Vol. 209, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 6, 1995 Pages 117-125

BIOCHEMICAL AND CALMODULIN BINDING PROPERTIES OF ESTROGEN RECEPTOR BINDING CYCLOPHILIN EXPRESSED IN *ESCHERICHIA COLI*

Thomas Ratajczak, Amerigo Carrello and Rodney F. Minchin[1]

Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, Nedlands, Australia
[1]Department of Pharmacology, University of Western Australia, The Queen Elizabeth II Medical Centre, Nedlands, Western Australia 6009

Received February 21, 1995

SUMMARY: Bovine estrogen receptor binding cyclophilin (ERBC), a cyclophilin component of the unactivated estrogen receptor, has been efficiently expressed in *Escherichia coli* as a fusion with glutathione S-transferase (GST) and purified by single-step chromatography on glutathione-agarose. Thrombin cleavage from GST allowed the isolation of purified, recombinant ERBC. The fusion protein, GST-ERBC, and recombinant ERBC were both characterised for peptidyl prolyl *cis-trans* isomerase activity. With N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide as substrate, GST-ERBC demonstrated a k_{cat}/K_M value of $5.1 \times 10^5\ M^{-1}s^{-1}$ at 5°C. The isomerase activity was inhibited by cyclosporin A with an IC_{50} value of 1030 nM. These values indicate that ERBC has a decreased catalytic efficiency and sensitivity to cyclosporin A relative to human cyclophilin. Retention of the GST-ERBC fusion protein on calmodulin-agarose in the presence of Ca^{2+} and subsequent elution with EGTA has provided evidence that ERBC is a calmodulin-binding protein.

We have previously reported on the cloning and sequencing of estrogen receptor binding cyclophilin (ERBC), a cyclophilin-related component of the unactivated estrogen receptor (1). ERBC (also known as cyclophilin-40, CyP-40) may facilitate the maintenance of a hormone-activatable receptor conformation by acting coordinately with other protein chaperones of the unactivated receptor complex: hsp90, hsp70 and FKBP59 (2). The immunophilins, ERBC and FKBP59, share significant homology in their C-terminal regions and belong to separate classes of peptidyl prolyl *cis-trans* isomerases characterised by a binding specificity for the immunosuppressants cyclosporin A (CsA) and FK506, respectively (3-5). The functional domains of ERBC and FKBP59 display a similar structural organisation in which the N-terminal region

Abbreviations: ERBC, estrogen receptor binding cyclophilin; CyP-40, cyclophilin-40; FKBP, FK506 binding protein; CsA, cyclosporin A; TPR, tetratricopeptide repeat; GST, glutathione S-transferase; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); IPTG, isopropyl β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; NK-TR, natural killer cell tumour-recognition molecule.

0006-291X/95 $5.00

harbours overlapping isomerase and ligand binding activities and the conserved C-terminal segment incorporates a tetratricopeptide repeat (TPR) domain predicted to mediate protein-protein interaction (1). On the basis of sequence homology with FKBP59, we have previously proposed that the TPR domain in ERBC is terminated by a potential site for calmodulin binding (1,6,7).

As a first step toward the biochemical characterisation of ERBC, leading to a better understanding of its role in estrogen receptor function, we describe here the overexpression of bovine ERBC as a glutathione-S-transferase (GST) fusion protein in *Escherichia coli*. The recombinant protein displayed a peptidyl prolyl *cis-trans* isomerase activity inhibitable by CsA and was specifically retained on calmodulin-agarose in the presence of Ca^{2+} confirming ERBC to be a calmodulin binding protein.

METHODS

Expression Plasmid Construction

Oligonucleotide primers identical to the 5' end and complimentary to the 3' end of the ERBC coding region (1) were synthesised with built-in *BamHI* (5' end) and *SmaI* (3' end) restriction sites. A pGEM-3Z plasmid containing a 1.3 kb ERBC cDNA was then used as template to amplify the ERBC gene using the polymerase chain reaction (PCR). After *BamHI* and *SmaI* digestion, the 1.1 kb amplified fragment was subcloned into pGEM-3Z. To minimise mutations introduced by PCR the 922 bp *XhoI* to *BclI* fragment was removed and substituted with the corresponding wild-type DNA. Sequence fidelity of the remaining PCR-derived DNA was confirmed by automated sequencing using dye-labelled dideoxy nucleotide terminators (Applied Biosystems). The 1.1 kb *BamHI* to *SmaI* insert from this modified plasmid was ligated into the procaryotic expression vector pGEX-2T (8) to produce the expression clone pGEX-2T-ERBC.

Expression and Purification of GST-ERBC Fusion Protein

The pGEX-2T-ERBC plasmid was transformed into *E.coli* strain XL1-Blue. Typically, 100 ml overnight cultures of the bacterial cells were diluted 1:10 with fresh medium and induced over 4 hr at 37°C with 0.1 mM IPTG. The cells were pelleted by centrifugation and resuspended in 1/100 culture volume of lysing buffer (16 mM disodium hydrogen orthophosphate, pH 7.4, containing 0.15 M NaCl, 1% Triton X-100, 5 mM dithiothreitol, 2 mM EDTA and the protease inhibitor mixture: 1 mM phenylmethylsulphonyl fluoride/5 mM benzamidine). After freezing at -70°C and thawing, the cells were lysed by sonication at 4°C. Centrifugation gave a clear supernatant which was mixed for 10 min by rotation at 4°C with 0.5 ml of glutathione-agarose (AMRAD-Pharmacia) previously equilibrated in lysing buffer. The gel was packed in a 8.5 x 90 mm column and washed repeatedly with lysing buffer (150 ml total). Bound fusion protein (5 mg) was recovered by competitive elution with 10 mM glutathione in 50 mM Tris pH 8.0 (2 ml). The protein was dialyzed against 10 mM potassium dihydrogen orthophosphate buffer containing 20% (w/v) glycerol and stored at -70°C.

Purification of ERBC

GST-ERBC fusion protein was equilibrated in 50 mM Tris, pH 8.0, containing 150 mM NaCl and 2.5 mM $CaCl_2$ and incubated for 1 hr at 25°C with thrombin (Sigma) at an enzyme : substrate ratio of 1:800. ERBC was isolated after removal of cleaved GST by glutathione-agarose chromatography and stored until further use in 20% (w/v) glycerol at -70°C.

Measurement of Peptidyl Prolyl *cis-trans* Isomerase Activity

The enzymatic activity of recombinant ERBC and GST-ERBC fusion protein was determined as described by Konfron et al (9) using the substrate N-succinyl-Ala-Ala-Pro-Phe-p-

nitroanilide (Sigma) dissolved in LiCl/trifluorethanol. The assay was performed at 5°C in 0.1 M Tris pH 8.0 buffer. Isomerization was monitored at 405 nm and data were collected every 0.5 s over 5 min. using a GBC spectrophotometer interfaced with an IBM personal computer. The Sigmaplot program (Jandel Scientific) was used to fit the data by nonlinear least squares regression analysis to the equation $A = A_{max} - A_o e^{-k.t}$ where A = absorbance at 405 nm, A_{max} = maximum absorbance, A_o = absorbance at time (t) = zero and k = apparent rate constant.

GST-ERBC fusion protein at 200 nM final concentration was used in isomerase inhibition studies with CsA. Aliquots of the protein were incubated on ice for at least 20 min with varying amounts of CsA (a gift from Sandoz Pharma) prior to assay.

Calmodulin Interaction Studies

Interaction studies between calmodulin and GST-ERBC fusion protein were performed batchwise according to the following protocol: fusion protein (50 μg) in 100 μl of binding buffer (20 mM Tris, pH 7.6, containing 100 mM KCl and 0.1 mM dithiothreitol) containing 2 mM Ca^{2+} was mixed by rotation for one hr at 4°C with 50 μl of calmodulin-agarose (Sigma) pre-equilibrated in the same buffer. The supernatant was collected after brief centrifugation and the gel was cleared of unbound protein by successive washes (5 x 200 μl aliquots) with binding buffer containing 2 mM Ca^{2+}. Elution of specifically retained protein was achieved by repetitive incubation of the gel with three 200 μl aliquots of binding buffer plus 2 mM EGTA for 30 min at 4° C with rotation. In control studies carried out in parallel, EGTA replaced Ca^{2+} throughout the experiment. Calmodulin-fusion protein interaction was monitored by SDS-PAGE.

RESULTS AND DISCUSSION

Our approach to the detailed biochemical characterisation of bovine ERBC has been to express ERBC as a GST fusion protein using the procaryotic pGEX-2T vector (8). Specific PCR primers, with built-in restriction sites to facilitate cloning into pGEX-2T, were used to amplify the entire open reading frame of wild type bovine ERBC cDNA (1). Yields of 5 mg/L of culture were obtained in *E.coli* and the 66 kDa fusion protein, GST-ERBC, was purified to homogeneity by single-step affinity chromatography on glutathione-agarose (Fig 1, lane 4). A thrombin cleavage site at the GST/ERBC junction, allowed the isolation of recombinant ERBC after thrombin digestion and GST removal with glutathione-agarose (Fig. 1, lane 6).

Both the GST-ERBC fusion protein and purified, recombinant ERBC were shown to be catalytically active in the chymotrypsin-coupled assay with an N-blocked Ala-Ala-Pro-Phe-p-nitroanilide chromogenic substrate. Fig. 2 shows representative profiles for substrate isomerization in the presence of zero, 50 and 200 nM GST-ERBC. An accelerated conversion of substrate is clearly demonstrated with increasing amounts of fusion protein. Similar rates of isomerization were observed with 50 nM GST-ERBC and recombinant ERBC (Fig. 2A) suggesting that the GST segment of the fusion protein does not contribute to the reaction. Assessment of isomerization profiles in the presence of varying amounts of GST-ERBC fusion protein enabled the generation of a linear k_{obs} versus [GST-ERBC] plot (not shown) from which a k_{cat}/K_M value of 5.1 x 10^5 $M^{-1}s^{-1}$ was calculated. The interaction of CsA with the GST-ERBC fusion protein was studied using an inhibition assay in which the isomerase activity was blocked

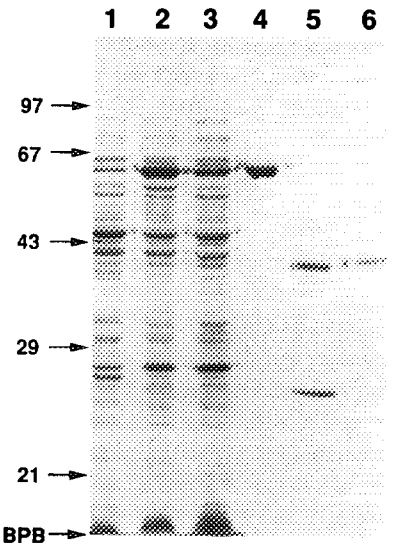

FIGURE 1. Analytical SDS-PAGE of recombinant ERBC at various stages of purification. Lanes 1 and 2, supernatants derived from lysed, uninduced (lane 1) and IPTG-induced (lane 2) cultures of XL1-Blue bacteria transformed with pGEX-2T-ERBC expression plasmid; lane 3, flow-through from glutathione-agarose chromatography of the supernatant represented in lane 2; lane 4, purified GST-ERBC fusion protein recovered from glutathione-agarose by elution with glutathione; lane 5, cleaved product from thrombin digestion of purified GST-ERBC fusion protein; lane 6, purified recombinant ERBC recovered after passage of the thrombin digest through glutathione-agarose. The positions of molecular weight markers (Pharmacia) are shown on the left side. BPB, bromophenol blue.

with increasing concentrations of CsA. The resulting isomerase inhibition curve (Fig. 2B) allowed an IC_{50} value of 1030 nM to be calculated for the CsA-fusion protein interaction. Corresponding values of $k_{cat}/K_M = 1.4 \times 10^7\ M^{-1}s^{-1}$ at 10° C and an $IC_{50} = 19$ nM, previously reported for human cyclophilin (10), suggest that in comparison the catalytic efficiency and CsA binding affinity of GST-ERBC is substantially decreased. for native bovine ERBC (CyP-40), Keiffer et al (11) have obtained values of $k_{cat}/K_M = 1.9 \times 10^6\ M^{-1}s^{-1}$ at 10°C and an $IC_{50} = 300$ nM for CsA isomerase inhibition. The reason for the observed difference in kinetic parameters between native ERBC and the GST-ERBC fusion protein is presently unknown, but might reflect post-translational modification. The results for GST-ERBC are closely comparable to those described for NK-TR, a surface cyclophilin-related protein specific to natural killer cells and thought to be involved in tumour recognition (12). Under similar assay conditions, a $k_{cat}/K_M = 7.4 \times 10^5\ M^{-1}s^{-1}$ at 5° C and an IC_{50} for CsA isomerase inhibition of 870 nM have been determined for a GST-fusion protein derived from the N-terminal cyclophilin-homologous domain of NK-TR (13).

An amino acid sequence alignment of ERBC and NK-TR cyclophilin domains with human cyclophilin and NinaA is shown in Fig. 3. Within a core domain represented by the human

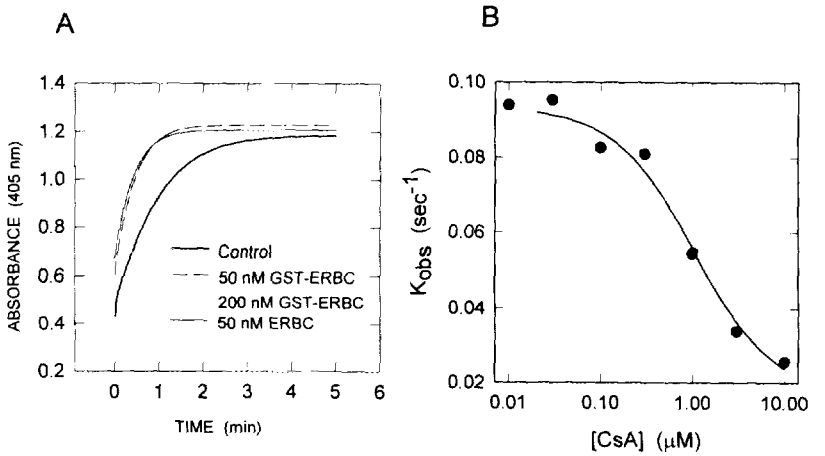

FIGURE 2. ERBC peptidyl prolyl *cis-trans* isomerase activity and sensitivity to CsA. **A.** Typical profiles obtained from the chymotrypsin-coupled assay in the absence of isomerase and in the presence of GST-ERBC fusion protein or recombinant ERBC. **B.** Inhibition of isomerase activity of the GST-ERBC fusion protein by CsA. The parameters of the equation $k_{obs}=k^{\circ}_{obs}/(1+[CsA]/IC_{50})$ where k°_{obs} is k_{obs} without inhibitor (13), were fitted to the data.

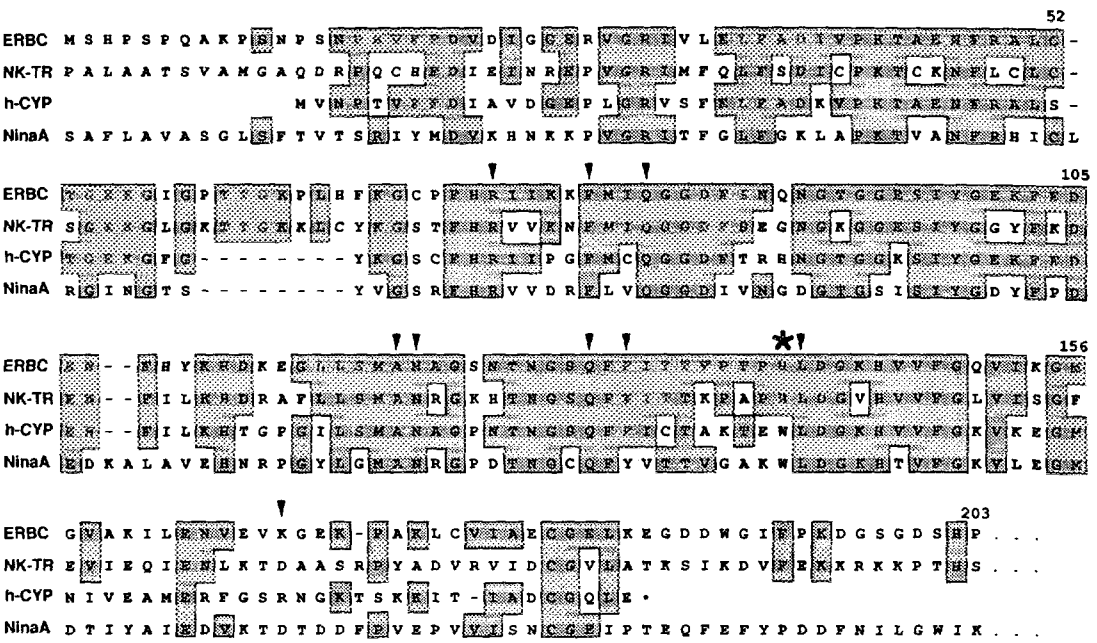

FIGURE 3. Amino acid sequence alignment of the ERBC cyclophilin domain with the homologs NK-TR, human cyclophilin and NinaA. The numbering applies to the ERBC sequence (1). Boxed, shaded regions signify residue identity. Residues which define the human cyclophilin catalytic site are notated by arrowheads (18). An asterisk indicates the tryptophan residue important for human cyclophilin -CsA binding (19).

cyclophilin sequence ERBC shares 57.5, 62 and 41.4% identity with NK-TR (12), human cyclophilin (14) and NinaA (15), respectively (Fig. 3). A chaperone function has been demonstrated for the latter three cyclophilins (13,16,17) lending support to the possibility that ERBC may exert a similar activity. The close equivalence in isomerase activity and sensitivity to inhibition by CsA, as displayed by ERBC and NK-TR, might reflect common features in their cyclophilin domains which are located toward the N-terminal ends of both proteins. The almost full conservation in ERBC and NK-TR of residues thought to define the enzyme active site in cyclophilin (18) (Fig. 3) is consistent with a significant, albeit lower, catalytic activity for the proteins. Although thought to play only a minor role in isomerase activity, the tryptophan residue W121, within the human cyclophilin catalytic site, has been implicated in CsA binding (19) (Fig. 3). In ERBC and NK-TR, the corresponding residue is substituted with histidine (Fig. 3). The reduced sensitivity to CsA inhibition for both cyclophilins then appears to be compatible with this alteration. A unique feature of the cyclophilin domains of ERBC and NK-TR is a partially conserved, 8 amino acid insert which is absent from human cyclophilin (Fig. 3). The conserved sequence is common among plant cyclophilins (20) and may constitute part of a metal binding region (21).

Our observation of *cis-trans* isomerase activity which is inhibitable by CsA confirmed that the recombinant ERBC was bioactive and might therefore be suitable for probing the protein interaction properties of the ERBC functional domains. The structural similarity between ERBC and FKBP59 (1) suggests a similar or perhaps competing role for both immunophilins in cellular function. Within unactivated steroid receptor complexes, FKBP59 is known to be associated with hsp90 and the interaction between the two heat shock protein components has been studied extensively (22,23). A recent report by Massol et al (7) has demonstrated the specific binding of FKBP59 to calmodulin and identified two candidate sites within the FKBP59 amino acid sequence for calmodulin interaction. One of these sites, situated between amino acids 398-415 and located C-terminal of the FKBP59 TPR domain is also highly conserved within ERBC (amino acid 353-370) both for residue identity and charge distribution (1). Protein targets for calmodulin are characterised by positively charged amphiphilic α-helical segments, approximately 20 residues in length (24). Secondary structure analysis (MacVector 3.5 program, International Biotechnologies) based on Chou-Fasman (25) and Garnier et al (26) methodologies indicated the putative site to be predominantly α-helical in both ERBC and FKBP59. A helical wheel representation of the sites in both proteins shows a segregation of hydrophobic and positively charged residues which may be conducive to calmodulin binding (Fig. 4A). Analysis of the ERBC amino acid sequence suggested the presence of a second putative calmodulin binding site between residues 240-253 (Fig. 4B). This α-helical segment resides within the first unit of the ERBC TPR

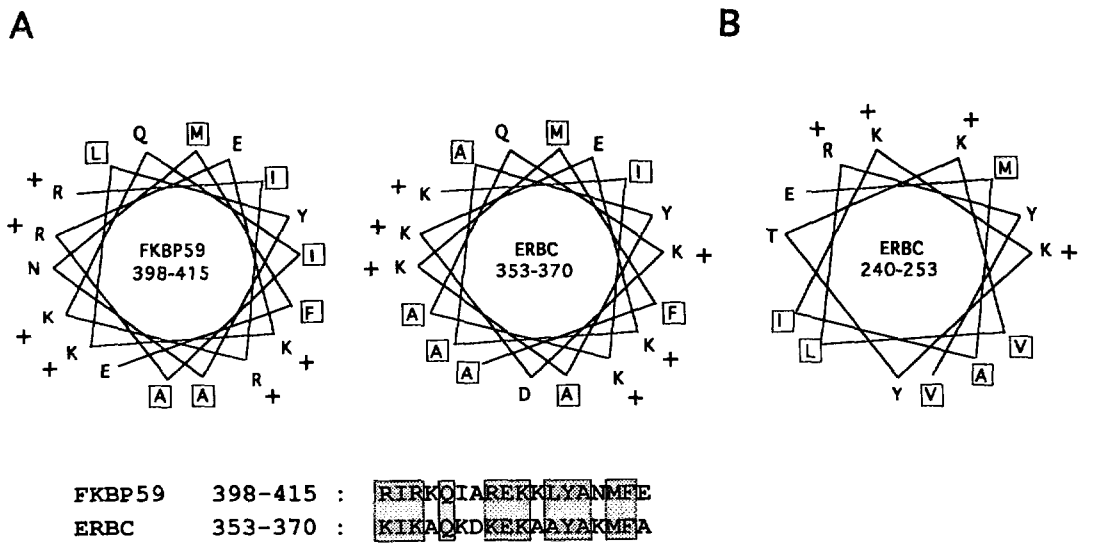

FIGURE 4. Calmodulin binding domains of FKBP59 and ERBC. **A.** The common, C-terminal region of identity between FKBP59 and ERBC is defined by alignment of residues 398-415 with 353-370, respectively (conserved residues are shown shaded). Both sites are depicted as helical wheel representations. **B.** A second calmodulin binding site for ERBC corresponds to amino acids 240-253. Basic residues are highlighted by positive charges and hydrophobic amino acids are enclosed by squares.

domain, a region predicted to mediate protein-protein interaction (1). It is of interest that a second putative calmodulin binding site identified within FKBP59 (7) is also located within the 3-unit TPR domain.

Incubation of GST-ERBC fusion protein with calmodulin-agarose in the presence of Ca^{2+} resulted in a complete retention of the protein on the resin (Fig. 5A). We found GST not to be retained under the same conditions (not shown). In a parallel, control experiment, in which EGTA replaced Ca^{2+} throughout, the majority of the loaded protein remained unabsorbed (Fig. 5B). The results suggested the specific, Ca^{2+}-dependent binding of the fusion protein to calmodulin via ERBC. Our observation that recombinant ERBC is a calmodulin binding protein complements evidence for calmodulin interaction by the additional non-hormone binding components of unactivated steroid receptors, namely hsp90 (27), hsp70 (28) and FKBP59 (7). Calmodulin may regulate the normal functions of these proteins and may inhibit binding to their specific protein targets either by direct steric blockade or via Ca^{2+} - induced conformational changes (24). In this regard, evidence suggests that the cytoplasmic localisation of the unactivated glucocorticoid receptor is dependent on the binding of hsp90 to actin filaments (30). Calmodulin has been shown to inhibit hsp90-actin interaction in a Ca^{2+} - specific manner (31).

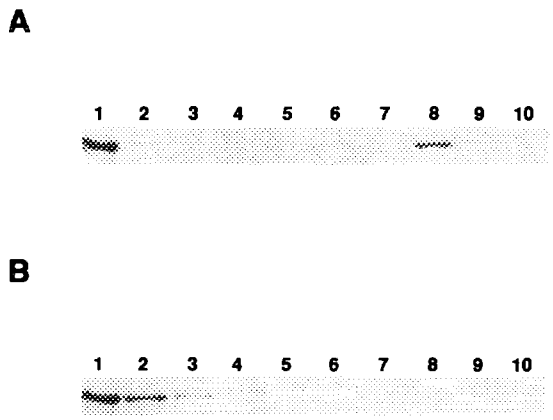

FIGURE 5. Calmodulin binding properties of ERBC. **A.** GST-ERBC fusion protein (50 μg) in binding buffer containing 2 mM Ca^{2+} (100 μl) was incubated with calmodulin resin (50 μl) and the supernatant was recovered by centrifugation. The gel was washed successively with binding buffer plus Ca^{2+} (5x200 μl aliquots) and bound protein was eluted with further 3x200 μl washes of binding buffer containing 2 mM EGTA. Aliquots (10 μl) of recovered fractions were submitted to analytical SDS-PAGE on 12.5% (w/v) acrylamide gels. Lane 1 represents the amount of GST-ERBC fusion protein applied to the calmodulin resin; lane 2, flow through; lanes 3-7, wash fractions with Ca^{2+}; lanes 8-10, wash fractions with EGTA. **B.** The experiment was conducted as in A except that 2 mM EGTA replaced Ca^{2+} throughout.

ACKNOWLEDGMENTS

This was supported by the National Health and Medical Research Council of Australia. We thank J. Fairweather for assistance with reagent preparation, A Scalzo for secondary structure analyses, P. Mark for help with illustrations and RL Martin for useful discussions throughout the work.

REFERENCES

1. Ratajczak, T., Carrello, A., Mark, P.J., Warner, B.J., Simpson, R.J., Moritz, R.L., and House, A.K. (1993) J. Biol. Chem. 268, 13187-13192.
2. Pratt, W.B. (1993) In Steroid Hormone Action: Frontiers in Molecular Biology (M.G. Parker, Ed.), pp 64-93, Oxford University Press, Oxford.
3. Schreiber, S.L. (1991) Science 251, 283 - 287.
4. Schreiber, S.L., and Crabtree, G.R. (1992) Immunol. Today 13, 136-142.
5. Fruman, D.A., Burakoff, S.J., and Bierer, B.E. (1994), FASEB J. 8, 391-400.
6. Lebeau, M.-C., Massol, N., Herrick, J., Faber, L.E., Renoir, J.-M, Radanyi, C., and Baulieu, E.-E. (1992) J. Biol. Chem. 267, 4281-4284.
7. Massol, N., Lebeau, M.-C., Renoir, J.-M., Faber, L.E., and Baulieu, E.-E. (1992) Biochem. Biophys. Res. Commun. 187, 1330-1335.
8. Smith, D.B., and Johnson, K.S. (1988) Gene 67, 31-40.
9. Konfron, J.L., Kuzmic, P., Kishore, V., Bonilla-Colon, E., and Rich, D.H. (1991) Biochemistry 30, 6127-6134.
10. Liu, J., Albers, M.W., Chen, C.-M., Schreiber, S.L., and Walsh, C.T. (1990) Proc. Natl. Acad. Sci. USA 87, 2304-2308.
11. Keiffer, L.J., Thalhammer, T., and Handschumacher, R.E. (1992) J. Biol. Chem. 267, 5503-5507.

12. Anderson, S.K., Gallinger, S., Roder, J., Frey, J., Young, H.A., and Ortaldo, J.R. (1993) Proc. Natl. Acad. Sci. USA 90, 542-546.
13. Rinfret, A., Collins, C., Menard, R., and Anderson, S.K. (1994) Biochemistry 33, 1668-1673.
14. Haendler, B., Hofer-Warbinek, R., and Hofer, E. (1987) EMBO J. 6, 947-950.
15. Shieh, B.-H., Stamnes, M.A., Seavello, S., Harris, G.L., and Zuker, C.S. (1989) Nature 338, 67-70.
16. Freskgard, P.-O., Bergenhem, N., Jonsson, B.-H., Svensson, M., and Carlsson, U. (1992) Science 258, 466-468.
17. Baker, E.K., Colley, N.J., and Zuker, C.S. (1994) EMBO J. 13, 4886-4895.
18. Kallen, J., and Walkinshaw, M.D. (1992) FEBS LETT. 300, 286-290.
19. Zydowsky, L.D., Elzkorn, F.A. Chang, H.Y., Ferguson, S.B., Stolz, L.A., Ho, S.I., and Walsh, C.T. (1992) Protein Sci. 1, 1092-1099.
20. Gasser, C.S., Gunning, D.A., Budelier, K.A., and Brown, S.M. (1990) Proc. Natl. Acad. Sci. USA 87, 9519-9523.
21. Keiffer, L.J., Seng, T.W., Wei, L., Osterman, D.G., Handschumacher, R.E., and Bayney, R.M. (1993) J. Biol. Chem. 268, 12303-12310.
22. Renoir, J.-M., Radanyi, C., Faber, L.E., and Baulieu, E.-E. (1990) J. Biol. Chem. 265, 10740-10745.
23. Radanyi, C., Chambraud, B., and Baulieu, E.-E. (1994) Proc. Natl. Acad. Sci. USA 91, 11197-11201.
24. O'Neil, K.T., and DeGrado, W.F. (1990) Trends in Biol. Sci. 15, 59-64.
25. Chou, P.Y., and Fasman, G.D. (1978) Ann. Rev. Biochem. 47, 251-276.
26. Garnier, J., Osguthorpe, D.J., and Robson, B. (1978) J. Mol. Biol. 120, 97-120.
27. Peattie, D.A., Harding, M.W., Fleming, M.A., DeCenzo, M.T., Lippke, J. A., Livingston, D.J., and Benasutti, M. (1992) Proc. Natl. Acad. Sci. USA 89, 10974-10978.
28. Minami, Y., Kawasaki, H., Suziki, K., and Yahara, I. (1993) J. Biol. Chem. 268, 9604-9610.
29. Stevenson, M.A., and Calderwood, S.K. (1990) Mol. Cell. Biol. 10, 1234-1238.
30. Miyata, Y., and Yahara, I. (1991) J. Biol. Chem. 266, 8779-8783.
31. Nishida, E., Koyasu, S., Sakai, H., and Yahara, I. (1986) J. Biol. Chem. 261, 16033-16036.