MOLECULAR AND CELLULAR BIOLOGY, Feb. 1993, p. 869–876
0270-7306/93/020869-08$02.00/0

Vol. 13, No. 2

Identification of a 60-Kilodalton Stress-Related Protein, p60, Which Interacts with hsp90 and hsp70

DAVID F. SMITH,[1] WILLIAM P. SULLIVAN,[2] TONY N. MARION,[3] KIYOSHI ZAITSU,[4] BENJAMIN MADDEN,[2] DANIEL J. McCORMICK,[2] AND DAVID O. TOFT[2]*

Department of Pharmacology, University of Nebraska Medical Center, Omaha, Nebraska 68198-6260[1]; Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota 55905[2]; Department of Microbiology and Immunology, University of Tennessee Medical Center, Memphis, Tennessee 36163[3]; and Kyushu University, 62 Maidashi, Higashi-ku, Fukuoka, Japan 812[4]

Received 16 September 1992/Returned for modification 8 October 1992/Accepted 23 October 1992

Immunoaffinity purification of hsp90 from chick oviduct cytosol reveals two major proteins, hsp70 and a 60-kDa protein (p60), copurifying with hsp90. A similar result is obtained when hsp90 is immunoaffinity purified from chick liver and brain cytosols, avian fibroblasts, and rabbit reticulocyte lysate. This p60 is the same protein previously identified in certain assembly complexes of chick progesterone receptor generated in a cell-free reconstitution system. Tryptic and cyanogen bromide peptide fragments were generated from gel-purified p60, and partial N-terminal sequences were determined from eight peptides. The sequences show a striking similarity to the sequence of a 63-kDa human protein (IEF SSP 3521) whose abundance is increased in MRC-5 fibroblasts following simian virus 40 transformation. A monoclonal antibody was prepared against avian p60; Western immunoblot analysis showed that p60 was present in each of eight chick tissues examined and in each of the human, rat, rabbit, and *Xenopus* tissues tested. Immunoaffinity purifications from both chick oviduct cytosol and rabbit reticulocyte lysate using anti-p60 and anti-hsp70 monoclonal antibodies confirm that there is a relatively abundant complex in these extracts containing hsp90, hsp70, and p60. This complex appears to comprise an important functional unit in the assembly of progesterone receptor complexes. However, judging from the abundance and widespread occurrence of this multiprotein complex, hsp90, hsp70, and p60 probably function interactively in other systems as well.

Numerous recent studies (2, 7, 8, 10, 34, 49, 54) have shown that one of the major heat shock proteins, hsp70, functions in an ATP-dependent manner through transient interactions to mediate folding or unfolding of polypeptide chains. Another major heat shock protein, hsp90, is thought to perhaps also function in some capacity related to folding or protein-protein interactions, but its function(s) remains poorly defined (1). Supporting its potential role in protein folding is a recent demonstration that hsp90 enhances renaturation of some proteins in vitro (52). Perhaps the most widely studied interaction of hsp90 is its identity as a stable component of several unactivated steroid receptor complexes (6, 38, 41). For glucocorticoid receptors, hsp90 binding to receptor is required to maintain high-affinity ligand binding (3, 26, 40), but other steroid receptors that have been examined do not show this same dependency on hsp90. In all hsp90-nuclear receptor complexes, ligand-dependent activation of the receptor DNA-binding ability is accompanied by dissociation of hsp90 (14, 17, 31, 43), and it appears likely that one hsp90 function is to repress DNA binding by receptor.

Steroid receptor-hsp90 interactions provide a model for understanding hsp90 function, but exploiting this model has been hindered by the inability to reversibly assemble receptor-hsp90 complexes in vitro. This drawback was recently overcome by establishing certain physicochemical conditions that permit the use of rabbit reticulocyte lysate as a cell-free medium for assembly of hsp90 with chick progesterone receptor (PR) (44, 45) and rat glucocorticoid receptor (40). By analyzing the in vitro assembly process of hsp90 with chick PR, we found evidence that hsp70 and a 60-kDa protein (p60) serve a transient role in establishing PR-hsp90 binding. Furthermore, preliminary evidence suggested that reticulocyte lysate contained a relatively abundant, preexisting complex between hsp90, hsp70, and p60.

In this report, we examine more closely the interaction of hsp90 with hsp70 and p60 and present evidence that p60 is a highly conserved and ubiquitous protein having sequence identity with a 63-kDa human protein that is sensitive to simian virus 40 transformation (15) and is related to the yeast heat shock-responsive STI1 gene product (27).

MATERIALS AND METHODS

Preparation of tissue and cell extracts. Oviducts were collected from estrogen-stimulated chicks and homogenized in 4 volumes of homogenization buffer (PTg; 50 mM potassium phosphate [pH 7.0], 10 mM monothioglycerol). Homogenates were centrifuged at 20,000 × *g* for 20 min; the supernatant was collected and centrifuged at 100,000 × *g* for 1 h. The high-speed supernatant was collected as the cytosol. Chick and *Xenopus* liver extracts were prepared similarly. Small-scale tissue extracts from chick lung, brain, kidney, spleen, heart, and breast muscle were prepared by homogenization in a glass-glass homogenizer followed by centrifugation at 18,000 × *g* in a microcentrifuge for 30 min. Whole cell extracts were prepared by suspending a cell pellet in 10 volumes of sodium dodecyl sulfate (SDS) sample buffer; the suspension was disrupted by forcing repeatedly through a 20-gauge needle. The cell extract was placed in a boiling water bath for 5 min and then spun in a microcentrifuge for 5 min to pellet insoluble material.

Tissue and cell sources. Rabbit reticulocyte lysate was

* Corresponding author.

obtained from Promega. *Xenopus* liver was obtained from Amy Smithgall. Rat liver extract was obtained from Patrick Iverson. MCF7, GH4, and CCL cells were obtained from James Shull.

Antibody reagents. Several mouse monoclonal immunoglobulin G (IgG) antibodies against hsp90 were used. D7α was prepared against hsp90 from chicken brain (5); D7α also cross-reacts with native rabbit hsp90. 4F3 was prepared against hsp90 from chicken oviduct (47); it reacts poorly with mammalian hsp90. AC88 (StressGen Biotechnology Corp.) was prepared against hsp90 from the water mold *Achlya ambisexualis* and cross-reacts with hsp90 from a variety of vertebrate species (32). Antibody N27 (N27F3-4; StressGen) was prepared against human hsp70 by W. J. Welch (2, 17).

Antibody DS14F5 is a mouse monoclonal IgG prepared against gel-purified avian p60. (The procedure for gel purification is described below.) BALB/c mice were given primary immunizations containing approximately 50 μg of p60 suspended in 100 μl of H_2O plus 100 μl of complete Freund's adjuvant. A secondary immunization, given after 3 weeks, consisted of 50 μg of p60 in incomplete adjuvant. The final immunization consisted of 50 μg of p60 in phosphate-buffered saline (PBS). Spleen cell fusions were performed 3 days after the final immunization according to a previously published protocol (23). The plasmacytoma line used was P3x63-Ag8.653 (16).

Immune sera and conditioned media from hybridomas were screened initially by Western immunoblot analysis against whole oviduct cytosol, using multiwell immunoblot devices (Immunetics MN45). Positive media were further screened for cross-reactivity with mammalian antigen by Western analysis of rabbit reticulocyte lysate. Positive media were also screened for the ability to recognize native p60 by indirect immunoprecipitation from oviduct cytosol, using protein G-Sepharose (Pharmacia). Monoclonal hybridomas producing specific antibody against p60 were obtained by limiting-dilution cloning of culture wells demonstrated to have specific antibody-producing hybrids.

Antibody BB70 is a mouse monoclonal IgG prepared against avian hsp70 complexed with hsp90. The hsp90 protein complexes were isolated from chick oviduct cytosol with antibody D7α:protein A. After washing, the resin-bound proteins (100 μg) were mixed with an equal volume of adjuvant and injected subcutaneously. Freund's complete adjuvant was used for primary injection, whereas Freund's incomplete adjuvant was used for secondary injections. For the final injection, the resin was resuspended in an equal volume of PBS and injected intraperitoneally. Splenocytes were prepared and fusion was accomplished by standard procedures (12), using cells of the myeloma cell line SpZ/O-Ag14 as fusion partners. Hybridomas were selected by culture in hypoxanthine-aminopterin-thymidine, and supernatants were screened by Western blots against antigen preparations.

Immunopurification of hsp90 and associated proteins. Immunoaffinity resin was prepared by incubating antibody with either protein G-Sepharose or protein A-Sepharose (Pharmacia). In some cases, immunoaffinity resins were prepared by covalently linking D7α and AC88 to Actigel (Sterogene, San Gabriel, Calif.) according to the manufacturer's suggestions, using 10 mg of antibody per ml of resin.

Immunoaffinity resin was added to cytosols prepared in PTg and incubated for 1 h on ice with occasional tube inversions. Typical proportions were 40 μg of antibody plus 50 μl of protein A-Sepharose or 50 μl of covalently coupled antibody resin added to 0.5 ml of cytosol. Resins were washed five times with 15 volumes of PTg. Bound proteins were extracted directly into either SDS or urea sample buffer.

Electrophoresis, electroelution, and Western blotting. SDS-polyacrylamide gel electrophoresis (PAGE) (19) was performed by using gels containing 8 to 10% acrylamide. Two-dimensional (2D) PAGE was performed essentially as described by O'Farrell (29); isoelectric focusing gels contained 2% pH 3.5 to 10 ampholines (Pharmacia LKB). Gels were stained by either Coomassie brilliant blue R-250 or silver nitrate (13). For SDS-PAGE, proteins bound to immunoaffinity resin were extracted into SDS sample buffer containing 2% SDS and 5% mercaptoethanol. For 2D PAGE, resins were extracted into urea sample buffer containing 9.5 M urea, 2% Triton X-100, 2% ampholines, and 5% mercaptoethanol.

Gel-purified p60 was prepared by using D7α to purify hsp90 complexes containing p60 from oviduct cytosol. After separation by SDS-PAGE, the p60 band could be visualized in the gels by light diffraction without fixing and staining; the p60 band was excised from the gel and sliced into pieces approximately 1 by 2 by 5 mm. Electroelution of protein from the gel pieces was performed by using a Six-Pac Electroeluter (Hoefer Scientific Instruments) according to the manufacturer's recommendations. Briefly, gel pieces in 300 μl of Laemmli buffer were eluted into 120 μl of 4× Laemmli buffer at 80 V for 2 h. For immunizations and cyanogen bromide digestion, eluates were pooled and dialyzed against 5 mM NH_4HCO_3 plus 0.02% SDS and then dried by vacuum centrifugation. For trypsin digestion, eluted p60 was collected by methanol-chloroform precipitation (50).

Procedures for Western blot analyses using a second antibody-alkaline phosphatase conjugate were the same as published previously (17) except that gels were electroblotted to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore).

Gel and blot images used for figures were produced either by normal photographic methods or by densitometric scanning of media with a Molecular Dynamics personal densitometer. Scanned images in a digitized format were handled by using image-processing software (Picture Publisher; Micrographx) and printed on a Hewlett Packard LaserJet III equipped with a LaserPix 4.0 board (XLI Corp., Woburn, Mass.).

p60 peptide generation and sequence analysis. For trypsin digestion, gel-purified p60 was dissolved in 100 μl of 0.4 M NH_4HCO_3–8 M urea and 50 μl of 50% acetonitrile. By using the method of Stone et al. (46), the protein was reduced, carboxymethylated, and then digested with trypsin for 16 h at room temperature. The resulting tryptic peptides were separated by reverse-phase high-pressure liquid chromatography (HPLC), using a Vydac C_{18} column (The Separations Group, Hesperia, Calif.) and trifluoroacetic acid-acetonitrile solutions. For cyanogen bromide digestion, the procedure of LeGendre and Matsudaira was used (22). Briefly, immunoaffinity-purified hsp90 complexes containing p60 were reduced and alkylated prior to separation on SDS-gels. p60 was electroeluted, dialyzed, and dried as described above and then resuspended in 70% formic acid containing 200 μg of cyanogen bromide. The sample was digested under N_2 overnight at room temperature and then dried by vacuum centrifugation. Protein fragments were separated on an SDS-gel by the procedure of Schagger and von Jagow (39) and electrotransferred to a PVDF membrane (Problott; Applied Biosystems, Inc.). Peptide fragments were visual-

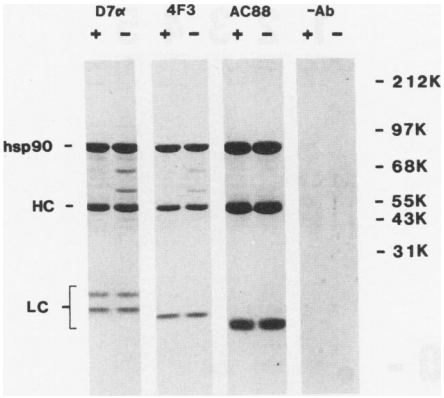

FIG. 1. Immunoaffinity purification of hsp90 from low- and high-ionic-strength oviduct cytosol. hsp90 purifications were performed on oviduct cytosol, using a mouse MAb bound to protein A-Sepharose. Purifications were performed in the presence of 0.3 M KCl (lanes labeled +) or with no added KCl (lanes labeled −). The antibodies used are indicated above each pair of lanes. Purification was also performed by using protein A-Sepharose lacking antibody (−Ab). The migration positions of hsp90 and of antibody heavy (HC) and light (LC) chains are labeled on the left. Molecular weight markers are indicated on the right.

ized by Coomassie blue staining, and fragment bands were excised and subjected to sequence analysis. The separated fragments were sequenced from the amino terminus by using either a Porton 2090E Integrated Microsequencing System (Porton Instruments Inc., Tarzana, Calif.) or an ABI 470A/120A Microsequencer (Applied Biosystems Inc., Foster City, Calif.). Both instruments perform automated gas-phase Edman degradation and identify the resulting phenylthiohydantoin amino acids, using on-line reverse-phase HPLC with narrow-bore C_{18} columns. The data from both instruments were collected into an Everex 286/12 computer and analyzed by using the Porton Instruments protein sequence analysis software.

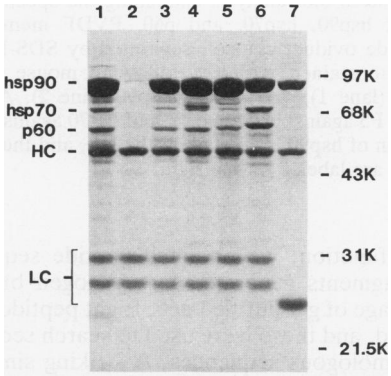

FIG. 2. Major proteins copurifying with hsp90 from several tissues. hsp90 was purified from crude tissue or cell extracts by using antibody D7α (lanes 1 to 6) or antibody AC88 (lane 7) covalently attached to an agarose resin. Extracts were prepared from the following sources: chick oviduct (lanes 1, 2, and 7), chick brain (lane 3), chick liver (lane 4), QT6 quail fibroblasts (lane 5), and rabbit reticulocyte lysate (lane 6). In one sample (lane 2), oviduct cytosol was pretreated with soluble D7α to block immunospecific binding to covalently coupled antibody resin. Samples were separated by SDS-PAGE and stained with Coomassie brilliant blue dye. Major protein bands (see legend to Fig. 1) are identified on the left; molecular weight markers are indicated on the right.

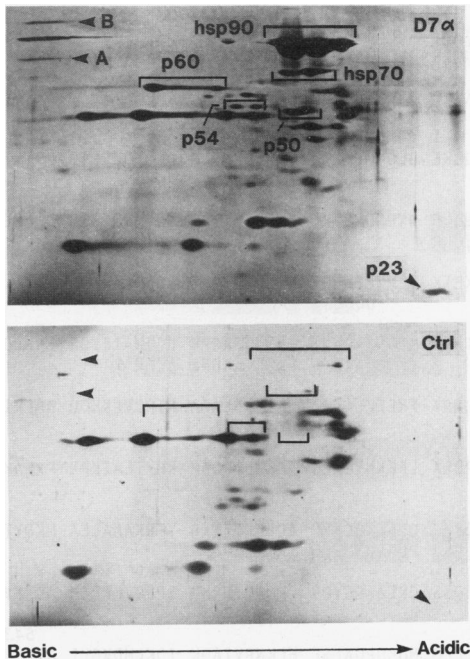

FIG. 3. 2D PAGE of proteins copurifying with hsp90. Using D7α covalently attached to an agarose resin, hsp90 was immunopurified from oviduct cytosol (a) or from oviduct cytosol pretreated with soluble D7α (b) to block immunospecific binding to covalently coupled antibody resin. Purified proteins from 170 μl of cytosol were separated by 2D PAGE and silver stained. Migration positions for hsp90 and major proteins that copurify in an immunospecific manner are labeled in panel a. A and B identify the two forms of PR.

RESULTS

In this study, complexes containing hsp90 were isolated by adsorption to antibody resins. Three different monoclonal antibodies (MAbs) recognizing hsp90 were used to immunoprecipitate hsp90 from chick oviduct cytosol (Fig. 1). At elevated ionic strength, only hsp90 was purified by antibody resins; however, at lower ionic strength, two of the resin precipitates (D7α and 4F3) contained proteins in addition to hsp90, suggesting that these antibodies recognize hsp90 in complexes with other proteins. By comparison, AC88 precipitated only hsp90 from both low- and high-ionic-strength cytosol. These results are consistent with earlier observations (32, 41, 47) that AC88 recognizes only free hsp90, whereas D7α and 4F3 can bind to hsp90 in PR complexes.

The gel images in Fig. 1 show two proteins that copurify with hsp90, one of which has been identified by Western blot analysis (45) as hsp70; the other has the mobility of a 60- to 64-kDa protein (p60). The dark bands around 55 and 25 kDa in each of the gel lanes are antibody heavy and light chains.

When D7α is used to immunoprecipitate hsp90 complexes from tissues other than oviduct, a similar pattern is seen (Fig. 2). Extracts from chick brain and liver, quail fibroblasts, and even rabbit reticulocyte lysate each yield hsp70 and p60 as the most abundant proteins copurifying with hsp90. Rabbit reticulocyte lysate was examined here because it has been used as the cell-free medium for studying assembly of PR complexes (44, 45). In those assembly

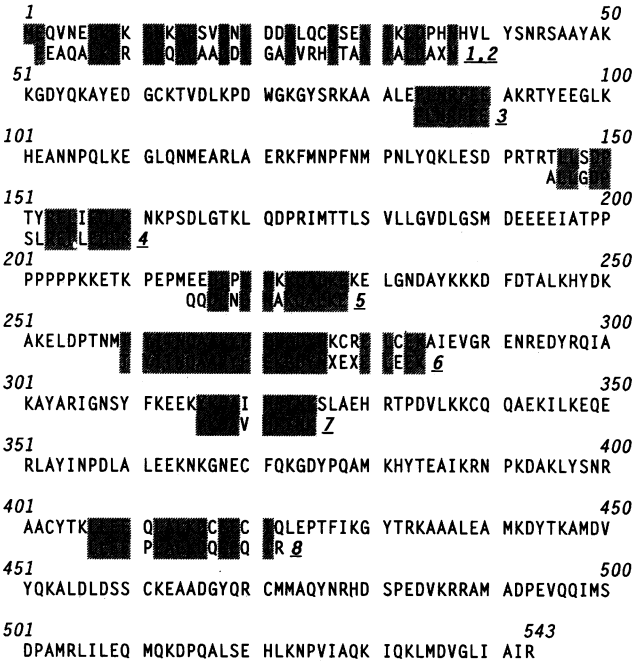

FIG. 4. Sequence comparison of p60 peptides with the 63-kDa human transformation-sensitive protein. Gel-purified p60 was digested with trypsin or cyanogen bromide, and fragments were separated and sequenced as detailed in Materials and Methods. In comparing the sequences obtained with sequence data bases, a striking similarity was observed with a recently cloned gene (15) coding for a 63-kDa protein from human MRC-5 fibroblasts whose abundance increases in response to simian virus 40 transformation. The published sequence for the 63-kDa protein is shown here aligned with p60 peptide sequences, with sequence identities indicated by shading. Avian peptide sequences are numbered sequentially from the amino terminus. Peptides 1, 5, and 6 were obtained as cyanogen bromide fragments, and the others were obtained as trypsin fragments. Note that peptides 1 (residues 2 to 34) and 2 (residues 25 to 37) overlapped and are combined as one sequence. X indicates a residue whose determination was ambiguous.

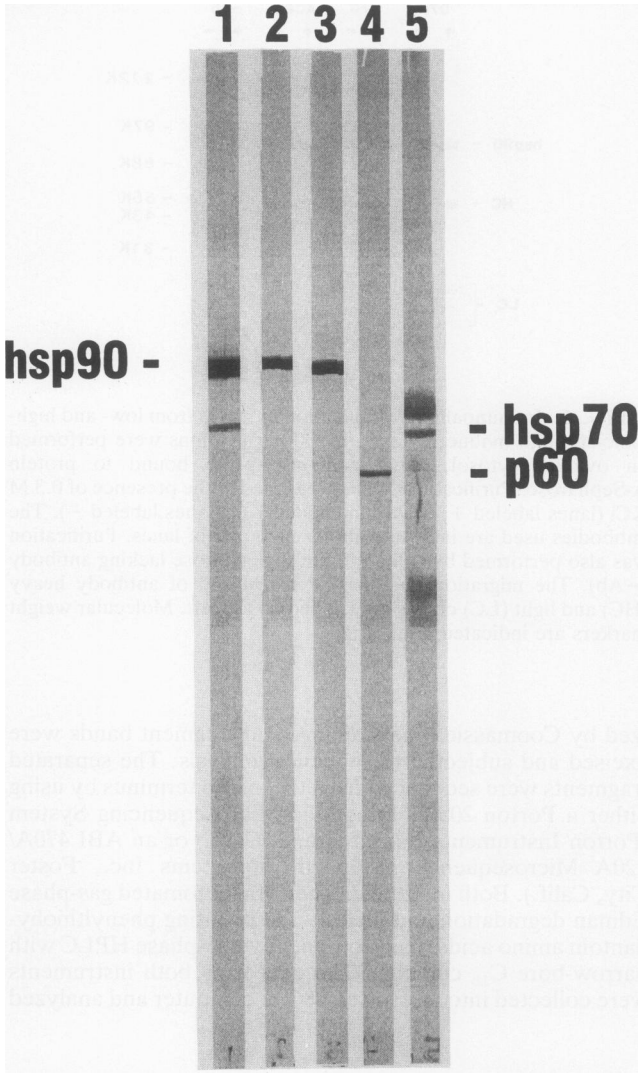

FIG. 5. Western blot analysis examining the specificity of antibodies against hsp90, hsp70, and p60. PVDF membrane strips containing crude oviduct cytosol, separated by SDS-PAGE, were Western immunostained with the following mouse MAbs: D7α against hsp90 (lane 1), 4F3 against hsp90 (lane 2), AC88 against hsp90 (lane 3), F5 against p60 (lane 4), and BB70 against hsp70 (lane 5). The position of hsp90 is labeled on the left, and the positions of hsp70 and p60 are labeled on the right.

studies, there was evidence that p60 plays a role in the binding of hsp90 to PR, though p60 was not identified as a component in purified, unactivated PR complexes from oviduct cytosol (43, 45).

Although hsp70 and p60 are the most abundant copurifying proteins in the gel lanes of Fig. 1 and 2, there are other, less abundant proteins that copurify with hsp90. By separating D7α immunoprecipitates from oviduct cytosol on 2D gels and silver staining, some of these other proteins are evident (Fig. 3). Among these are PR forms A and B, which do not enter the isoelectrofocusing gel, and proteins that we have previously identified (43) as components of PR complexes (p54, p50, and p23). A few minor, unidentified protein spots can also be seen in Fig. 3a that are absent from Fig. 3b. Judging from these data and from studies in other laboratories on hsp90 interactions (4, 18, 24, 33), it is likely that additional proteins less abundant than the identified spots in Fig. 3 associate with hsp90.

Again, the most abundant copurifying proteins are hsp70 and p60. hsp70 resolves as a doublet, which is typical for constitutively expressed members of the hsp70 gene family (also referred to as hsc70, or the heat shock cognate protein). The pI of p60 is around pH 6.5, but charge heterogeneity is apparent. As one approach to identifying p60 and understanding its function, we obtained peptide sequence data from p60 fragments generated by cyanogen bromide and trypsin cleavage of gel-purified p60. Eight peptide sequences were obtained, and these were used to search sequence data bases for homologous sequences. A striking similarity was discovered between the avian p60 sequences and the sequence reported recently by Honoré et al. (15) for cDNA coding for a 63-kDa human protein termed IEF SSP 3521; this protein is up-regulated in simian virus 40-transformed fibroblasts. As pointed out in that report, the human 63-kDa protein has significant homology to the yeast STI1 gene product, a protein reported by Nicolet and Craig (27) to be a stress-inducible mediator of the heat shock response. The sequences of the human protein and of the corresponding chicken peptides are shown in Fig. 4. The extent of identity among the eight peptides ranges from 47% (peptides 1 and 2)

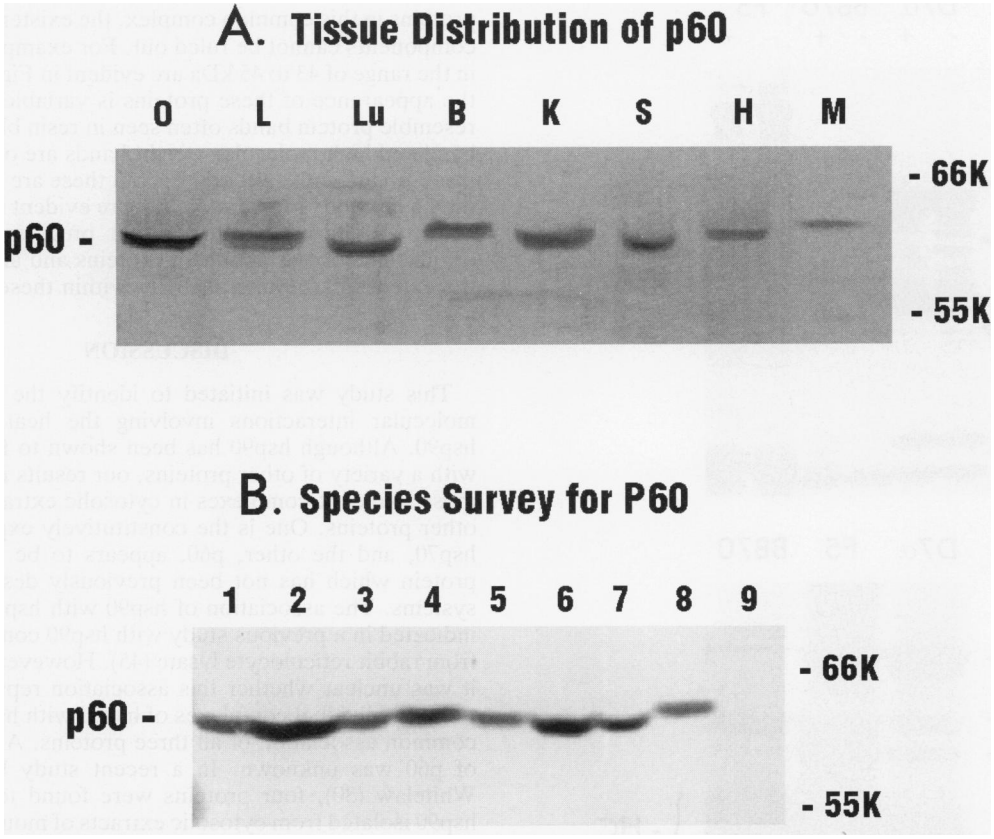

FIG. 6. (A) Avian tissue survey for p60, using MAb F5. Crude cytosolic extracts were prepared from eight different chick tissues, separated by SDS-PAGE, and transferred to a PVDF membrane. The membrane was immunostained with MAb F5 prepared against protein p60 from oviduct. Tissues surveyed: O, oviduct; L, liver; Lu, lung; B, brain; K, kidney; S, spleen; H, heart; M, breast muscle. Only the region of the immunostained membrane containing reactive bands is shown. Molecular weight markers are indicated on the right. (B) Species survey for antigens cross-reactive with MAb F5. Crude cytosolic or total cell extracts were prepared, separated by SDS-PAGE, transferred to a PVDF membrane, and then immunostained with MAb F5 against avian p60. Samples surveyed: 1, chick oviduct cytosol; 2, human HeLa cell extract; 3, human MCF7 cell extract; 4, rabbit reticulocyte lysate; 5, rat liver cytosol; 6, rat GH4 cell extract; 7, mink CCL cell extract; 8, *Xenopus* liver cytosol; 9, *S. cerevisiae* cell extract. Migration positions for prestained molecular weight markers are indicated on the left.

to 100% (peptide 3). Overall, the extent of identity between the p60 peptides and the human protein is 70%. Thus, p60 is clearly related to the human protein and probably represents the avian homolog of this protein.

We have developed a MAb (DS14F5, or simply F5) against gel-purified avian p60. This antibody has been used in preliminary studies to characterize the distribution of p60 and its interaction with hsp90 and hsp70. An additional MAb (BB70) was developed against hsp70. The specificities of these antibodies and antibodies to hsp90 were examined by Western analysis against crude oviduct cytosol (Fig. 5). In addition to its interaction with hsp90, antibody D7α recognizes a minor protein band of 75 kDa. This protein is unrelated to hsp70 or p60, and it is believed to be a degradation product of hsp90. Antibody F5 reacts with a single 60-kDa band, but BB70 exhibits reactivity with two bands, the lower of which corresponds to hsp70, as identified by reaction with another hsp70 antibody, N27. Although BB70 reacts with denatured, non-hsp70 antigens (diffuse bands above and below the indicated hsp70 band in Fig. 5) on Western blots of crude tissue extracts, this antibody displays specificity for native proteins by reacting only with hsp70 in indirect immunoaffinity precipitation from crude cytosol.

As shown in Fig. 6A, p60 was present in extracts from eight different chick tissues, as determined by Western analysis using MAb F5. Though slightly different mobilities for the antigen are evident in Fig. 6A, such small differences could be artifacts due to electrophoretic influences of other components in the individual tissue extracts. However, we cannot rule out that there may be tissue-specific heterogeneity in p60 forms.

Antibody F5 was also used to survey tissue extracts from various species (Fig. 6B). The p60 antigen is present in human, rat, chicken, and *Xenopus* extracts. No cross-reacting antigen was detected in *Saccharomyces cerevisiae* extract, but genetic data have shown that the STI1 gene product in yeast cells has 42% sequence identity to a human homolog of p60. Probably, sequences in the F5 epitope of vertebrate p60 differ from the homologous region of the yeast protein.

In a closer examination of the interactions between hsp90, hsp70, and p60, we have used MAbs to separately immunoaffinity purify hsp90, hsp70, and p60 from both oviduct cytosol and rabbit reticulocyte lysate (Fig. 7). In Fig. 7A, proteins were purified from both low- and high-ionic-strength oviduct cytosol. Consistent with the results in Fig. 1, the anti-hsp90 antibody D7α coprecipitated hsp70 and p60 at low ionic strength but not at elevated ionic strength. A similar pattern of copurification is seen with antibodies

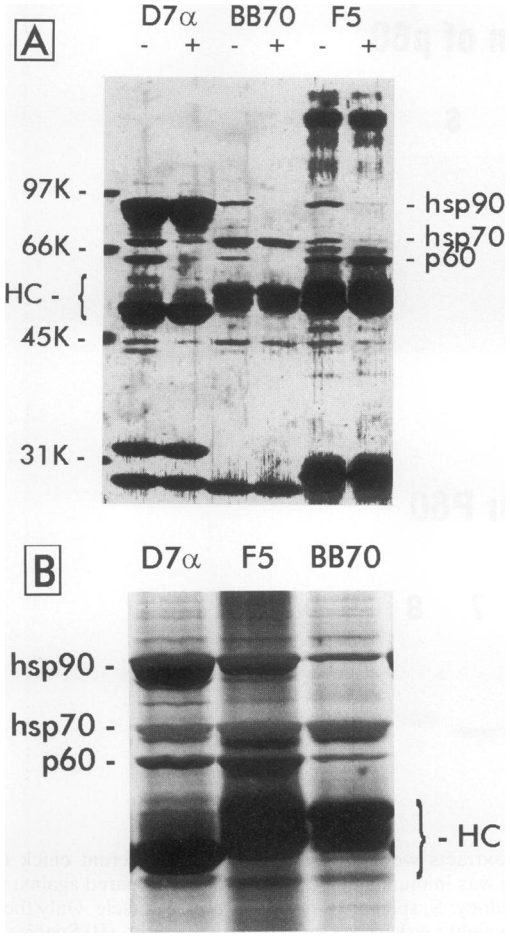

FIG. 7. (A) Immunoaffinity purification of the hsp90-hsp70-p60 protein complex from oviduct cytosol, using antibodies against individual polypeptides. Shown is a region from an SDS-gel loaded with immunopurified proteins and visualized by Coomassie staining. Samples were immunoaffinity purified from chick oviduct cytosol with no added salt (−) or adjusted to 0.4 M KCl (+). As indicated, antibodies used for purification were D7α against hsp90, BB70 against hsp70, and F5 against p60. In each lane shown, protein was purified from 0.5 ml of cytosol, using 20 to 25 μl of IgG bound to 20 μl of protein G-Sepharose. Relevant protein bands are indicated on the left; molecular weight markers and the bands corresponding to antibody heavy chains (HC) are indicated on the right. (B) Purification of the hsp90-hsp70-p60 protein complex from rabbit reticulocyte lysate. Conditions were similar to those in panel A except that proteins were purified from 0.3 ml of rabbit reticulocyte lysate without added salt. Proteins were visualized by silver staining of the SDS-gel.

against hsp70 and p60; the most abundant proteins coprecipitating with hsp70 are hsp90 and p60; likewise, the most abundant proteins coprecipitating with p60 are hsp90 and hsp70. The immunoprecipitation data from rabbit reticulocyte lysate (Fig. 7B) are similar to those seen for chicken oviduct cytosol, though the relative ratios of each protein appear to differ between the tissues. Stoichiometry of the apparent complex between hsp90, hsp70, and p60 cannot be determined from the data in Fig. 7, since it is not presently known what percentage of the total for each antibody-targeted protein is in this complex versus free protein or protein in complexes other than hsp90-hsp70-p60. It should be stressed that while these appear to be the three major proteins in this common complex, the existence of additional components cannot be ruled out. For example, two proteins in the range of 43 to 45 kDa are evident in Fig. 7A. However, the appearance of these proteins is variable, and the bands resemble protein bands often seen in resin blanks. A 66-kDa band and high-molecular-weight bands are observed in samples isolated with antibody F5, but these are contaminants in the F5 antibody preparation that are evident at high antibody loads. Additional studies are in progress to assess the significance of less abundant proteins and also to determine the extent of size heterogeneity within these complexes.

DISCUSSION

This study was initiated to identify the most prevalent molecular interactions involving the heat shock protein hsp90. Although hsp90 has been shown to form complexes with a variety of other proteins, our results indicate that the most abundant complexes in cytosolic extracts contain two other proteins. One is the constitutively expressed form of hsp70, and the other, p60, appears to be a stress-related protein which has not been previously described in avian systems. The association of hsp90 with hsp70 and p60 was indicated in a previous study with hsp90 complexes isolated from rabbit reticulocyte lysate (45). However, in that report, it was unclear whether this association represented a mixture of individual complexes of hsp90 with hsp70 or p60 or a common association of all three proteins. Also, the identity of p60 was unknown. In a recent study by Perdew and Whitelaw (30), four proteins were found to copurify with hsp90 isolated from cytosolic extracts of mouse Hepa 1 cells. These proteins included hsp70 and proteins of 63, 56, and 50 kDa. It seems likely that the 63-kDa protein observed by Perdew and Whitelaw is the mouse equivalent of p60. The p50 protein was further identified as a 50-kDa protein associated with $pp60^{v\text{-}src}$-hsp90 complexes (51), and p56 was shown to be another heat shock protein, hsp56 (35, 36), which is identical to a p59 immunophilin that binds FK 506 (21, 48, 53). p50 and p56 are not as abundant in our preparations of hsp90 complexes. They may represent separate complexes with hsp90 that are less stable, less abundant, or poorly recognized by our antibodies.

The cellular functions of hsp90 remain unclear. It has been shown to interact with steroid receptors (6, 38, 41), with a variety of protein kinases (4, 24, 25, 33), and with actin (18) and tubulin (37). Some evidence exists for its possible function as a protein kinase (9, 28), and one report indicates a molecular chaperone activity of hsp90 in protein renaturation in vitro (52).

hsp70 appears to play a role in several cellular processes that involve protein folding or unfolding. These include the folding of newly synthesized protein (2), the uncoating of clathrin-coated vesicles (7, 49), and the transport of proteins through membranes (8, 10, 54). hsp70 contains sites for peptide binding and for ATP hydrolysis that are believed to be required for its activities.

There are few indications as to the function of the third protein in this complex, p60. Previous reports of p60 association with hsp90 allowed speculation on its identity with either the receptor-associated protein, p59 (hsp56), or the common heat shock protein, hsp60, that is predominantly in mitochondria. The present sequence analysis shows there to be no relationship between p60 and these proteins. p60 is closely related to a human 63-kDa protein reported earlier (15). In addition, these two proteins have similar molecular weights and isoelectric points, as judged by 2D gel electro-

phoresis. The human protein has been reported to contain isoforms that differ in pI, and this also appears to be true for p60 from chicken (Fig. 3). While we have not yet studied p60 in stressed avian cells, the human protein is up-regulated by stress and by viral transformation of cells (15). The human protein contains several 34-residue repeats, termed TPR motifs (42), that indicate a structural relationship to a number of other proteins (15). However, its closest identity is with the yeast stress-induced protein STI1 (27), whose function is unknown. Yeast cells lacking this protein can grow at 30°C but are growth impaired at higher and lower temperatures.

The observation that hsp90, hsp70, and p60 interact suggests that they cooperate in function. This view is consistent with recent studies showing that heat shock proteins can function together to promote protein folding and assembly. For example, Gaitanaris et al. (11) have shown that the renaturation of denatured λ repressor in *Escherichia coli* is dependent on the three heat shock proteins DnaK, DnaJ, and GrpE. Another study by Langer et al. (20) presents evidence for a chaperone-mediated protein-folding pathway involving sequential actions of DnaK, DnaJ, GrpE, and GroEL/ES. In this regard, we have recently developed a cell-free system in reticulocyte lysate which allows the assembly of PR with hsp90 and hsp70 (45). This assembly appears to be quite complex and requires ATP hydrolysis in addition to other factors in reticulocyte lysate. The final receptor complex does not contain appreciable amounts of p60. However, if the source of ATP is limited, p60 is observed in the complex, suggesting that it may be part of an intermediate form. Further characterization of this protein should be facilitated by the structural identity and antibody probes described in this report.

ACKNOWLEDGMENTS

We thank Nita Maihle for providing QT6 cells, William Welch for providing antibody N27, and Joan Brugge for providing antibody D7α. We thank Ronald Corbisier and Jill Johnson for assistance in the isolation of p60. Technical assistance by Nancy McMahon and Bridget Stensgard and manuscript preparation by Sherry Linander are greatly appreciated. We thank Barbara Switzer (University of Nebraska Medical Center Antibody Core) for assistance in cloning cells for antibody F5.

These studies were supported by NIH grants HD 09140 (D.O.T.) and DK 44923 (D.F.S.).

REFERENCES

1. **Ang, D., K. Liberek, D. Skowyra, M. Zylicz, and C. Georgopoulos.** 1991. Biological role and regulation of the universally conserved heat shock proteins. J. Biol. Chem. **266**:24233–24236.
2. **Beckman, R. P., L. A. Mizzen, and W. J. Welch.** 1990. Interaction of hsp70 with newly synthesized proteins: implications for protein folding and assembly. Science **248**:850–854.
3. **Bresnick, E. H., F. C. Dalman, E. R. Sanchez, and W. B. Pratt.** 1989. Evidence that the 90-kDa heat shock protein is necessary for the steroid binding conformation of the L cell glucocorticoid receptor. J. Biol. Chem. **264**:4992–4997.
4. **Brugge, J.** 1986. Interaction of the Rous sarcoma virus protein $pp60^{src}$ with the cellular proteins pp50 and pp90. Curr. Top. Microbiol. Immunol. **123**:1–22.
5. **Brugge, J., W. Yonemoto, and D. Darrow.** 1983. Interaction between the Rous sarcoma virus transforming protein and two cellular phosphoproteins: analysis of the turnover and distribution of this complex. Mol. Cell. Biol. **3**:9–19.
6. **Catelli, M. G., N. Binart, I. Jung-Testas, J.-M. Renoir, E.-E. Baulieu, J. R. Feramisco, and W. J. Welch.** 1985. The common 90-kd protein component of non-transformed '8S' steroid receptors is a heat-shock protein. EMBO J. **4**:3131–3135.
7. **Chappell, T. G., W. J. Welch, D. M. Schlossman, K. B. Palter, M. J. Schlesinger, and J. E. Rothman.** 1986. Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. Cell **45**:3–13.
8. **Chirico, W. J., M. G. Waters, and G. Blobel.** 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. Nature (London) **332**:805–810.
9. **Csermely, P., and C. R. Kahn.** 1991. The 90-kDa heat shock protein (hsp-90) possesses an ATP binding site and autophosphorylating activity. J. Biol. Chem. **266**:4943–4950.
10. **Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman.** 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature (London) **332**:800–805.
11. **Gaitanaris, G. A., A. G. Papavassiliou, P. Rubock, S. J. Silverstein, and M. E. Gottesman.** 1990. Renaturation of denatured λ repressor requires heat shock proteins. Cell **6**:1013–1020.
12. **Harlow, E., and D. P. Lane.** 1988. Antibodies: a laboratory manual, p. 208–211. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Heukeshoven, J., and R. Dernick.** 1985. Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis **6**:103–112.
14. **Holbrook, N. J., J. E. Bodwell, M. Jeffries, and A. Munck.** 1983. Characterization of nonactivated and activated glucocorticoid-receptor complexes from intact rat thymus cells. J. Biol. Chem. **258**:6477–6485.
15. **Honoré, B., H. Leffers, P. Madsen, H. H. Rasmussen, J. Vanderkerckhove, and J. E. Celis.** 1992. Molecular cloning and expression of a transformation-sensitive human protein containing the TPR motif and sharing identity to the stress-inducible yeast protein STI1. J. Biol. Chem. **267**:8485–8491.
16. **Kearney, J. F., A. Radbruch, B. Liesegang, and K. Rajewsky.** 1979. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cells. J. Immunol. **123**:1548.
17. **Kost, S. L., D. F. Smith, W. P. Sullivan, W. J. Welch, and D. O. Toft.** 1989. Binding of heat shock proteins to the avian progesterone receptor. Mol. Cell. Biol. **9**:3829–3838.
18. **Koyasu, S., E. Nishida, T. Kadowaki, F. Matsuzaki, K. Iida, F. Harada, M. Kasuga, H. Sakai, and I. Yahara.** 1986. Two mammalian heat shock proteins, hsp90 and hsp100, are actin-binding proteins. Proc. Natl. Acad. Sci. USA **83**:8054–8058.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Langer, T., C. Lu, H. Echols, J. Flanagan, M. K. Hayer, and F. U. Hartl.** 1992. Successive action of DnaK, DnaJ, and GroEL along the pathway of chaperone-mediated protein folding. Nature (London) **356**:683–689.
21. **Lebeau, M.-C., N. Massol, J. Herrick, L. E. Faber, J.-M. Renoir, C. Radanyi, and E.-E. Baulieu.** 1992. P59, an hsp 90-binding protein. J. Biol. Chem. **267**:4281–4284.
22. **LeGendre, N., and P. Matsudaira.** 1989. Purification of proteins and peptides by SDS-PAGE, p. 49–69. *In* P. T. Matsudaira (ed.), A practical guide to protein and peptide purification for microsequencing. Academic Press, San Diego, Calif.
23. **Marion, T. N., and D. E. Briles.** 1981. Analysis of autoimmune DNA antibody responses using somatic cell hybridization, p. 251–258. *In* G. J. Hammerling and J. F. Kearney (ed.), Monoclonal antibodies and t cell hybridomas. Elsevier/North-Holland, Amsterdam.
24. **Matts, R. L., and R. Hurst.** 1989. Evidence for the association of the heme-regulated eIF-2α kinase with the 90-kDa heat shock protein in a rabbit reticulocyte lysate *in situ*. J. Biol. Chem. **264**:15542–15547.
25. **Miyata, Y., and I. Yahara.** 1992. The 90-kDa heat shock protein, HSP90, binds and protects casein kinase II from self-aggregation and enhances its kinase activity. J. Biol. Chem. **267**:7042–7047.
26. **Nemoto, T., G. G. F. Mason, A. Wilhelmsson, S. Cuthill, J. Hapgood, J.-A. Gustafsson, and L. Poellinger.** 1990. Activation of the dioxin and glucocorticoid receptors to a DNA binding

state under cell-free conditions. J. Biol. Chem. **265:**2269–2277.
27. **Nicolet, C. M., and E. A. Craig.** 1989. Isolation and characterization of STI1, a stress-inducible gene from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9:**3638–3646.
28. **Nygard, O., A. Nilsson, U. Carlberg, L. Nilsson, and R. Amons.** 1991. Phosphorylation regulates the activity of the eEF-2-specific Ca^{2+}- and calmodulin-dependent protein kinase III. J. Biol. Chem. **266:**16425–16430.
29. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
30. **Perdew, G. H., and M. L. Whitelaw.** 1991. Evidence that the 90-kDa heat shock protein (hsp90) exists in cytosol in heteromeric complexes containing hsp70 and three other proteins with M_r of 63,000, 56,000, and 50,000. J. Biol. Chem. **266:**6708–6713.
31. **Raaka, B. M., M. Finnerty, E. Sun, and H. H. Samuels.** 1985. Effects of molybdate on steroid receptors in intact GH_1 cells. J. Biol. Chem. **260:**14009–14015.
32. **Riehl, R. M., W. P. Sullivan, B. T. Vroman, V. J. Bauer, G. R. Pearson, and D. O. Toft.** 1985. Immunological evidence that the nonhormone binding component of avian steroid receptors exists in a wide range of tissues and species. Biochemistry **24:**6586–6591.
33. **Rose, D. W., R. E. H. Wettenhall, W. Kudlicki, G. Kramer, and B. Hardesty.** 1987. The 90-kilodalton peptide of the heme-regulated eIF-2α kinase has sequence similarity with the 90-kilodalton heat shock protein. Biochemistry **26:**6583–6587.
34. **Rothman, J. E.** 1989. Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell **59:**591–601.
35. **Sanchez, E. R.** 1990. Hsp56: a novel heat shock protein associated with untransformed steroid receptor complexes. J. Biol. Chem. **265:**22067–22070.
36. **Sanchez, E. R., L. E. Faber, W. J. Henzel, and W. B. Pratt.** 1990. The 56-59 kilodalton protein identified in untransformed steroid receptor complexes is a unique protein that exists in cytosol in a complex with both the 70- and 90-kilodalton heat shock proteins. Biochemistry **29:**5145–5152.
37. **Sanchez, E. R., T. Redmond, L. C. Scherrer, E. H. Bresnick, M. J. Welsh, and W. B. Pratt.** 1988. Evidence that the 90-kilodalton heat shock protein is associated with tubulin-containing complexes in L cell cytosol and in intact PtK cells. Mol. Endocrinol. **2:**756–760.
38. **Sanchez, E. R., D. O. Toft, M. J. Schlesinger, and W. B. Pratt.** 1985. Evidence that the 90-kDa phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat shock protein. J. Biol. Chem. **260:**12398–12401.
39. **Schagger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl-sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range of 1 to 100 kDa. Anal. Biochem. **166:**368–379.
40. **Scherrer, L. C., F. C. Dalman, E. Massa, S. Meshinchi, and W. B. Pratt.** 1990. Structural and functional reconstitution of the glucocorticoid receptor-hsp90 complex. J. Biol. Chem. **265:** 21397–21400.
41. **Schuh, S., W. Yonemoto, W. Brugge, V. J. Bauer, R. M. Riehl, W. J. Sullivan, and D. O. Toft.** 1985. A 90,000-dalton binding protein common to both steroid receptors and the Rous sarcoma virus transforming protein, $pp60^{v\text{-}src}$. J. Biol. Chem. **260:**14292–14296.
42. **Sikorski, R. S., M. S. Boguski, M. Goebl, and P. Hieter.** 1990. A repeating amino acid motif in *CDC23* defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis. Cell **60:**307–317.
43. **Smith, D. F., L. E. Faber, and D. O. Toft.** 1990. Purification of unactivated progesterone receptor and identification of novel receptor-associated proteins. J. Biol. Chem. **265:**3996–4003.
44. **Smith, D. F., D. B. Schowalter, S. L. Kost, and D. O. Toft.** 1990. Reconstitution of progesterone receptor with heat shock proteins. Mol. Endocrinol. **4:**1704–1711.
45. **Smith, D. F., B. A. Stensgard, W. J. Welch, and D. O. Toft.** 1992. Assembly of progesterone receptor with heat shock proteins and receptor activation are ATP mediated events. J. Biol. Chem. **267:**1350–1356.
46. **Stone, K. L., M. B. LoPresti, J. M. Crawford, R. DeAngelis, and K. R. Williams.** 1989. Enzymatic digestion of proteins and HPLC peptide isolation, p. 31–47. *In* P. T. Matsudaira (ed.), A practical guide to protein and peptide purification for microsequencing. Academic Press, San Diego, Calif.
47. **Sullivan, W. P., B. T. Vroman, V. J. Bauer, R. K. Puri, R. M. Riehl, G. R. Pearson, and D. O. Toft.** 1985. Isolation of steroid receptor binding protein from chicken oviduct and production of monoclonal antibodies. Biochemistry **24:**4214–4222.
48. **Tai, P.-K. K., M. W. Albers, H. Chang, L. E. Faber, and S. L. Schreiber.** 1992. Association of a 59-kilodalton immunophilin with the glucocorticoid receptor complex. Science **256:**1315–1318.
49. **Ungewickell, E.** 1985. The 70-kd mammalian heat shock proteins are structurally and functionally related to the uncoating protein that releases clathrin triskelia from coated vesicles. EMBO J. **4:**3385–3391.
50. **Wessel, D., and U. I. Flügge.** 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. **138:**141–143.
51. **Whitelaw, M. L., K. Hutchison, and G. H. Perdew.** 1991. A 50-kDa cytosolic protein complexed with the 90-kDa heat shock protein (hsp90) is the same protein complexed with $pp60^{v\text{-}src}$ hsp90 in cells transformed by the Rous sarcoma virus. J. Biol. Chem. **266:**16436–16440.
52. **Wiech, H., J. Buchner, R. Zimmerman, and U. Jakob.** 1992. Hsp90 chaperones protein folding in vitro. Nature (London) **358:**169–170.
53. **Yem, A. W., A. G. Tomasselli, R. L. Heinrikson, H. Zurcher-Neely, V. A. Ruff, R. A. Johnson, and M. R. Deibel, Jr.** 1992. The hsp56 component of steroid receptor complexes binds to immobilized FK506 and shows homology to FKBP-12 and FKBP-13. J. Biol. Chem. **267:**2868–2871.
54. **Zimmerman, R., M. Sagstetter, M. J. Lewis, and H. R. B. Pelham.** 1988. Seventy-kilodalton heat shock proteins and an additional component from reticulocyte lysate stimulate import of M13 procoat protein into microsomes. EMBO J. **7:**2875–2880.