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Characterization of a Novel 23-Kilodalton Protein of Unactive Progesterone Receptor Complexes

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Immunoprecipitation of unactivated avian progesterone receptor results in the copurification of hsp90, hsp70, and three additional proteins, p54, p50, and p23. p23 is also present in immunoaffinity-purified hsp90 complexes along with hsp70 and another protein, p60. Antibody and cDNA probes for p23 were prepared in an effort to elucidate the significance and function of this protein. Antibodies to p23 detect similar levels of p23 in all tissues tested and cross-react with a protein of the same size in mice, rabbits, guinea pigs, humans, and *Saccharomyces cerevisiae*, indicating that p23 is a conserved protein of broad tissue distribution. These antibodies were used to screen a chicken brain cDNA library, resulting in the isolation of a 468-bp partial cDNA clone encoding a sequence containing four sequences corresponding to peptide fragments isolated from chicken p23. This partial clone was subsequently used to isolate a full-length human cDNA clone. The human cDNA encodes a protein of 160 amino acids that does not show homology to previously identified proteins. The chicken and human cDNAs are 88% identical at the DNA level and 96.3% identical at the protein level. p23 is a highly acidic phosphoprotein with an aspartic acid-rich carboxy-terminal domain. Bacterially overexpressed human p23 was used to raise several monoclonal antibodies to p23. These antibodies specifically immunoprecipitate p23 in complex with hsp90 in all tissues tested and can be used to immunoaffinity isolate progesterone receptor complexes from chicken oviduct cytosol.

Unactivated steroid hormone receptors exist in low-salt tissue extracts as 8 or 9S complexes that are composed of distinct cellular proteins in addition to the hormone receptor itself. In the presence of hormone, these complexes dissociate, leaving the 4S form of the receptor that is capable of dimerization and binding to DNA. In the absence of hormone, the native avian progesterone receptor (PR) associates with hsp90, hsp70, and three additional proteins, p54, p50, and p23 (30). The binding of these proteins is salt dependent and stabilized by molybdate (30).

As the components of the various steroid receptor complexes have been examined (reviewed in references 22 and 34), hsp90 has been found in association with glucocorticoid, androgen, estrogen, and mineralocorticoid receptors, as well as PR. Comparative studies on the other receptor-associated proteins are less complete. hsp70 has been observed with chicken and mammalian PR (12, 20) and with some forms of the glucocorticoid receptor (27). A 59-kDa heat shock protein (p59, hsp56) (26) associates with mammalian progesterone, estrogen, androgen, and glucocorticoid receptors (37). p59 has been shown to bind the immunosuppressant drug FK506 (14, 36, 39). It belongs to the family of FK506-binding proteins (FKBPs) and has also been called FKBP52 (21). The p54 and p50 proteins seen in the chick PR complex have recently been shown to belong to the same family of FKBPs, with p50 more closely related to p59 (29). Thus, with the exception of p23, the major receptor-associated proteins have been identified as either heat shock proteins or immunophilins even though their significance to receptor structure and function remains unclear.

In this report we characterize p23, the 23-kDa protein originally found in the chicken PR complex. p23 is also present in some hsp90 complexes. p23 is a highly conserved, novel protein with no structural homology to known proteins. In addition, antibodies which specifically immunoprecipitate p23 coprecipitate the chicken PR along with hsp90 and hsp70.

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MATERIALS AND METHODS

Preparation of tissue cytosol. Oviduct cytosol was freshly prepared from estradiol-stimulated chicks (31). Soluble tissue homogenates from chicken brain, liver, oviduct, and spleen; mouse liver; and guinea pig liver were prepared in 4 volumes of 50 mM potassium phosphate (pH 7.4)–10 mM thioglycerol homogenization buffer as previously described (31). Yeast (*Saccharomyces cerevisiae*) extract was prepared by vortexing the yeast pellet in 10 volumes of sodium dodecyl sulfate (SDS) sample buffer in the presence of glass beads. HeLa cell S100 extract was a gift from Jay Tichelaar. Untreated rabbit reticulocyte lysate was purchased from Green Hectares (Oregon, Wis.).

Antibodies. Mouse monoclonal immunoglobulin G (IgG) antibody PR22 against the avian PR has been described (35). Antibody D7α against avian hsp90 was prepared by Brugge and coworkers (3). Antibodies to p23 were prepared as described below. These include an IgM mouse monoclonal antibody, R3; four mouse monoclonal IgG antibodies, JJ3, JJ5, JJ6, and JJ10; and a rabbit antiserum against a p23 peptide, p23t2.

Immunoprecipitation of PR, hsp90, and p23 complexes. PR22 or D7α monoclonal antibody (20 μg) or 10 μl of p23 monoclonal antibody ascites was preincubated with 25 μl of protein A–Sepharose CL-4B (Pharmacia, Piscataway, N.J.) in 100 mM Tris-HCl (pH 8.0) for 30 min at room temperature and then washed with homogenization buffer. One milliliter of cytosol was added to each 25-μl resin pellet. After 1 h on ice, with gentle resuspension every 5 min, the pellet was washed

four times with 1 ml of homogenization buffer, and extracted directly into SDS sample buffer before analysis by SDS-polyacrylamide gel electrophoresis (PAGE). For the peptide competition studies with the JJ3 and JJ5 antibodies, 75 μg of peptide was added to the cytosol during the 1-h immunoprecipitation.

Gel electrophoresis and Western blot analysis. Samples were extracted into SDS sample buffer containing 2% SDS and 5% β-mercaptoethanol. SDS-polyacrylamide gels were prepared as described previously (13). Gels were stained with Coomassie brilliant blue R-250. For Western blotting (immunoblotting), SDS-polyacrylamide gels were transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, Mass.) as described elsewhere (12). For blotting p23, the membranes were incubated with primary antibody at a 1:2,000 dilution overnight at 4°C, washed, incubated with the appropriate alkaline phosphatase-conjugated goat anti-mouse second antibody for 30 min, washed, and stained with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The p23t2 antiserum was used at a 1:1,000 dilution with alkaline phosphatase-conjugated goat anti-rabbit IgG second antibody.

Obtaining peptide sequence for p23. Immunoprecipitated PR and hsp90 complexes from chicken oviduct cytosol were resolved on a 10% gel. After the position of p23 was determined by staining the edges of the gel with Coomassie blue, p23 was excised and electroeluted with either a Centricon Electroeluter (Amicon, Beverly, Mass.) or a Six-Pac Electroeluter (Hoefer Scientific Instruments) according to the manufacturer's suggestions. The electroeluted protein was dialyzed into 5 mM NH_4HCO_3 and precipitated with either ethanol (40) or chloroform-methanol (38). The pellet was resuspended and digested with either trypsin or cyanogen bromide (CNBr) essentially as described previously (33).

Microsequencing of peptide fragments. Protein sequencing and peptide synthesis were conducted in The Protein Sequencing/Peptide Synthesis Resource Laboratory, Mayo Clinic, directed by Daniel J. McCormick. After treatment of p23 with trypsin or CNBr, peptide fragments were resolved by high-performance liquid chromatography with an ABI Aquapore OD-300 C18 column. Individual peptides were sequenced from the amino terminus by gas-phase Edman degradation.

Synthetic peptide synthesis. Peptides were synthesized by solid-phase methods, using the 9-fluorenylmethyloxycarbonyl protection scheme. The two internal peptides, p23t2 (residues 49 to 63) and surf1 (residues 60 to 75), were synthesized on Rink resin, and the C-terminal peptide, surf2 (residues 143 to 160), was synthesized on Wang resin. Cysteines were added to the C terminus of the purified internal peptides and the N terminus of the C-terminal peptide for conjugation to maleimide-activated keyhole limpet hemocyanin (Pierce Chemical, Rockford, Ill.).

Development of polyclonal antisera to p23. The synthetic peptide p23t2, LNEIDLFSNIDPNE, was conjugated to keyhole limpet hemocyanin to raise rabbit polyclonal antiserum. The second test bleed was reactive with p23 on a Western blot of chick oviduct cytosol. The polyclonal antiserum was affinity purified on an antigen column. Briefly, bovine serum albumin-conjugated peptide was covalently cross-linked to ACTIGEL-ALD according to the manufacturer's instructions (Sterogene Bioseparations, Arcadia, Calif.). Polyclonal antiserum diluted 1:10 in 10 mM Tris-Cl (pH 7.5) was bound to the column and washed extensively with 10 mM Tris-Cl (pH 7.5) and then with 500 mM NaCl–10 mM Tris-Cl (pH 7.5). The p23-specific antibodies were eluted into 1 M Tris-Cl (pH 8.0) with 100 mM glycine-Cl (pH 2.5) and then with 100 mM triethylamine-Cl (pH 11.5), pooled, and dialyzed into phosphate-buffered saline by the method of Harlow and Lane (6).

Immunoscreening of cDNA expression libraries. A Clontech chicken 5′-Stretch cDNA brain library (catalog number CL1016b) was screened with R3 antibody and purified polyclonal antiserum by conventional methods. Plaques positive on tertiary screens were used for large-scale phage preparations performed by the glycerol gradient step method (25). The phage DNA was digested with *Eco*RI, and the insert was subcloned into pGEM 7zf(−) (Promega, Madison, Wis.) by conventional methods (25). Double-stranded sequencing was performed on two independent subclones by using [^{35}S]dATP and the Sequenase 2.0 kit (U.S. Biochemicals).

Library screening with labeled RNA probe from a partial clone. A Clontech human testis cDNA library (catalog number HL1010b) was screened with an [α-^{32}P]GTP-labeled RNA probe synthesized from the partial chicken clone. The prehybridization and hybridization of the labeled RNA probe to nylon filters (Amersham, Inc.) were carried out in 5× SSPE (1× SSPE is 0.18 M NaCl, 0.01 M NaH_2PO_4, and 0.001 M EDTA), 50% formamide, 5× Denhardt's solution (1× Denhardt's solution is 0.02% [wt/vol] bovine serum albumin, 0.02% [wt/vol] Ficoll [Pharmacia], and 0.02% [wt/vol] polyvinylpyrrolidone), 0.5% (wt/vol) SDS, and 0.25 mg of denatured sonicated salmon sperm DNA at 40°C according to the manufacturer's instructions.

Bacterial expression of human p23. A full-length human p23 PCR product was generated by using the forward p23-specific primer 5′-CGTTCACAATGGATCCTGCTTCTTGC-3′ and a reverse vector-specific SP6 primer. The 650-bp PCR product was digested with *Bam*HI and subcloned into the Invitrogen (San Diego, Calif.) pTrcHis expression vector. The resulting 26-kDa fusion protein replaces the original two amino acids of the native sequence with the additional amino-terminal sequence MGGSHHHHHHGMASMTGGQQMGRDLYDD DDKD. *Escherichia coli* TOP10 (Invitrogen) containing the expression vector was grown at 37°C to an optical density at 600 nm of 0.3, induced with 1 mM isopropylthio-β-D-galactoside and harvested 4 h later. Soluble cell extract was prepared by resuspending and sonicating the pellet in 20 mM sodium phosphate–500 mM sodium chloride (pH 7.8). Recombinant p23 elutes from a DEAE-cellulose column during a 0.4 M KCl step elution as a highly purified protein. Collection and dialysis of peak fractions yield approximately 10 mg of 50 to 70% pure p23 per liter of culture.

Monoclonal antibody production. Mouse monoclonal antibody R3 was prepared by the intrasplenic immunization method of Nilsson and Larsson (19). PR complexes immunoprecipitated from chick oviduct cytosol were resolved by SDS-PAGE, transferred to an Immobilon membrane (Millipore), and stained with Coomassie blue. The p23 band was excised and inserted directly into the spleens of three mice. This was repeated after 1 month, and cell fusion was performed 5 days later. Hybridoma culture supernatants were tested for p23 antibodies by Western blotting against preparations of PR complex. Additional antibodies were prepared by conventional subcutaneous injection with p23 expressed in *E. coli* and purified on DEAE-cellulose. Hybridoma supernatants were tested on enzyme-linked immunosorbent assay plates coated with recombinant p23, yielding nine positive hybridomas.

Reconstitution of PR complex with ^{35}S-labeled p23. [^{35}S]methionine-labeled p23 was produced by using the TNT T7-coupled reticulocyte lysate system (Promega, Madison, Wis.) according to the manufacturer's instructions. Briefly, 0.5 μg of the Hp23C clone was transcribed and translated in Promega

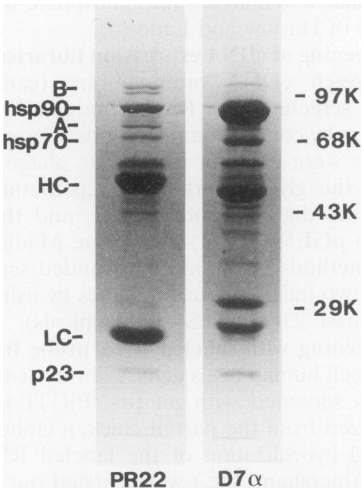

FIG. 1. p23 is part of the unactivated chicken PR complex. Immunopurified complexes were isolated from chicken oviduct cytosol as described. (Left panel) Immune isolation of chicken PR complex with antibody PR22; (right panel) immune isolation of hsp90 complex with antibody D7α. The protein A pellets were washed with homogenization buffer, boiled in sample buffer, run on an SDS–10% polyacrylamide gel, and stained with Coomassie blue. The positions of molecular mass standards (Diversified Biotech) are shown on the right. K, kilodalton.

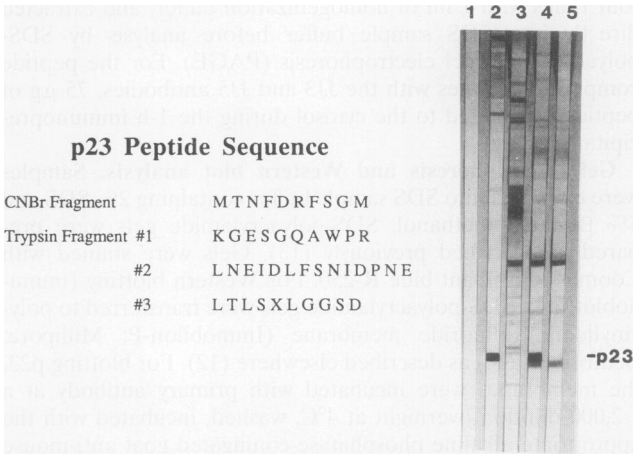

FIG. 2. Peptide fragments of p23 and development of polyclonal antiserum to p23. One CNBr fragment and three trypsin fragments obtained from gel-purified p23 were sequenced. Polyclonal antiserum p23t2 was raised against the second trypsin fragment. Chick oviduct cytosol was run on a 10% gel, transferred to Immobilon, and Western blotted with antibodies against p23. Lane 1, anti-mouse IgM second antibody alone; lane 2, IgM monoclonal antibody R3; lanes 3 to 5, 1:1,000 dilution of rabbit serum; lane 3, rabbit preimmune serum; lane 4, reactive p23t2 polyclonal antiserum; lane 5, 23t2 polyclonal antiserum inhibited with 100 μg of peptide.

lysate with T7 polymerase in a 50-μl volume. After 2 h the lysate was divided into three reaction mixtures, and Promega untreated lysate (catalog number L4150) was added to a volume of 200 μl. An ATP regenerating system (32) was also added at this time.

Chick oviduct cytosol was prepared as described above, adjusted to 0.5 M KCl, and incubated on ice for 30 min. One and a half milliliters of this salt-stripped cytosol was added to each 25-μl protein A-PR22 antibody resin mixture. After a 1-h incubation on ice, the pellets were washed four times (1 ml each wash) in 10 mM Tris-Cl (pH 7.5). The reticulocyte lysate mixture was added to the PR resin pellet and incubated at 30°C for 30 min with mixing every 5 min. To activate the receptor, 2×10^{-7} M (final concentration) progesterone was added as indicated and the 30°C incubation was continued for an additional 30 min. The samples were washed five times (1 ml each wash) with 10 mM Tris-Cl (pH 7.5) and analyzed by SDS-PAGE.

Computer analysis of sequence. All computer analyses of DNA and protein sequences were done with the Genetics Computer Group (GCG; Madison, Wis.) sequence analysis software package.

Nucleotide sequence accession number. The human p23 cDNA and chicken p23 cDNA sequences have been submitted to GenBank with the accession numbers L24804 and L24898, respectively.

RESULTS

p23 was first observed as a component of the unactivated complex of the avian PR (30). However, its existence is not confined to receptor complexes, since it has also been isolated in complexes with the 90-kDa heat shock protein, hsp90 (33). A typical immunoprecipitation of chick oviduct PR with the monoclonal antibody PR22 is shown in Fig. 1 (left panel). The PR22 antibody recognizes the B and A forms of the receptor (35). Five additional proteins, hsp90, hsp70, p54, p50, and p23, have been shown to specifically copurify with the PR (30). The positions of PR B and A, hsp90, hsp70, and p23 are shown. The identities of these proteins were confirmed by Western blotting (data not shown). The heavy chain of the antibody obscures the p54 and p50 proteins. From densitometric analysis of Coomassie blue-stained gels, the molar ratio of p23 to PR appears to be approximately 1:1 (30). The proteins copurifying with the chick oviduct hsp90 immunoprecipitated with D7α are also shown in Fig. 1 (right panel). These proteins have been identified on Western blots as hsp70, p60, and p23 (33; data not shown). The major proteins that specifically copurify with hsp90 are hsp70 and a stress-related protein termed p60 (33). p60 is a homolog of the yeast stress protein, STI1 (18), and this protein has also been shown to be up-regulated by viral transformation of human cells (9). p23 is less abundant than the above proteins but is still very evident.

Immunoscreening of chicken cDNA expression libraries. Peptide fragment sequences obtained from gel-purified chicken p23 digested with trypsin or CNBr are shown in Fig. 2 (left panel). These peptide fragments indicated that p23 was a novel protein. p23 in chick oviduct cytosol can be detected on Western blots with the mouse IgM monoclonal antibody, R3, which was raised against gel-purified p23 (Fig. 2, lane 2). Initial screening of Clontech 5′-stretch liver and brain libraries with antibody R3 resulted in the isolation of numerous false-positive clones, prompting us to generate polyclonal antiserum against the longest trypsin fragment, LNEIDLFSNIDPNE. This antiserum, p23t2, specifically recognizes p23 on a Western blot with chick oviduct cytosol, and this interaction can be inhibited by free peptide (Fig. 2, lanes 3 to 5). Western blotting of a two-dimensional gel confirmed that the peptide antiserum was specific for p23 (data not shown). After peptide affinity column purification to remove unrelated antibodies, this antiserum was used to screen a Clontech 5′-stretch brain library. One clone, c23e, that was positive and competed with free peptide was isolated. This clone was also detected with antibody R3.

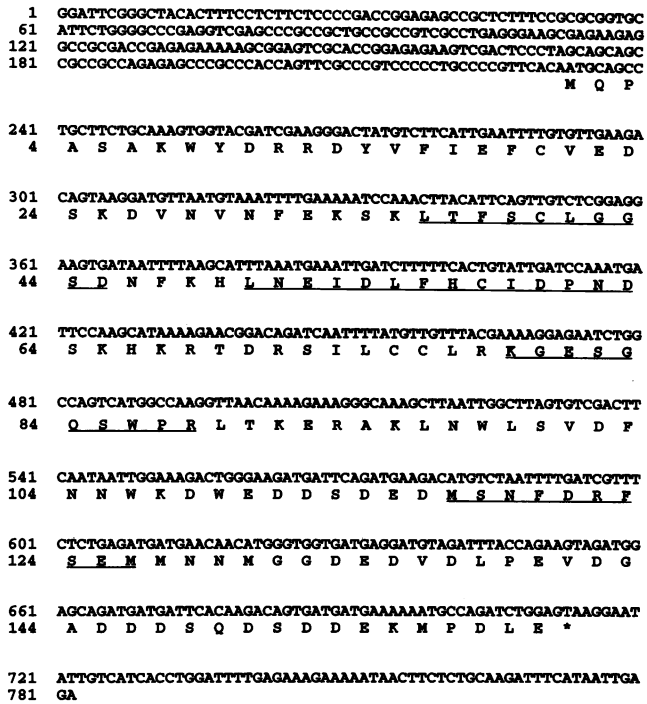

FIG. 3. Human p23 cDNA sequence. The 782-bp full-length p23 cDNA clone encodes the complete 160-amino-acid p23 open reading frame. The human sequences corresponding to the peptide fragments obtained from gel-purified chicken p23 are underlined. The DNA homology between chicken and human p23 DNA is 88%.

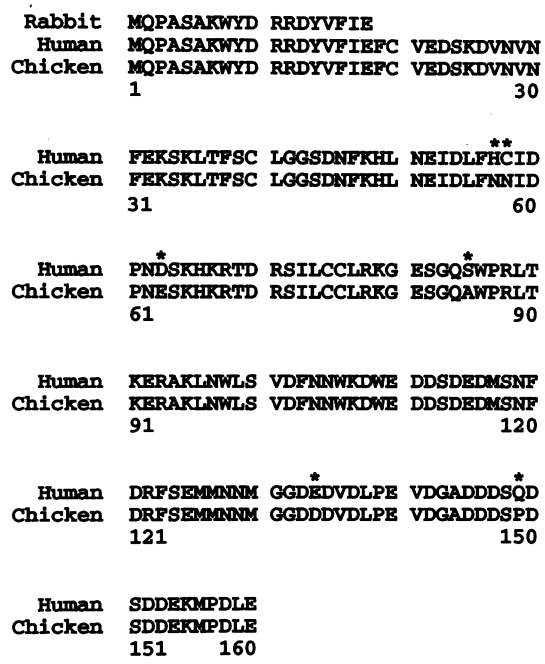

FIG. 4. p23 protein sequence is highly conserved. Protein sequences for rabbit, human, and chicken p23 are shown. The full-length human coding sequence is deduced from the cDNA sequence. The chicken sequence is a composite of amino-terminal sequence and partial cDNA sequence (residues 16 to 160). The rabbit sequence was obtained by amino-terminal sequencing of gel-purified rabbit p23 transferred to Problott. The chicken and human sequences are 96.3% identical, with the differences marked with asterisks. Searches revealed no homologies with known proteins in either the Swiss-Prot or NBRF-PIR data bases.

p23 cDNA and protein sequence. The 468-bp partial clone, c23e, encodes an open reading frame of 145 amino acids, including the four peptides obtained from sequenced fragments of chicken p23 (data not shown). An [α-^{32}P]GTP-labeled c23e RNA probe was used to screen a human testis cDNA library. Three clones, all with inserts of approximately the same size, were isolated. One of these clones was selected for further characterization and found to contain an insert 782 nucleotides in length. This clone, Hp23C, represents the complete coding sequence of p23 and also contains 232 bp of 5′ untranslated sequence and 70 bp of 3′ untranslated sequence (Fig. 3).

The human cDNA encodes an open reading frame of 160 amino acids (Fig. 3). The four peptide sequences obtained from chicken p23 (Fig. 2) correspond closely to the human sequence and are underlined in Fig. 3. p23 is highly conserved, with the chicken and human sequences being 96.3% identical at the amino acid level (Fig. 4). p23 is a very hydrophilic protein with an aspartic acid-rich carboxy terminus. The predicted pI of 4.2 is in good agreement with the observed pI of 4.5 to 5.2 from SDS-polyacrylamide and isoelectrofocusing gels (30). The predicted molecular mass of p23 is only 18.7 kDa, whereas p23 migrates at approximately 23 kDa on SDS-polyacrylamide gels. To determine whether we had correctly identified the amino terminus of p23, we obtained amino-terminal peptide sequence from SDS-PAGE-purified chicken and rabbit p23. The chicken and rabbit amino-terminal p23 sequences are identical to the derived amino terminus of the human p23 clone (Fig. 4). Thus, the size discrepancy is due to either decreased mobility of p23 on SDS-polyacrylamide gels or posttranslational modification.

A search against GenBank (1) nucleic acid sequences revealed extensive homology between the Hp23C clone and the opposite strand of the 5′ untranslated region of the human zinc finger 6 (ZNF6) locus (16) (GenBank accession number X56465). As published, this sequence contains multiple frameshifts in the p23 coding sequence. Relative to the 3′ end of the p23 coding sequence, the ZNF6 sequence and the Hp23C clone are >98% identical, with only 7 nucleotide differences over 565 nucleotides, at which point the sequences diverge. The location of the p23 sequence in the untranslated region of the ZNF6 locus raises the possibility that the sequence represents a p23 pseudogene. Because the homology is over 98% identical throughout the p23 coding region and the sequences have a distinct divergence point, the ZNF6 locus most likely represents a cloning artifact in which the p23 cDNA and the ZNF6 cDNA became ligated in a head-to-head manner, although the possibility of the ZNF6 representing a pseudogene has not been eliminated. Portions of the p23 coding sequence were recently submitted to GenBank (accession number T11431) as an expressed sequence tag isolated from human pancreatic islet.

Comparisons of p23 sequence to protein sequences listed in the Swiss-Prot and NBRF-PIR data bases do not reveal any significant homologies to known proteins, indicating that p23 is a novel protein. p23 has been observed to be a phosphoprotein (4), and multiple conserved candidate phosphorylation sites are present, as detected by the GenBank Motifs (1) program. No other common structural motifs were detected.

Tissue and species distribution of p23. Recombinant p23 was expressed in *E. coli* and used to prepare a series of monoclonal antibodies (see Materials and Methods). Antibody

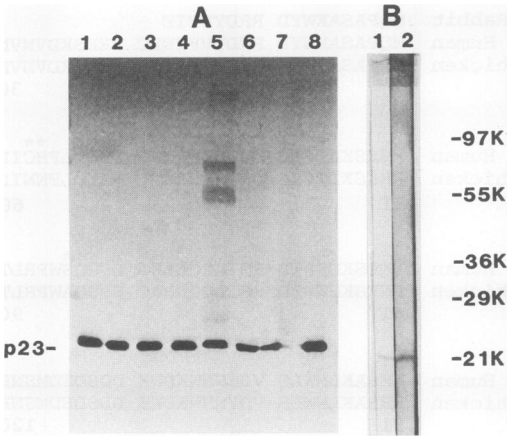

FIG. 5. Tissue and species cross-reactivities of the monoclonal antibody JJ5. (A) Ten microliters of various tissue and cell cytosols was run on a 12% gel, transferred to Immobilon, and Western blotted with the antibody JJ5 (1:5,000 dilution). Lane 1, chicken oviduct cytosol; lane 2, chicken brain cytosol; lane 3, chicken spleen cytosol; lane 4, chicken liver cytosol; lane 5, mouse liver cytosol; lane 6, guinea pig liver cytosol; lane 7, HeLa cell extract; lane 8, rabbit reticulocyte lysate. (B) Western blot of *S. cerevisiae* whole-cell extract with monoclonal antibodies JJ6 (lane 1) and JJ10 (lane 2) at a 1:500 dilution. Positions of molecular mass markers are shown on the right. K, kilodalton.

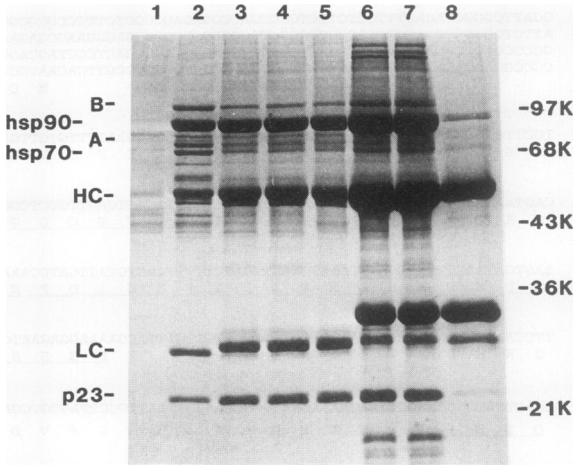

FIG. 6. Monoclonal antibodies to p23 immunoprecipitate PR. p23 complexes were immunoaffinity isolated, run on an 11% gel, and stained with Coomassie blue. Lane 1, mock immune precipitation with protein A-Sepharose; lane 2, immune isolation with monoclonal antibody PR22 against the PR; lane 3, immune isolation with the monoclonal antibody JJ3; lane 6, immune isolation with the monoclonal antibody JJ5. The surf2 peptide corresponding to the 18 carboxy-terminal amino acids effectively blocked the immune precipitation of p23 complexes by the JJ5 antibody (lane 8) but had no effect on the JJ3 antibody (lane 5). The surf1 peptide (amino acids 60 to 75) had no effect on either JJ3 (lane 4) or JJ5 (lane 7). Positions of molecular mass markers are shown on the right. K, kilodalton.

JJ5 was used to screen various chicken and mammalian tissues to identify the tissue and species distribution of p23. Per gram of tissue, similar levels of p23 are seen in chicken oviduct, brain, spleen, and liver cytosols (Fig. 5A). In a comparison of chicken oviduct cytosol versus crude chicken oviduct nuclear extract, the majority of p23 appears to be present in the cytosol, although there are detectable levels in the nuclear extract (data not shown). The JJ5 monoclonal antibody also cross-reacts with 23-kDa antigens in mouse liver, guinea pig liver, rabbit reticulocyte lysate, and human HeLa cell extract (Fig. 5A). Although JJ5 does not recognize a 23-kDa protein in *S. cerevisiae* (data not shown), two additional monoclonal antibodies, JJ6 and JJ10, do recognize a 21 to 22-kDa protein in *S. cerevisiae* (Fig. 5B). These studies clearly show that p23 is a protein with a broad tissue and species distribution. In addition, a panel of antibodies against p23, including the monoclonal antibodies JJ3, JJ5, and R3, were found not to share cross-reactivity with any proteins other than p23, suggesting that p23 is a unique protein.

Immunoprecipitation of p23. Immunoprecipitations from chicken oviduct cytosol with two monoclonal antibodies against p23 are shown in Fig. 6. Upon immunoprecipitation with antibodies JJ3 and JJ5 against p23 (lanes 3 and 6, respectively), the pattern of bands is quite similar to that with PR22 immunoprecipitation (lane 2). The bands corresponding to the PR B and A forms, hsp90, and hsp70 have been confirmed by Western blot analysis (data not shown). Two peptides, surf1 (DPNESKHKRTDRSILC) and surf2 (GADD DSPDSDDEKMPDLE), were prepared because their sequences were predicted to be highly antigenic. These peptides were tested for their ability to block the interaction of p23 with antibodies JJ3 and JJ5. The binding of antibody JJ5 but not antibody JJ3 to p23 complexes is inhibited by the peptide surf2, which corresponds to the carboxy-terminal 18 amino acids of p23 (Fig. 6, lane 8). In addition to inhibiting binding of p23 to the antibody, the surf2 peptide also specifically eliminates the copurification of hsp90, PR B and A, hsp70, and additional proteins of lesser abundance: one migrating at approximately 200 kDa, a few proteins in the 35- to 40-kDa range, and two lower-molecular-mass proteins of about 15 kDa. The identities of the latter proteins are unknown. The surf1 peptide does not have an effect on immunoprecipitation of p23 with either antibody.

In Fig. 7, antibody JJ3 was used to immunoprecipitate p23 from mouse liver, rabbit reticulocyte lysate, chicken brain, spleen, liver, and oviduct. hsp90 and hsp70 specifically coprecipitate with p23 in each tissue tested, although the ratios of p23 to hsp90 and hsp70 vary depending on the tissue. A number of other proteins coprecipitate with p23 antibodies in various tissues, the most prominent being a 42-kDa protein seen precipitating from chick liver (lane 6). Only the bands corresponding to hsp90 and hsp70 have been identified by Western blot (data not shown). A similar pattern of immunoprecipitation was seen with the JJ5 antibody (data not shown).

Reconstitution of p23 into PR complexes. Immunoaffinity-isolated chicken PR that has been stripped of associated proteins in high salt will reassociate with rabbit hsp90, hsp70, and p23 when incubated in rabbit reticulocyte lysate (31, 32). This is not a simple binding event but occurs through a temperature- and ATP-dependent process that is still unclear. Once reassembled, the addition of progesterone leads to activation of the PR, evident in the dissociation of hsp90 and p23 (31, 32). To demonstrate that the full-length cDNA clone Hp23C is sufficient to produce functional p23, we transcribed and translated the clone in vitro, producing [^{35}S]methionine-labeled human p23. The translation mixture was then added to a standard chicken PR reconstitution (32), shown in Fig. 8. On the Coomassie blue-stained gel (lanes 1 to 5), lane 1 shows the proteins in the intact receptor complex isolated from chick oviduct cytosol, and lane 2 shows the isolated PR after treatment with 0.5 M KCl to remove hsp90 and p23. When samples identical to that of lane 2 (salt stripped) were incu-

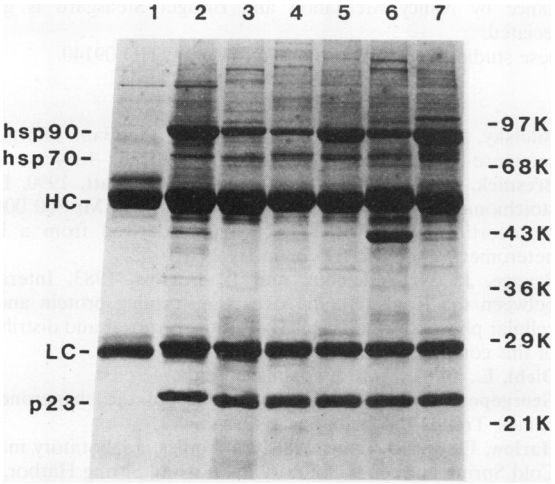

FIG. 7. Tissue and species specificities of immunoisolated p23 complexes. The antibody JJ3 was used to immunoprecipitate p23 from different tissues and species. One milliliter of tissue cytosol was used per 25-μl protein A-Sepharose pellet. Lane 2, mouse liver cytosol; lane 3, 0.5 ml of rabbit reticulocyte lysate; lane 4, chick brain cytosol; lane 5, chick spleen cytosol; lane 6, chick liver cytosol; lane 7, chick oviduct cytosol; lane 1, control immunoprecipitation from mouse liver, using PR22-bound protein A-Sepharose control. The samples were run on an 11% gel and stained with Coomassie blue. Positions of molecular mass markers are shown on the right. K, kilodalton.

bated in rabbit reticulocyte lysate at 30°C for 30 min, this results in the binding of rabbit hsp90 and p23 to the receptor, as evident in lane 4. The subsequent addition of 2×10^{-7} M progesterone promotes a partial dissociation of hsp90 and p23 (lane 5). The extent of [^{35}S]methionine-labeled p23 in these reconstituted samples was shown by autoradiography (lanes 6 to 8). The [^{35}S]methionine-labeled p23 comigrates with rabbit p23 and specifically associates with PR in the presence of reticulocyte lysate (lane 7). Further treatment with progesterone to activate the PR leads to decreased recovery of both rabbit p23 in the stained gel and human p23 in the autoradiograph (lane 8).

DISCUSSION

In this report we have identified p23 as a novel, ubiquitous, and conserved protein that associates with unactivated PR complexes. Although p23 has only been studied in detail in relation to PR complexes, it may be a component of other steroid receptors complexes, as well as other complexes containing hsp90. A 23-kDa protein associated with mouse L-cell glucocorticoid receptor (2) cross-reacts with the monoclonal antibody R3 (10), indicating that it is a related protein. The 22-kDa protein associated with bovine estrogen receptor (24) is a likely candidate for a p23 homolog, although this has not yet been tested. Recent studies with the unactivated human PR from T47D cells also show p23 copurifying with human PR (17). Despite these reports, p23 has not been recognized as a major component common to steroid receptor complexes, in part because of the lack of alternative methods for showing a relationship between p23 and steroid receptors. The demonstration that antibodies to p23 specifically coprecipitate the PR complements studies showing p23 copurifying with immunoprecipitated PR, providing further evidence that p23 is indeed a structural component of the PR complex.

p23 is a highly conserved protein that has been found in every tissue tested to date. The high amino acid homology (96.3%) between chicken and human p23 equals that of hsp90, which shows 96% identity at the amino acid level between chicken and human forms (8). p23 is not one of the major heat shock proteins, and its sequence shows no relatedness to those of the family of 22- to 28-kDa heat shock proteins (15). Incubation of minced chick oviduct at 43°C for 1 h produces a substantial increase in the synthesis ([^{35}S]methionine labeling)

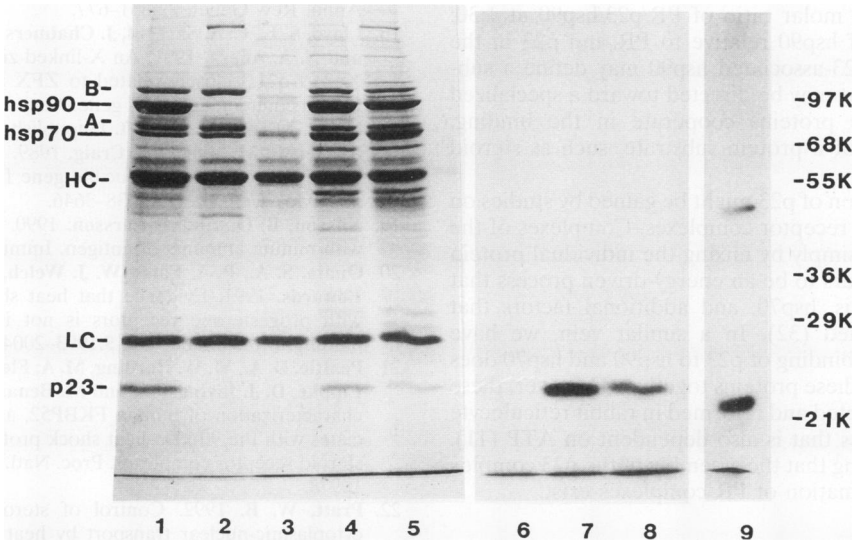

FIG. 8. Reassociation of p23 with PR complexes. [^{35}S]methionine-labeled p23 was transcribed and translated from the Hp23C clone in vitro and assayed for its ability to associate with PR. Samples were run on an 11% gel and stained with Coomassie blue. Lane 1, native PR complex immunoisolated from chicken oviduct cytosol; lane 2, PR stripped of associated proteins by high salt; lanes 3 to 5, rabbit reticulocyte lysate containing [^{35}S]methionine-labeled p23 was added to salt-stripped PR; lane 4, PR reconstitution in rabbit reticulocyte lysate; lane 5, PR reconstitution followed by treatment with progesterone; lane 3, mock reconstitution (resin without PR); lanes 6 to 8, autoradiographs of lanes 3 to 5, respectively; lane 9, autoradiograph of the total translation mixture. Positions of molecular mass markers are shown on the right. K, kilodalton.

of hsp90, hsp70, and hsp24, with no increase in the synthesis of p23 (data not shown).

The common identity of p23 in receptor complexes and in hsp90 complexes and the protein encoded by the cloned p23 cDNA was demonstrated by uniform recognition by monoclonal antibodies prepared against p23 from chick oviduct or from bacterially expressed protein. Also, p23 synthesized in vitro was able to bind to PR and to partially dissociate from the receptor complex upon progesterone treatment. hsp70 and hsp90 appear to bind directly to the PR (12, 28), but it is not known whether p54, p50, and p23 bind to the PR through hsp90 or hsp70 or whether they bind to the receptor itself. From the present work, it appears that most p23 in oviduct cytosol is bound to hsp90, suggesting that p23 is binding to the receptor in conjunction with hsp90. This does not rule out direct binding of p23 to the PR.

While it seems likely that p23 is a common component of steroid receptors, this has not yet been established. The function of this protein is completely unknown. No clues are provided from its sequence, which lacks any identity to known proteins. The only common functional motifs present in p23 are multiple potential phosphorylation sites. Although p23 is readily labeled with $^{32}P_i$ in rabbit reticulocyte lysate (4), it has not yet been determined whether the phosphorylation state of p23 is important for binding to hsp90 or the PR. We have found that the simple treatment of extracts with phosphatase or phosphatase inhibitors does not affect the appearance of p23 in PR complexes (11).

p23 appears to be structurally unrelated to the two major classes of proteins previously shown to bind steroid receptors, those being heat shock proteins (22, 34) and immunophilins (14, 23, 36, 39). However, the interaction of p23 with hsp90 and hsp70 observed in a variety of tissues suggests that its function relates to these two proteins that are believed to act as chaperones in the folding and processing of proteins (5, 7). In many tissues, p23 appears to be almost totally bound to hsp90. On the basis of immunodepletion studies in chick oviduct cytosol (data not shown), we estimate the concentration of p23 in the cytosol to be 5 to 7 μg/ml. By using estimated levels of receptor (1 μg/ml) and hsp90 (150 μg/ml), this allows the rough estimate for the molar ratio of PR/p23/hsp90 at 1:30:175. The abundance of hsp90 relative to PR and p23 in the cytosol suggests that p23-associated hsp90 may define a subpopulation of hsp90 that may be directed toward a specialized function in which the proteins cooperate in the binding, folding, or processing of a protein substrate, such as steroid receptors.

Clues as to the function of p23 might be gained by studies on the assembly of steroid receptor complexes. Complexes of the avian PR do not form simply by mixing the individual protein components. This appears to be an energy-driven process that requires ATP hydrolysis, hsp70, and additional factors that have not been identified (32). In a similar vein, we have recently found that the binding of p23 to hsp90 and hsp70 does not occur upon mixing these proteins together. However, these complexes can be disrupted and reformed in rabbit reticulocyte lysate through a process that is also dependent on ATP (11). Thus, parallels suggesting that the assembly of the p23 complex is a key step in the formation of PR complexes exist.

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