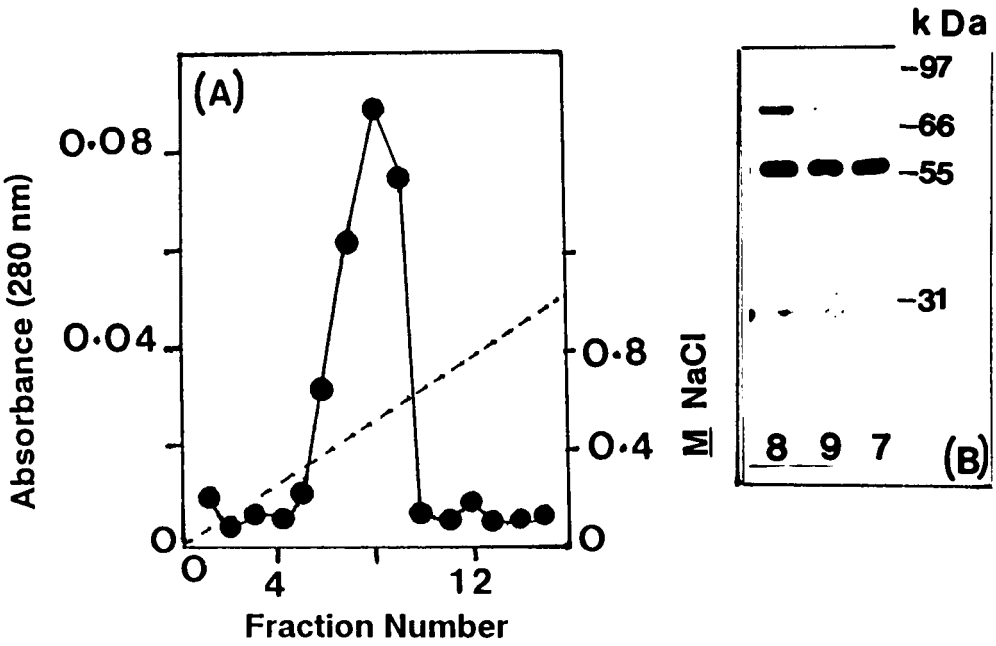

Fig. 7. hER-HBD peptide Sepharose chromatography of goat uterine cytosolic proteins. **A**: The hER-HBD peptide contained the amino acids 302 to 320 of hER, representing pNLS1 and HBD. Goat uterine cytosol was chromatographed over the HBD peptide column, which was equilibrated and washed with TEMN buffer. Elution of the HBD-bound proteins was achieved using a linear salt gradient. The fractions collected were examined for their absorbance at 280 nm. **B**: The fractions belonging to the absorbance peak were subjected to SDS-PAGE in 10% gels. The proteins were stained with silver nitrate. The numbers on the lanes correspond to the fractions in the absorbance peak of (A).

(0.5 μg), and goat uterine nuclei was incubated with increasing concentrations of free NLS peptide (0–6 μg) at 30°C for 30 min. The transport was inhibited progressively along with the increase in the concentration of the NLS peptide added, reaching the lowest level in the presence of 2.5 μg of the peptide (Fig. 12A). The assay was repeated using a system that contained 2.5 μg free NLS peptide, a 10-fold excess of ER, and the nuclei in the presence or absence of p55. A control set without the peptide was also incubated under similar conditions. No transport of ER took place in the presence of 2.5 μg free NLS peptide. The inhibition could be overcome only by the addition of excess p55 and not by excess gER or nuclei (Fig.12B). This was yet more supporting evidence that the p55 interacts with the NLS on the gER.

DISCUSSION

A mechanism of regulated entry of gER from the cytoplasm to the nucleus under the influence of estradiol has been identified. The nuclear transport is mediated by a cytosolic 55-kDa protein, p55, which recognizes the NLS on the gER, binds to it, and transports it to the nucleus. It contributes to the predominant nuclear localization of the gER. Ylikomi et al. [1992] observed an additional increase in the nuclear entry of hER under hormonal stimulation. Here we present a possible regulatory mechanism that takes care of the estradiol-stimulated nuclear transport of the ER. It appears from the studies presented in this report that in the absence of estradiol bound to the HBD of the gER, the NLS site on the gER is masked by a 28-kDa protein, p28, which shares several functional features with p55. It appears that p55 dimerizes with p28, which apparently results in the masking of the NLS binding domain on the p55. Because the NLS binding domain of p28 is not masked, it is free to interact with the NLS on the gER. Thus, the association of p28 with the NLS in the ER may be the prime internal regulatory mechanism that checks an uncontrolled nuclear entry of ER and the consequent stimulation of transcription of estrogen-responsive genes.

It was the experimental observation that p73 was present along with p55 and p28 in the active fraction of uterine cytosol eluted from the gER Sepharose that identified the future

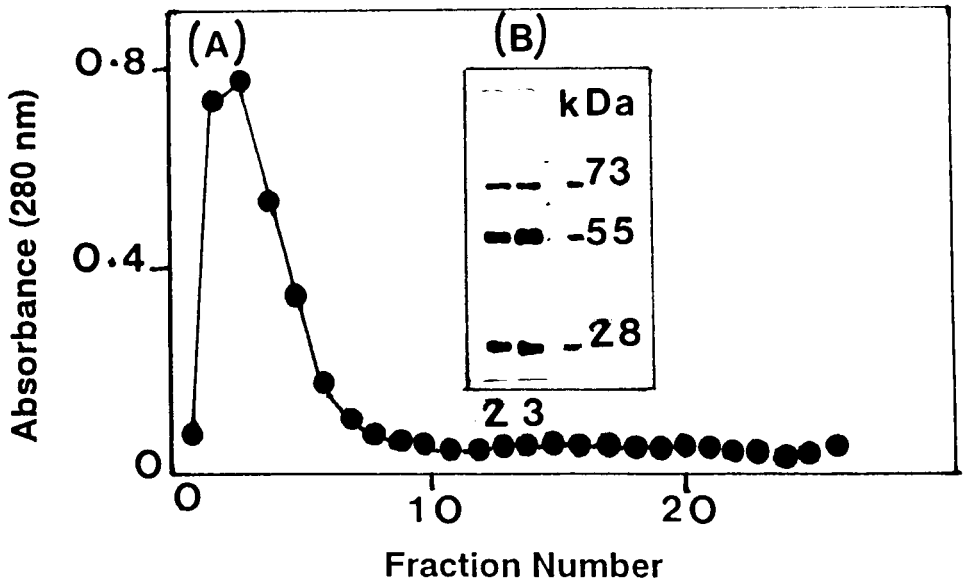

Fig. 8. Estradiol-dependent release of proteins bound to gER Sepharose. **A**: Goat uterine cytosol was chromatographed over a column of gER Sepharose equilibrated with TEMN buffer; the column was washed extensively with the same buffer. Elution of bound proteins was achieved using TEMN buffer containing 20 nM estradiol. The absorbance of the fractions at 280 nm was measured. **B**: The fractions that represented the absorbance peak were subjected to SDS-PAGE on a 12% gel. The gel was stained with silver nitrate. The lane numbers correspond to the fraction numbers given in (A).

direction in which the studies could be pursued. There were several fractions where p55 was found to be present in appreciable quantities. These fractions apparently did not display the transport function. A closer look at the SDS gel revealed that these fractions contained, in addition, the p28 as well. The fractions that displayed peak transport activity alone contained p73, in addition to p55 and p28. The results pointed to the possibility that p28 inhibited p55 binding to the gER and also that the p28-mediated inhibition was reversed by p73.

Chromatography of a mixture of proteins containing p55, p28, and p73 on HBD Sepharose or gER Sepharose and the subsequent elution using either high salt or 20 nM estradiol showed the presence of all three proteins in the protein fraction eluted from the column. The HBD-peptide sequence contained two lysine residues. These lysine residues could have contributed to the specificity in the binding of p55 and p28 to the HBD. In the SV 40 large T-antigen NLS, a point mutation resulting in the substitution of threonine for lysine at the codon 128 position abolished its ability to localize in the nucleus [Kalderon et al., 1984]. This is indicative of the possibility that the position and presence of lysine residues are very important for the NLS function. Estradiol-dependent elution of gER-bound proteins clearly showed that when chromatographed in isolation, p73 alone was released by the hormone from the matrix. On the other hand, simultaneous availability of the three proteins on the affinity column promoted the release of all three proteins from the column on elution with estradiol. This could be due to the presence of p28 and p55 as a protein aggregate bound to the lysine residues. p73 binding to the p28 and the subsequent dissociation of the latter from the matrix could have caused a simultaneous release of the p55 as well. The studies serve to give credibility to the assumption that p73 binds to the HBD of the gER.

It is clear that p55 and p28 aim at the common NLS target on the gER. This and the immunological relationship displayed by the two proteins with hsp70 and hsp25 could be a basis for the belief that p28 is a proteolytic product of p55. It is true that p55 has functional features that are not found in p28, which include the ability of p55 to interact with actin and p12. This could be attributed to the loss of the actin and p12 binding domains of p55 during its transformation to p28. One observation that can counter the theory that p28 is derived from p55 is that p73 brings about dissociation

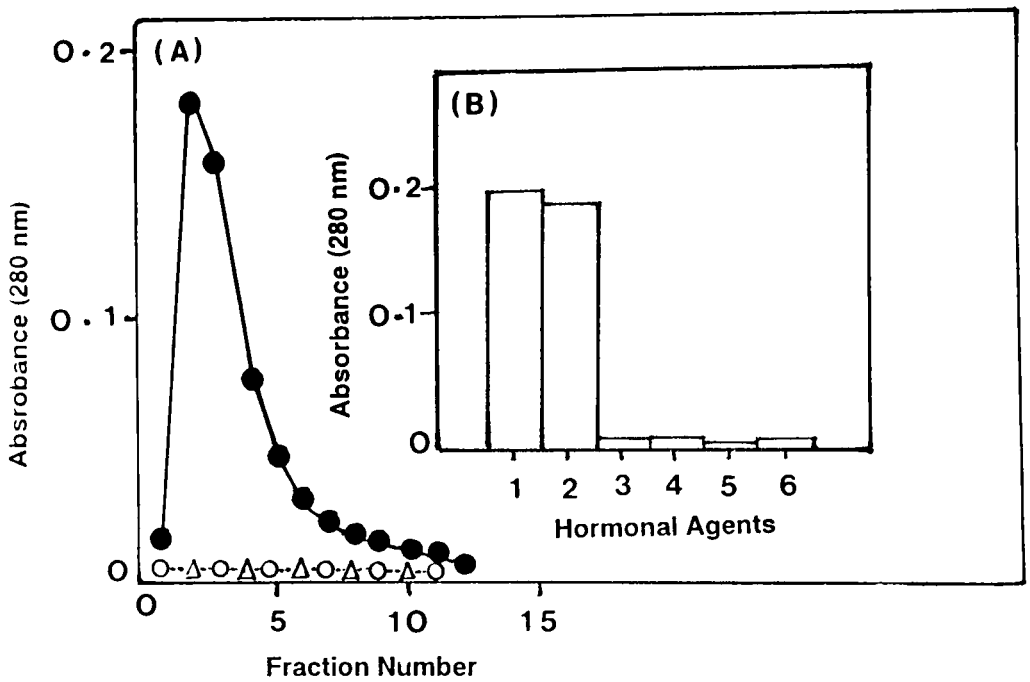

Fig. 9. Evidence to show that estradiol releases p73 alone from the gER Sepharose and also that the hormonal response is estrogen specific. **A**: This is a repetition of the experiment given in Figure 8A, except that cytosol was substituted by the purified proteins p73, p55, or p28. The proteins were chromatographed independently over gER Sepharose. The column was washed with the buffer, and the proteins bound to the column were eluted by TEMN buffer containing 20 nM estradiol. Filled circles: p73; open circles: p55; open triangles: p28. **B**: Hormone specificity associated with the release of p73 from the gER Sepharose. Purified p73 was chromatographed over a column of gER Sepharose and elution was achieved using TEMN buffer containing one of the following hormones, at 20 nM concentration: 1, diethylstilbestrol; 2, estradiol-17β ; 3, dihydrotestosterone; 4, progesterone; 5, dexamethasone; 6, testosterone.

of p28 from the NLS and not of p55. An affinity column containing p73 immobilized on Sepharose 4B binds p28 but not p55 (data not shown). Had this not been the case, p55-dependent ER transport would not have taken place in the presence of p73. It is possible that the proteolytic transformation of p55 to p28 could have generated a new functional domain on the p28 for its interaction with p73. It may be stated that the formation of p28 is a physiological necessity to regulate the nuclear transport of the ER. The signal for the removal of p28 from the NLS needs to originate from the binding of estradiol to the HBD. It is difficult at this stage to speculate on the mechanism by which estradiol brings about a close association between p73 and p28 presumably bound at two locations on the gER. The two possibilities are: 1) dissociation of p73 from HBD after hormone binding and its subsequent interaction with p28; and 2) conformational change on the HBD as a result of estradiol binding, which brings the HBD-bound p73 close to the p28 located at the neighboring NLS site. An observation that highlights the actin involvement in the ER transport is that p55 and not p28 binds to actin, and also that p28 is incapable of transporting ER into the nucleus. Another observation that supports the independent status of p28 is that if p28 is the product of limited proteolysis of p55, it is logical to anticipate detection of the remaining fragment during the chromatographic analysis of cytosol either on actin Sepharose or on p12-Sepharose. It has not been possible to detect the "other" 27-k fragment. The possibility, nevertheless, remains that the "other" fragment could have been the target of extensive proteolysis. The p28, on the other hand, appeared in the flow-through factions derived from actin-Sepharose and p12 Sepharose (data not shown).

The data are summarized in Figure 13. Under hormone-free conditions, p73 remains bound to the HBD on the ER, while the p28-p55 heterodimer remains associated with the NLS on the ER. The heterodimerization is

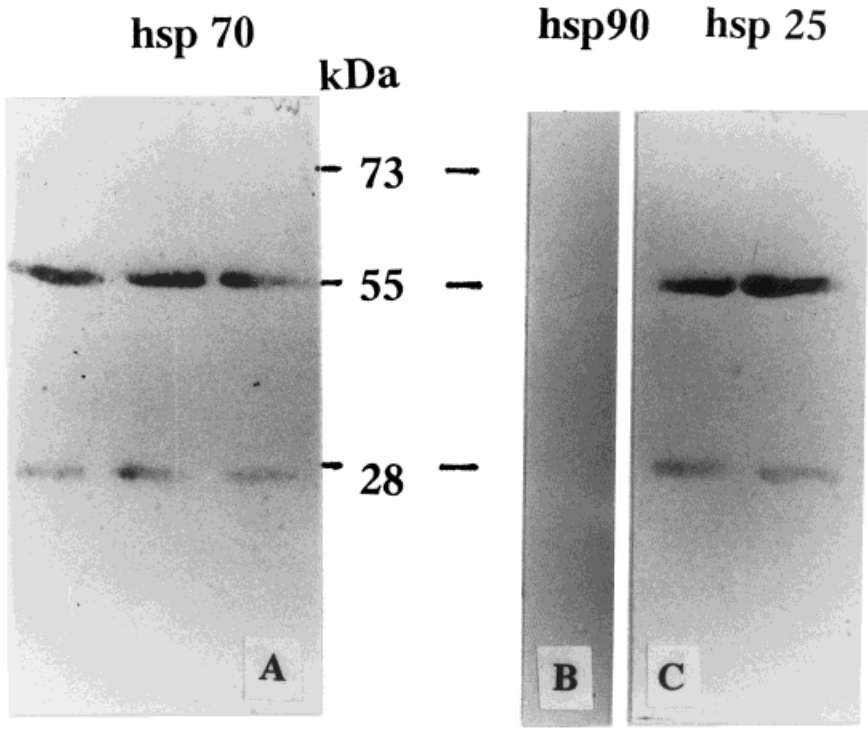

Fig. 10. Immunological cross-relatedness among p28, p55, and the heat shock proteins 70 and 25. Purified p73, p55, and p28 were subjected to SDS-PAGE. The proteins were transferred to nitrocellulose sheets that, in turn, were exposed to anti-hsp 90 IgG, monoclonal anti-hsp 70, or to monoclonal anti-hsp 25. The blots were reexposed to alkaline phosphatase–coupled anti-mouse IgG and stained with BCIP/NBT. **A**: Cross-reactivity with anti-hsp 70 IgG . C: Cross-reactivity with anti-hsp 25 IgG. **B**: The blot that was exposed to anti-hsp 90 IgG.

shown to result in the making of the NLS-binding domain of the p55, while that of p28 remains free to interact with the NLS. Hormone binding to HBD and the resultant conformational change in the ER brings p73 close to p28, located at the NLS. p73-p28 interaction serves to dissociate p28 from p55 as well as from its binding site on the ER-NLS. The free p55 interacts with the NLS and transports the ER to the nucleus.

The limiting factor is p28. In the absence of p28, p55–ER interaction remains uninterrupted and the subsequent ER transport to the nucleus is independent of the presence of estradiol. Possibly, in the early studies reported by Gorski [Welshons et al., 1984] and Greene [King and Greene, 1984] a similar situation could have existed in which the concentration of p28 was not sufficient to block the p55-mediated nuclear entry of the ER. Under these conditions, the ER entry into the nucleus will remain independent of the presence of estradiol.

The immunological cross-reactivity observed between p55 and p28 on the one hand and anti hsp-70 and anti hsp-25 on the other warrants attention. The observation is interesting for two reasons:

1. Possible structural similarity between estrogen receptor associated proteins and heat shock proteins.
2. Projected immunological similarity between hsp-70 and hsp-25. The possibility exists that the four proteins involved might have some common motif in their primary structure. The amino acid homology may suggest an evolutionary conservation of domains critical to the function of the protein. The cross-reactivity with the monoclonal antibodies is again indicative of the high sequence homology of the proteins, p55 and p28, between themselves and also between them and the heat shock proteins, giving a clue to their functions as molecular chaperones. Studies reported by Faucher et al. [1993] also projected similar results.

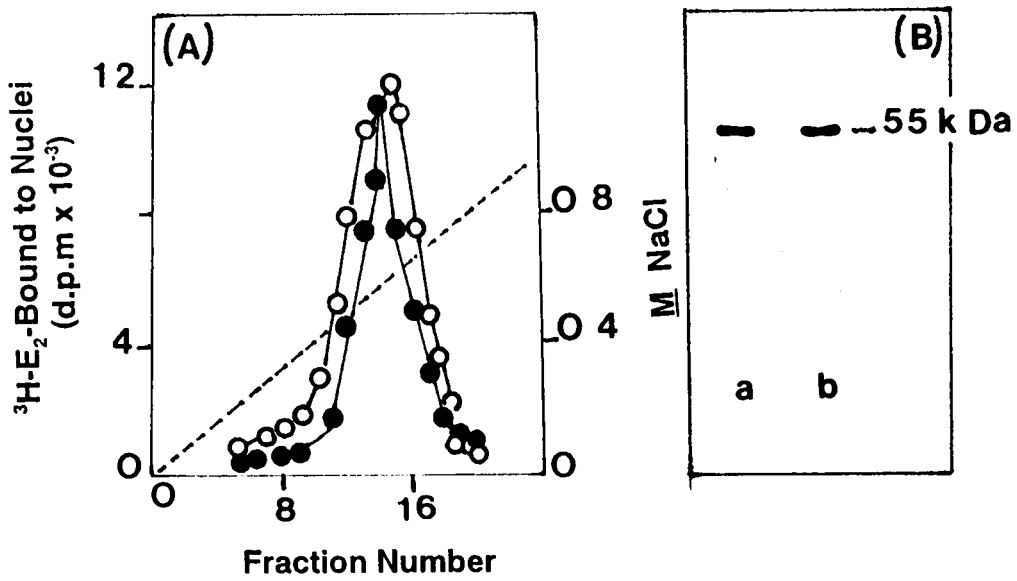

Fig. 11. Evidence to show that p55 but not p28 has the capacity to interact with actin and p12. **A**: A (1:1) mixture of purified p55 and p28 was chromatographed over a column of actin Sepharose (filled circles) or p12-Sepharose (open circles) equilibrated with TEMN buffer. Elution was achieved with a linear salt gradient. The fractions collected were subjected to the nuclear transport assay. **B**: The fractions belonging to the absorbance peak were subjected to SDS-PAGE. The gels were stained with silver nitrate. In both cases, p28 appeared in the flow-through fractions (results not shown).

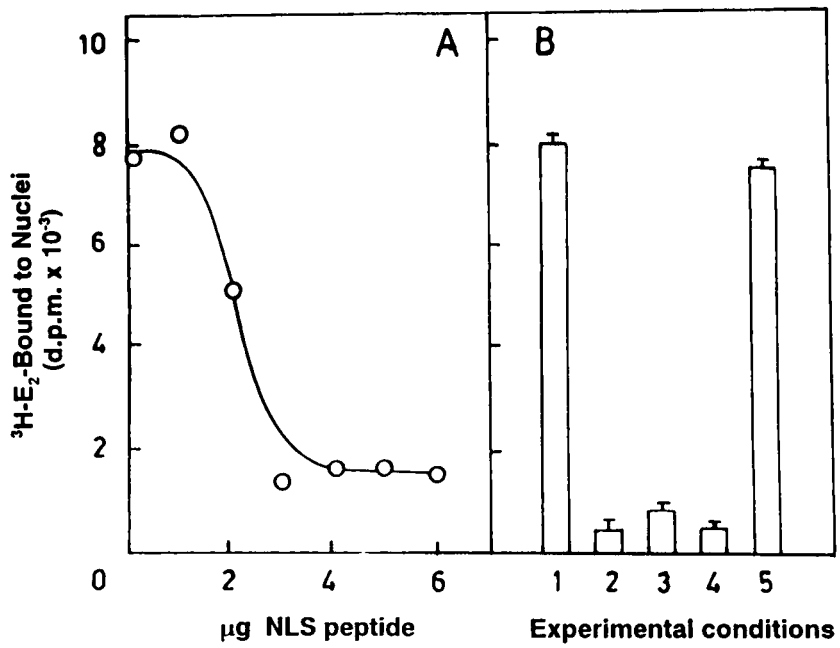

Fig. 12. Inhibition of p55-mediated nuclear transport of ^{3}H-E_2-gER by free NLS peptide. **A**: The nuclear transport assay mixture consisting of ^{3}H-E_2-gER, nuclei, and p55 was incubated with increasing concentrations of free NLS peptide, and the assay was performed as described in Materials and Methods. ^{3}H-E_2-gER bound to the nuclei was measured. **B**: The nuclear transport assay of (A) was carried out under different experimental conditions: 1, Control, without external NLS peptide; 2, NLS peptide added in a 1:10 ratio in relation to the gER; 3) the incubation medium of (2) with 10-fold excess of gER; 4, the incubation medium of (2) with 10-fold excess of nuclei; 5) the incubation medium of (2) with 10-fold excess of p55.

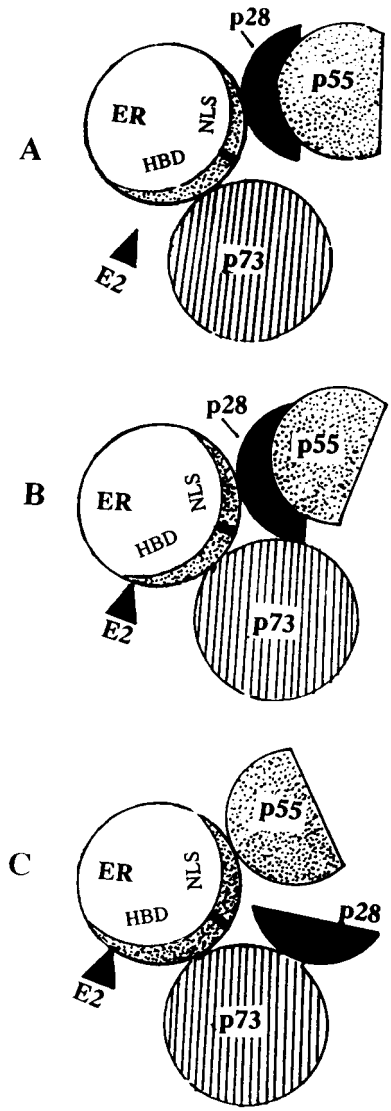

Fig. 13. A model presented to explain the protein–protein interactions that the gER undergoes before its nuclear entry. **A:** The p55 remains as a heterodimer with p28. p28 recognizes the NLS on the ER. The NLS-binding domain of p55 is masked by the p28, thereby preventing p55 interaction with the NLS. Under these conditions, the p73 remains bound to the hormone binding domain (HBD) of the ER. Estradiol is not bound to the HBD. **B:** Estradiol binds to the HBD. The possible conformational changes that result in the ER after hormone binding bring p73 close to p28 that is localized at the NLS. **C**: p73–p28 interaction causes dissociation of p28 from the NLS as well as from its state of dimerization with p55. The free p55 interacts with the NLS on the ER.

There is a general consensus that the protein to be transported into the nuclei needs to recognize the NLS receptor that is being identified as importin α [Gorlich and Mattaj, 1996]. The importin α in turn heterodimerizes with importin β that, in turn, docks at the nuclear pore complex (NPC) [Paschal and Gerace, 1995; Percipall et al., 1997]. The p55 identified by us represents the importin α, whereas the p12 represents importin β. We have not looked into the role of Ran GTPase and a p15 in the nuclear transport of estrogen receptor. Therefore, we are not in a position to rule out their possible involvement in the estrogen transport at the level of the NPC.

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REFERENCES

Baeuerle PA, Baltimore D. 1988. IkB: a specific inhibitor of the NF-kB transcription factor. Science 242:540–546.

Blum H, Beier H, Gross HJ. 1987. Improved silver staining of proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8:93–99.

Chard T. 1987. In: Burton RH, van Knippenberg PH, editors. Laboratory techniques in biochemistry and molecular biology: an introduction to radioimmunoassay and related techniques, vol. 6: part 2. Amsterdam: Elsevier. p 68–87.

Faucher C, Capdevielle J, Canal I, Ferrara P, Mazarguil H, McGuire WL, Darbon JM. 1993. The 28-kDa protein whose phosphorylation is induced by protein kinase C activators in MCF-7 cells belongs to the family of low molecular mass heat shock proteins and is the estrogen regulated 24 kDa protein. J Biol Chem 268:15168–15173.

Ghosh S, Baltimore D. 1990. Activation in vitro of NF-kB by phosphorylation of its inhibitor IkB. Nature 344:678–682.

Gilmore TD. 1990. NF-kB, KBF1, dorsal and related matters. Cell 62:841–843.

Gorlich D, Mattaj IW. 1996. Nucleocytoplasmic transport. Science 271:1513–1518.

Kalderon D, Richardson WD, Markham AF, Smith AE. 1984. Sequence requirement for nuclear localization of simian virus 40 large T-antigen. Nature 311:33–38.

King WJ, Greene GL. 1984. Monoclonal antibodies localize oestrogen receptor in the nucleus of target cells. Nature 307:745–747.

Laemmli UK. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680–685.

March SC, Parikh I, Cuatrecasas P. 1974. A simplified method of cyanogen bromide activation of agarose for affinity chromatography. Anal Biochem 60:149–152.

Moll T, Tebb G, Surane U, Robitsch H, Nasmyth K. 1991. The phosphorylation and the cdc 28 protein kinase in cell cycle regulated nuclear import of the S. cerevisiae transcription factor sw.15. Cell 66:743–758.

Nirmala PB, Thampan RV. 1995a. A 55 kDa protein (p55) of the goat uterus mediates nuclear transport of the estrogen receptor. 1. Purification & characterization. Arch Biochem Biophys 319:551–561.

Nirmala PB, Thampan RV. 1995b. A 55 kDa protein (p55) of the goat uterus mediates nuclear transport of the estrogen receptor II. Details of the transport mechanism. Arch Biochem Biophys 319:562–569.

Pardee JD, Spudich JA. 1982. Purification of muscle actin. Methods Enzymol 85:164–181.

Paschal BM, Gerace L. 1995. Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62. J Cell Biol 129:925–937.

Percipalle P, Clarkson WD, Kent HM, Rhodes D, Stewart M. 1997. Molecular interactions between the importin alpha/beta heterodimer and proteins involved in vertebrate nuclear import. J Mol Biol 266:722–732.

Picard D, Yamamoto KR. 1987. Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. EMBO J 6:3333–3340.

Renil M, Nagaraj R, Pillai VNR. 1994. Gel-phase peptide synthesis on a new high-capacity tetraethylene cross linked polystyrene support: synthesis of pardoxin (16-33). Tetrahedron 50:6681–6688.

Sai Padma A, Thampan RV. 2000. Interdependence between a 55 kDa protein (p55) and a 12 kDa protein (p12) in facilitating the nuclear entry of goat uterine estrogen receptor under cell-free conditions. Biol Chem 381:(in press).

Sweet DJ, Gerace L. 1995. Taking from the cytoplasm and giving to the pore: soluble transport factors in nuclear protein import. Trends Cell Biol 5:444–447.

Thampan RV. 1985. The nuclear binding of estradiol stimulates ribonucleoprotein transport in the rat uterus. J Biol Chem 260:5420–5426.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Van der Hoeven T. 1981. Isolation of hepatic microsomes by polyethylene glycol 6000 fractionation of the post mitochondrial fraction. Anal Biochem 115:398–402.

Welshons WN, Lieberman ME, Gorski J. 1984. Nuclear localization of unoccupied estrogen receptors. Nature 307:747–749.

Ylikomi T, Bocquel MT, Berry M, Gronenmeyer H, Chambon P. 1992. Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. EMBO J 11:3681–3694.

Zafar A, Thampan RV. 1993. A four step inexpensive protocol for large scale purification of goat uterine estrogen receptor. Protein Expr Purif 4:534–538.