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An immunophilin that binds M_r 90,000 heat shock protein: Main structural features of a mammalian p59 protein

(hydrophobic cluster analysis/steroid receptors/FK506 binding protein)

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ABSTRACT **In the rabbit, a p59 protein included in the untransformed, non-DNA binding, "8–9S," steroid receptor complexes binds heat shock protein M_r ≈90,000 (hsp90). Sequence data [Lebeau, M. C., Massol, N., Herrick, J., Faber, L. E., Renoir, J. M., Radanyi, C. & Baulieu, E. E. (1992) *J. Biol. Chem.* 267, 4281–4284] and hydrophobic cluster analysis delineate, from the N terminus, two successive domains closely related to the immunosuppressant FK506 binding immunophilin FKBP (FK506 binding protein), consistent with recent purification of the human p56 immunophilin cognate protein by FK506 affinity chromatography [Yem, A. W., Tomasselli, A. G., Heinrikson, R. L., Zurcher-Neely, H., Ruff, V. A., Johnson, R. A. & Deibel, M. R., Jr. (1992) *J. Biol. Chem.* 267, 2868–2871]. The first FKBP-like domain demonstrates all structural characteristics known to be necessary for immunosuppressant binding and for peptidylprolyl cis–trans isomerase (rotamase) activity. Hence, p59 is a "hsp binding immunophilin" (HBI). It is thus speculated that hsp binding immunophilin may help the assembly/disassembly mechanisms involved in steroid receptor trafficking and activity and participate in the poorly understood hsp90 function. ATP/GTP binding likely occurs within the second FKBP-like domain, near the FK506 binding site on the FKBP template. A third domain detected by the hydrophobic cluster analysis method is distantly structurally related to the two first FKBP-like domains and is followed by the C-terminal part of the protein, which contains a calmodulin binding consensus sequence. Hsp binding immunophilin may be involved in a number of immunological, endocrinological, and chaperone-mediated pathways.**

The non-DNA binding "untransformed" "8–9S" forms of all steroid hormone receptors are heterooligomeric complexes that include the heat shock protein hsp90 (1) and a p59 protein detected by the monoclonal antibody EC1 and the polyclonal antibody 173 in all mammalian cells tested so far (2–4). p59 does not bind to the receptors themselves, but to hsp90 (3), and in fact is also found in receptor-free complexes containing hsp90 and possibly other associated proteins (5). The cDNA of rabbit p59 has recently been obtained and sequenced (4). The corresponding amino acid sequence revealed no homology with the hsp60 family, but it has been suggested that the human p56 equivalent of p59 is a heat shock protein (6).

Sequence alignment processings and hydrophobic cluster analysis (HCA) (7, 8) were applied to the amino acid sequence of rabbit p59.

From the N-terminal extremity of p59, we find three domains [hsp binding immunophilins I, II, and III (HBI-I, HBI-II, and HBI-III)] structurally related to FKBP-12 (9), a cytoplasmic binding protein for FK506 and other immunosuppressive drugs (10, 11), which also functions as a peptidylprolyl cis–trans isomerase (rotamase) (10) and the three-dimensional structure of which has recently been solved (12, 13). HBI-I structure is so similar to that of FKBP that it is highly probable that it is responsible for the recent successful purification of p59 by FK506 affinity chromatography (14). HBI-II and HBI-III diverge progressively from the FKBP-like structure, and the three domains may have evolved from a common ancestral gene. An ATP/GTP binding site (S. Le Bihan, personal communication) is likely located in HBI-II, near the site occupied by FK506 in the FKBP template, and a calmodulin binding site may be present in the C-terminal region of the protein (4).

HBI may be a prototype for a new category of immunophilins interacting with a chaperone (15) and therefore interfering, in a ligand-dependent manner, with the function of associated proteins such as steroid receptors.

METHODS

One-dimensional sequence programs (16) were used to perform initial searches in the MIPS protein data bank (MIPS: Martinsrieder Institut für Protein Sequenzen; D-8033 Martiensried).

Two dimensional HCA strategies and applications (7) have been recently reviewed (8). Rather than first maximizing sequence identity (a procedure that becomes unrealistic when levels fall under 25%), HCA uses the general principles of protein folding and works on the detection of successive correspondences of hydrophobic clusters that are relevant of the secondary structure and three-dimensional folding of protein domains. In related proteins, a precise sequence alignment can be deduced from the HCA plots by proceeding outward from the hydrophobic core of similar clusters toward cluster-linking regions (loops) where insertions/deletions are allowed. For these HCA alignments, a numerical value (HCA score) can be calculated between clusters to assess the alignments (7, 8). The power of the HCA method has recently been illustrated for a number of proteins, for which detailed predictions have been experimentally verified (17–20), although sequence identities allowing predictions have frequently been <13% through all β structures.

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Abbreviations: HBI, heat shock protein binding immunophilin; FKBP, FK506 binding protein; p59, protein of M_r ≈59,000; hsp90, heat shock protein M_r ≈90,000; HCA, hydrophobic cluster analysis.

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Three-dimensional studies were performed with the MANOSK software (21) running on an Evans and Sutherland PS390 display.

RESULTS

HCA Studies: Detection of Immunophilin-Like Domains (HBI-I, HBI-II, and HBI-III). Starting at the N terminus, two successive stretches of p59, about 100 amino acids long, were found clearly related to the FKBP-12 immunophilin (Fig. 1) (7, 8, 16). Fig. 2*a* shows the HCA plot patterns of FKBP and that of HBI split into domains HBI-I and HBI-II according to the relationship with FKBP. The classical segmentation of globular domains into regular secondary structures (β strands and α-helix) statistically centered on hydrophobic clusters of the HCA plots and in loops connecting these structures is clearly conserved for FKBP, HBI-I, and HBI-II. HBI-I shares 49% sequence identity with FKBP without introduction of any deletion or insertion. HBI-II exhibits 28% sequence identity with FKBP with four areas of insertion or deletion totaling nine amino acids (Fig. 1). Such sequence identity should lead to an average root mean square between Cα of HBI-I and HBI-II with respect to FKBP of 0.9 Å and 1.2 Å, respectively (28). Consequently, these two first domains would have, as their FKBP homologous structure, an antiparallel β sheet folding topology accompanied by one α-helix and an extra β strand (Fig. 3). We refer hereafter to β sheet as A (strands A1 to A5).

HBI-I and HBI-II share 34% sequence identity. The homology observed between FKBP and HBI-I and HBI-II domains is also found for other already recognized FKBP-like proteins (Fig. 1).

It is interesting to stress the overall similarity, centered on the S4 segment, that could be detected on the HCA plot between HBI-I, HBI-II, and a stretch of sequence following HBI-II (Fig. 2*a*). This stretch could correspond to a third globular domain, HBI-III, possibly related to HBI-I and HBI-II. Thus, HBI could have evolved from the triplication of an ancestral FKBP-like gene.

This hypothesis was assessed as follows. First, an amino acid per amino acid alignment was done, primarily using the S4, S5, and S6 segments of the HBI-I and HBI-II domain HCA plots as anchors (shown in Fig. 2*b*). This demonstrated 13% sequence identity between HBI-II and HBI-III. With HBI-I, HBI-III shares 10% sequence identity. Although these values are low, the relationship between HBI-III and

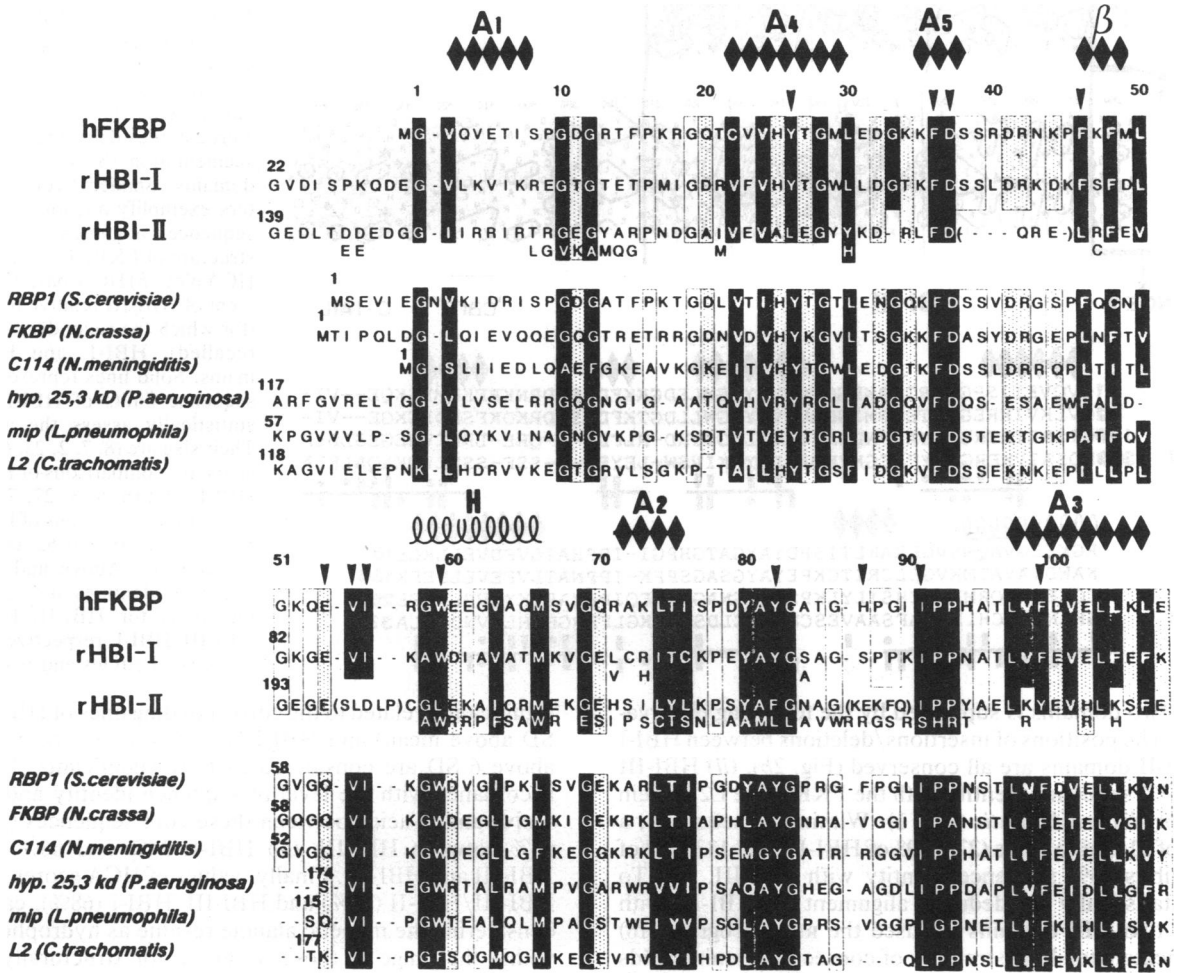

FIG. 1. Overall linear alignment of human FKBP (hFKBP) (22), rabbit HBI-I and HBI-II domains (rHBI-I, rHBI-II) (4), and FKBP-like domains already recognized in other proteins. Proteins are *Saccharomyces cerevisiae* rapamycin binding protein RBP1 (23), *Neurospora crassa* FKBP (24), a cryptic sequence from *Neisseria meningiditis* (25), a 25.3-kDa hypothetical protein from *Pseudomonas aeruginosa* (accession no. JQ0140), the cell surface protein mip of *Legionella pneumophila* (26), and the L2 protein of *Chlamydia trachomatis* (27). Below the sequence of HBI-I and HBI-II are indicated the nonidentical amino acids in the corresponding domains of GenBank accession no. X17069 protein (4, 16). β Strands are depicted as ◆◆◆ and α-helix is depicted as ⟅⟅⟅. Black boxes indicate conserved residues; grey and dotted ones indicate hydrophobic and hydrophilic homologous residues, respectively, and open ones indicate positions that are particularly rich in P, G, S, T, and/or A. Amino acids (main and/or side chains) that are critical for FK506 binding to FKBP and are fully conserved for HBI-I are highlighted by arrowheads. Brackets are introduced when amino acid to amino acid alignment is not possible.

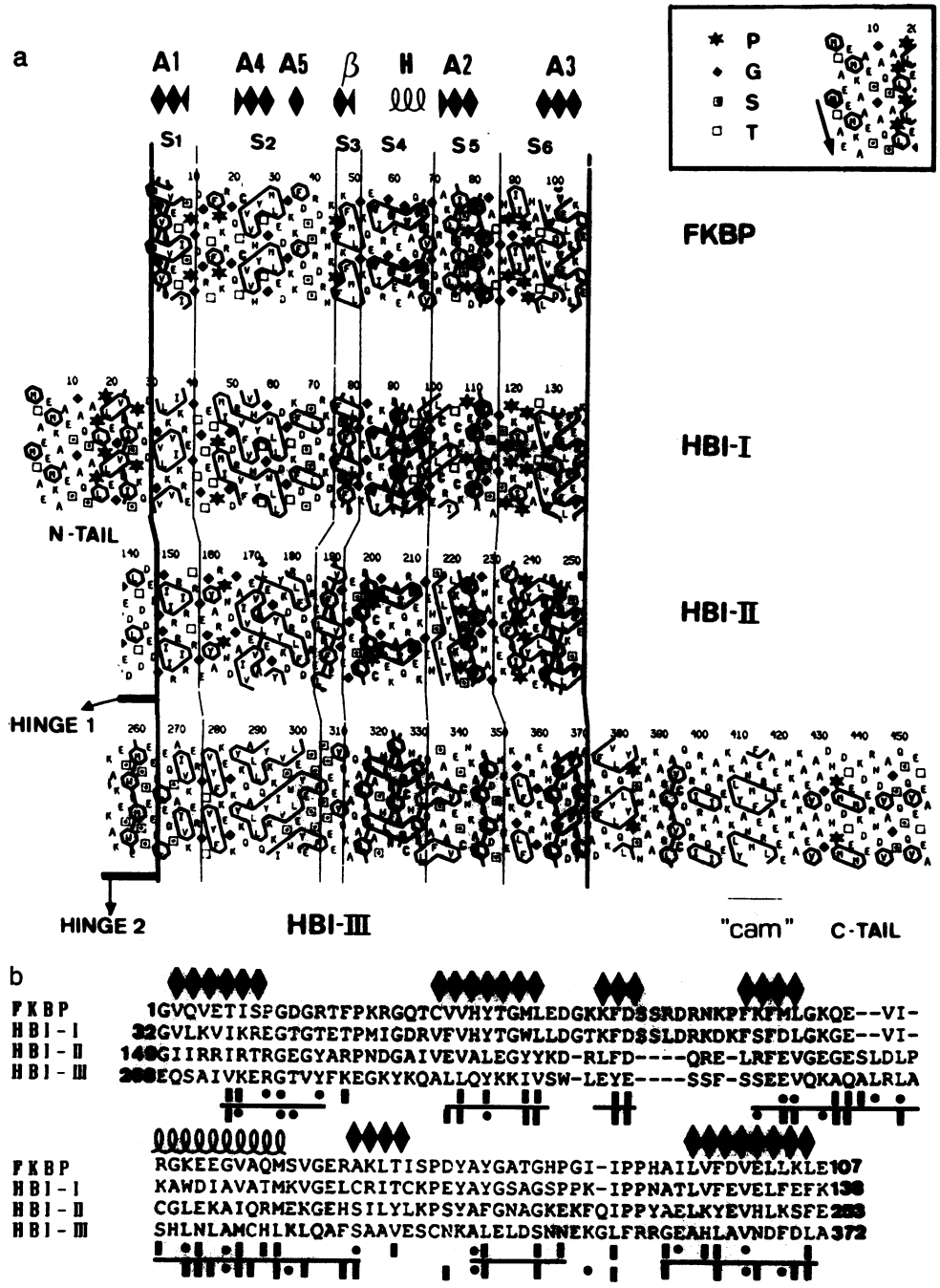

FIG. 2. HCA studies of rabbit HBI. (*a*) HCA plots of human FKBP and rabbit HBI. The protein sequence, split into three parts, is drawn on a classical α-helix smoothed on a cylinder, which is then cut parallel to its axis and unrolled to provide a convenient two-dimensional representation of the sequence. The plot is duplicated vertically to restore the full two-dimensional environment of each amino acid. Sets of hydrophobic amino acids (VILFWMY) are encircled into clusters. The classical one-letter code is used for all amino acids except (see box) for P (stars), which is considered as a cluster breaker; G (diamond-shaped symbol), which shows a large conformational flexibility; and T and S (open and dotted squares, respectively), which, although frequently encountered in loops, can also be found in hydrophobic environments where the hydrophilicity of their hydroxyl groups is neutralized through H bonds with main chains. The box reproducing the N-terminal part of HBI also indicates how to read the sequence linearly (nearly vertical arrow) and it reveals the medium range of neighboring amino acids (horizontal "reading"). Vertical lines delineate the structural segmentation (S1 to S6) within the domains. Shaded hydrophobic clusters exemplify anchor points for the sequence's alignment. The secondary structure of FKBP is shown above its HCA plot. (*b*) HCA-based linear alignment of HBI-III relative to the FKBP (for which the secondary structure is recalled), HBI-I, and HBI-II domains. Solid lines represent the core sequence limits of HBI-III used to statistically assess the alignments. Their size are (8, 7, 2, 23, 6, 12 amino acids) for comparisons of HBI-III and HBI-II and (6, 8, 3, 27, 7, 11 amino acids) for comparisons of HBI-III and HBI-I—i.e., 58 and 62 amino acids, respectively. Above and below are sequence identities and conservative mutations for HBI-III/HBI-II and HBI-III/HBI-I, respectively (indicated by solid circles and bars).

the first two domains is supported by the following observations. (*i*) The positions of insertions/deletions between HBI-I and HBI-II domains are all conserved (Fig. 2*b*). (*ii*) HBI-III shares 13% sequence identity with the FKBP-like L2 protein of *Chlamydia trachomatis* (Fig. 1). Within this sequence, a stretch of 21 amino acids (273–293 of HBI-III and 133–152 of L2) exhibits 45% sequence identity with HBI-III. (*iii*) To assess statistically the deduced alignment for HBI-III with the first two HBI domains we used the RDF2 program (16) with concatenated long stretches of consecutive amino acids centered around conserved positions for all segments S1 to S6 and mainly including the residues taking part in the regular secondary structures (Fig. 2*b*). The HBI-I and HBI-II concatenated core sequences (62 and 58 amino acids, respectively) were each compared to the corresponding ones in HBI-III and to 500 sequences resulting from randomization of the HBI-III core sequences to get a good estimation of alignment background score (mean) and standard deviation (SD). The results show that the HBI-III core sequence is significantly related to the corresponding ones of HBI-II (7.27 SD above mean) and HBI-I (9.59 SD above mean) (values above 6 SD are considered as fully significant). This is in accordance with the level of sequence identity and conservative amino acids between these core sequences (19% and 68% between HBI-III and HBI-I; 24% and 66% between HBI-III and HBI-II). Finally, values of HCA scores between HBI-III/HBI-II (74%) and HBI-III/HBI-I (68%), calculated considering the mimetic alanine residue as hydrophobic, are within the range of those observed for structurally related domains (8).

Main Features of the HBI Domains. *HBI-I.* There are no insertions/deletions in HBI-I relatively to FKBP, and all of the main or side chains involved in FK506 binding by FKBP are conserved within the HBI-I domain (Figs. 1 and 3). Indeed, one notes that replacement of FKBP Q^{53} by a glycine in HBI-I does not modify the corresponding CO main chain interaction with a putative ligand. The replacement of H^{87} by a serine appears to have little influence since it interacts

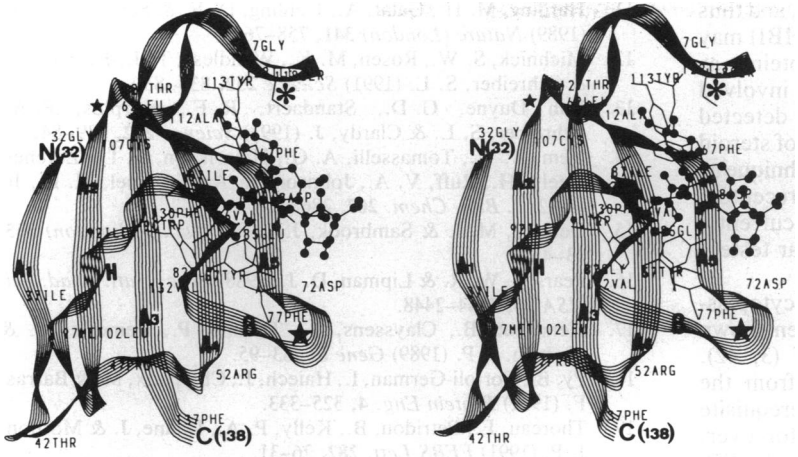

FIG. 3. FKBP main chain ribbon template and proposed structure of HBI-I. FKBP (Protein Data Bank entry 1FKF) and HBI (there are no insertions/deletions between FKBP and HBI) are represented with bound FK506 (black dots). Amino acids critical for ligand binding are indicated (see also Fig. 1). On the same figures the specificities of the HBI-II domain are highlighted: (*i*) shortening (four amino acids) of the 38–45 loop (FKBP numbering; ★), (*ii*) lengthening (three amino acids) of the 55–56 loop (putative ATP binding site; ◆), (*iii*) insertion (one amino acid) within the 87–90 loop (possible ATP phosphate stabilization; *), (*iv*) deletion (one amino acid) within the 31–35 loop (⋆).

through Van der Waals contact at a rather long distance (3.8–4 Å). A similar mutation (alanine) occurs at the same place for the human FKBP13 protein (9). Consequently, all structural requirements that would occur in the putative catalytic site of FKBP are conserved.

HBI-II. Although the HBI-II domain is clearly related to the FKBP template, there are noticeable differences (Figs. 1, 2*a*, and 3): (*i*) 8 of 13 of the amino acids critical for the FK506 binding to FKBP are not conserved; (*ii*) the V^{55}–I^{56} loop (β-like/α loop FKBP numbering) of FKBP (large solid arrow in Fig. 3) is lengthened by 3 amino acids, making impossible the maintenance of the binding of a ligand similar to FK506 as these residues are in contact with the very important FK506 pipecolinyl ring (deeper part of the active site); (*iii*) the 87–90 loop (A2–A3 loop) is largely modified with lengthening of 1 amino acid and introduction of basic residues (large asterisk in Fig. 3). This area is situated in contact with FK506 in the FKBP template (H^{87}); (*iv*) the well-exposed Ω loop 38–45 (A5–β loop), which also binds part of FK506, is shortened by 4 amino acids (large star), conducting to a direct link between positions 39 and 45, through a probable continuous β strand concatenating A5 and β (Figs. 1 and 3); (*v*) the 31–35 loop (A4–A5 loop) is slightly modified by one deletion (G^{33}; small star).

HBI-III. Although HBI-III shares with HBI-II the same distribution of insertions/deletions relative to the first domain and has a sequence significantly related to it, it appears to have lost many characteristics of FKBP yet conserved for the two first domains. All but one of the critical amino acids for FK506 binding have been mutated and the sequence identity with FKBP itself is very weak (5.7%).

Hinge regions and N and C extremities. Figs. 1, 2*a*, and 4 show hinge regions linking the globular domains described above. Hinge 1 between HBI-I and HBI-II is made up of 10 highly hydrophilic and acidic amino acids, and hinge 2 between HBI-II and HBI-III is made up of 13 mainly hydrophilic residues.

The small N-terminal sequence before HBI-I is 31 amino acids long and also is found in human p56 (29), and a longer C-terminal segment after HBI-III shows a consensus calmodulin binding site (30).

DISCUSSION

HBI is a hsp90 binding protein of M_r ≈59,000, originally detected with a monoclonal antibody in rabbit steroid receptors (2). In fact, it binds not only to hsp90 included in untransformed, non-DNA binding, 8–9S mammalian steroid receptors (3) but also to hsp90 not associated with steroid receptors (5).

Here, we show, on the grounds of the amino acid sequence (4), that HBI embodies structural domains related to the FKBP structure (12, 13, 31). Immunophilin FKBP-12 is a cytoplasmic protein (10, 11) of M_r ≈12,000, which binds at least two macrolides, FK506 and rapamycin, demonstrating immunosuppressive activity (32). Like cyclophilin, another non-structurally related immunophilin binding cyclosporin A (33), FKBP is a peptidylprolyl cis–trans isomerase (rotamase) (10). Enzymatic activity is abrogated by the respective ligands, but it has been demonstrated that this inhibition is not directly responsible for the immunosuppressive property of the drugs (34, 35). However, the rotamase activity of FKBP may exert a wide range of regulatory effects on important intracellular proteins (36, 37), as could be the case for HBI.

The binding of ATP is frequently observed in proteins involved in the folding and trafficking of other proteins (15). Within the HBI-II domain, the sequence 199–222 may include two already recognized nucleotide binding consensus sequences. Furthermore, the binding of ATP in the FK506 binding sites is consistent with the lengthening of the V^{55}–I^{56} loop and the presence of Lys-232 (Fig. 3; unpublished data).

In conclusion, HBI appears to be mainly constituted of a string of three similar-sized domains, HBI-I, HBI-II, and HBI-III, separated by two short hydrophilic hinge regions. The C terminus of each domain predicted from the FKBP template allows an easy linking of the successive domains (Fig. 4). The fact that HBI has been purified by affinity chromatography using an FK506 adsorbant (14) is remarkably consistent with our findings. It is likely, but not yet demonstrated, that only HBI-I binds FK506.

Several large proteins have been preliminarily detected by their binding to immunosuppressant drugs (14, 37). The rabbit analog of human "hsp56" described by Yem *et al.* (14) is the

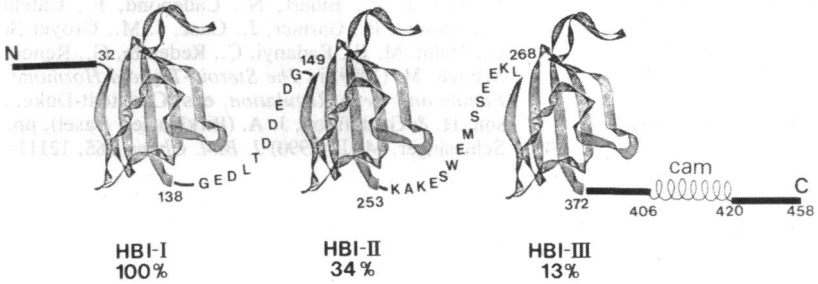

FIG. 4. Schematic representation of the main structural features of HBI. Amino acid identity, relative to HBI-I (100%), is indicated for HBI-II and HBI-III.

only one duly characterized (4). It binds hsp90 (3, 5), and thus we suggest that this hsp90 binding immunophilin (HBI) may influence the function of other hsp90 binding proteins, as possibly for steroid receptors, and consequently be involved in a molecular cascade. Indeed, HBI was originally detected as "p59" in heterooligomeric untransformed forms of steroid receptors (2), following the same immunological technique as for the discovery of hsp90 binding to all steroid receptors (38–41). With the antibodies EC1 and 173 (4), it is currently observed that HBI is present in all mammals so far tested, with a molecular mass in the 56- to 59-kDa range.

HBI (designated originally as p59) is nuclear and cytoplasmic (42) and in heterooligomeric complexes has been shown to bind to hsp90 but not to the receptor itself (3, 42). Hormone, or any other agent detaching hsp90 from the receptor, releases HBI, and this separation is a prerequisite for DNA binding and thus hormone action (1, 42). Moreover, the binding of immunosuppressant drugs to HBI, as to FKBP, may create a link between immunologically active molecules and hormone receptor function.¶

Besides steroid receptors, hsp90 interacts with many other cellular proteins, such as tyrosine kinase oncogenes, heme-regulated eIF2α kinase, etc. (reviewed in ref. 43), and it remains to be determined whether HBI, which is far less abundant than hsp90, is really involved in complexes that have already been detected and whether it has a biological function. HBI may represent a key protein receptor for immunologically active ligands and influence the trafficking and function of part of hsp90 and thus secondarily the function of some proteins interacting with this heat shock protein, including steroid receptors. HBI may be a molecular crossroad between chaperones, endocrinologically related proteins, and immune function.

¶Actually recent results obtained by C. Radanyi, J.-M.R., and E.-E.B. in the rabbit uterus progesterone receptor cell-free system show a FK506- and rapamycin-dependent increase of hormone and antihormone binding (unpublished data).

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