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Rapid report

Free leucine dissociates homo- and heterodimers formed between proteins containing leucine heptad repeats

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Abstract

A highly specific method for the dissociation of protein dimers has been developed. The method involves exposure of the dimers to free leucine at a concentration ranging between 3 and 10 mM. Using this method it has been possible to dissociate goat uterine oestrogen receptor homodimers, heterodimers formed between the non-activated oestrogen receptor (naER) and the oestrogen receptor activation factor (E-RAF) of the goat uterus, c-jun homodimers derived from bovine bone marrow and also glucocorticoid receptor homodimers isolated from rat liver cytosol. The pattern of dimer dissociation by leucine clearly differentiates two classes of proteins. The first is represented by steroid hormone receptors where dimerization is apparently contributed by both coiled-coil dimerization interfaces and the conserved heptad repeats of leucine. The second is represented by oncoproteins like c-fos and c-jun which dimerize through the exclusive involvement of leucine zippers. The patterns of dissociation of these two groups of proteins from the concerned affinity columns are distinctly different. This indicates a possibility that the elution pattern may be used as a yardstick to determine whether two proteins dimerize through the exclusive involvement of leucine zippers or whether coiled-coil interfaces are also involved in the dimerization process.

Keywords: Dissociation of dimers; Leucine zipper; Coiled-coil interaction

Leucine-rich regions of proteins, known as leucine zippers, have been proposed as possible sites of protein dimerization [1]. They are a special class of heptad repeats which contain leucine at every seventh amino acid residue [1]. The leucine zipper motif was first described in eukaryotic transcription regulators and also in nuclear oncoproteins such as jun, fos and myc [2]. It is believed that leucine zipper motif participates in the dimerization and creates a DNA-binding site. The importance of leucine zipper regions in homo and heterodimerization of proteins has been recognized [1,3]. The activity of these proteins, identified as bZip proteins, is regulated not only by the interactions between the protein and the DNA, but also by the protein–protein interactions among the leucine zipper domains [4]. This versatile structural motif is known to be involved in many cell functions such as receptor dimerization, domain repulsion, and the regulation of adhesion and binding events [5].

Analysis of sequences in the steroid binding domain of mouse oestrogen receptor revealed a heptad repeat of hydrophobic residues in this region which

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are well conserved in all members of the nuclear receptor superfamily [6]. The oestrogen receptor exists as a homodimer, bound to the oestrogen responsive element, and there is a distinct possibility of leucine zipper involvement in the dimerization and DNA-binding functions of the receptor. This prompted us to explore the feasibility of using leucine in the dissociation of oestrogen receptor homodimer and converting this as a potential method for the purification of oestrogen receptor. In the process we also experimented with the goat uterine oestrogen receptor heterodimer system that involves the non-activated oestrogen receptor (naER) and the oestrogen receptor activation factor (E-RAF). naER is a non-DNA-binding, high-affinity oestrogen-binding protein primarily localized at the plasma membrane [7] while E-RAF is a DNA-binding protein with no hormone-binding function to perform [8]. Both naER and E-RAF are 66 kDa proteins. Since the primary structure of these proteins is not yet known, it is not possible to say at this stage whether they contain leucine zipper motifs. Our studies clearly indicate that the homo- and heterodimers involving goat uterine oestrogen receptor, the oestrogen receptor-associated proteins, the c-jun homodimers and the glucocorticoid receptor homodimers do dissociate in the presence of free leucine. It is being proposed that this method may be projected as a general protocol for the purification of proteins which use leucine zippers also in the dimerization process and for the isolation of unknown dimerization partners of nuclear proteins which employ leucine heptad repeat either as the sole or partial structural motifs in dimerization.

Goat uterine oestrogen receptor (ER) was purified following the method developed by Zafar and Thampan [9]. Goat uterine non-activated oestrogen receptor (naER) was isolated and purified as described by Karthikeyan and Thampan [7] and the oestrogen receptor activation factor (E-RAF) was purified from the goat uterus following a method published earlier [8]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli [10] and the gels were stained with silver nitrate as described by Blum et al. [11]. Western transfer of proteins was achieved following the method developed by Towbins et al. [12]. Hydroxylapatite (HAP) binding assay for oestrogen receptor was carried out as described by Peck and Clark [13] after overnight exposure of the receptor to 20 nM [^{3}H]estradiol (2,4,6,7-[^{3}H]estradiol-17β, spec. act. 91 Ci/mmol, purchased from Amersham, UK). Anti-ER, anti-naER and anti-E-RAF IgGs were raised in rabbits. Anti-GR IgG and recombinant c-jun (79) sc-4113 were purchased from Santa Cruz Biotechnology, California. Anti-c-jun IgG was obtained from Medac, Hamburg, Germany. Rat liver glucocorticoid receptor (GR) was isolated and purified using an immunoaffinity column consisting of anti-GR IgG (Santa Cruz) coupled to CNBr-activated Sepharose. HAP assay for GR was carried out using GR incubated overnight with 20 nM [^{3}H]dexamethasone (1,2,4,6,7 [^{3}H]dexamethasone, spec. act. 90 Ci/mmol, obtained from Amersham). The AP-1 oligonucleotide sequence: 5′-CGC TTG ATG AGT CAG CCG GAA-3′; 3′-GCG AAC TAC TCA GTC GGC CTT-5′ was synthesized commercially (Gibco-BRL, USA) and immobilized on CNBr-activated Sepharose. The ER, naER, E-RAF, GR and AP1 oligonucleotide chains were coupled to CNBr-activated Sepharose 4B (Pharmacia, Sweden) to create five affinity columns, following the method of March et al. [14].

In the first study three different affinity columns were used: ER-Sepharose, naER-Sepharose and E-RAF Sepharose. Goat uterine cytosol prepared in TEM buffer (10 mM Tris–HCl (pH 7.6) containing 1 mM EDTA and 12 mM monothioglycerol) was chromatographed over the affinity matrices independently, following which the columns were washed extensively using TEM buffer containing 2 M NaCl. The column was washed again with salt-free TEM buffer following which elution of bound proteins was accomplished using a 0–10 mM leucine gradient in TEM buffer. The fractions collected from the naER-Sepharose column were analysed for absorbance at 280 nm while those collected from ER-Sepharose and E-RAF Sepharose were assayed for oestrogen binding using the HAP-adsorption method. Individual fractions representing peak activity were subjected to SDS–PAGE and subsequently to immunoblot analysis.

The basis on which the studies were carried out was the assumption that the ER in the crude cytosol should homodimerize with the immobilized ER and get eluted after exposure to the leucine gradient. The

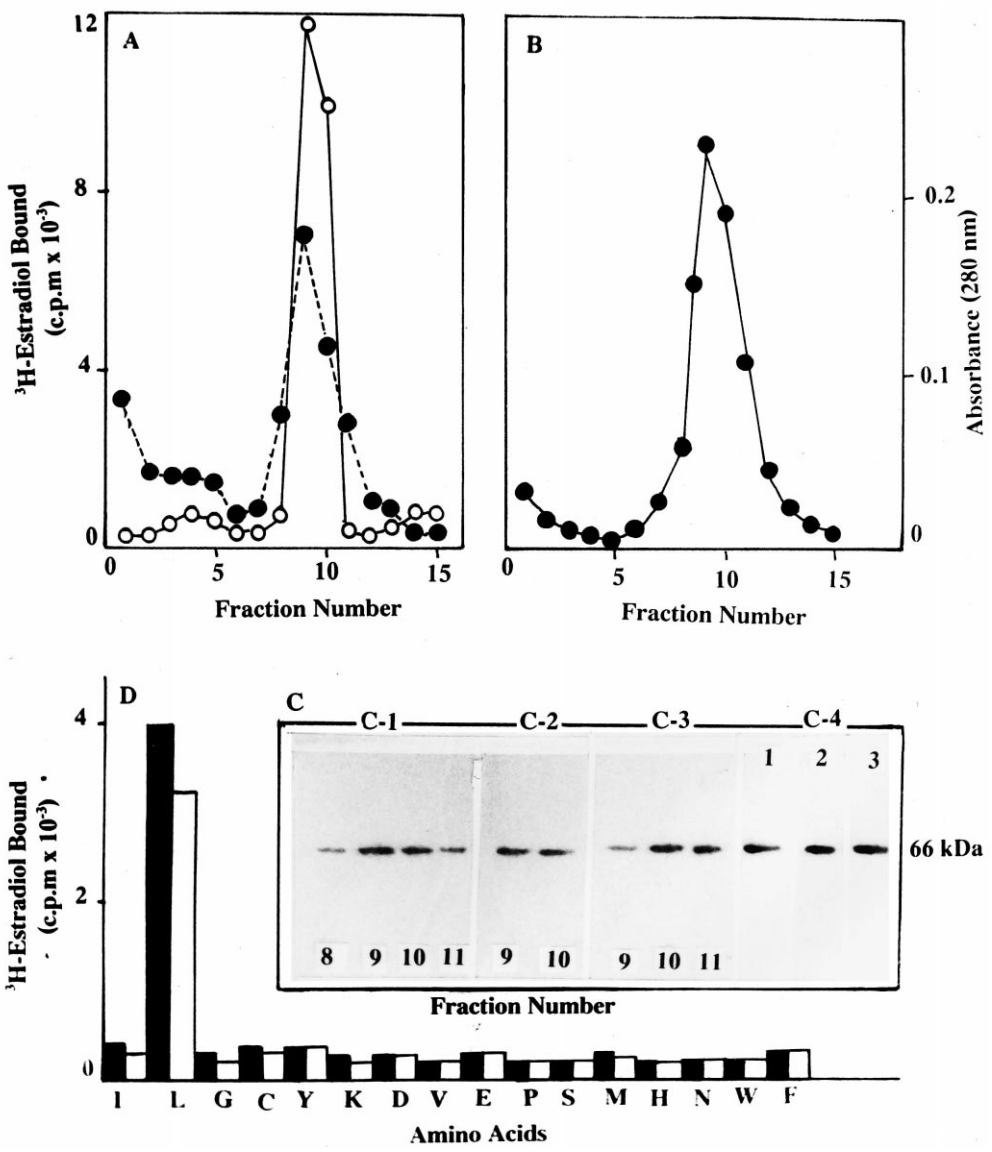

Fig. 1. Isolation of oestrogen receptor (ER), non-activated oestrogen receptor (naER) and oestrogen receptor activation factor (E-RAF) through leucine elution from affinity columns. (A) Goat uterine cytosol was chromatographed on 5-ml columns of ER-Sepharose (●) and E-RAF Sepharose (○). The columns were washed first with buffer containing 2 M NaCl and subsequently with salt-free TEM buffer. Elution was achieved using a linear 0–10 mM leucine gradient in TEM buffer. Fifteen 1-ml fractions were collected and the individual fractions were assayed for estradiol-binding activity using a HAP adsorption assay. (B) Uterine cytosol was chromatographed over a 5-ml column of naER-Sepharose equilibrated with TEM buffer. The column was washed extensively with TEM buffer containing 2 M NaCl followed immediately with salt-free buffer. Elution was achieved using a 0–10 mM leucine gradient in TEM buffer. Individual fractions collected were analysed for absorbance at 280 nm. (C) SDS–PAGE of the peak fractions (the numbers shown represent the fraction numbers of A and B) from (C-1) ER-Sepharose, (C-2) E-RAF Sepharose and (C-3) naER-Sepharose. C-4 presents the immunoblot data on the proteins. Recognition of the protein derived from (1) ER-Sepharose by anti-ER IgG, (2) that from E-RAF Sepharose by anti-naER IgG and (3) that from naER-Sepharose by anti-E-RAF IgG has been shown. Pre-exposure of the blots to free antigen completely inhibited the recognition by the concerned antibody (data not shown). (D) Effect of various free amino acids on the release of (open histograms) naER from the E-RAF Sepharose and (closed histograms) ER from ER-Sepharose. Goat uterine cytosol was adsorbed to both ER-Sepharose and E-RAF Sepharose. The matrices were washed with both 2 M NaCl-TEM and salt-free TEM following which elution was carried out using three gel volumes of TEM buffer containing the amino acid at 10 mM concentration. The eluted materials were subjected to HAP assay for estradiol binding.

same basis was applied for the isolation of naER and E-RAF following chromatography of the cytosol over E-RAF Sepharose and naER-Sepharose, respectively. Prior to chromatography of the cytosol the column was washed with buffer containing 10 mM leucine in order to remove the free proteins remaining in a dimeric state. The leucine was removed following extensive washing of the column with TEM buffer prior to the chromatography of cytosol. Since E-RAF does not bind estradiol, detection of E-RAF in the fractions cannot be accomplished using a HAP assay. In all the three cases elution of matrix-bound proteins could be achieved using the leucine gradient (Fig. 1A,B). That the proteins isolated were highly homogeneous is indicated by the silver-stained SDS gel represented in Fig. 1C. In order to confirm the identity of the three proteins, the isolated proteins were exposed to anti-ER, anti-naER and anti-E-RAF IgG. The results, presented in Fig. 1C (C-4) serve to confirm the assumption that the protein eluted from ER-Sepharose is ER itself (C-1) while those eluted from the E-RAF Sepharose and naER-Sepharose represent naER (C-2) and E-RAF (C-3), respectively. It was also necessary to confirm the specificity of the amino acid involved in the dissociation process. Elution of ER and naER, bound to ER-Sepharose and E-RAF Sepharose, respectively, was attempted using amino acids belonging to different categories. It was observed that the only amino acid that succeeded in the process was leucine (Fig. 1D). Isoleucine remained ineffective.

Studies were also carried out using two other unrelated systems: c-jun homodimers and glucocorticoid receptor (GR) homodimers. Ovine bone marrow was used as the tissue source for the purification of c-jun. The bone marrow nuclei were isolated following a published procedure [15] and solubilization and isolation of nuclear proteins was achieved as described by Sambucetti and Curran [16]. The solubilized nuclear proteins were chromatographed over a column of AP-1 Sepharose equilibrated with TEM buffer. The column was washed with this buffer following which elution was carried out using a linear salt gradient in TEM buffer. Measurement of absorption at 280 nm in the fractions generated a near symmetrical peak of absorption values (Fig. 2A). Fractions belonging to the two halves of the peak, A_1 and A_2, were separated, pooled, concentrated and subsequently immobilized on CNBr-activated Sepharose. The two affinity columns thus formed, A_1 Sepharose and A_2 Sepharose, were utilized in the remaining stages of the experiment. The columns were washed with TEM buffer containing 10 mM leucine and subsequently with leucine-free TEM buffer before chromatography of the nuclear extract. Bone marrow nuclear extract was chromatographed independently over the two columns. The columns were washed first with TEM buffer containing 2 M NaCl and subsequently with salt-free buffer. Elution of the bound proteins was achieved using a leucine gradient in TEM buffer. Measurement of absorption at 280 nm in the fractions collected from A_1 Sepharose and A_2 Sepharose clearly indicated the presence of proteins in the fractions (Fig. 2B). The leucine-eluted fractions were pooled, concentrated and subjected to SDS–PAGE. A single protein band of 39 kDa was observed in the fractions eluted from both A_1 and A_2 Sepharose (Fig. 2B). The protein that was transferred to a Western blot cross-reacted with anti-c-jun IgG (Fig. 2C).

Additional studies, in order to confirm the results mentioned above, were carried out using rat liver cytosol chromatographed over GR-Sepharose. GR-Sepharose was washed with TEM buffer containing 10 mM leucine and subsequently with leucine-free TEM-buffer prior to the chromatography of rat liver cytosol over the column. The column was washed, as before, with TEM buffer containing 2M NaCl and subsequently with salt-free TEM buffer. Elution of GR-Sepharose-bound proteins was achieved using a 0–10 mM leucine gradient. Measurement of [^{3}H]dexamethasone binding in the fractions using a HAP assay displayed a prominent peak of activity (Fig. 2D). SDS–PAGE of the peak fraction showed a single protein band of 95 kDa that was recognized by anti-GR-IgG (Fig. 2D).

The dimer dissociation by leucine was given a closer look in the subsequent studies. Goat uterine cytosol was chromatographed over a column of single-stranded DNA Sepharose equilibrated with TEM buffer. Following extensive washing the DNA bound proteins were eluted using TEM buffer containing 10 mM ATP. This step was employed in the original procedure that dealt with goat uterine ER purification [9]. The proteins in the ATP-eluted fraction were precipitated with ammonium sulphate at 70%

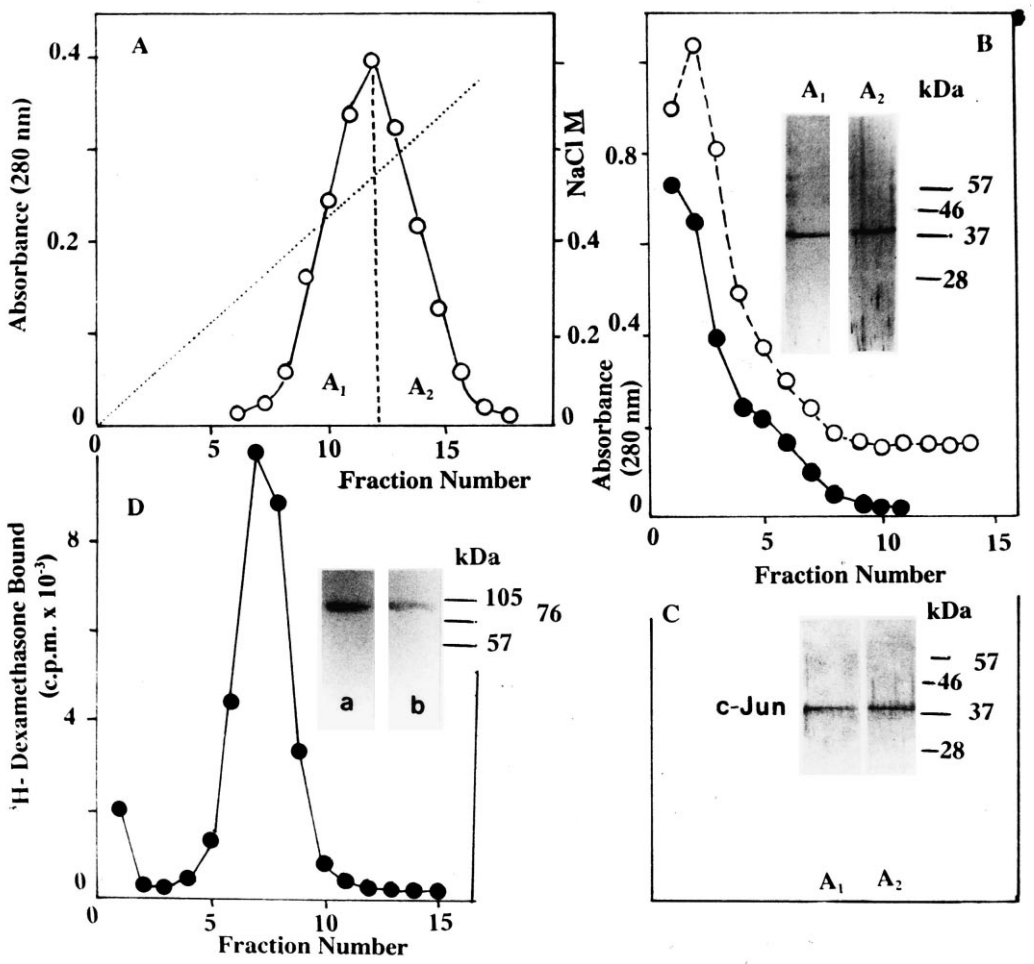

Fig. 2. Isolation of c-jun from goat bone marrow and glucocorticoid receptor from rat liver through leucine elution from affinity columns. (A) Isolation of c-jun from goat bone marrow nuclei. The nuclear extract, prepared following the method of Sambucetti and Curran [16], was chromatographed on a column of AP-1 Sepharose equilibrated with TEM buffer. The column was washed with the buffer and elution was achieved using a linear salt gradient. The fractions belonging to the two halves (A_1 and A_2) of the 280 nm absorption peak were collected, pooled separately and concentrated. A_1 and A_2 were immobilized on Sepharose. (B) Isolation of c-jun from crude nuclear extracts through chromatography on A_1 and A_2 Sepharose columns and elution with leucine gradient. The crude, bone marrow nuclear extract was chromatographed independently over A_1 Sepharose (○) and A_2 Sepharose (●). The columns were washed with TEM buffer (both 2 M NaCl containing buffer and salt-free TEM) following which elution was achieved using a 0–10 mM leucine gradient in TEM buffer. The fractions collected were analysed for absorbance at 280 nm. The fractions showing absorbance were pooled, concentrated and subjected to SDS–PAGE. Both A_1 and A_2 displayed a clear 39 kDa band (shown in inset). (C) The protein that was subjected to SDS–PAGE was Western transferred to nitrocellulose and exposed to anti-c-jun IgG. The 39 kDa protein was seen to cross react with the c-jun antibody. (D) Elution of glucocorticoid receptor (GR) bound to GR Sepharose using leucine. Rat liver GR was purified using an immunoaffinity column consisting of anti-GR IgG immobilized on Sepharose 4B. The 95 kDa GR protein was, in turn, coupled to CNBr-activated Sepharose. Crude rat liver cytosol was chromatographed over a 5-ml GR Sepharose column equilibrated with TEM buffer. The column was first washed with TEM buffer containing 2 M NaCl and subsequently with salt-free TEM buffer. Elution was achieved using a 0–10 mM leucine gradient in TEM. The fractions collected were subjected to [^{3}H]dexamethasone-binding assay using a HAP adsorption method. The inset shows (1) SDS–PAGE of the peak fraction and (2) immunorecognition of the protein by anti-GR IgG on a Western blot.

saturation. The precipitate was redissolved in the buffer and dialysed overnight (4°C) to remove the excess salt. This preparation was divided into two. While one was retained as the control the other was exposed overnight to 10 mM leucine at 4°C; both preparations contained 20 nM estradiol. The two samples were subjected to sucrose density gradient analysis on 5–20% sucrose density gradients in TEM buffer. Following centrifugation at 36000 rpm for 16 h in the SW 41 rotor of a Beckman ultracentrifuge the fractions collected were subjected to HAP assay for estradiol binding. While the control sample displayed an activity that sedimented at 4.8S, the leucine-treated sample was seen to sediment at 3.8S, clearly demonstrating dimer dissociation (Fig. 3A). The fractions belonging to the two peaks were subjected to chromatography on a leucine-washed ER-Sepharose column and elution was achieved using a leucine gradient. While the 4.8S peak failed to bind to ER-Sepharose, the 3.8S peak succeeded in binding to the column, to be eluted by a leucine gradient (Fig. 3B). Prior washing of the column with leucine gradient before the chromatography revealed that there was no contamination of proteins dissociated from the immobilized proteins.

The specificity of the c-jun–c-jun interaction was

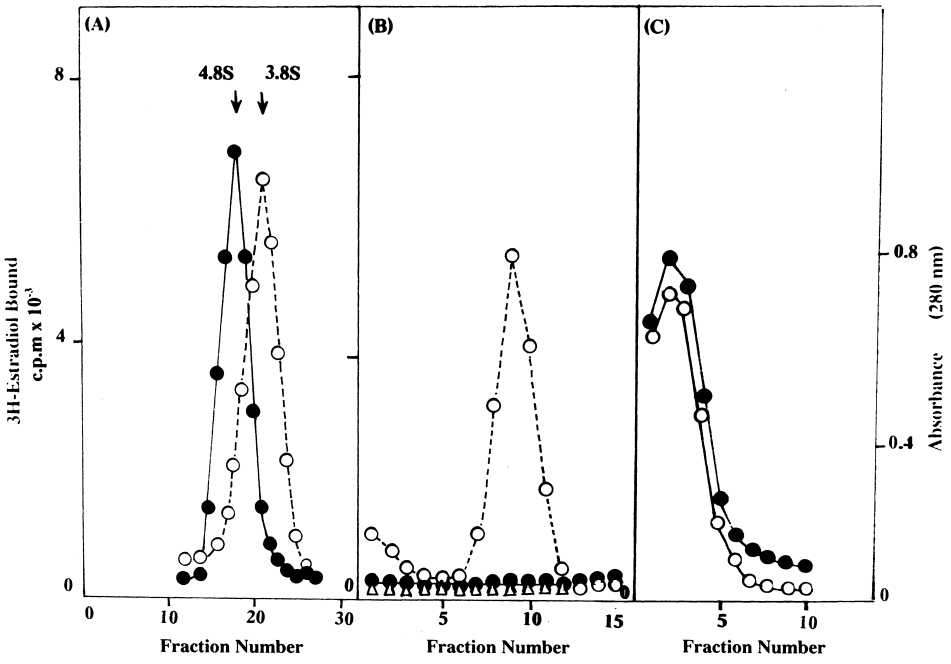

Fig. 3. Additional studies on leucine mediated dissociation of protein dimmers. (A) Sucrose density gradient analysis of ER α monomers (○) and dimers (●). Goat uterine cytosol was chromatographed over a column of single-stranded DNA Sepharose. The column was washed with TEM buffer and elution of the DNA-bound proteins was achieved using the TEM buffer containing 10 mM ATP. The protein was precipitated with $(NH_4)_2SO_4$ at 70% saturation; the precipitate was redissolved in TEM buffer and dialysed overnight against the same. This fraction was partitioned into two. While one remained as the control, the other was incubated overnight with 10 mM leucine; both samples contained 20 nM estradiol. The samples were subjected to sucrose density gradient centrifugation (15–20% sucrose in TEM; 36000 rpm, 16 h, 4°C). Bovine serum albumin and human γ-globulin were used as the sedimentation markers. The fractions collected were subjected to HAP assay for estradiol binding. (B) ER-Sepharose chromatography of the 4.8S fractions (●) and 3.8S (○) fractions collected from the sucrose gradient. The two fractions were chromatographed independently over ER-Sepharose that was previously washed with TEM buffer containing 10 mM leucine and subsequently with TEM buffer alone. Elution was achieved using a 0–10 mM leucine gradient after washing the column first with TEM buffer containing 2 M NaCl and subsequently with salt-free TEM buffer. The fractions collected were assayed for estradiol binding. The triangles indicate the estradiol-binding activity associated with the fractions in the 10 mM leucine wash prior to chromatography of the density gradient fractions. (C) Chromatography of the affinity-purified c-jun of ovine bone marrow (○) and recombinant c-jun (●) on c-jun Sepharose. The column was made using commercially available, recombinant c-jun immobilized on Sepharose 4B. The two batches of c-jun were chromatographed independently over the column following prior washing of the column with TEM buffer both in the presence and absence of 10 mM leucine. The column was washed with TEM and elution was achieved using 0–10 mM leucine gradient in TEM buffer.

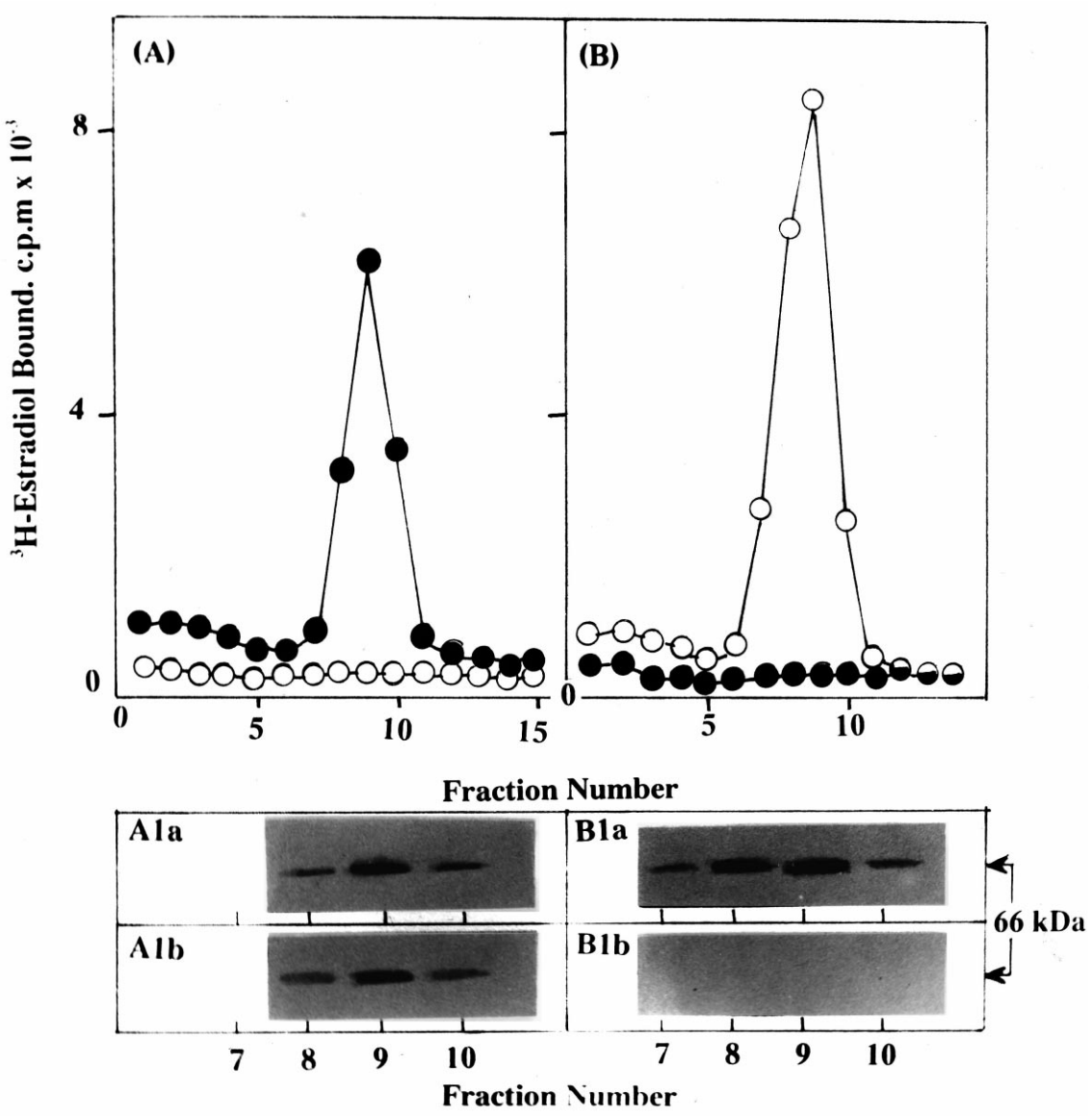

Fig. 4. Display of the specificity associated with the dimerization process. Chromatography of goat uterine cytosol over ER-Sepharose (A) or E-RAF Sepharose (B). ER-Sepharose and E-RAF Sepharose were first exposed for one hour to either ER-E_2 complex (○) or naER E_2-complex (●), both having been prepared by incubating the concerned receptor (9 μg protein) with 20 nM estradiol overnight at 4°C. (The free hormone was removed following dextran-coated charcoal treatment.) The matrices which were exposed to one of the two receptor hormone complexes were washed with TEM buffer both in the presence and absence of 2 M NaCl. These gels were re-exposed to goat uterine cytosol at 4°C for 1 h. The gel was removed following brief centrifugation and the supernatant was chromatographed on a fresh ER-Sepharose or E-RAF Sepharose, that were washed previously with TEM buffer both in the presence and absence of 10 mM leucine. Elution was achieved using a 0–10 mM leucine gradient and estradiol-binding activity in the fractions was examined using a HAP adsorption assay. The fractions belonging to the peaks were subjected to SDS–PAGE followed by Western blotting and exposure to either anti-ER or anti-naER IgGs. The fraction numbers indicated on the immunoblots represent the corresponding numbers of A or B. The blots were exposed to (A1a) anti-ER α polyclonal IgG; (B1a) anti-naER polyclonal IgG; (A1b,B1b) anti-ER α monoclonal IgG. A1a and A1b represent fractions from the ER-Sepharose, and B1a and B1b represent fractions from the E-RAF Sepharose.

also put to an additional test. This time commercially available c-jun was immobilized on Sepharose 4B and both the commercial protein as well as the protein purified from bovine bone marrow were chromatographed independently on the affinity column. Following washing, the proteins bound to c-jun Sepharose were eluted using a leucine gradient. The pattern of elution observed was identical to the one displayed in Fig. 2B (Fig. 3C).

The data presented in Fig. 4 provided additional evidence concerning the specificity associated with the oestrogen receptor dimer formation. ER-Sepharose and E-RAF Sepharose were exposed to ER-E_2 or naER-E_2 complexes prior to their exposure to goat uterine cytosol. Following exposure of these affinity matrices to cytosol, the matrices were subjected to centrifugation at low speed and the medium was collected. These media were chromatographed over either ER-Sepharose or E-RAF Sepharose which was washed previously with TEM buffer both in the presence and absence of 10 mM leucine. Proteins bound to the matrices were eluted using a leucine gradient. Exposure of the ER-Sepharose to ER E_2 complex prevented additional binding of cytosolic ER to the matrix while prior to exposure to naER-E_2 complex remained ineffective (Fig. 4A). The reverse appeared to be true for the E-RAF Sepharose where prior exposure of the matrix to naER-E_2 prevented additional binding of cytosolic naER to E-RAF Sepharose. Exposure to ER-E_2 was of no consequence. That the protein which dimerized with ER-Sepharose was ER α itself was demonstrated by the immunoblots wherein monoclonal anti-ER α IgG recognized the 66 kDa protein eluted from the ER-Sepharose and not that eluted from the E-RAF Sepharose (Fig. 4A1b,B1b).

This is possibly the first experimental report wherein the subject matter of leucine zipper dissociation is addressed. From the very early studies on steroid receptors it has been recognized that the receptor dimers (nuclear receptors) do not dissociate in the presence of high salt. If the dimer formation is the result of an exclusive hydrophobic interaction, hydrophobic amino acids other than leucine could have caused the dissociation of the complexes. This, apparently, is not the case. There appears to be a greater degree of specificity involved in leucine zipper formation since leucine is the only amino acid that causes dissociation of the dimers. It is not, however, possible at this stage to predict the exact molecular mechanism that operates in the leucine-dependent dissociation of the protein dimers.

It is known that hydrophobic amino acids in the protein interior contribute to protein stability through two distinct mechanisms [17–19]. In the first case hydrophobic amino acids are buried in the protein interior, thereby removing them away from the solvent. The second mechanism involves van der Waals interactions between the amino acid chains of interest and other hydrophobic amino acids in the interior. Pace et al. [20] observed that contribution of the second mechanism to protein stability is twice that of the first. The leucine zipper dimerization domain has a characteristic amino acid sequence with an amphipathic α-helical structure and near variant heptad repeat of leucines [4,21]. Crystallographic studies reveal the structure of leucine zipper to be that of a dimeric parallel coiled coil [22,23]. The 'd' position leucine interacts intrahelically with neighbouring hydrophobic amino acids in the 'a' position and interhelically with the 'd' position in the opposite helix. Possibly, the free leucine introduced into the system, through its interaction at both intrahelical and interhelical levels, brings about a local disturbance in the helical structure and organization, eventually causing the dissociation of monomeric units.

Whether the dimerization mechanisms detected in the ER and GR were influenced by the concerned hormone is not clear at this stage. The cytosols were prepared from tissues which are known to contain circulating endogenous hormone. In case hormone binding is necessary for dimerization, the endogenous hormone should have contributed the required influence for the process.

As mentioned earlier, the primary structure of E-RAF and naER have not been identified. In view of this it is not possible to state unequivocally that the two proteins contain leucine heptad repeats. Nevertheless, since the pattern of dissociation of these proteins from the concerned affinity matrices using leucine gradient is identical to that displayed by ER and GR, it may be suggested that the heterodimerization between naER and E-RAF is also contributed, at lease partially, by leucine heptad repeats.

The results raise a few provocative questions.

(1) Could this effect be reproduced in an in vivo system? (2) Will an artificially created enhancement in the intracellular concentration of leucine have a negative effect on the gene expression mechanisms mediated by leucine zipper proteins? Needless to say the immediate beneficiary of such an intervention will be the cancer patient. Even though the response is not leucine-specific, recent studies on the amino acid-mediated changes in gene expression deserves attention [24]. While leucine starvation was found to enhance transcription, an increase in the plasma leucine concentration was found to have the reverse effect [24]. Could the negative effect be at least partly attributed to the leucine-mediated inhibition of dimerization of proteins which influence the transcription process?

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