An Active FK506-Binding Domain of 17 000 Daltons Is Isolated Following Limited Proteolysis of Chicken Thymus HSP56

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ABSTRACT: We have previously identified hsp56, a protein component of steroid receptor complexes, as an FK506 binding protein [Yem *et al.* (1992) *J. Biol. Chem. 267,* 2868–2871]. We now report that hsp56 is also found to be a major immunophilin in chicken thymus, by virtue of binding to FK506-Affi-Gel-10 as well as positive cross-reactivity with a polyclonal antiserum directed against human hsp56. Limited digests of purified chicken hsp56 with endoproteinase Lys C result in the production of a unique polypeptide having a mass of about 17 kDa (p17), as judged by Western blotting. Peptide mapping provided additional proof that p17 is a fragment which comprises the entire FK506 binding domain I of chicken hsp56, terminating with an Arg-Lys which might represent a processing site. Binding of radiolabeled dihydro FK506 to p17 is saturable with a calculated K_D of 42 nM. Since size exclusion chromatography of drug–p17 complexes indicates that the active species is a homodimer with a mass of 30–40 kDa, the stoichiometry calculated for the drug–protein complex is approximately 1:1. Furthermore, unlike FKBP-12, chicken p17 bound to FK506 does not bind to calcineurin–calmodulin complexes. This work demonstrates the excision of a domain from an hsp56 protein that is active in binding FK506 and functionally distinct from FKBP-12, a protein of similar size and structure.

Hsp56[1] is a protein which was originally identified as a component of steroid receptor complexes (Renoir *et al.*, 1990; Sanchez *et al.*, 1990; Tai *et al.*, 1986). Recently, the same protein was isolated and characterized as an immunophilin following FK506 affinity chromatography (Yem *et al.*, 1992); specific peptide sequences within this protein were shown to have strong homology to FKBP-12 and FKBP-13. The cloning of hsp56 from rabbit liver cDNA was subsequently reported, and the derived amino acid sequence of hsp56 confirmed our original inference that hsp56 contained an FK506-binding domain (Lebeau *et al.*, 1992). Additionally, a human form of hsp56 has now been cloned and expressed in *Escherichia coli* (Peattie *et al.*, 1992). This immunophilin has been referred to in the literature by a variety of acronyms, such as hsp56 (Yem *et al.* 1992), p59 (Lebeau *et al.*, 1992), FKBP-52 (Peattie *et al.*, 1992), HBI (Callebaut *et al.*, 1992), FKBP-51 (Wiederrecht *et al.*, 1992), and FKBP-59 (Tai *et al.*, 1992), yet it is becoming clear that all of these terms relate to the same or derivative protein species. The original finding that hsp56 associates with various steroid receptor complexes (Renoir *et al.*, 1990; Sanchez *et al.*, 1990; Tai *et al.*, 1986), taken together with the recent demonstration that hsp56 binds to FK506 (Yem *et al.*, 1992), suggests that hsp56 *in vivo* may have a dual role in mammalian biochemistry. Hsp56 also binds to hsp90 (Renoir *et al.*, 1990; Tai *et al.*, 1986; Rexin *et al.*, 1991), another heat shock protein component of steroid receptor complexes, but the physiological functions and significance of these protein complexes are not understood at present.

We[2] and others (Callebaut *et al.*, 1992; Tai *et al.*, 1992; Trandinh *et al.*, 1992) have examined the primary structure of mammalian hsp56 for the presence of domains within the polypeptide, with special emphasis on regions which might show homology to known FK506-binding proteins such as FKBP-12 (Harding *et al.*, 1989; Siekierka *et al.*, 1989; Standaert *et al.*, 1990), FKBP-13 (Jin *et al.*, 1991), and FKBP-25 (Galat *et al.*, 1992; Jin *et al.*, 1992). A region with clear homology to FKBP-12, one we have designated FK506-binding domain I, is located near the N-terminus of hsp56, comprising residues 31–135 of the 59-kDa protein (Lebeau *et al.*, 1992; Callebaut *et al.*, 1992; Tai *et al.*, 1992). In fact, this domain is roughly 50% identical in sequence to FKBP-12 and FKBP-13 (Callebaut *et al.*, 1992; Tai *et al.*, 1992). Furthermore, there is a second domain in hsp56 (residues 145–252) which is about 25% identical to FKBP-12 and FKBP-13 (Callebaut *et al.*, 1992; Tai *et al.*, 1992), but this second domain lacks nearly all of the conserved residues found both in FK506-binding domain I of hsp56 and FKBP-12 itself. The presence of yet a third FK506-binding domain in hsp56 has been proposed (Callebaut *et al.*, 1992), even though its degree of similarity to well-characterized FK506-binding proteins is only 5%.

The fact that hsp56 was isolated by binding to immobilized FK506 and the existence of one or more domains within hsp56 with homology to FK506-binding proteins have led to efforts to determine the binding affinity of hsp56 for the drug. The binding of FK506 to human hsp56 was investigated by Tai *et al.* (1992), who generated a Scatchard plot both to determine the binding constant and to calculate the number of drug binding sites. In their study, a direct binding constant of 66 nM was determined for FK506, but only after the hsp56 was bound to an anti-hsp56-IgG-Sepharose matrix (MAb EC1).

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[1] Abbreviations: hsp, heat shock protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography; EKC, endoproteinase Lys C; TFA, trifluoroacetic acid; RPC, reverse-phase HPLC.

[2] A. G. Tomasselli, I. M. Reardon, A. W. Yem, V. A. Ruff, K. L. Leach, M. R. Deibel, Jr., and R. L. Heinrikson, manuscript in preparation.

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Nevertheless, a single high-affinity binding site was suggested by Scatchard analysis, which might be taken as evidence that only one FK506 binding domain, presumably domain I, is operational. Since hsp56 also has rotamase activity, which is potently inhibited by FK506, several investigators have utilized kinetic analysis to estimate the affinity of the protein for FK506. In particular, K_i values of 116 nM (Wiederrecht *et al.*, 1992) and 10 nM (Peattie et al., 1992) were determined kinetically on the basis of the competition of FK506 for a small peptide used as a rotamase substrate.

In this communication we describe an avian form (chicken thymus) of hsp56.[3] This protein binds to FK506-Affi-Gel-10 in a manner similar to its mammalian counterpart (Yem *et al.*, 1992) and shows a high degree of similarity to mammalian hsp56 in the N-terminal 20–25 amino acid residues. Upon digestion of the purified hsp56 with endoproteinase Lys C, a 17-kDa polypeptide is released which represents the entire structure of FK506-binding domain I. The isolated FK506-binding domain exhibits saturation binding kinetics with [^{3}H]-dihydro-FK506 and exists in its active binding form as a homodimer of 30–40 kDa.

The existence of a small FK506-binding domain in hsp56 has been predicted on the basis of sequence similarity studies by ourselves and others as defined above. Our study indeed verifies those predictions and also confirms the recent identification of hsp56 in chicken tissue reported by Smith *et al.* (1993).

MATERIALS AND METHODS

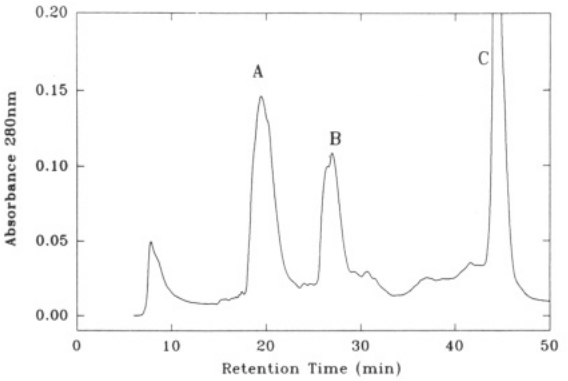
Electrophoresis and Blotting. SDS–PAGE was carried out with a mini-Protean II slab gel system (Bio-Rad) as described by Laemmli (1970). Gels were stained with Coomassie Blue G-250 or with silver (Bio-Rad). Western blots were conducted on nitrocellulose or PVDF papers. UPJ56 antibody [rabbit anti-human hsp56; see Ruff *et al.* (1992)] was diluted in 10 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl (TN buffer) and 5% BSA (fraction V powder). Detection was performed using a secondary antibody (goat anti-rabbit IgG) coupled to alkaline phosphatase (Promega) (1:750) diluted into TN buffer with 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine (BCIP) and nitro blue tetrazolium chloride (NBT) (Bio-Rad) as substrates.

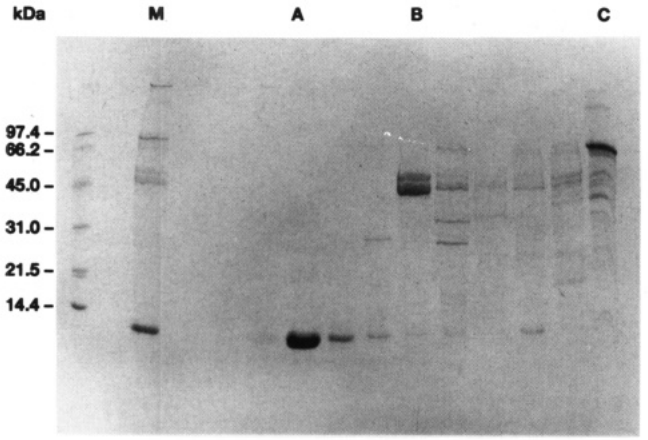
Preparation of Chicken Thymus p17. One hundred grams of frozen chicken thymus (Pel-Freez, Rogers, AR) was minced in 1 × PBS, pH 7.5, followed by extraction at 4 °C in a Waring blender containing 200–250 mL of chilled extraction buffer (25 mM sodium phosphate, 50 mM NaF, 5 mM mercaptoethanol, 1 mM PMSF). The supernatant, recovered by centrifugation at 20 000 rpm (SS-34 rotor) at 4 °C for 40 min, was then filtered through cheese cloth. Supernatants were recycled over a 2-mL column of the 32-(β-amino)-propionate analogue of FK506-Affi-Gel-10 matrix (1.5 cm diameter Econo column, Bio-Rad) (Yem *et al.*, 1992; Harding *et al.*, 1989; Fretz *et al.*, 1991) for 1–2 h at 4–8 °C (FK506 was prepared at the Upjohn Company from fermentation broths of *Streptomyces tsukubaenis).* The affinity matrix was retrieved and washed with 17 column volumes of extraction buffer, once with 25 mM sodium phosphate, pH 7.5, and twice with deionized water alone. Proteins retained on the matrix were dissociated with four aliquots each of 3 mL of TFA (0.1%), and these solutions were filtered through a 0.45-μm filter and injected directly onto a C4 reverse-phase HPLC column (Vydac; 0.46 × 25 cm) equilibrated with 0.1% TFA. FK506-binding proteins were eluted using a linear gradient of increasing acetonitrile concentration (24–48%) in 0.1% TFA, developed over a time period of 38 min. The effluent stream was monitored with a photodiode array detector (Hitachi), and protein fractions were collected manually during peak elution. These fractions were freeze dried by vacuum centrifugation (Savant). Hsp56 (4 nmol) was resuspended in 150–300 μL of 10 mM Tris-HCl, pH 8.7, and to this solution was added 0.5 unit of endoproteinase Lys C (EKC) (Promega, Madison, WI). After incubation at 37 °C for 3–4 h, the peptide products were resolved on a C18 reverse-phase column (0.46 × 15 cm; Vydac) in 0.1% TFA using an increasing concentration gradient of acetonitrile (0–36% over an 87-min period) (Figure 3, top). The column profile was monitored by a photodiode array detector. For peptide mapping, p17 generated from the first native EKC digest (above) was reduced in 1% 2-mercaptoethanol under denaturing conditions (25 mM Tris-HCl, 6 M guanidine HCl, 1 mM EDTA, pH 8.5) for 30 min at 37 °C. 4-Vinylpyridine (0.5 μL) (Aldrich) was added, and the reaction was allowed to proceed for 3 h at room temperature. To prepare the sample for EKC digestion, salts and excess reagent were removed by precipitation. EKC digestion and peptide mapping (see Figure 3, bottom) were as defined above.

Protein Compositional and Sequence Analysis. Amino acid compositions of proteins and peptides were determined by automated ion-exchange chromatography on a Beckman amino acid analyzer (Beckman 6300). Samples were hydrolyzed for 24 h *in vacuo* at 110 °C in 6 N HCl. Dried hydrolysates were dissolved in buffer at pH 2.2 (NaS; Beckman) prior to application to the analyzer. Automated Edman degradation of protein and peptide samples was performed in an Applied Biosystems Sequencer (Model 470) fitted with an on-line HPLC analyzer (Model 120-A) for identification and quantitation of phenylthiohydantoin amino acids. Quantitation was afforded by the Nelson Analytical Turbochrom chromatography data system connected in parallel with the recorder to the output of the HPLC system.

Size Exclusion FPLC for Detection of [^{3}H]Dihydro-FK506–Protein Complexes. Hsp56 or the 17-kDa fragment of hsp56 was incubated at 4 °C for up to 16 h with variable amounts of [^{3}H]dihydro-FK506 in a buffer system at pH 7.5 consisting of 40 mM Tris-HCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM DTT, and BSA (0.1 mg/mL). Bound drug was resolved from free drug by a Superose 12 preparative grade FPLC column (1.0 × 30.0 cm) preequilibrated with the same buffer. The column was developed isocratically at a flow rate of 1.0 mL/min (at 4 °C) and 1.0-mL fractions were collected. Fractions (0.5 mL) were mixed with Ultima Gold cocktail and counted in a Packard scintillation counter. Binding of [^{3}H]dihydro-FK506 to p17 was measured by varying the concentration of the drug at a constant protein concentration of 39.1 pmol, the latter of which was determined by Edman sequencing. A determination of bound + free drug was made by direct counting of reaction mixture. Data were analyzed by a one site binding model equation using the ENZFITTER program. Similarly, to determine whether calcineurin and calmodulin affect drug binding, parallel experiments were conducted using excess calcineurin and calmodulin (Sigma, bovine brain) with saturating concentrations of [^{3}H]dihydro-FK506 and p17. Chicken thymus FKBP-12 was purified similarly to hsp56 (retention time of 19–21 min by the RPC step; see Figure 1).

[3] A portion of this work was described in *FASEB J.*, Vol. 7, No. 7, 1993, abstract 1375.





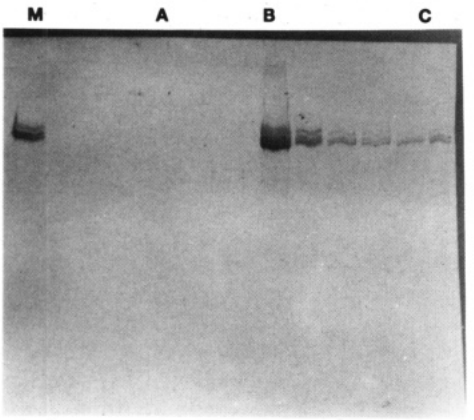


FIGURE 1: Purification of chicken thymus hsp56 by FK506-Affi-Gel-10 and C4 reverse-phase HPLC. C4 reverse-phase HPLC profile (top) showing the peak positions of FKBP-12 (A), hsp56 (B), and hsp90 (C); SDS–PAGE (middle) and Western blot (bottom) of fractions from the C4 column profile showing that hsp56, but not FKBP-12, is immunoreactive to the UPJ56 antibody. Lane M represents the TFA eluate from the FK506-Affi-Gel-10 column.

RESULTS AND DISCUSSION

Chicken Thymus Extracts Contain hsp56. We have applied methods which were successful in purifying hsp56 from mammalian tissues to a search for other sources of the protein. In the case of chicken thymus extracts, we have also been able to identify an hsp56 protein. Following affinity chromatography by FK506-Affi-Gel-10 and dissociation of bound proteins with TFA, the eluted proteins were resolved further by C4 reverse-phase HPLC as shown in Figure 1(top). SDS–PAGE of specific protein peaks, especially those which had retention times similar to that of human hsp56, showed the presence of a protein corresponding in molecular mass to human hsp56 (Figure 1, middle, lane B). Western blotting of the same fractions with an antiserum developed against human hsp56 (Ruff et al., 1992) showed the presence of strong cross-reactivity in the vicinity of 55–60 kDa (Figure 1, bottom). N-terminal sequence analysis of the purified chicken thymus hsp56 provided the sequence shown in Figure 2. Comparison of chicken and several mammalian hsp56 sequences showed that the chicken protein is identical to the others over the first six residues. The five residues encompassing 7–11 in chicken hsp56 fit somewhere in the region encompassing 7–18 (12 residues) in mammalian hsp56 proteins, but from residues 19 to 27 the proteins are again nearly identical (Figure 2).

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                5   10   15   20   25
Human:      TAEEMKATESGAQSAPLPMEGVDISPK....
Bovine:     TAEEMKAAESGAQSAPLRL....
Rabbit:     TAEEMKAAESGAQSAPLPLEGVDISPK....

Chicken:    TAEEMK-----ADGAP--LEGKDITPK....
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FIGURE 2: N-Terminal sequence of purified chicken hsp56 and comparison to human, bovine, and rabbit hsp56 sequences.

It is clear that chicken thymus hsp56 represents a major protein species/immunophilin that interacts with immobilized FK506. In our attempt to determine spectrophotometrically the relative amount of hsp56 present in chicken thymus relative to FKBP-12, considered to be the most abundant immunophilin in T cells, we calculated a stoichiometry of 1:1 for FKBP-12 and hsp56. A 90-kDa protein, determined to be hsp90 by sequence analysis, was also observed among the affinity-purified proteins from chicken thymus (Figure 1, top, peak C). It is likely that hsp90 and hsp56 are associated in a macromolecular complex in chicken thymus extracts; hsp56 binds to both the FK506-Affigel-10 matrix as well as to hsp90.

Limited Proteolysis of Chicken hsp56 Releases a 17-kDa Fragment Representing FK506-Binding Domain I. The chicken thymus hsp56 employed for limited digestion with EKC (see Figure 1, top, peak B) does not appear homogeneous when analyzed by SDS–PAGE and Western blotting using the UPJ56 antisera. Instead, as shown in Figure 1 (middle), several bands are observed by immunodetection ranging in molecular mass from 52–57 kDa. However, N-terminal sequence analysis of this same protein preparation indicates the presence of a single protein species with clear homology to the N-terminus of human hsp56 (Figure 2). Thus, it is likely that the protein, during isolation and purification steps, has been proteolyzed to generate multiple C-terminal truncations.

Purified chicken thymus hsp56 subjected to limited proteolysis with EKC shows only a single 17-kDa polypeptide when visualized by Western blotting of SDS–polyacrylamide gels (data not shown). In control experiments, crude extracts of chicken thymus, representing hsp56 which had not been subjected to purification, were also analyzed by digestion and blotting to verify that the 17-kDa band is, indeed, the principal immunoreactive protein generated by EKC. This 17-kDa protein, unlike either chicken or human FKBP-12, is immunoreactive with our polyclonal serum against human hsp56. Digestion of native hsp56 (nonreducing and nonalkylating conditions) with EKC generated several peptides that were resolved by HPLC. Fractions corresponding to each of the peaks shown in Figure 3(top) were probed with anti-human antibody, and only one, that labeled p17, was found to be

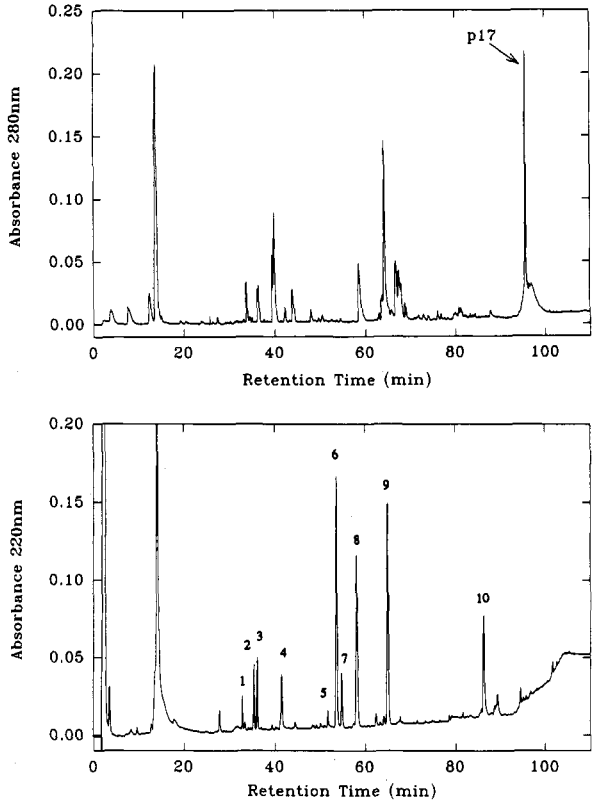

FIGURE 3: (Top) C18 reverse-phase HPLC peptide map of the EKC digest of native purified chicken hsp56. The arrow indicates the position of the 17-kDa fragment also identified by Western blotting using the UPJ56 antibody. (Bottom) EKC peptide map of the reduced and carboxymethylated chicken 17-kDa degraded fragment of hsp56. The numbered peaks refer to peptides which were sequenced (see Figure 4). The experimental procedures for both column runs are defined under Materials and Methods.

immunoreactive. Edman degradation of p17 occasionally gave two sequences, one which initiated at Arg 39 and the other, at Ala 12, of chicken thymus hsp56. Moreover, the amino acid analysis of p17 was in close accord with that expected from the first 150 residues of mammalian hsp56 proteins. Since the N-terminal sequence of purified p17 is similar to the beginning of a region of rabbit and human hsp56 which defines the first FK506-binding domain at of chicken hsp56, and since the size and composition of p17 fit well with the corresponding region of the mammalian proteins which encompasses the first FK506-binding domain (Callebaut *et al.*, 1992), it seemed likely that p17 represented an excised FK506-binding domain of chicken hsp56. Significantly, the liberation of a similarly sized polypeptide from human hsp56 under identical experimental conditions does not occur. We therefore considered that chicken thymus hsp56 must either be homologous but conformationally dissimilar to its human counterpart or differ in sequence from the human hsp56 at the site where processing occurs. To address this issue, we subjected the 17-kDa protein to peptide mapping as described below.

Peptide Mapping, Sequence Alignments, and a Protease Processing Site of Chicken Thymus P17. To determine the entire sequence of p17, the purified fraction was denatured, reduced, and alkylated with vinyl pyridine and then subjected to digestion once again with EKC. As shown in Figure 3 (bottom), 10 major peptides were resolved by HPLC; each numbered peak in the chromatogram was analyzed by Edman degradation and amino acid analysis. The resulting peptide sequences were aligned against the published sequences of

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                10        20        30        40        50        60

Hu   TAEEMKATESGAQSAPLPMEGVDISPKQDEGVLKVIKREGTGTEMPMIGDRVFVHYTGWL
Rb   TAEEMKAAESGAQSAPLPLEGVDISPKQDEGVLKVIKREGTGTETPMIGDRVFVHYTGWL
Ck   TAEEMK-----ADGAP--LEGKDITPKRDEGVLK??KREGTGTESPMIGDKVQVHYTGWL

EKC              <---------4------><---1--> <------3--------><------9--

                70        80        90        100       110       120
Hu   LDGTKFDSSLDRKDKFSFDLGKGEVIKAWDIAIATMKVGEVCHITCKPEYAYGSAGSPPK
Rb   LDGTKFDSSLDRKDKFSFDLGKGEVIKAWDIAVATMKVGELCRITCKPEYAYGSAGSPPK
Ck   LDGTKFDSSLDRK??FSFDLGK?????AWDIAVATSKVGELCRITCKPEYAYGSAGSPPK

EKC  ----><---2---><---7--->   <----8----><---------6------------>

                130       140       150       160       170       180
Hu   IPPNATLVFEVELFEFKGEDLTEEEDGGIIRRIQTRGEGYAKPNEGAIVEVALEGYYKDK
Rb   IPPNATLVFEVELFEFKGEDLTDDEDGGIIRRIRTRGEGYARPNDGAIVEVALEGYYKDR
Ck   IPPNATLIFEIELFEFKGEDLTDDEDGGIIRK

EKC  <-------------10----------------->
                    <--------5-------->
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FIGURE 4: Alignment of the 17-kDa chicken hsp56 fragment sequences with mammalian hsp56's. Chicken hsp56 residues in boldface differ from corresponding residues in both mammalian proteins. Peptides indicated by numbers are defined in Figure 3, bottom.

rabbit (Lebeau *et al.*, 1992) and human (Peattie *et al.*, 1992) hsp56, as shown in Figure 4. In fact, the high level of similarity of these peptides from chicken hsp56 to corresponding peptides from the mammalian proteins made the alignment simple and unambiguous. We were able to account for all but seven residues in the first FK506-binding domain, which, as we have defined this region, encompasses residues 31–135 of the mammalian hsp56. The missing residues are in what would be predicted to be small peptides that may have passed immediately through the reverse-phase HPLC column; there also may be deletions in these regions. In any case, given the gaps which appear in the first 20 residues (Figures 2 and 4), there is a surprising degree of identity among the several hsp56 proteins throughout the first FK506-binding domain. Both compositional and sequence analysis identified the EKC peptide beginning with Ile121 as extending to Lys152. From the size of p17, the specificity of EKC for lysine, and the absence of peptides C-terminal to Lys152, we conclude that Lys152 is the COOH-terminal residue in p17. The p17 domain exhibits 79% identity with human hsp56 (Peattie *et al.*, 1992) and 83% identity with rabbit hsp56 in comparisons of 138 residues in p17 with the first 152 residues of the mammalian proteins (Figure 4). Since we did not sequence the chicken hsp56 beyond the first FK506-binding domain, we are not in a position to identify or characterize further the overall degree of similarity or identity with mammalian hsp56's. In a similar comparison, p17 showed 41% identity to human FKBP-12 and 36% identity to FKBP-13.

Protein domains are often connected by extended regions which show enhanced susceptibility to proteolysis, so it is, perhaps, not surprising that we were able to remove the first domain with EKC. The failure of this enzyme to carry out the same process with mammalian hsp56 may be explained by the existence of a Lys in place of Arg at position 152 (Figure 4). Attempts to cleave the mammalian hsp56 with the Arg-specific clostrapain failed to yield comparable results, but this could be due to destruction of the peptide corresponding to FK506-binding domain I via cleavages at Arg residues within this N-terminal region. Because of the high degree of sequence similarity among all of these proteins, it seems unlikely that the conformation of chicken hsp56 will differ substantially from that of the mammalian proteins. It may be important to note here the existence in all of these hsp56 proteins of a pair of dibasic amino acids, Arg-Arg or Arg-Lys, at residues 151–152. Such pairs might constitute sites of processing, and this sequence may serve as a signal for intracellular breakdown of hsp56.

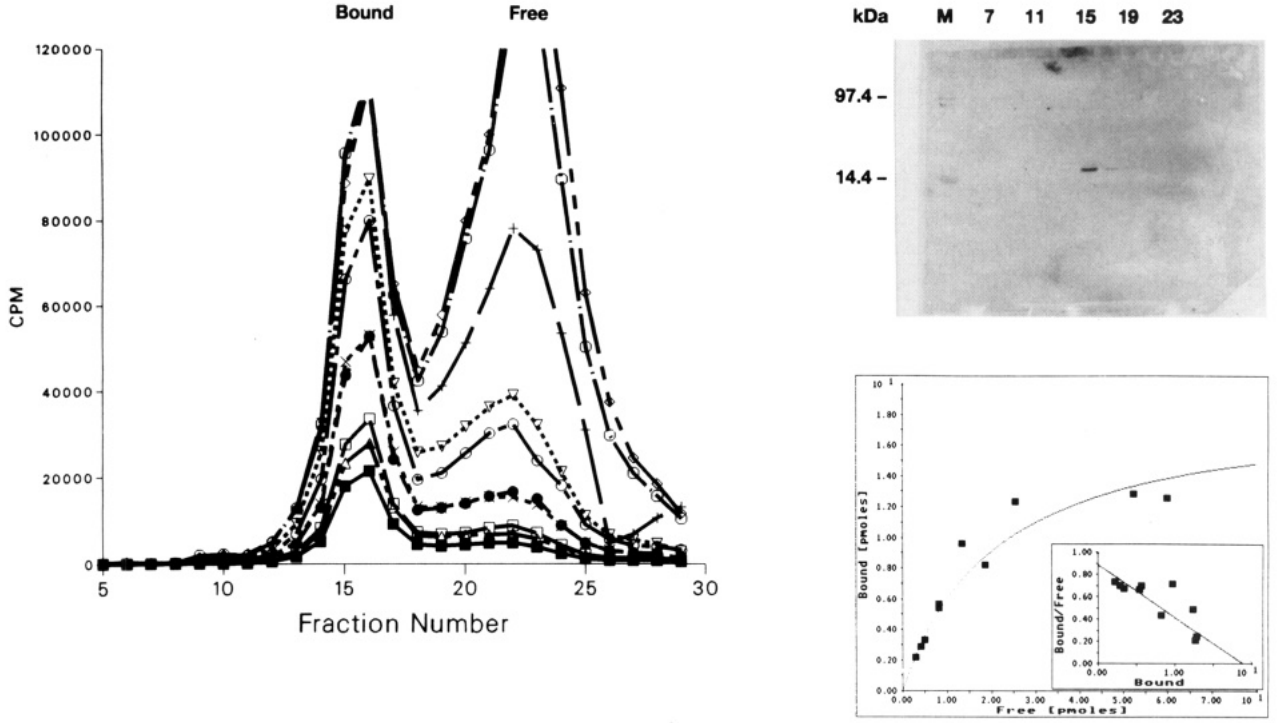

FIGURE 5: Assay for determination of the binding of [^{3}H]dihydro-FK506 to purified chicken p17. (Left) Gel filtration profiles to resolve bound and free drug when varying drug concentrations at a fixed p17 concentration. The [^{3}H]dihydro-FK506 concentrations utilized in the assays were: (■) 10.2 nM, (△) 13.9 nM, (□) 16.6 nM, (●) 26.9 nM, (×) 27.4 nM, (▽) 46.1 nM, (○) 53.5 nM, (+) 75.6 nM, (octagons) 130.0 nM, and (◇) 144.5 nM. (Top right) Western blot analysis of fractions from the gel filtration profile showing that p17 is immunodetected in fractions coincident with "bound" drug. (Bottom right) Saturation curve for a single site model for [^{3}H]dihydro-FK506 binding to purified chicken p17 (inset, Scatchard plot).

Binding Properties of p17 to [^{3}H]Dihydro-FK506. The binding properties of the chicken thymus hsp56-derived p17 FK506-binding domain were examined by incubation with [^{3}H]dihydro-FK506 as described under Materials and Methods. When purified chicken p17 was incubated with radiolabeled FK506, a radioactive peak was observed at a mass approximating 30–40 kDa (fraction 15–16; Figure 5, left). These fractions were subjected to Western blot analysis using UPJ56 polyclonal anti-hsp56 serum to reveal only one cross-reactive band at 17 kDa (Figure 5, top right). These data suggest that the proteolytically cleaved p17 fragment associates to form dimers under native conditions. For comparison, when radioactive dihydro-FK506 is complexed with FKBP-12, a peak is observed at a mass approximating 12 kDa, corresponding to fraction 19 in the gel filtration profile shown in Figure 5 (data not shown). These results provide evidence that FKBP-12 and the p17 derived from hsp56 have dissimilar biophysical properties.

There is precedence for dimerization of immunophilins. Wiederrecht *et al.* (1992) suggested that FKBP51, an N- and probably C-terminally truncated form of human hsp56, may dimerize in its native state or in the presence of drug. These authors were able to show that the protein migrated at an approximate mass of 110 kDa in the native state and as a 51-kDa protein on SDS–polyacrylamide gels in the denatured state. The role of the drug in dimerization was not clearly demonstrated since the protein as a monomer or as a homodimer was not detected in the absence of drug. The chicken p17 protein is analogous to their human hsp56 protein with respect to dimerization: it exists as a 30–40-kDa protein using native gel filtration and as a monomer of 17 kDa by SDS–PAGE.

The hsp56-derived p17 immunophilin shows saturation binding of the drug FK506 (Figure 5, bottom right), and the K_D from Scatchard binding analysis is 42 nM. This number, significantly higher than that for FKBP-12, is, however, comparable to a K_D value published for human hsp56 [approximately 66 nM (Tai *et al.*, 1992)]. Surprisingly, the parent 55–60-kDa chicken hsp56 protein isolated by FK506-Affi-Gel-10 chromatography and by C4 reverse-phase HPLC exhibited less than 1% of the activity in binding [^{3}H]dihydro-FK506 than was observed for the 17-kDa protein derived from it. Nevertheless, saturable binding of [^{3}H]dihydro-FK506 is observed following digestion with EKC and subsequent purification of p17. A determination of the stoichiometry indicates that 2.1 molecules of p17 bind to 1.0 molecule of [^{3}H]dihydro-FK506. Since the p17 protein exists in an active form as a homodimer, the corrected stoichiometry is approximately 1:1. These data imply that processing of chicken hsp56 can give rise to individual domains with attributed new functions. For example, does the increased activity observed post processing suggest that the hsp56 behaves as a precursor to a true drug binding form of the protein? Since the hsp56 was isolated by reverse-phase HPLC, an alternative explanation might be that the protein was denatured. However, the copurification of hsp90 and hsp56 by affinity chromatography on FK506-Affi-Gel-10 (see Figure 1) suggests that hsp90, a known chaperonin which binds to hsp56, might be responsible for the proper presentation of hsp56 to FK506. Removal of hsp90 by reverse-phase HPLC might then be responsible for loss of FK506 binding activity by hsp56. Nevertheless, subsequent exogenous protease processing of hsp56 could then allow excision of a single domain and its conformational transformation by refolding into an active drug-binding polypeptide. Why and how the p17 protein recovers this drug-binding function is still not clear.

Since the FKBP-12–FK506 complex can associate with calcineurin in the presence of calcium and calmodulin to form a 110-kDa complex (Wiederrecht *et al.*, 1992; Liu *et al.*, 1991), we investigated the potential for the p17 protein–FK506

complex to also interact with these other proteins in a pentameric complex. Using size exclusion chromatography to discriminate between p17 and a calcineurin–p17 complex, we found that the addition of excess calcineurin and calmodulin does not effect the migration mass of the p17–FK506 complex (data not shown). Alternatively, we show in the same experiment that in the presence of calcineurin–calmodulin, the drug–protein complex with purified chicken thymus FKBP-12 is shifted from a retention time of 19 to 12 min. These results demonstrate a clear functional distinction between the p17 protein and FKBP-12, in that the p17 protein cannot replace FKBP-12 in the drug-induced calcineurin–calmodulin complex.

CONCLUSION

The physiological importance of the degradation and subsequent production of individual domains of hsp56 from mammalian or avian sources cannot be assessed at present. For instance, since we have shown that the 17-kDa protein is easily formed quantitatively from hsp56 under native conditions in the presence of EKC, could such events also occur *in vivo* or during the normal course of metabolism of hsp56? If an active degraded form of the protein is generated *in vivo*, it would be of interest to consider whether such a polypeptide might mimic FKBP-12, FKBP-13, FKBP-25, or none of these in its biological or toxic effects on target cells. To address part of this issue *in vitro*, we have shown that the 17-kDa cleaved product does not bind to calcineurin in the presence of FK506, a clear distinction from FKBP-12. Of course, because of the relatively high binding constant for dihydro-FK506 for chicken p17, it is possible that the interaction of p17–dihydro-FK506 with calcineurin might be thermodynamically unfavorable. Precedents for lack of interference of drug-loaded FK506 binding immunophilins with the enzymatic activity of calcineurin are documented. For example, although FK506 binds to FKBP-13, FKBP-25, and hsp56, these drug–protein complexes, in the presence of calcium, do not appear to interact with calcineurin, in the presence or absence of calmodulin. It is conceivable that a p17–drug complex might bind to yet another protein involved in one or more signal transduction pathways. Alternatively, p17 might interact with steroid receptor complexes in a manner analogous to intact hsp56 itself. Investigations of steroid receptors have shown that they interact with several proteins (*e.g.*, hsp90 and hsp56), the latter of which likely participate as molecular chaperones in one or more folding, assembly, or disassembly pathways. However, the exact role of hsp56 in mammalian and/or avian physiology is still uncertain, so that the identification of a proteolytic fragment which retains the characteristic of drug binding will also require additional biochemical studies to verify or refute its importance or function *in vivo*.

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