P59, an hsp 90-binding Protein

CLONING AND SEQUENCING OF ITS cDNA AND PREPARATION OF A PEPTIDE-DIRECTED POLYCLONAL ANTIBODY*

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The primary sequence of the rabbit liver cDNA coding for protein p59 has been determined. The protein binds to the 90-kDa heat shock protein (hsp 90) and is associated with it, including when hsp 90 participates in hetero-oligomeric complexes of untransformed mammalian steroid receptors that sediment at 8–10 S. The cloned cDNA codes for an open reading frame of 458 amino acids defining a yet unknown protein. However, 55% amino acid homology to peptidyl-prolyl isomerase is found between amino acids 41 and 137, suggesting rotamase activity for p59, which speculatively may apply to bound hsp 90 and thus be implied in the intracellular trafficking of hetero-oligomeric forms of steroid hormone receptors. A polyclonal antibody derived from the COOH-terminal peptide 441–458 demonstrates a good affinity for rabbit, rat, and human "p59" protein. It interacts with at least one epitope, available in 8–10 S untransformed steroid receptor complexes and different from that recognized by the monoclonal antibody KN382/EC-1.

Non-transformed steroid hormone receptors are recovered in cytosol from target tissues in the form of hetero-oligomers, sedimenting as 8–10 S entities in low salt density gradients (reviewed in Refs. 1 and 2). Among the proteins that are associated with the receptors, the non-hormone-binding heat shock protein hsp 90 has been largely described (3–6). In rat Hepa 1 cells, p59 also associates with hsp 90, along with other proteins (10). A human 56-kDa protein, which reacts with EC-1 and is probably the human equivalent of rabbit p59, has been described as a heat shock protein (11), and two-dimensional gel analysis has shown two major and four minor isomorphs (12). Here we describe the cloning and sequencing of the rabbit p59 protein, which should bring some information concerning its as yet unknown function. We also report the preparation of a polyclonal antibody which recognizes other epitope(s) than EC-1, and unlike EC-1, interacts with p59 in rodents.

MATeRIALS AND METHODS

Cloning—Screening by the antibody EC-1 of a rabbit liver cDNA library cloned in the expression vector Agt11 (Clontech) allowed us to isolate four positive clones (out of 300,000 recombinants), two of which were identical. They were used both to generate recombinant forms of rabbit liver mRNA (12 pmol/spot) and to isolate larger clones in the same library. The largest cDNA, of approximately 2.1 kb, in clone B, was used for sequencing and was also subcloned into pGEM 7Zf (Promega Biotec). Transcription and translation were as described by Promega Biotec, and immunoprecipitation was performed with a goat anti-mouse antibody. In order to find the nucleotides which correspond to the NH2-terminal sequence determined by protein sequencing, and which were missing in the cDNA library, a rabbit genomic DNA library, cloned in the EMBL 3 vector (Clontech) was screened. The five positive clones were digested by appropriate restriction endonucleases, and the resulting Southern blots were probed with oligonucleotides complementary to the 5’-end of the cDNA. A positive clone, digested by Sall (EMBL 3 cloning site) and SauI gave a 1.1-kb fragment that contained 480 additional base pairs upstream of the 5’-end of clone B cDNA. The fragment was subcloned into M13 MP18/19 vectors and sequenced as described below.

Sequencing—Clone B was digested by restriction endonucleases EcoRI and SacI to produce two cDNA inserts of 0.8 and 1.3 kb. These were subcloned into M13 MP 18/19 vectors, and both strands were sequenced using the Sequenase 2.0 kit (U. S. Biochemical Corp.) and a series of synthetic oligonucleotide primers. Alternatively, the cDNA subcloned in the pGEM 7Zf vector was digested by exonuclease III to generate deletion subclones (13), which were then sequenced using the T7 RNA polymerase promoter as a primer.

NH2-terminal Amino Acid Sequence of p59—The protein was immuno purified as described (9), and the purified protein (30 pmol) was electrophoresed in 8.5% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane (Immobilon, Millipore Corp., Bedford, MA), and stained with Coomassie Blue. The p59-stained spot was excised and put into an automated gas-phase sequencer (Applied Biosystems model 470 A) equipped with an on-line phenylthiohydantoin analyzer (model 120 A). Amino acid data were obtained using an IPC computer.

Gel Electrophoresis and Immunoblotting—SDS-PAGE and immunoblotting were performed as previously described (9, 17), using rainbow molecular weight standards (Amersham Corp.).

Density Gradient Analysis—Samples (200 μl) of charcoal-extracted cytosol incubated with 15–20 nM [3H]RU 486 (50 Ci/mmol, Roussel-UCLAF, Romainville, France) were loaded on a 5–20% sucrose gradient in PGW buffer (phosphate 10 mM, glycerol 10%, tungstic acid 20 mM, HCl, pH 7.8). Extracts were incubated with antibodies during 4 h at 0°C and then centrifuged as previously described (9, 17).

Antipeptide Antibody Preparation—From immunogenicity predictions, the COOH-terminal peptide corresponding to amino acids 441–458, as deduced from the sequence of the cDNA, was synthesized, coupled to KLH, and injected into two rabbits. IgG fractions were purified by ammonium sulfate precipitation and ion exchange chromatography at each bleeding.

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The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; kb, kilobase pair(s).
RESULTS AND DISCUSSION

P59 cDNA was isolated from a rabbit liver cDNA library cloned in the expression vector Agt11 (Clontech) by screening with the antibody EC-1. A Northern blot of rabbit liver mRNA probed with labeled inserts of the positive clones, showed two bands, at approximately 2.2 and 1.6 kb (Fig. 1). The largest cDNA isolated was sequenced. The nucleotide sequence and the derived amino acid sequence of the whole open reading frame are shown in Fig. 2. Protein sequence of the NH2-terminal region, as determined by us, showed 100% identity between the first 25 amino acids and the nucleotide-derived sequence, and 15/19 amino acids identical to the sequence of human p56 (12).

cDNA from clone B was subcloned into pGEM 7Zf (Promega Biotec), transcribed, and translated in a rabbit reticulocyte lysate system. The protein synthesized was recognized by EC-1 and migrated like authentic p59 protein in a 10% Laemmli gel (Fig. 3). Rabbit reticulocyte lysate contains an EC-1 reactive 59-kDa protein, which conveniently represents an internal molecular mass standard. Consistent with our observation, Mats et al. (14) have recently shown that, in rabbit reticulocyte lysate and in the presence of hemin, EC-1...
Fig. 3. Panel a, p59 cDNA was subcloned into plasmid pGEM 7Zf(+) and transcribed and translated in a rabbit reticulocyte lysate, in the presence of [35S]methionine. Lanes: 1, protein markers; 2, authentic p59 protein; 3, complete reaction mix without mRNA; 4, reaction mix with correctly oriented mRNA; 5, reaction mix with antisense mRNA. Panel b, corresponding autoradiography. Panel c, same as in a, followed by immunoprecipitation with antibody EC-1 and anti-mouse IgG. The sizes of the molecular mass standards are indicated to the left of a and c; arrowheads point to p59 protein.

FIG. 4. Western blot of samples from different sources with anti-p59 antibody 173. Rabbit uterus cytosol (lane 1, 10 µl), rat hepatoma cell (lane 2), and HeLa cell (lane 3) cytosols were electrophoresed after ion exchange chromatography. Position of markers are indicated on the left of the figure; HC indicates rabbit immunoglobulin heavy chains detected by the second anti-rabbit antibody.

antibody co-adsorbs a heme-regulated protein kinase (HRI), hsp 90, hsp 70, and a 58-kDa protein (which is equivalent to the p59 protein described here).

It appeared, however, that a small number of nucleotides were missing at the 5' end of our largest cDNA since both Sanchez' (12) and our results indicated the presence of at least 3 amino acids upstream of the first ATG in the nucleotide sequence we determined. A rabbit genomic library cloned in the EMBL 3 vector (Clontech) was screened with a probe corresponding to the 5' region of the cDNA. Sequencing the positive clone most likely to contain the nucleotides missing in clone B revealed the 9 nucleotides that code for Met, Thr, and Ala. The last 2 amino acids corresponded to the NH2 terminus of the protein sequence we had previously determined, confirming that the whole open reading frame was sequenced. After consultation of GenBank and EMBL data bank, GenBank revealed that p59 cDNA presents 82% homology to a mouse mRNA which codes for an unknown protein (GenBank accession number X17069). P59 therefore appears to be a new, yet undefined protein. It does not bear significant sequence homology with rat hsp 60 (15), in spite of the fact that it has been characterized as a heat shock protein (11). However the region comprised between amino acids 41-137 bears 55% homology to peptidyl-prolyl isomerase. Screening for consensus sequences that correspond to particular features of the protein also revealed possible phosphorylation sites, a putative ATP-binding site, and a calmodulin-binding site (Fig. 2), all of which are currently being investigated for functional confirmation. There is no consensus sequence for a nuclear localization signal, although our immunocytochemical studies in rabbit uterus have shown p59 to be localized in majority in the nucleus (16).
Using the nucleotide deduced amino acid sequence, a rabbit polyclonal antibody, 173, was derived from peptide 441–458, corresponding to the carboxyl terminus of the protein. This polyclonal antibody was used either in immunoblotting or density gradient analysis to demonstrate its ability to recognize p59 in different tissues and species, as well as in the hetero-oligomeric structure of PR. A Western blot of rabbit uterus cytosol allowed visualization by antibody 173 of a single band at 59 kDa, at the same position as that recognized by EC-1 (9) (Fig. 4). Interestingly, the epitope recognized by antibody 173 is available in the 8–10 S PR hetero-oligomer; the addition of EC-1 to preformed antibody 173–8–10 S PR complexes shifts its sedimentation coefficient from 10.5 to 12 S, arguing in favor of different epitopes from 10.5 to 12 S, arguing in favor of different epitopes recognized by the two antibodies, all accessible in the hetero-oligomeric rabbit uterus PR (Fig. 5). We cannot yet precisely localize the EC-1 epitope, but we can deduce from the immunoreactivity of truncated clones that the epitope cannot be situated within the first 155 amino acids of the p59 NH$_2$ terminus.

Although p59 has only recently been identified, its association with hsp 90 has been observed in a number of laboratories (8–10, 14), using different approaches. However, only 1 part of hsp 90 is associated with p59, since the latter protein is much less represented than hsp 90, a universal and fairly conserved as hsp 90-binding protein and peptidyl-prolyl isomerase adds another unexpected feature to p59 structure. Furthermore, even if p59 has been found in many mammalian tissues (7, 9), it may not be as well represented as hsp 90, since it has been detected neither in the Service de Génie Cytogénétique de Bicêtre for technical help.

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