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†

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ABSTRACT: It has previously been shown that 9S, untransformed progestin, estrogen, androgen, and glucocorticoid receptor complexes in rabbit uterine and liver cytosols contain a 59-kDa protein [Tai, P. K., Maeda, Y., Nakao, K., Wakim, N. G., Duhring, J. L., & Faber, L. E. (1986) *Biochemistry 25*, 5269–5275]. In this work we show that the monoclonal antibody KN 382/EC1 raised against the rabbit 59-kDa protein reacts with 9S, untransformed glucocorticoid receptor complexes in cytosol prepared from human IM-9 lymphocytes but not with 4S salt-transformed receptors. The human protein recognized by the EC1 antibody is a 56-kDa protein (p56) of moderate abundance located predominantly in the cytoplasm by indirect immunofluorescence. There are at least six isomorphs of p56 by two-dimensional gel analysis. N-Terminal sequencing (20 amino acids) shows that p56 is a unique human protein. When p56 is immunoadsorbed from IM-9 cell cytosol, both the 70- and 90-kDa heat shock proteins are coadsorbed in an immune-specific manner. Neither heat shock protein reacts directly with the EC1 antibody. We conclude that p56 exists in cytosol in a higher order complex containing hsp70 and hsp90, both of which in turn have been found to be associated with untransformed steroid receptors.

When cytosols are prepared from cells that have not been exposed to hormone, most of the steroid receptors are recovered as large 9S complexes containing the receptor in a non-DNA-binding form [see Pratt (1987) for review]. When the complexes are dissociated, the DNA binding function of the receptor is derepressed and the receptor exists in a 4–5S state that behaves as a monomer or dimer depending on the receptor and the conditions. In glucocorticoid receptor systems, there is rather strong evidence that the large receptor complex is derived from the normal inactive form of the receptor that becomes activated by binding of hormone in the intact cell (Mendel et al., 1986; Pratt et al., 1988; Rexin et al., 1988a). An important direct correlation between the size of the cytosolic receptor complex and receptor activity has been made in cells transfected with mutant receptor cDNAs (Pratt et al., 1988). It was found that, in the absence of hormone, steroid-inducible forms of the human GR were recovered in molybdate-stabilized COS cell cytosol as large 9S complexes whereas mutant receptors with constitutive activity were recovered only in the dissociated 4S form.

In addition to the receptor itself, the 9S comlex contains another well-characterized phosphoprotein, the 90-kDa heat shock protein (hsp90)[1] (Sanchez et al., 1985, 1987a,b; Shuh et al., 1985; Catelli et al., 1985; Denis et al., 1987). In the case of the glucocorticoid receptor, the 9S, M_r 300000 complex contains a single molecule of the steroid-binding protein (Gehring & Arndt, 1985; Okret et al., 1985) and two molecules of hsp90 (Mendel & Orti, 1988). It has been shown that the GR binds to hsp90 either at or very close to the termination of receptor translation in vitro (Dalman et al., 1989), and the newly translated receptor is in the 9S form (Denis & Gustafsson, 1989). Another protein with which the receptor interacts either directly or indirectly in the 9S complex has been identified in rabbit cytosols by Tai et al. (1986). This is a 59-kDa protein of unknown function. A monoclonal antibody (KN 382/EC1) prepared against this protein shifts rabbit estrogen, progestin, androgen, and glucocorticoid receptors from 9S to 10–11S on sucrose gradient centrifugation.

Although there is evidence (cited above) that the 9S form of the GR is derived from the biologically inactive state of the receptor in the intact cell, it is not known if the 9S, M_r 300000 form represents the entire complex or if the receptor is associated with other molecules, some of which may be important in getting it to its ultimate site of action. Recent studies using very gentle immunoadsorption and washing procedures designed to preserve as much of the structure of the native complex as possible suggest that the 9S glucocorticoid receptor complex may be a core unit derived from a larger complex that contains multiple molecules of hsp90, a protein about 55 kDa with multiple isomorphs, and a 23-kDa protein (Bresnick et al., 1990). Similar studies with the progesterone receptors suggest that a large untransformed[2] receptor complex contains hsp70 in addition to hsp90 (Estes et al., 1987; Kost et al., 1989) as well as three other proteins of apparent M_r 54000, 50000, and 23000 (Smith et al., 1990).

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[1] Abbreviations: GR, glucocorticoid receptor; TA or triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-2,30-dione 16,17-acetonide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; hsp70 and hsp90, the 70- and 90-kDa heat shock proteins.

[2] In this work, we will use the word "untransformed" to describe the 8–9S form of the receptor that does not bind to DNA and the word "transformed" to describe the 4S, DNA-binding form.

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If we are going to understand both how steroid receptors are transported to their nuclear sites of action and the nature of the structure with which the receptors are associated while they are in their biologically inactive state prior to hormone binding, it will ultimately be necessary to define higher order structure than the 9S complex. One approach to this goal is to use the same gentle immunoadsorption and washing procedures that have been employed with antireceptor antibodies in order to determine what proteins are immunoadsorbed by monoclonal antibodies directed against the known receptor-associated proteins. If common components can be defined, a picture of the composition of a higher order structure may develop. Perdew has prepared a very useful IgM monoclonal antibody against hsp90 that reacts with the heat shock protein even when it is associated with other proteins in a complex (Perdew, 1988), and he is now using this approach to identify hsp90-associated proteins (G. H. Perdew, submitted for publication). In the work described in this paper, we use the EC1 antibody against the other known nonreceptor component of the 9S complex, which is recognized by the EC1 antibody, in order to determine with what proteins it associates in human lymphocyte (IM-9) cytosol. In addition, we determine a number of physical properties of the 56-kDa protein recognized by the EC1 antibody and demonstrate by N-terminal sequencing that the protein is unique.

Experimental Procedures

Materials

[6,7-^{3}H]Triamcinolone acetonide (42.5 Ci/mmol), L-[^{35}S]methionine, and ^{125}I-conjugates of goat anti-mouse IgG and goat anti-rabbit IgG were from New England Nuclear (Boston, MA). Ampholines came from LKB Instruments, Inc. Nonimmune mouse IgG, fluorescein-conjugated goat anti-mouse IgG, goat anti-mouse IgG–horseradish peroxidase conjugate, TES, Hepes, protein A–Sepharose CL-4B, Tween 20, and monoclonal antibodies against α-tubulin and β-tubulin were from Sigma Chemical Co. (St. Louis, MO). Immobilon P membranes were from Millipore Corp. (Bedford, MA). The AC88 monoclonal antibody against hsp90 (Riehl et al., 1985) was kindly provided by Dr. David Toft. The KN 382/EC1 antibody, which will be referred to as simply EC1, is an IgG monoclonal antibody raised against partially purified 9S rabbit uterine progesterone receptor (Nakao et al., 1985) and shown to be directed against a 59-kDa receptor-associated protein (Tai & Faber, 1985). The rabbit anti-hsp70 serum prepared against the C-terminal 21 amino acid sequence from human hsp70 (Ehrhart et al., 1988) was kindly provided by Dr. Ettore Appella.

Methods

Cell Source and Fractionation. IM-9 human lymphocytes were obtained from the American Type Culture Collection and were grown in stationary suspension culture at 37 °C in RPMI 1640 medium containing iron-supplemented calf serum as previously described (Wheeler et al., 1981). Cells were harvested by centrifugation and washed by resuspension in Earle's saline and recentrifugation at 600*g* for 5 min. Cells were ruptured by resuspension in 1.5 volumes of 10 mM Hepes, 1 mM EDTA, pH 7.35, and 10 mM sodium molybdate at 4 °C and Dounce homogenization. The homogenate was centrifuged at 100000*g* for 1 h, and the resulting cytosol supernatant was either used immediately (Figures 1, 2, and 7–9) or frozen at −70 °C. For ^{35}S-labeling experiments, cells were grown for 24 h at 37 °C in methionine-free medium containing 10% iron-supplemented calf serum and [^{35}S]methionine at 2 μCi/mL.

Immunoadsorption. Aliquots of cytosol were mixed with 1 volume of TEG buffer [10 mM TES, 4 mM EDTA, 10% (w/v) glycerol, 50 mM NaCl, pH 7.6], EC1 hybridoma fluid was added at 5–15% of the final volume as noted in the figure legends, and the mixture was incubated for 2 h at 0 °C. Each sample was then added to a protein A–Sepharose pellet (10 μL of pellet/0.1 mL of sample) and mixed by rotation for 2.5 h at 4 °C. Protein A–Sepharose pellets were washed six times by resuspension in 1-mL aliquots of TEG buffer.

Gel Electrophoresis and Immunoblotting. SDS–polyacrylamide gel electrophoresis was performed in 7% slab gels as described previously (Bresnick et al., 1989). For two-dimensional gel electrophoresis, immunoadsorbed complexes were dissolved by incubating them in 9.5 M urea, 2% (w/v) NP-40, 5% ampholines (pH range 5–8 only for Figures 3 and 4, or a mixture of 4% pH range 5–8 and 1% pH range 3–10), and 5% β-mercaptoethanol. For immunoblotting, proteins were transferred to Immobilon P membranes and the membrane was incubated first overnight with the indicated probe antibody at 1% followed by the appropriate (anti-mouse or anti-rabbit) ^{125}I-conjugated anti-IgG as previously described (Bresnick et al., 1990).

Glycerol Gradients. Samples (90 μL) of charcoal-extracted cytosol containing [^{3}H]triamcinolone acetonide-bound receptor in Hepes buffer with 30 mM sodium molybdate were incubated for 4 h on ice with 20 μL of nonimmune mouse IgG or EC1 hybridoma fluid and centrifuged in 10–35% glycerol gradients with internal glucose oxidase (7.9S) and horseradish peroxidase (3.6S) markers as described previously (Pratt et al., 1988).

Indirect Immunofluorescence. Human choriocarcinoma (JAR) cells were grown in monolayer on glass cover slips in MEM medium supplemented with 10% calf serum in 95% air/5% CO_2. The immunofluorescence procedure was essentially as described in Sanchez et al. (1988). Briefly, after fixation in 3.7% paraformaldehyde in phosphate-buffered saline, the cover slips were immersed in acetone at −20 °C, blotted, inverted onto a 15-μL drop of primary antibody solution, either 1% EC1 or 0.1% anti-α-tubulin antibody for 1 h, and then rinsed in phosphate-buffered saline. Samples were then inverted for an additional 1 h over 15 μL of fluorescein-labeled goat anti-mouse IgG, washed, mounted, and photographed as described.

Amino Acid Sequence Determination. Sequence data were obtained on an automated gas-phase sequencer (Applied Biosystems Model 470A) equipped with an on-line PTH analyzer (Model 120A). Data interpretation was performed on a VAX 11/785 computer (Henzel et al., 1987). Aliquots (0.5 mL) of IM-9 cytosol were immunoadsorbed with 100 μg of EC1, and the immunoadsorbed proteins were resolved by two-dimensional isoelectric focusing–SDS–PAGE, transferred to Immobilon paper, and stained with Coomassie blue (Matsudaira, 1987). The stained spots corresponding to the major p56 isomorphs were excised from the Immobilon sheet, and sequence analysis was performed directly on the Immobilon-bound protein as described by Matsudaira (1987). The FASTP program of Lipman and Pearson (1985) was used to search a combined EMBL, GenBank, Dayhoff (Release 20.0, March 31, 1989), and in-house (Genentech) protein sequence data base for homology to other proteins.

Results

Interaction of EC1 with Complexes Containing Human GR and hsp90. The EC1 monoclonal antibody was raised against a protein component of the untransformed rabbit progesterone receptor (Nakao et al.; 1985). The study of the protein rec-

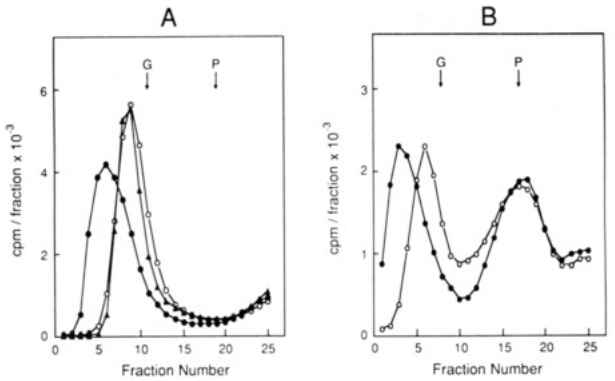

FIGURE 1: The EC1 antibody binds to the untransformed glucocorticoid receptor complex. Panel A: IM-9 cytosol containing molybdate and [^{3}H]TA-bound receptors was incubated with EC1 antibody (●), nonimmune mouse IgG (○), or buffer (▲) and centrifuged in 10–35% glycerol gradients as described under Methods. Panel B: Cytosol containing [^{3}H]TA-bound receptors without molybdate was incubated for 1.5 h with 200 mM KCl to permit partial receptor transformation, molybdate was added, and the cytosol was incubated with EC1 (●) or nonimmune IgG (○) prior to glycerol gradient centrifugation. Internal markers: (G) glucose oxidase, 7.9S; (P) peroxidase, 3.6S.

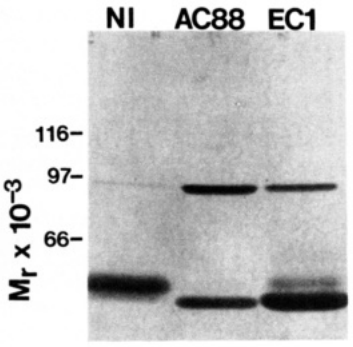

FIGURE 2: The 90-kDa protein which is immunoadsorbed from cytosol by the EC1 antibody is hsp90. Aliquots (270 μL) of IM-9 cell cytosol were diluted 1:1 with TEG buffer containing sodium molybdate and incubated with 5 μL of the AC88 monoclonal antibody against hsp90, 30 μL of EC1, or 30 μL of nonimmune mouse IgG as control (NI). The mixtures were immunoadsorbed to protein A–Sepharose and washed four times with TEG buffer containing 10 mM sodium molybdate, and the bound proteins were eluted with SDS sample buffer. The samples were resolved by SDS–PAGE and immunoblotting by using the AC88 antibody as probe and developing with peroxidase.

ognized by this antibody has been held back because EC1 does not cross-react with cytosolic proteins in some commonly studied receptor systems, such as the mouse, rat, and chicken. We found that the EC1 antibody does cross-react with a human protein, and Figure 1A shows that it recognizes a component of the untransformed 9S GR complex in human IM-9 lymphocyte cytosol. The reaction with the GR complex is detected by a shift in the average sedimentation value of the receptor from 8.7 ± 0.1 (SE) to 10.2 ± 0.2 (SE) S, determined from four separate experiments. Consistent with previous observations in rabbit cytosols (Tai et al., 1986), the EC1 antibody does not shift the receptor when it is in the dissociated 4S form (Figure 1B).

Tai et al. (1986) also reported that immunoadsorption of rabbit uterine or liver cytosol with EC1 was accompanied by the coabsorption of an unidentified M_r 92000 protein that did not react with the EC1 antibody on western blot. As shown in Figure 2, immunoabsorption of IM-9 cytosol with EC1 causes the immune-specific absorption of a 90-kDa protein that reacts on immunoblot with the AC88 monoclonal antibody directed against hsp90. The EC1 antibody does not react

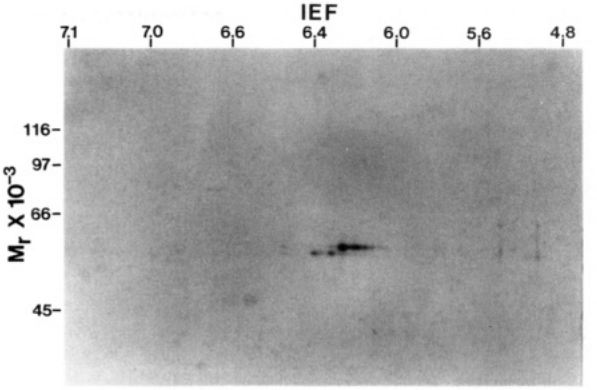

FIGURE 3: Two-dimensional gel analysis of protein isomorphs immunoadsorbed by EC1. IM-9 cell cytosol (200 μL) was incubated with 15 μL of EC1 antibody, adsorbed to protein A–Sepharose, and washed with TEG buffer as described under Methods. The bound proteins were eluted with O'Farrell's lysis buffer and subjected to isoelectric focusing (IEF) with 5% (pH 5–8 only) ampholytes. After the IEF tube gel was run on a 7% SDEs–polyacrylamide gel, the proteins were transferred to Immobilon paper and probed with EC1 antibody, followed by ^{125}I-goat anti-mouse IgG.

directly with hsp90 that has been immunopurified with AC88 (data not shown). These data suggest that the human system is much like the rabbit system in that the EC1 antibody recognizes a protein that is a component of the 9S GR complex and that is associated with a 90-kDa protein that is now established to be hsp90. It should be noted that when we use the same immunoadsorption protocol as in Figure 2, we isolate only very small amounts of GR from IM-9 cytosol containing untransformed receptors labeled with [^{3}H]dexamethasone 21-mesylate (data not shown). It is not clear why the glycerol gradient approach allows us to detect a complex with the GR that one cannot readily isolate by this method of immunoadsorption.

Apparent M_r and pI of the Protein Recognized by EC1 in IM-9 Cytosol. The apparent M_r of the rabbit protein recognized by EC1 is 59000 (Tai et al., 1986). However, the human protein has a slightly lower M_r, and when it is immunoadsorbed and run on one-dimensional SDS–PAGE, its presence is obscured by the heavy chain of EC1. Figure 3 shows the isomorphs of the protein containing the epitope recognized by EC1. In this experiment, IM-9 cytosol was first immunoadsorbed with EC1 and the immunoadsorbed proteins were immunoblotted with EC1 after resolution on two-dimensional gels. The protein recognized by EC1 resolves into two major isomorphs with p*I*s at about 6.3 and at least four minor isomorphs. Two of the minor forms have a slightly lower M_r than the major forms and could represent cleavage products.

Both hsp90 and hsp70 (which we show below to be immune-specifically adsorbed by EC1) have been found by indirect immunofluorescence to localize with microtubules in intact cells (Sanchez et al., 1988; Redmond et al., 1989; Weller, 1988). As the human protein recognized by EC1 has an M_r similar to tubulin, it is important to demonstrate that it is not tubulin. In the experiments of Figure 4, IM-9 cytosol was immunoadsorbed with either EC1 or monoclonal antibodies directed against α- or β-tubulin, and the immunoadsorbed proteins were then resolved on two-dimensional gels and immunoblotted. As shown in panel I (section E), the two major isomorphs immunoadsorbed by EC1 have an apparent M_r that is slightly larger than that of α-tubulin (MW 55000). On the basis of this reference and calculation from a standard marker curve, we assign an apparent M_r of 56000 for the major isomorphs recognized by EC1, and we will refer to the human isomorphs collectively as p56. It is clear from sections

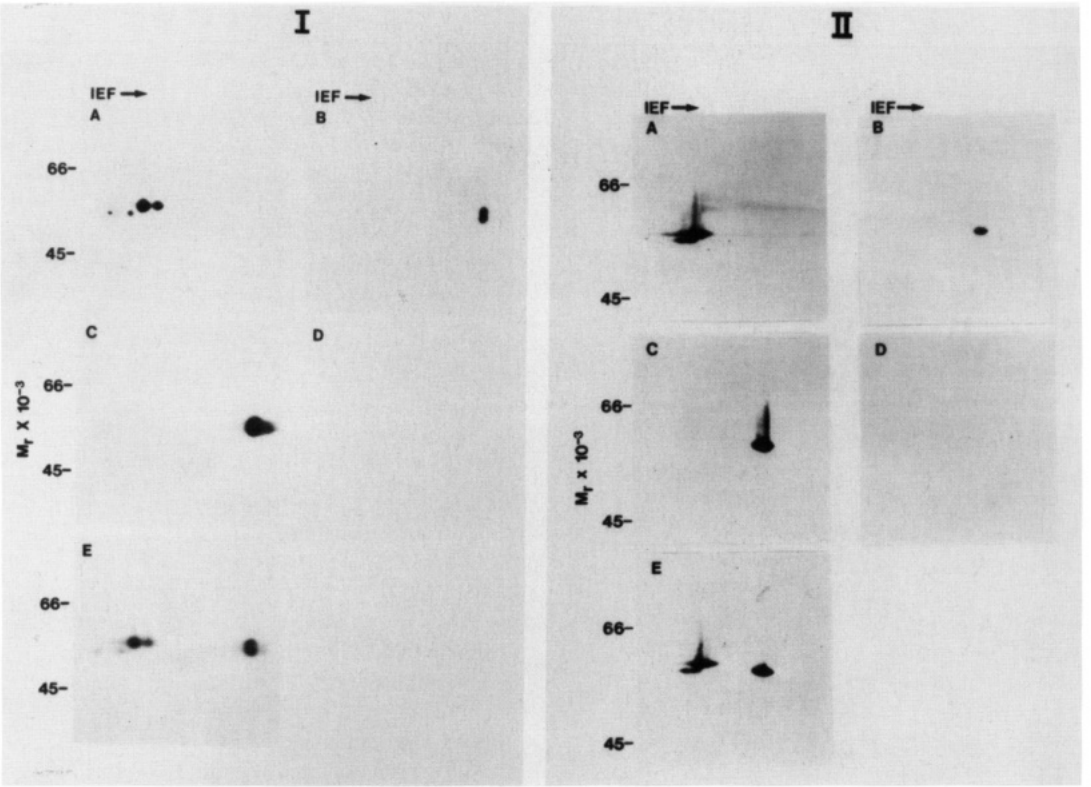

FIGURE 4: M_r and p*I* of protein isomorphs immunoadsorbed by EC1 relative to those of α- and β-tubulin. Panel I: Aliquots (200 μL) of IM-9 cell cytosol were incubated with the antibodies indicated below and immunoadsorbed to protein A–Sepharose as described under Methods. After washing of the Sepharose beads with TEG buffer containing 450 mM NaCl, the bound proteins were eluted with O'Farrell's lysis buffer, subjected to isoelectric focusing (IEF) with pH 5–8 ampholytes, followed by SDS–PAGE, and transferred to Imobilon paper. Immunoblots were probed with the indicated antibodies and developed with ^{125}I-conjugated goat anti-mouse IgG. Sections: (A) cytosolic proteins were immunoadsorbed with EC1 and immunoblot probed with EC1; (B) cytosolic proteins were adsorbed with EC1 and probed with anti-α-tubulin; (C) cytosolic proteins were adsorbed with anti-α-tubulin and probed with anti-α-tubulin; (D) cytosolic proteins were adsorbed with anti-α-tubulin and probed with EC1; (E) EC1 and anti-α-tubulin antibodies were used simultaneously during immunoadsorption and blotting. Panel II: Aliquots of cytosol were processed as described in panel I, except that anti-β-tubulin antibody was used in the place of anti-α-tubulin antibody.

E of panels I and II of Figure 4 that p56 has a more basic p*I* than α- or β-tubulin. Also, from sections B and D, it is clear that EC1 does not recognize tubulin and antibodies against tubulin do not recognize p56. As will be shown later in Figure 9, the presence of α- or β-tubulin in EC1 immunoadsorbates reflects nonspecific adsorption to protein A–Sepharose.

Cellular Localization and Relative Abundance of p56. Human JAR choriocarcinoma cells were chosen for performing indirect immunofluorescence localization with EC1 (Figure 5A) because these cells spread out flat on the culture dish, providing a large area of cytoplasm that is optimal for visualization of cytoskeletal elements and determining cytoplasmic versus nuclear distribution. The distribution of EC1-directed immunofluorescence shown in Figure 5A is predominantly cytoplasmic without any obvious cytoskeletal localization. The EC1-directed immunofluorescence is more concentrated in the perinuclear region and does not extend all the way to the cell periphery like the anti-tubulin immunofluorescence in Figure 5B. As nucleolar shadows can be seen within the nuclei in Figure 5A, it is very likely that some of the EC1-directed immunofluorescence lies within the nucleus. This field was chosen for presentation because it also shows the diffuse intense fluorescence seen in a mitotic cell where the nuclear membrane is not present.

Figure 6 shows that p56 is present in IM-9 cytosol at moderate abundance. In this experiment small aliquots of IM-9 cytosol were either submitted directly to two-dimensional gel electrophoresis (panel A) or first immunoadsorbed either with nonimmune mouse IgG (panel B) or with EC1 to deplete p56 (panel C) prior to resolution of the supernatant proteins by the two-dimensional procedure. Each panel shows the stained (Coomassie blue) cytosolic proteins with the two major isomorphs of p56 present within the brackets in panels A and B. Although p56 associates with hsp90, which is one of the most abundant cytosolic proteins, p56 itself is present at only moderate abundance in comparison with the cytosolic proteins that can be visualized by this method. On the basis of our experience with the Coomassie blue staining of GR isolated from mouse L cell cytosol [e.g., Bresnick et al. (1990)], which has a glucocorticoid binding capacity similar to IM-9 cytosol, we estimate that p56 is present in considerable stoichiometric excess over the receptor.

Evidence That p56 Is Present in a Cytosolic Complex Containing Two Heat Shock Proteins. Figure 7A shows the proteins immunoadsorbed from IM-9 cytosol by EC1 and stained with Coomassie blue. Both p56 and hsp90 are present in a completely immune-specific manner. Some hsp70 is present in the control sample adsorbed with nonimmune mouse IgG (Figure 7B), but considerably more is present in the EC1 immunoadsorbate in panel A. In addition, there is a weakly staining 35-kDa protein with a more acidic p*I* than hsp90 which cannot be seen in the photograph but is also present in a completely immune-specific manner.

In the experiment of Figure 8, the proteins immunoadsorbed by EC1 (panel A) or nonimmune IgG (panel B) were immunoblotted with a rabbit antiserum that reacts with both hsp70 and hsp90. This antibody was raised against a peptide representing the C-terminal 21 amino acids of human hsp70, and the cross-reactivity probably reflects the fact that the four C-terminal amino acids of hsp70 and hsp90 are identical

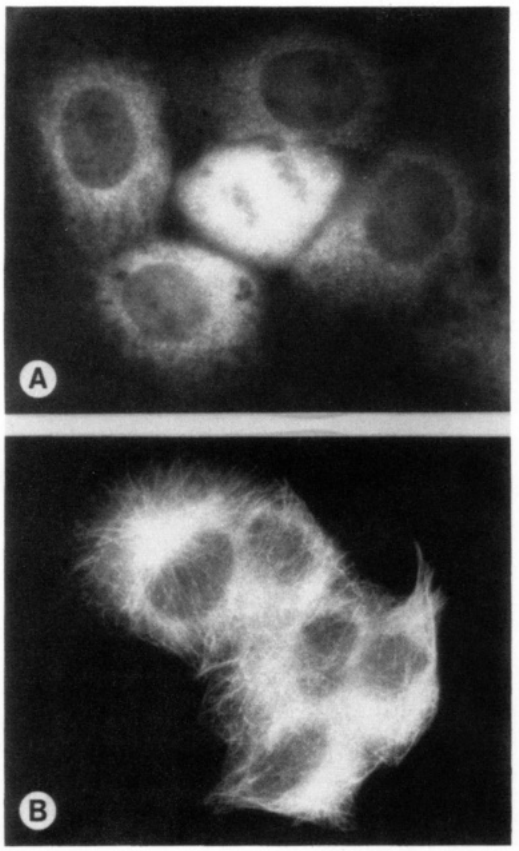

FIGURE 5: Localization of p56 in intact human JAR choriocarcinoma cells by indirect immunofluorescence. JAR cells growing on glass cover slips were fixed in formaldehyde, permeabilized with acetone, and subjected to indirect immunofluorescence with EC1 or anti-α-tubulin monoclonal antibody as described under Methods. Panel A: Four interphase cells and one mitotic cell (center) showing fluorescence pattern obtained with EC1 antibody. Panel B: Five interphase cells showing fibrilar fluorescence pattern obtained with anti-α-tubulin antibody.

(Ehrhart et al., 1988). Again, hsp90 is present only in the EC1-immunoadsorbed material, and one can appreciate a clear increase in hsp70 over the nonimmune control. As immunoadsorption of p56 with EC1 results in coisolation of both hsp90 and hsp70, neither of which react directly with EC1 on western blot, it is reasonable to propose that p56 exists in cytosol in a heteromeric complex that contains both of these abundant heat shock proteins.

Other Potential Components of the Heteromeric p56-Containing Complex. In order to look for other proteins that may be associated with the p56-containing complex, cytosol was prepared from [^{35}S]methionine-labeled IM-9 cells and immunoadsorbed with EC1 (Figure 9A) or nonimmune mouse IgG (Figure 9B). In addition to the immune-specific presence of hsp70 and hsp90 we note three other [^{35}S]methionine-labeled spots that are present in an immune-specific manner. The acidic protein p35 is the faintly staining protein mentioned above. Another protein, p23, migrates just to the basic side of hsp90 but is not visible by staining with Coomassie blue. The identities of p23 and p35 are unknown.

N-Terminal Sequence of p56. Because the p53 transformation-related protein, which consists of several isomorphs with isoelectric focusing properties similar to p56, also associates with hsp70 (Ehrhart et al., 1988), it is reasonable to ask if p53 and p56 are identical. We determined from N-terminal sequence, however, that the two proteins are different. The N-terminal sequence of p56 shown in Figure 10 is unique with respect to other known human proteins.

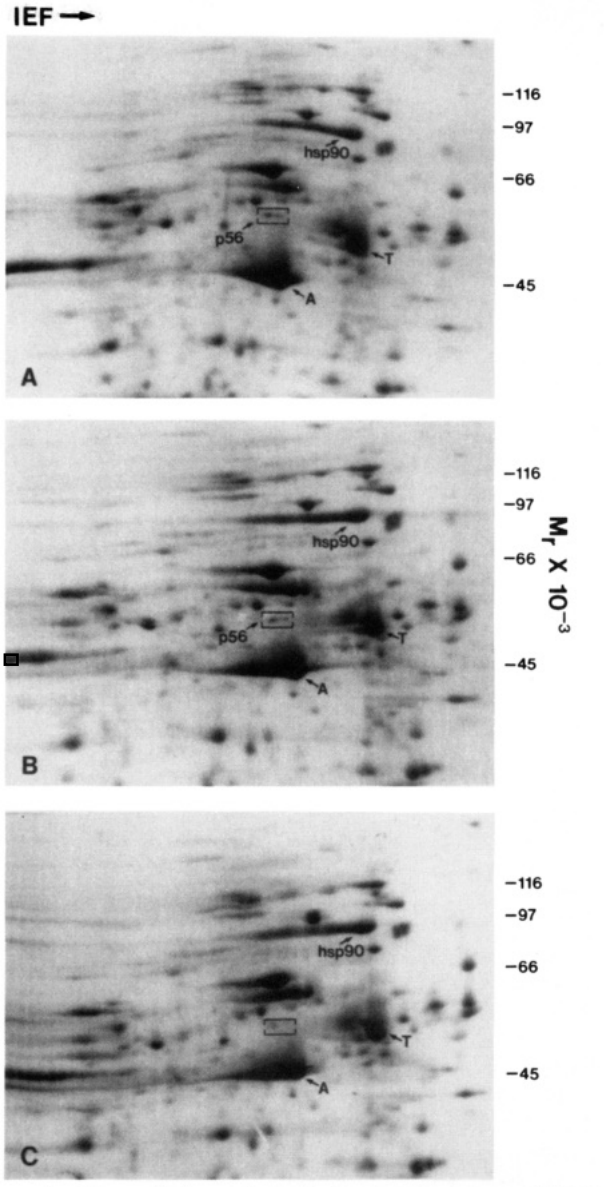

FIGURE 6: p56 protein is a moderately abundant component of IM-9 cell cytosol. Aliquots (25 μL) of IM-9 cell cytosol were diluted with 20 μL of TEG buffer, incubated with 5 μL of EC1 or with nonimmune mouse IgG, and adsorbed to protein A–Sepharose. After centrifugation, the supernatants over the immunoadsorbed pellets were mixed with 100 μL of O'Farrell's lysis buffer. A third aliquot of IM-9 cytosol was simply diluted with 25 μL of TEG buffer containing no antibody and mixed with lysis buffer. All samples were subjected to isoelectric focusing and SDS–PAGE, followed by staining with Coomassie blue. Panels: (A) IM-9 cytosol not immunoadsorbed; (B) supernatant of IM-9 cytosol immunoadsorbed with nonimmune mouse IgG; (C) supernatant of IM-9 cytosol immunoadsorbed with EC1 antibody. The brackets localize the stained p56 isomorphs in panels A and B. Symbol A indicates actin, and T is tubulin.

Discussion

There is now evidence from three laboratories that the EC1 monoclonal antibody reacts with the untransformed form of steroid receptors (Figure 1), including those for progestins, glucocorticoids, androgens, and estrogens (Tai et al., 1986; Renoir et al., 1989; Faber et al., 1989). In addition to indirect immunologic evidence, such as shift of 9S receptor to larger forms with EC1 and immunoadsorption by matrix-immobilized EC1 (Tai et al., 1986), Faber et al. (1989) have provided direct physicochemical evidence for the presence of p56 by cross-linking the 9S calf uterine estrogen receptor with dimethyl pimelimidate and demonstrating a cross-linked p59-hsp90

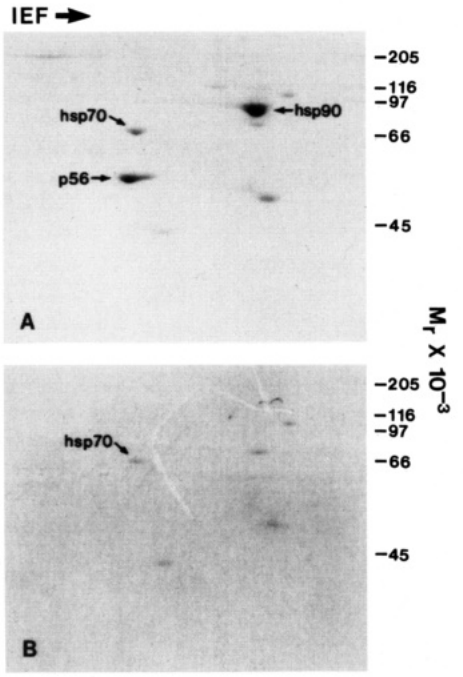

FIGURE 7: Immunopurification of p56 results in copurification of 70- and 90-kDa proteins. Aliquots (500 μL) of IM-9 cell cytosol were immunoadsorbed with 100 μL of EC1 antibody (panel A) or nonimmune mouse IgG (panel B) as described under Methods. Each sample was resolved by two-dimensional isoelectric focusing–SDS–PAGE followed by staining with Coomassie blue.

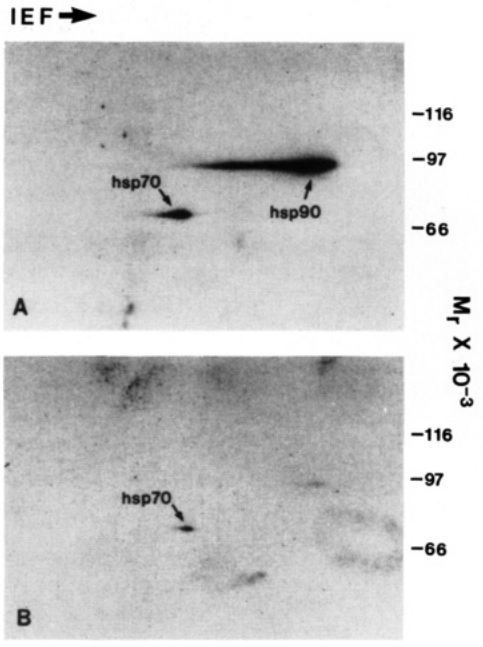

FIGURE 8: 70- and 90-kDa proteins that coimmunoadsorb with p56 are hsp70 and hsp90. Aliquots (150 μL) of IM-9 cell cytosol were immunoadsorbed with 15 μL of EC1 antibody (panel A) or nonimmune mouse IgG (panel B). After two-dimensional isoelectric focusing–SDS–PAGE and transfer to Immobilon paper, the blots were probed with 1% rabbit anti-hsp70 serum. The blot was developed with ^{125}I-conjugated goat anti-rabbit IgG. As described in the text, this antibody reacts with the carboxy terminus of both hsp70 and hsp90 (Ehrhart et al., 1988).

product (in calf uterine cytosol p56 migrates at 59 kDa) after SDS–PAGE and immunoblotting with EC1 and with the AC88 antibody against hsp90. The presence of p56 in the 9S form of steroid receptors suggests that this unit has a more complex composition than is accounted for by a model containing one steroid-binding protein and two molecules of hsp90

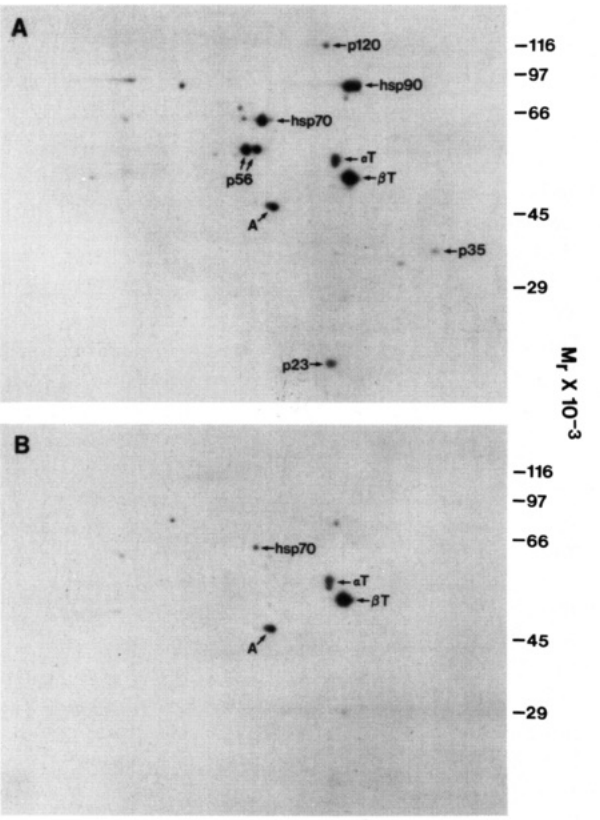

FIGURE 9: Small amounts of three other proteins of M_r 23 000, 35 000, and 120 000 also copurify with p56. Aliquots (200 μL) of cytosol prepared from IM-9 cells labeled for 24 h with [^{35}S]methionine were immunoadsorbed with 20 μL of EC1 (panel A) or nonimmune mouse IgG (panel B). The figure presents autoradiograms of two-dimensional gels of the immunoadsorbed pellets. A is actin, and T is tubulin.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
X	[A]	E	E	M	K	A	T	E	[S]	[G]	A	Q	X	A	P	L	P	M	E
-	ALA	GLU	GLU	MET	LYS	ALA	THR	GLU	SER	GLY	ALA	GLN	-	ALA	PRO	LEU	PRO	MET	GLU

FIGURE 10: N-Terminal amino acid sequence of the major p56 isomorph. Parentheses denote uncertain residues; X, residue not identified.

(Renoir et al., 1984; Mendel & Orti, 1988; Denis et al., 1988; Radanyi et al., 1989).

Experiments such as those of Figures 2 and 7 strongly suggest that p56 is associated with hsp90. It is quite clear that the amounts of p56 and hsp90 that are copurified in Figure 7 are much larger than the amount of GR that exists in the same volume of cytosol. Thus, p56 and hsp90 must be associated with each other in manner that is independent of the presence of the GR. It is important to note that EC1 shifts all of the 9S receptor peak to a larger form [Figure 1 and Tai et al. (1986)], suggesting that all 9S complexes may contain p56. As shown in Figure 6, hsp90 is present in cytosol in large excess with respect to p56. Thus, relatively few molecules of hsp90 can be directly associated with p56. The question then arises as to whether the GR becomes associated with only a subclass of hsp90 that is bound to p56.

The way in which the steroid binding protein, the hsp90 dimer, and p56 interact with each other in the 9S complex is not known. The p56 could be oriented in the complex such that it also interacts with the receptor, or it could be separated from it by the intervening hsp90. The cross-linking approaches being developed by Rexin et al. (1988a,b) and Arányi et al. (1988) may eventually help in determining the geometry of the complex. Rexin et al. (1988b) have treated both mouse lymphoma cytosol and intact cells with dimethyl suberimidate and then imunopurified the [^{3}H]dexamethasone mesylate-la-

beled GR and resolved its cross-linked products by SDS–PAGE. Analysis of the size of the cross-linked products revealed complexes consistent with a GR linked to two molecules of a 90-kDa protein and one molecule of a 50-kDa protein and subcombinations thereof (GR + p50, GR + p90, GR + p50 + p90, GR + 2p90). If the protein calculated by Rexin et al. to be about 50 kDa in molecular mass is the murine analogue of the p56 protein we have described here, then the recovery of GR cross-linked to the 50-kDa protein would suggest that the receptor and p56 may lie proximate to each other in the larger complex.

Toft and his co-workers (Kost et al., 1989; Smith et al., 1990) have suggested that hsp70 is associated with both untransformed and transformed progesterone receptors. This proposal would also be consistent with the data of Estes et al. (1987) who found that extensive purification of chick oviduct progesterone receptor without hsp90 resulted in copurification of approximately equivalent amounts of a 76-kDa non-steroid-binding protein. The data of Toft and his co-workers would suggest that hsp70 must also be present in the 9S complex, although to our knowledge that has not been directly demonstrated with gradient shift experiments, such as that of Figure 1, as it has for both hsp90 [e.g., Radanyi (1983)] and p56 (Tai et al., 1986).

The data of Figures 7–9 rather strongly suggest that hsp70 is also a component of the same cytosolic protein complex that contains p56 and hsp90. Again, like hsp90, only a small portion of the total cytosolic hsp70 would be present in such a complex because p56 is present in much lower amounts than hsp70. It should be noted, however, that hsp70 is sticky and there is always some present in nonimmune samples, although there is a clear increase in samples immunoadsorbed with EC1. [^{35}S]Methionine labeling experiments like that of Figure 9 raise the possibility that at least two other proteins with apparent M_r 23 000 and 35 000 may be present in the complex. The amount of p35 appears to be very low by the criterion of Coomassie stain, and p23 is not visible by stain at all. It is not known if the p23 observed here with high specific activity [^{35}S]methionine label is the same as the 23-kDa protein we have previously found to be coimmunoadsorbed with GR from L cell cytosol (Bresnick et al., 1990). In that work the 23-kDa protein was identified by staining with Coomassie blue and was not detected by [^{35}S]methionine, which was present at low specific activity in the labeling protocol.

Perdew has used the 8D3 IgM monoclonal antibody to immunoadsorb large amounts of hsp90 from [^{35}S]-methionine-labeled Hepa 1c1c7 cytosol and finds proteins of about 49, 54, 63, 68, and >200 kDa that are immunospecifically adsorbed in addition to hsp90 (G. H. Perdew, submitted for publication). Two of the proteins in this complex have been identified by western blotting: p68 is hsp70, and p54 cross-reacts with a polyclonal antibody raised against the rabbit 59-kDa protein, which is also the antigen for the EC1 antibody used in this work. Thus, both the Perdew work and our findings reported here strongly suggest that p56, hsp70, and hsp90 can exist as a heteromeric complex in cytosols prepared with low-salt buffer. It is of interest that Milarski et al. (1989) have reported that immunoadsorption of S-phase HeLa cell lysates with a monoclonal antibody to hsp70 is accompanied by coisolation of an unidentified 51-kDa protein. As it seems the heteromeric complex of p56, hsp70, and hsp90 exists independent of the presence of steroid receptors, it is likely that the steroid receptors or steroid receptor–hsp90 units formed during receptor translation (Dalman et al., 1989) bind to a preexisting complex structure that exists in great stoichiometric excess over the receptors themselves.

A computer scan of protein amino acid sequences showed that the N-terminal sequence of p56 (Figure 10) is unique. One would expect a protein that enters into a heteromeric complex with highly conserved proteins, such as hsp70 and hsp90, to be itself conserved. In this respect, it is interesting that N-terminal amino acids 2–13 of the human p56 show 83% homology with N-terminal amino acids 2–13 of the rabbit p59 (Faber and Toft, unpublished results).

Because p56 is a unique human protein that exists in heteromeric complexes containing two heat shock proteins and because it appears to be a component of a higher order structure with which a number of steroid-binding proteins associate prior to their transformation to the DNA binding state, it is reasonable to predict that p56 may prove to be of considerable interest to laboratories investigating cellular regulatory mechanisms. The search for steroid receptor associated proteins is analagous to the parallel search for proteins associated with some avian and mammalian transforming virus gene products, such as pp60^{src} (Brugge, 1986) and the T antigens of simian virus 40 (SV40) and polyomavirus [e.g., Murphy et al. (1986), Grossenmeyer et al. (1987), and Walter et al. (1988)]. In both of these situations a complete picture of the mechanisms involved in the intracellular transport, targeting, and "docking" of the proteins will require definition of the higher order structures with which the proteins must associate during these complex events.

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