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OVEREXPRESSION OF p59-HBI (FKBP59), FULL LENGTH AND DOMAINS, AND CHARACTERIZATION OF PPIase ACTIVITY

Béatrice Chambraud[1], Nathalie Rouvière-Fourmy[1], Christine Radanyi[1], Kathy Hsiao[2], Debra A. Peattie[2], David J. Livingston[2], and Etienne Emile Baulieu[1]*

[1] INSERM U33, 80 Rue du Général Leclerc, 94276 Kremlin Bicêtre Cedex, France

[2] Vertex Pharmaceuticals Incorporated, 40 Allston Street, Cambridge, MA 02139-4211

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Summary : It has been previously proposed that the rabbit p59-HBI (Heat shock protein Binding Immunophilin) or *r*FKBP59 (FK506 Binding Protein), found associated with the 90 kDa heat shock protein in nontransformed steroid receptor complexes, has three domains structurally related to *h*FKBP12 (Callebaut, I., Renoir, J.M., Lebeau, M.C., Massol, N., Burny, A., Baulieu, E.E. and Mornon, J.P. (1992) Proc. Natl. Acad. Sci., USA 89, 6270-6274). Here we report the overexpression, as fusion proteins in E. coli, of the full length p59-HBI and a series of p59-HBI mutants delimiting these domains and their respective peptidyl prolyl *cis trans* isomerase (PPIase) activity. The PPIase activity of p59-HBI is comparable to that of *h*FKBP12 and is due to domain p59-HBI I which displays the highest homology with this immunophilin. The residual enzymatic activity found in domain p59-HBI II is discussed.

Immunophilins are a family of proteins which bind immunosuppressant ligands such as FK506, rapamycin or cyclosporin and exhibit a peptidyl prolyl *cis-trans* isomerase (PPIase) or rotamase activity *in vitro*. They are divided into two categories, the cyclophilins and the FK-Binding-Proteins. FKBP12, an FKBP of MW ~ 12 kDa, is the best characterized and is an abundant cytosolic protein found in many tissues and in a large variety of organisms. FK506 and rapamycin complexed to FKBP12 inhibit the activation of T lymphocytes by distinct signal transduction pathways (1). Inhibition of FKBP PPIase activity by FK506 and rapamycin is not sufficient for immunosuppression however (2).

Additional immunophilins have been cloned including the rabbit p59-HBI (Heat shock protein Binding Immunophilin of MW ~ 59 kDa) or *r*FKBP59 (3) which, besides its capacity to bind FK506 (4, 5), interacts with the 90 kDa heat shock protein in the heterooligomeric form of steroid receptors (6, 7). The human analog, *h*FKBP52, has 91% identity with rabbit p59-HBI (8) ; as FKBP12, p59-HBI has been identified in many tissues and species (7, 9).

* To whom correspondence should be addressed.

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The deduced amino acid sequence of rabbit p59-HBI cDNA has defined three domains structurally related to *h*FKBP12 and a putative calmodulin binding site located at the C terminal extremity of the protein (3, 10). The functions of the different domains are not clearly defined, and the possible role of p59-HBI present in the multimeric steroid receptor complex remains unclear (11,12).

In order to investigate the different functions of this protein, we have overexpressed in E. coli the full length p59-HBI and a series of p59-HBI mutants delimiting the domains. We describe this work and also report on the kinetic parameters for the PPIase activity of p59-HBI, full length and domains. Generation of the mutants also allowed us to localize the epitope recognized by EC1, a monoclonal antibody previously used to detect FKBP59 in heterooligomeric receptor complexes (9) and for cloning its cDNA (3).

Material & Methods

Construction of the p59-HBI domains :

All DNA constructions were performed using standard procedures (13). Site directed mutagenesis was used to construct the p59-HBI domains. Single strand DNA templates were prepared by using either the viral vector M13 mp19 or M13 mp18 containing the EcoR1-Sst1 fragment (from 1 bp to 783 bp) and Sst1-EcoR1 fragment (from 783 bp to 2070 bp) of the p59-HBI cDNA respectively. The oligonucleotide primers were chosen in order to allow precise introduction of EcoRV, Hpa1, Nru1, Eco47III and Bal1 sites, as shown in figure 1. The EcorV restriction site was introduced just after the initiation codon ATG of the p59-HBI cDNA, and the Bal1 restriction site was introduced just before the stop codon TAG of the p59-HBI cDNA. All mutations were checked by sequencing before subcloning the mutant DNA into the vector pGEM-7Zf$^+$ (Promega Corporation, Madison, WI USA). Using this strategy, cassettes corresponding to the different domains of wild type rabbit p59-HBI, separated or combined, were obtained after cleavage by the appropriate enzymes as shown in figure 1.

Overexpression of GST-HBI proteins :

Full length and truncated mutants of p59-HBI were inserted at the EcoR1 restriction site of the vector pGEX-1λT (Pharmacia LKB Biotechnology, Inc. Piscataway, NJ USA). For large scale purification of glutathione S-transferase (GST) fusion proteins, the strain E. coli (UT 5600) was grown in L-broth to an A_{600} of 0.5 and induced with 1 mM isopropyl β-D-thiogalactopyranoside for one hour at room temperature. Affinity purification on glutathione-sepharose (Pharmacia) was performed according to procedures described in (14).

PPIase assay :

The peptidyl-prolyl *cis-trans* isomerization rate was determined via isomerization of a prolyl-containing peptide N-succinyl-Ala-Leu-Pro-Phe-p-nitroanilide (15,16). Measurement of k_{cat}/K_m for the PPIase reaction was performed essentially according to procedures described in (17).

SDS-PAGE and immunoblotting :

Eluates of glutathione-sepharose were boiled in SDS sample buffer and resolved by electrophoresis on 10% SDS gels (18) prior to Coomassie brilliant blue staining or immunoblotting. Proteins were transferred to Immobilon-P membranes and probed with EC1 (10 µg/ml). Antigen-antibody complexes were visualized using the vectastain ABC immunoperoxidase system (Burlingame, CA) according to the recommendation of manufacturer.

Results & Discussion

Overexpression of the full length protein [following the introduction of specific enzyme restriction sites (fig.1)] was achieved using a GST vector pGEX-1λT. The GST-HBI fusion

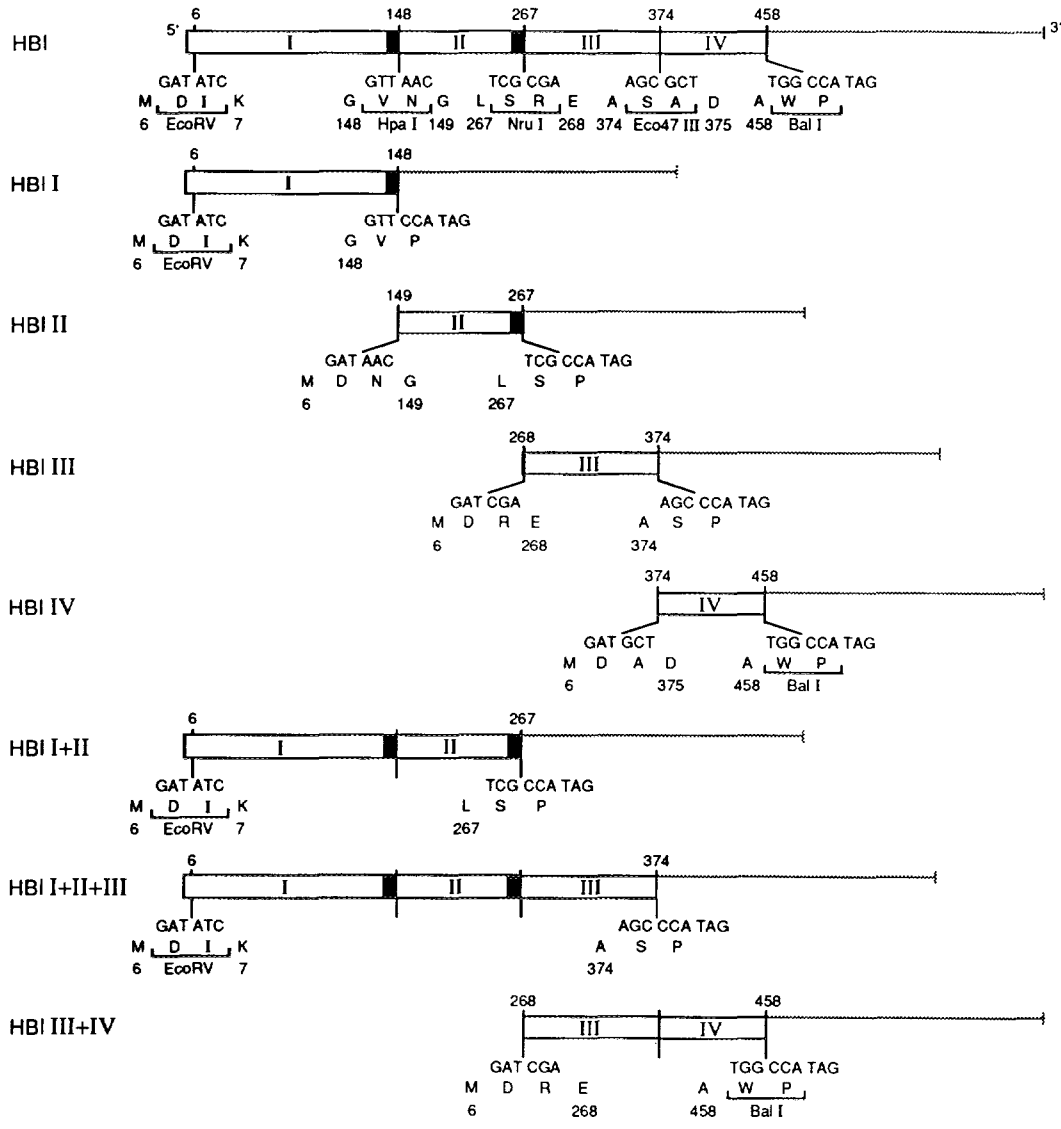

Fig.1 . Construction of p59-HBI mutants
The structure of p59-HBI cDNA is shown schematically, the numbers refer to amino acids positions. The EcoR V, Hpa1, Nru1, Eco47III and Bal1 sites were created at the extremity of p59-HBI different domains to allow the deletion of each of them. Two criteria were retained to choose these restriction sites : 1°) Absence of these sites in the sequence of the cDNA and vector (pGEM-7Zf+), 2°) all of them had to be compatible with each others. Hinges are represented by black boxes.

protein had an expected molecular weight of ~77 kDa [staining by Coomassie blue (fig.2)], and we characterized its PPIase activity.

The peptidyl-prolyl *cis-trans* isomerization rate of the substrate N-succinyl-Ala-Leu-Pro-Phe-p-nitroanilide was determined and results are presented in Table I. The catalytic activity value of the whole protein, as expressed by the second order rate constant k_{cat}/K_m, is 1.4 ± 0.2 (10^6 $M^{-1}s^{-1}$), showing that the p59-HBI displayed peptidyl prolyl *cis-*

Table I . Catalytic activity of the p59-HBI domains

Protein	Specific activity : k_{cat}/K_m (10^6 $M^{-1}.s^{-1}$)
*h*FKBP12	4.3 ± 0.4*
*h*FKBP52	0.39**
p59-HBI	1.4 ± 0.2
p59-HBI I	1.2 ± 0.1
p59-HBI II	0.02 ± 0.01
p59-HBI III	undetectable
p59-HBI IV	undetectable
p59-HBI I + II	0.9 ± 0.1
p59-HBI I + II + III	1.3 ± 0.1
p59-HBI III + IV	undetectable

Experimental conditions : Assays were performed in 0.1 M Tris buffer, pH 7.8 with Suc-Ala-Leu-Pro-Phe-p-nitroanilide as substrate, at 15°C ; k_{cat}/K_m was determined from K_{obs}-$k_{non\text{-}enz}$/[Enz] (15,16). k_{cat}/K_m values represent the average of 11 to 30 determinations on at least two independent preparations.

* Data from ref.17.
** Data from ref.8.

trans isomerase activity, as already suggested by comparing its deduced amino acid sequence to those of several FKBPs showing the enzymatic activity (3, 8) and by FK506 binding (4). This PPIase activity cannot be attributed to a contaminating cyclophilin since all the experiments were performed in the presence or absence of 2.5 µM cyclosporin, an inhibitor of cyclophilin PPIase activity, and no effect on PPIase activity was seen. Moreover, it has been checked that GST alone did not exhibit any PPIase activity (data not shown). These results demonstrate that the p59-HBI presents an FKBP-like PPIase activity.

A previous investigation by Hydrophobic Cluster Analysis (HCA) (10) allowed us to set up a model revealing three domains structurally related to *h*FKBP12. These domains called p59-HBI I, p59-HBI II and p59-HBI III, are separated by two short hydrophilic hinges: hinge I and hinge II. We called p59-HBI IV the C terminal part of the protein bearing the putative calmodulin binding site. On the basis of this model, and to investigate the independent roles of these different domains, and also to study the influence of each domain on the others, p59-HBI deletion mutants were obtained as shown in figure 1. They were expressed in E. coli as GST fusion proteins in the vector pGEX-1λT, and in all cases, proteins of the expected sizes were obtained (fig.2). Several other bands of lower Mr were visible after Coomassie blue staining, likely representing proteolytic fragments of the expressed fusion proteins despite the use of protease inhibitors during the purification procedure. The ability of these mutants to exhibit PPIase activity was checked.

The C terminal region of the p59-HBI expressed either from aa 268 or from aa 374 (giving p59-HBI III + IV an p59-HBI IV, respectively), and a segment of protein delimited

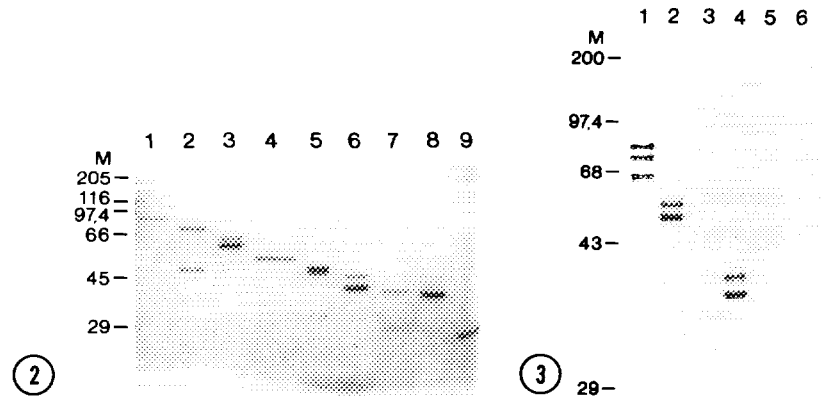

Fig.2 . SDS PAGE analysis of full length and p59-HBI deletion mutant overproduced in E. coli
Between one and two µg of full length and deletion mutant HBI fused to GST were electrophoresed on 10% SDS gels and stained by Coomassie blue : lane 1 full length GST-HBI (~77 kDa), lane 2 GST-HBI I + II + III (~68 kDa), lane 3 GST-HBI I + II (~56 kDa), lane 4 GST-HBI III + IV (~48 kDa), lane 5 GST-HBI I (~43 kDa), lane 6 GST-HBI II (~39 kDa), lane 7 GST-HBI III (~38 kDa), lane 8 GST-HBI IV (~36 kDa), lane 9 GST (~26 kDa). The position of molecular mass markers (Sigma) are shown along the left side.

Fig.3 . Immunoblotting analysis of full length and p59-HBI deletion mutant overproduced in E. coli
Western blotting was performed as described in material and methods with 10 µg/ml of antibody EC1 : lane 1 full length GST-HBI, lane 2 GST-HBI I + II, lane 3 GST-HBI I, lane 4 GST-HBI II, lane 5 GST-HBI III, lane 6 GST-HBI IV. The position of molecular mass marker (BRL) are shown along the left side.

between aa 268 and 373 (giving p59-HBI III), did not display measurable PPIase activity (Table I). Thus, the C terminal part of p59-HBI plays no detectable role in the catalysis of the PPIase reaction and it appears likely that the N terminal extremity is responsible for the catalytic activity demonstrated by the full length protein.

The k_{cat}/K_m value of the p59-HBI I expressed from aa 6 to aa 148 is 1.2 ± 0.1 (10^6 $M^{-1}s^{-1}$), a value identical, within experimental error, to that of the full length protein (Table I). This result demonstrates that the PPIase activity of the immunophilin is mainly due to the first domain which is the most closely structurally related to the *h*FKBP12 (10).

Moreover, the p59-HBI I PPIase activity was similar to that of *h*FKBP12 under identical experimental conditions, thus demonstrating that the GST fusion protein retained enzymatic activity after one step purification from crude lysates and was indeed functionally comparable to the *h*FKBP12. This result was expected since comparison of several sequences of FKBPs including *h*FKBP12 (8, 10), whose three-dimensional structure is well-known (19-21), revealed that the structures of the active sites of p59-HBI I and *h*FKBP12 are very similar. Twelve of 14 residues of *h*FKBP12 involving hydrogen bonding or hydrophobic interactions required for FK506 binding are maintained in p59-HBI I domain. The two residues Gln^{53} and His^{87} of the *h*FKBP12 which are neither

conserved in p59-HBI I, nor in *h*FKBP52, are not essential for catalysis (D. Livingston, unpublished).

As mentioned in Table I, the very weak PPIase activity of p59-HBI II, delineated between aa 149 and 267, represents only ~1.5% of either the full length protein or p59-HBI I activity, and thus is difficult to interpret. Nevertheless, this activity cannot be due to a contamination since 1) as mentioned above all enzymatic assays have been performed in the presence or absence of 2.5 µM of cyclosporin excluding a cyclophilin contamination and 2) p59-HBI III, IV, and III + IV expressed and assayed under similar conditions displayed undetectable levels of activity. Thus it seems that this very low PPIase activity represents a residual activity due to a partially conserved active site as compared to that of p59-HBI I and *h*FKBP12. The second domain shares 28% identity with *h*FKBP12 as deduced from HCA plots (10), but only four of 14 residues of *h*FKBP12 probably involved in FK506 binding (19-21) are maintained. Among the residues not conserved is the residue homologous to Trp^{59} of FKBP12, which is changed to Leu in p59-HBI II, but is maintained as Trp in p59-HBI I. Mutation of Trp^{59} to Leu in *h*FKBP12 decreases PPIase specific activity ten fold (D. Livingston, unpublished). We also cannot exclude that the residual activity observed could also be due to a low affinity of p59-HBI II for the peptide substrate used in these experiments and that, alternatively p59-HBI II could display another, yet unidentified, distinct enzymatic activity.

According to the results described above, the deletion of the C terminal part from aa 267 or from aa 374 to give the p59-HBI I + II and the p59-HBI I + II + III constructs respectively, has no influence on the PPIase activity of the p59-HBI. Both of them exhibit an activity similar to that of full length p59-HBI (Table I) since the differences observed between p59-HBI, p59-HBI I, p59-HBI I + II and p59-HBI I + II + III are probably not significant.

The PPIase activities found for p59-HBI I and p59-HBI I + II *in vitro* are similar, and suggest that if the low PPIase activity displayed by p59-HBI II domain is significant, these two domains did not present any cooperative effect.

We also took advantage of the overexpression of the different domains to determine which part of the protein reacted with the Mab EC1 used for the identification of p59-HBI in the heterooligomeric structure of steroid hormone receptors as well as for cloning its cDNA. As shown in Figure 3, all constructs which include p59-HBI II are recognized by EC1, allowing us to deduce that p59-HBI II bears the EC1 epitope (this construction includes the FKBP12 related core and hinge II). Again, bands of lower Mr likely represent degradation products of the overexpressed proteins.

In this report, we have characterized the PPIase activity of p59-HBI and on the basis of these observations, as p59-HBI was found associated in the heterooligomeric structure of steroid hormone receptors, it is of interest to determine whether HSP90 and/or the hormone binding unit could be substrate(s) of p59-HBI *in vivo*. In addition, p59-HBI may be involved in HSP90 folding and assembly of the inactive steroid receptor complex. Moreover, we have recently observed an increased steroid binding capacity of the non

activated progesterone receptor including p59-HBI following incubation of rabbit uterus cytosol with FK506 and rapamycin (22). Finally expression of the p59-HBI domains as GST fusion proteins provides a number of technical advantages and will be a useful tool to study interactions of p59-HBI with HSP90 and other potential functions of the protein.

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