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The serpins alpha-1-antitrypsin and alpha-1-antichymotrypsin specifically interact with immunophilins

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Received April 1, 2003, accepted April 30, 2003

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Pharmazie 58: 811–816 (2003)

In a yeast two-hybrid screen FKBP13, a member of the FK506 Binding Protein (FKBP) family, was detected to interact with the serpin alpha-1-antichymotrypsin (ACT). The specificity of the interaction was confirmed *in vitro* and by the lack of interaction of ACT with FKBP25 and FKBP52. Mutational analysis of ACT revealed that the entire protein is necessary to interact with FKBP13. ACT but also different unrelated small regions of the ACT protein were able to interact with the smaller FKBP12, demonstrating a rather nonspecific interaction with this immunophilin. Naturally occuring mutants of ACT were able to interact as well. Antitrypsin (AT) closely related to ACT did only interfere with FKBP12 a protein that does presumably not reside in the same cellular compartment with AT and ACT. Both serpins interacted with the unrelated immunophilin cyclophilin A. In conclusion the serpin alpha-1-antichymotrypsin physiologically interacts with the ER-immunophilin FKBP13 and the secreted immunophilin cyclophilin A *in vivo* whereas alpha-1-antitrypsin might only react with cyclophilin A; both serpins may be controlled thereby in their genuine function.

1. Introduction

Serine protease inhibitors (serpins) are a superfamily of proteins that were detected in plants, viruses, invertebrates and vertebrates. Data base searching provides evidence for about 500 serpins [1]. According to phylogenetic relationships serpins are divided into 16 different clades arbitrarily lettered A to P and more than 40 are listed herein [2]. These monomeric proteins with various glycosylations consist of 350 to 500 amino acids and fold into three beta-sheets and nine alpha-helices. Selectivity for a particular protease is determined by the amino acid sequence surrounding the reactive site loop, which is a stretch of about 17 residues. Serpins neutralize overexpressed activity of their physiological substrates, serine proteases, and thus regulate diverse physiological processes as coagulation, fibrinolysis, complement activation, angiogenesis, apoptosis, inflammation and neoplasia (for review see [3]). Serine proteases and their inhibitors were identified in invertebrate parasites of mammals and represent a conserved parasitic survival strategy to interfere with the host immune response. Serpins inhibit serine proteases by an irreversible suicide substrate mechanism when the interaction proceeds down the inhibitory arm of a branched pathway. Noncovalent binding of the substrate to the serpin leads to an attack of the active site serine and to covalent ester linkage. The proteinase is subsequently translocated and its active site is distorted. The net result of this conformational rearrangement is kinetic trapping and irreversible inhibition of the protease. In a noninhibitory pathway the enzyme successfully cleaves the inhibitor yielding an active proteinase and an inactive serpin (for review see [4]).

Alpha-1-antitrypsin (AT, SERPINA1) and alpha-1-antichymotrypsin (ACT, SERPINA3) belong to the alpha-1-proteinase inhibitor clade. AT is the main archetypal member of the serpin superfamily. It is primarily synthesized in the liver, and its concentration in human plasma is increased in acute phases of inflammation and infection. AT inhibits pancreatic trypsin and controls effectively the activity of a number of other serine proteolytic enzymes such as neutrophil elastase, plasmin and thrombin. Its major physiological function is the protection of the lower respiratory tract against proteolytic destruction of human leucocyte elastase. In fact, a hereditary deficiency is associated with a 20 to 30 fold increased risk of developing chronic obstructive pulmonary disease. More than 30 variants of AT that may be associatd with emphysema or liver zirrho-

Abbreviations used in this work are: Serpin, serine protease inhibitor; AT, alpha-1-antitrypsin; ACT, alpha-1-antichymotrypsin; CsA, Cyclosporin A; CypA, cyclophilin A; FKBP, FK506 Binding Protein; FKBP13w/oS, FKBP 13 kDa without signalpeptide; β-gal, beta-galactosidase; DBD, DNA binding domain; GAL4AD, GAL4 activation domain; GST, Glutathion-S-transferase; GSH, Glutathion

sis have been described so far. A point mutation (M358R) changes the specificity of AT. This AT-Pittsburgh is an inhibitor of thrombin [5].
Alpha-1-antichymotrypsin (ACT), another serpin of clade A shares 42% homology to AT and its production and regulation are similar. Target proteases of ACT are neutrophil cathepsin G, mast cell chymase, and pancreatic chymotrypsin. Intriguingly, ACT is found within the fibrillary amyloid plaques from pancreatic patients with Alzheimer's disease [6] and appears to facilitate fibril formation by serving as a chaperon for the Aβ-(1–42)-peptide which is released into the extracellular milieu [7]. ACT additionally has the unique property amongst the serpins in that it is able to bind DNA in a stretch of three basic lysine residues [8]. ACT inhibits DNA synthesis either by inhibition of DNA polymerase [9] and/or by inhibition of DNA primase [9] albeit the physiological significance of this *in vitro* observation is not yet known. At least five different mutations were detected in patients with either occlusive cerebrovascular disease [10], Alzheimer's disease [11] or obstructive lung disease with reduced plasma levels of ACT [12–14].
Immunophilins are known to mediate the immunosuppressive action of Cyclosporin A (CsA) and FK506/Tacrolimus. These structurally unrelated drugs bind to Cyclophilin A (CypA) and to the FK506 Binding Protein 12 kDa (FKBP12), respectively. The drug immunophilin complex inhibits the protein phosphatase calcineurin preventing cytosolic signal transduction and thus mediates immunosuppression in T-cells [15]. The pharmacological action of e.g. rapamycin and everolimus is mediated by FKBPs, too, but the mechanism of action differs [16]. Cyclophilins and FKBPs can be found in every species analyzed so far and are classified into two families of at least five and ten members in humans. In general, they are peptidyl prolyl isomerases (E.C. 5.2.1.8) that catalyze the cis trans isomerization of proline imidic peptide bonds. Distinct effects were observed, for example FKBP12 is functionally related to ryanodine calcium channels [17], FKBP52 has been shown to play a role in steroid receptor complexes [18] and CypA is a chemoattractant for leucocytes [19] and a growth factor secreted upon oxidative stress [20].
FKBP13 is an immunophilin located in the endoplasmic reticulum [21], its mRNA is overexpressed upon heat shock and missfolding of proteins after tunicamycin treatment. However, specific interaction with other proteins was detected, indicating that FKBP13 is not a mere chaperon [22, 23]. The physiological function of immunophilins in endocrine systems is not well characterized. It was the aim to identify new functions of FKBPs; FKBP13 was used as a bait in a yeast two-hybrid screen and one of the preys detected was alpha-1-antichymotrypsin. Interaction of other FKBPs and Cyclophilin A with alpha-1-antichymotrypsin and alpha-1-antitrypsin was investigated as well.

2. Investigations and results

2.1. Yeast two-hybrid screen

The physiological function of immunophilins in endocrine systems is not well characterized. A commercially available fetal human liver cDNA expression library was used to identify proteins that interact with the immunophilin FKBP13 which is localized in the endoplasmic reticulum. Plasmids encoding FKBP13 without signal peptide fused to the LexA-DBD (pBTM-FKBP13w/oS) and the liver cDNA library were sequentially transformed into yeast L40. Ten million yeast double transformants (corresponding to 3.5×10^6 independent clones) were screened and selected for histidine prototrophy. Among 216 colonies isolated as His^+, 21 were found to display beta-galactosidase (β-gal) activity. Plasmids were isolated from each of these clones and were used for retransformation of yeast L40 pretransformed with pBTM-FKBP13w/oS. Six clones remained positive (clone A3, B1, B31, B55, C8, C30) and turned out to code for five different cDNAs after sequence analysis. Two of the six clones (B55, C30) representing the cDNA of alpha-1-antichymotrypsin (ACT) corresponding to amino acids 126–408 (B55) and the full length 408 amino acids wild type protein (C30, 1.5 kb) were selected for the present study.

2.2. Interaction of ACT with other FKBPs (specificity of interaction)

FKBPs share a common binding motif for the immunosuppressant FK506. To check the specificity of the FKBP13/ACT interaction, yeast L40 was cotransformed with the full length ACT clone (C30) and different plasmids each of them coding for FKBP12, FKBP25, and FKBP52 fused in frame to the DBD of LexA (pBTM-FKBP12, 25, and 52). Cotransformants were checked for histidine prototrophy and β-gal activity. FKBP25 and FKBP52 did not interact with ACT in the yeast two-hybrid system, whereas FKBP12 was detected positively to interfere with ACT.

2.3. Interaction of ACT with FKBP13 in vitro (GST pull-down assay)

In order to verify the results obtained with the yeast two-hybrid screen, the interaction of ACT with FKBP12 or FKBP13 was analyzed in a GST pull-down experiment. The cDNA of FKBP12 and FKBP13 was subcloned into a bacterial expression vector and both immunophilins were expressed as GST-fusion proteins. ACT was expressed with an *in vitro* translation system and labeled with [^{35}S]methionine. An unrelated protein, luciferase, was used as a negative control. Labeled proteins were incubated with GSH-Sepharose beads loaded with GST-FKBP12 or GST-FKBP13. After incubation, the supernatant and proteins adsorbed on GSH-Sepharose beads were analyzed. Staining and autoradiography of the SDS-PAGE demonstrate that FKBP12 and FKBP13 bind [^{35}S]ACT *in vitro* whereas luciferase does not interact with the FKBPs (Fig. 1). [^{35}S]ACT is not bound to GSH-Sepharose beads alone (data not shown).

2.4. Interaction of ACT with cyclophilin A

Cyclophilins are the second family of immunophilins. To check for cross reactivity, binding of ACT to the prototype of cyclophilins, cyclophilin A (CypA), was investigated in the yeast two-hybrid system. Yeast L40 was cotransformed with a plasmid expressing LexA-CypA hybrids (pBTM-CypA) and clone 30 and was tested for β-gal activity and histidine prototrophy. ACT and CypA interact in the yeast two-hybrid system as did ACT with FKBP12 and FKBP13w/oS. Interactions of ACT with the selected immunophilins are shown in Table 1.

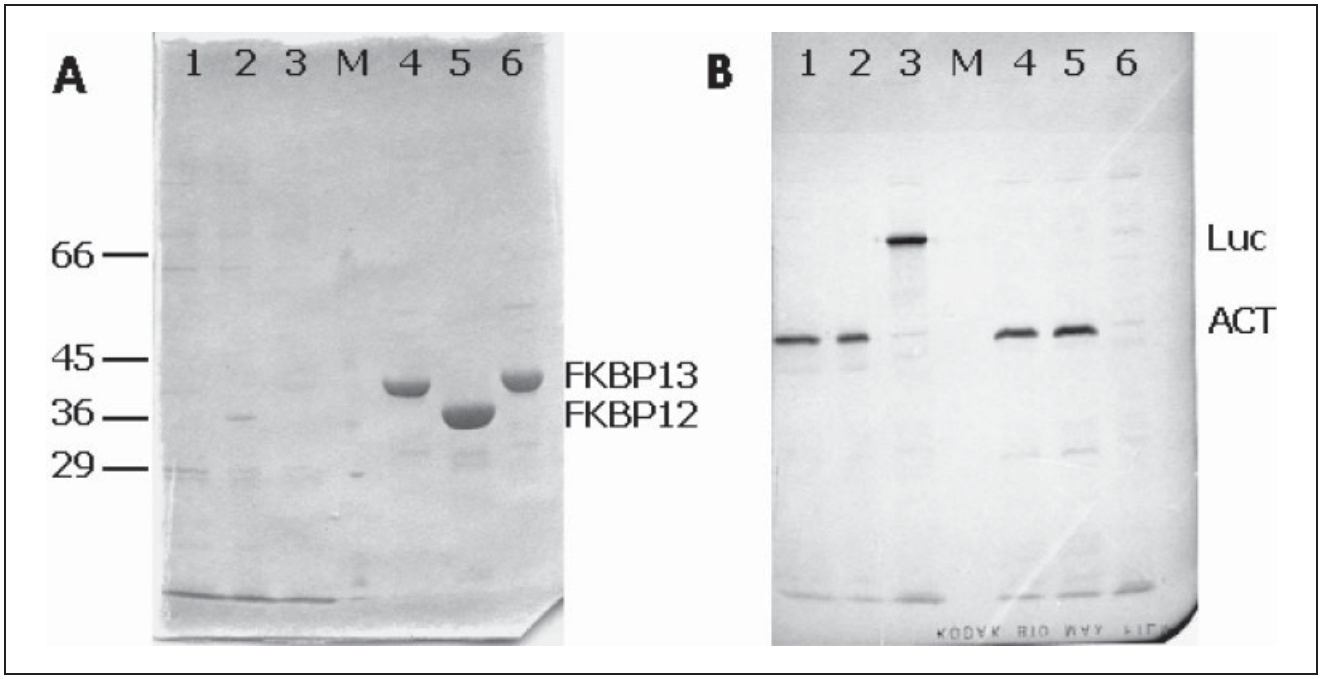

Fig. 1: Interaction of alpha-1-antichymotrypsin (ACT) with immunophilins *in vitro*.
ACT and luciferase were prepared and [^{35}S]-labelled *in vitro* and GST-FKBP12 and GST-FKBP13 fusion proteins were prepared as described in experimental procedures. GST-FKBP was trapped on GSH-Sepharose beads and incubated with the labelled proteins. The supernatant (line 1–3) and the adsorbed proteins (line 4–6) were analysed with SDS-PAGE and labelled proteins were visualized by autoradiography. Staining of the gel demonstrates binding of the GST-FKBP fusion proteins to GSH-Sepharose beads (A). The autoradiography demonstrates that labelled ACT and luciferase can be detected in the supernatant but only labelled ACT can be bound to FKBPs (B). Migration positions of molecular size markers in kDa are indicated.

2.5. Interaction of ACT with FKBP12 and FKBP13 in the presence of FK506

The crystal structure of FK506/FKBP12 complexes predicts that the recognition site in the endogenous ligand(s) equivalent to FK506 would be best emulated by Iso-Pro or Leu-Pro motifs [24]. FK506 might mimic the binding motif of ACT to FKBP12 and FKBP13. For this reason, the influence of the drug on the interaction of ACT with FKBP12 and FKBP13 was investigated in the yeast two-hybrid system. Cotransformants of yeast L40 with clone C30 and pBTM-FKBP12 or pBTM-FKBP13w/oS were incubated in the presence or absence of FK506. Cells were collected and measured for β-gal activity. As shown in Fig. 2a, 1 μM FK506 abolished the interaction of ACT with FKBP12. On the other hand, interaction of ACT with FKBP13w/oS could not be suppressed to more than 44%, even in the presence of 50 μM FK506 (Fig. 2b).

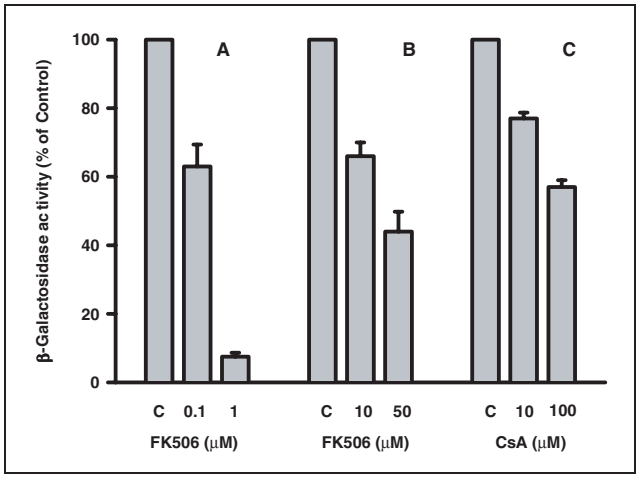

Fig. 2: Influence of immunosuppressants on the interaction of alpha-1-antichymotrypsin (ACT) with immunophilins.
Yeast L40 was cotransformed with pACT2-ACT (clone C30) and either with pBTM-FKBP12, -FKBP13w/oS, or -CypA. Cotransformants were incubated in the presence or absence of FK506 (FKBP12, A, FKBP13w/oS, B) or cyclosporin A (CypA, C) and analysed as described in experimental procedures. Shown are the results of 3–6 independent experiments ± S.E.M. The control was defined as 100%; the solvent ethanol was without influence.

2.6. Interaction of ACT with cyclophilin A in the presence of cyclosporin A

The structurally unrelated immunosuppressant cyclosporin A (CsA) might mimic the binding motif of an endogeous ligand to cyclophilin A (CypA). Therefore, the influence of the drug on the interaction of ACT with CypA was investigated in the yeast two-hybrid system. Cotransformants of yeast L40 with clone C30 and pBTM-CypA were incubated in the presence or absence of CsA and analyzed as described above. CsA only weakly suppresses the interaction of ACT with CypA expressed as percent β-gal activity. In the presence of 100 μM CsA β-gal activity is reduced to 57% (Fig. 2c).

Table 1: Comparison of the interactions of alpha-1-antichymotrypsin (ACT) and alpha-1-antitrypsin (AT) with immunophilins.

	FKBP12	FKBP13	FKBP25	FKBP52	CypA
ACT	+	+	−	−	+
AT	+	−	−	−	+

Yeast L40 was cotransformed with pACT2-ACT (clone C30) or pACT2-AT and with either pBTM-FKBP12 or -FKBP13w/oS, -FKBP25, -FKBP52, -CypA. Cotransformants were analyzed for β-gal activity and histidine prototrophy as described in Experimental. Cotransformants displaying positive β-gal activity within 4 h and histidine prototrophy were regarded as positive (+). Each combination represents the results of at least two independent transformations.

Table 2: Interaction of deletion mutants of alpha-1-antichymotrypsin (ACT) with immunophilins

		FKBP12	FKBP13	CypA
Full length ACT	1 (−25) 1312 (408)	+	+	+
ACT-M1	462 (126) 1312 (408)	+	+	+
ACT-M2	1 (−25) 966 (293)	+	−	−
ACT-M3	462 (126) 966 (293)	+	−	−
ACT-M4	967 (294) 1312 (408)	+	−	+

Yeast L40 was cotransformed with pACT2-ACT (wild type, clone C30) or with pACT2-M1 (clone B55), pACT2-ACTM2, -M3, or -M4 and with either pBTM-FKBP12, -FKBP13w/oS, or -CypA. Cotransformants were analysed for β-gal activity and histidine prototrophy as described in Experimental procedures. Cotransformants displaying positive β-gal activity within 4 h and histidine prototrophy were regarded as positive (+). Each combination represents the results of at least two independent transformations. Column 2 is the graphic representation of the deletion mutants used indicating the number of the first and last base (or amino acid). Numbers are according to [32].

2.7. Interaction of alpha-1-antitrypsin with immunophilins

Alpha-1-Antitrypsin (AT) and alpha-1-antichymotrypsin (ACT) share 40% homology and are most closely related to each other within the serpin superfamily. To check for a common binding motif the plasmid coding for a AT-Gal4AD hybrid protein (pACT2-AT) was cotransformed with pBTM-FKBP12, 13w/oS, 25, 52, or -CypA into yeast L40. The five cotransformants were checked for β-gal activity and histidine prototrophy. AT did not interact with FKBP13w/oS, 25, 52 in the yeast two-hybrid system, whereas FKBP12 and CypA were detected positively to interfere with AT (Table 1).

2.8. Interaction of deletion mutants of ACT with immunophilins

To further characterize the binding properties of ACT to the immunophilins, deletion mutants were prepared. Truncated cDNA of ACT coding for amino acids −25 to 293, 126 to 293, and 296 to 408 (ACTM2, M3, and M4) was subcloned into pACT2 to express ACTM2, 3, and 4-GAL4AD hybrids. Clone B55 is ACTM1 coding for amino acids 126 to 408. Yeast L40 was cotransformed with the prepared plasmids and with pBTM-FKBP12, -FKBP13w/oS and -CypA, respectively. The transformed reporter strain was assayed for β-gal activity and for histidine prototrophy (Table 2). FKBP13 interacts with the wild type ACT and the B55 clone (ACTM1) corresponding to amino acids 126 to 408. None of the other truncated forms of ACT alone was sufficient to interact with FKBP13. On the other hand, each of the short peptides of ACT interacts with FKBP12 in the yeast two-hybrid system. The wild type ACT and clone B55 interact with CypA as described above, additionally the 112 amino acids carboxyterminal part of ACT is sufficient to interfere with CypA in the yeast two-hybrid system. Amino acids −25 to 293 and 126 to 293 alone are not sufficient to interact with CypA.

2.9. Interaction of naturally occuring mutants of ACT with immunophilins

Four different point mutations and one two-base pair deletion of ACT were detected in patients with occlusive-cerebrovascular disease or obstructive lung disease with reduced plasma levels of ACT. Two point mutations Bonn (Pro229Ala) and Isehara1 (Met389Val) and the two-base pair deletion from codon 391 (Isehara2) were introduced into clone C30 to give pACT2-ACTBonn, -Isehara1, and -Isehara2. The two other known mutations were not checked as they are not part of the short B55 clone that is sufficient to interact with FKBP12, FKBP13w/oS and CypA. Yeast L40 was cotransformed with each of these plasmids and with pBTM-FKBP12, FKBP13w/oS or CypA, respectively. The constructs were analyzed in the yeast two-hybrid system for β-gal activity and histidine prototrophy. None of the mutations influenced the interaction of ACT with FKBP12 or with CypA. The point mutations Bonn and Isehara1 did not influence the interaction of ACT with FKBP13, too. The combination of Isehara2 and FKBP13 was the only one of the nine combinations tested that did not interact in the yeast two-hybrid system (Table 3)

Table 3: Interaction of described mutants of alpha-1-antichymotrypsin (ACT) with immunophilins

	Mutation	FKBP12	FKBP13	CypA
ACT	−	+	+	+
Bonn	Pro229Ala	+	+	+
Isehara1	Met389Val	+	+	+
Isehara2	2 bp deletion at Lys390	+	−	+

Yeast L40 was cotransformed with pACT2-ACT (wild type, clone C30) or pACT2-Bonn, -Isehara1, -Isehara2 and with either pBTM-FKBP12, -FKBP13w/oS, or -CypA. Cotransformants were analysed for β-gal activity and histidine prototrophy as described in Experimental procedures. Cotransformants displaying positive β-gal activity within 4 h and histidine prototrophy were regarded as positive (+). Each combination represents the results of at least two independent transformations. Column 2 indicates the mutation tested.

3. Discussion

3.1. Alpha-1-antichymotrypsin interacts with FKBP13 and CypA

A yeast two-hybrid screen was carried out using the FK506 Binding Protein 13 kDa (FKBP13) as a bait and a human liver expression library as a prey. FKBP13 is located in the endoplasmic reticulum and at least one specific binding protein for FKBP13 is known demonstrating a special function of this immunophilin [23]. Our screen revealed alpha-1-antichymotrypsin (ACT) to inter-

act with FKBP13. Two other FKBPs, namely FKBP25 and FKBP52 do not interact under the same conditions excluding that the conserved FK506 binding pocket of the FKBPs could solely be responsible for the interaction. Additionally, ACT and FKBP13 interact in an *in vitro* system, too, stressing the specificity of the interaction. These results show that other factors of the yeast system do not contribute to the interaction of the two proteins. Another explanation for the detected interaction could rely on the ability of ACT to bind to DNA [8]. DNA binding cannot be the only reason for the positive reaction of ACT in the yeast two-hybrid system because of the missing interaction with FKBP25 and FKBP52 and because of the positive results *in vitro*.

Another immunophilin, cyclophilin A (CypA) that binds the immunosuppressive drug cyclosporin A (CsA), turned out to interact with ACT, too. This kind of "cross reaction" between the two families of immunophilins has alredy been shown for the transcription factor YY1 that binds to FKBP12 and CypA [25]. Interestingly Yang et al. demonstrated that the carboxy-terminal 83 amino acids of YY1 are important for YY1-FKBP12 interaction, a region dispensible for YY1-CypA interaction. This clearly demonstrates that possible binding of a FKBP family member and a cyclophilin to one common protein differs on behalf of the binding properties. Deletion mutation analysis demonstrates that only the first 126 amino acids of ACT are dispensible for the interaction with FKBP13 and no short amino acid region of ACT could be detected to exert the interaction with FKBP13. On the other hand, the carboxyterminal 112 amino acids of ACT are sufficient to bind to CypA. This demonstrates that binding of ACT to the two immunophilins differs with regard to the binding regions needed.

Binding of ACT to FKBP13 and CypA was analyzed in the presence of FK506 or CsA, respectively. The rationale of this approach was to check whether the binding pocket of the immunophilins for their drug contributes to the interaction with ACT. Binding of ACT to FKBP13 and CypA could be reduced but not entirely suppressed by the immunosuppressive drugs even if a concentration of the drugs in the higher micromolar range was used. If the binding pocket of the immunophilins for their drugs contributes to the interaction with ACT this amino acid motif will not be sufficient to manage the interaction. Finally, naturally occuring mutations of ACT were checked for their interaction with the immunophilins. These mutations were detected in patients who suffered from occlusive cerebrovascular disease [10] or obstructive lung disease [13, 14] and who had reduced ACT levels. Of the mutations tested, only a two-base pair deletion at position Lys391 led to a loss of interaction of FKBP13 (but not CypA) with ACT. The two-base pair deletion led to a frame shift and loss of one of the two lysine rich DNA binding motifs of ACT at positions Lys210 to Lys212 and Lys391 to Lys393. As already discussed, DNA binding of ACT as a rationale for a false positiv reaction in the yeast two-hybrid system was excluded but the physiological relevance of the loss of interaction cannot be explained yet.

3.2. ACT interacts with FKBP12

Further analysis of the interaction of ACT with other FKBPs has shown that ACT and FKBP12 interact in the yeast two-hybrid system, too, and the interaction was confirmed *in vitro*. Unlike FKBP13, binding of FKBP12 to ACT in the yeast two-hybrid system can be prevented by FK506 in the lower micromolar range indicating that the FK506 binding pocket of the immunophilin plays an important role for the interaction. The affinity of FK506 to FKBP12, expressed by the drug's ability to inhibit the rotamase activity of the protein ($Ki = 2-4$ nM) is about ten times that of FK506 to FKBP13 ($Ki = 50$ nM). If binding of ACT to FKBP12 was similar to that of FKBP13 one would expect that binding to the latter could be suppressed by the drug as well, at least in a 50 fold higher concentration of FK506. The immunosuppressive drug is expected to bind to endogenous yeast-FKBPs as well and its influence on the interaction of proteins in the yeast two-hybrid system might be diminished thatway. However, binding of FKBP13 to other proteins, namely a homologue of the erythrocyte membrane cytoskeletal protein 4.1 and FAP48, can be suppressed by 50 μM FK506 [22, 23] demonstrating that the influence of the drug on protein complexes can be examined in the yeast two-hybrid system, too. Another approach to narrow the FKBP12 binding region within ACT failed as each region of about 100 to 300 amino acids demonstrated binding to FKBP12 in the yeast two-hybrid system. Indeed, FKBP12 might "recognize" ACT at different, unrelated parts of the peptide albeit this multiple interaction demonstrates non-specificity, too. ACT and FKBP13 are two peptides that are transported into the endoplasmic reticulum because of their amino-terminal signal peptide of about 25 amino acids. FKBP13 resides in this cellular compartment due to its carboxy-terminal putative ER retention signal (RRTEL) whereas ACT is secreted to find its physiological substrate in plasma. Interaction of ACT with FKBP13 is likely because of their appearance in the same cellular compartment. Otherwise, interaction of ACT with the cytoplasmic FKBP12 appears to be less reasonable and the interaction of different unrelated deletion mutants of ACT with FKBP12 underlines a rather non-specific interaction of these two proteins. Complexes of ACT with CypA might be possible on a physiological background as both peptides appear in plasma but a possible influence of CypA on the serine protease inhibitory action of ACT is yet unknown.

3.3. Alpha-1-antitrypsin interacts with FKBP12 and CypA

Alpha-1-antitrypsin (AT) and ACT are the most closely related serpins and share 40% homology. Because of their similarity, AT was checked for interacting with the immunophilins used in this work. AT does not interact with FKBP13, FKBP25, and FKBP52 in the yeast two-hybrid system confirming the results of the screen as AT mRNA would be expected to be more abundantly expressed than ACT mRNA. Again, FKBP12 interacts with AT but the physiological relevance is doubtful because of the different distribution of the two proteins within the cell. Like ACT, AT interacts with CypA in the yeast two-hybrid system. Binding of ACT with CypA can be narrowed to a carboxy-terminal 113 amino acids region of the ACT protein. Sequence alignment of this short region with AT using the BLAST 2 Sequences tool [25] confirmes a 39% identity with amino acids 321 to 417 of ACT (data not shown) and it may be speculated that the same short region of AT is sufficient to mediate the interaction of AT with CypA. On the other hand, two other proteins known to interact with CypA, namely the transcription factor YY1 [26] and the HIV-1 gag protein [27] do not

have any significant similarity to the 113 amino acids region of ACT excluding a general motif displayed by this peptide.

In our work the interaction of two serpins, namely alpha-1-antitrypsin (AT) and alpha-1-antichymotrypsin (ACT), with different immunophilins was examined. Both serpins interact with the cytosolic protein FKBP12 when tested in the yeast two-hybrid system. With regard to a different distribution within the cell and the results of our deletion mutant experiments interaction of FKBP12 with ACT or AT seems to be non-specific. On the other hand it is concluded that AT and ACT interact with cyclophilin A (CypA). Since these proteins are secreted into plasma the physiological relevance of this interaction is likely. ACT, but not AT, interacts with the FK506 binding protein FKBP13 and since both proteins are present in the endoplasmic reticulum a physiological interaction is likely, too.

4. Experimental

4.1. Expression vectors

The cDNA encoding FKBP13 amino acids 16–142 was amplified from pBluescript-FKBP13 (a gift from Dr. S. Burakoff, Dana-Faber Cancer Institute, Boston, MA) using specific primers CGCCGGAATTCATGCTGAGCGCCGTG and CGGCTGGATCCGAACAGTCTGGTC. The cDNA encoding full-length FKBP25 was amplified from pBluescript-FKBP25 (a gift from Dr. S. Burakoff, Dana-Faber Cancer Institute, Boston, MA) using the specific primers CGCGCGAATTCAACAGTCTGGTCCCTGATG and GGCGTAGGATCCGGGGTTGACTCCGGGGGC. The cDNA encoding full length cDNA of CypA was amplified from I.M.A.G.E clone 5264185 by using the specific primers GGTCCGGAATTCATGGTCAACCCCACCGTGTTC and GGCAGCTGGATCCACAAGTCAAACTTATTCGAG. After restriction digest, cDNAs were fused to the DNA binding domain of LexA by inserting the EcoRI/BamHI fragments into pBTM116 [28] to give pBTM-FKBP13w/oS, pBTM-FKBP25, and pBTM-CypA, respectively. pBTM-FKBP12, pBTM-FKBP52, and pGEX-FKBP12 (a gift from Dr. B. Chambraud, INSERM U488, Bicêtre, France) are described elsewhere [29]. The cDNA encoding full length FKBP13 was amplified from pBluescript-FKBP13 using specific primers GGCGTAGGATCCGGGGTTGACTCCGGGGGC and CGCGCGAATTCAACAGTCTGGTCCCTGATG. After restriction digest, the cDNA was fused to GST by inserting the BamHI/EcoRI fragment into pGEX-2T (Pharmacia Biotech, Uppsala, Sweden) to give pGEX-FKBP13. The cDNA of full lenght ACT was amplified from clone C30 with the specific 3′-primer GCCGGTAGCGGCCGCGATTCATAGATCTCTCGAG, digested with EcoRI/NotI and subcloned into pGEM11Zf(+) (Promega, Madison, WI) to give pGEM-ACT. Truncated cDNA of ACT was prepared from clone C30, or B55 by XhoI-digestion and religation of the plasmids to give pACT-ACTM2 and pACT-ACTM3, respectively. Note, there is an internal XhoI-site in ACT at position 966. The cDNA of ACTM4 was amplified from clone C30 using the specific 5′-primer GAGCCTGGAATTCCGAGGGACTATAACCTG, digested and recloned into the EcoRI/XhoI-site of pACT2 to give pACT-ACTM4. Three specific mutations were introduced in pGEM-ACT using the GeneEditor™ *in vitro* site directed mutagenesis system (Promega, Madison, WI) according to the manufacturer's instructions. Fragments bearing the mutations were excised with PmaCI and BsmI and religated into the C30 clone to give pACT-Bonn, pACT-Isehara1 and pACT-Isehara2. The cDNA encoding full length AT was excised from I.M.A.G.E clone 4767527 and was ligated into the EcoRI/XhoI-site of pACT2 to give pACT-AT. All inserts and mutations were verified by sequencing.

4.2. Two-hybrid screen

The human fetal liver MATCHMAKER cDNA library (Clontech, Palo Alto, CA) was a gift from Dr. C. Sorg (Institute of Experimental Dermatology, Münster, Germany). The yeast reporter strain L40, containing two reporter genes, *HIS3* and *LacZ*, was sequentially transformed with pBTM-FKBP13w/oS and the cDNA library using the lithium acetate method [30]. Double transformants were plated on Minimal SD Base containing the -Leu/-Trp/-His DO supplement (Clontech, Palo Alto, CA). The plates were incubated at 30 °C for 5 days. His^+ colonies were patched and assayed for β-gal activity. Positive clones, inserted into the pACT2 vector, were rescued and tested for specificity by retransformation into yeast L40 with pBTM-FKBP13w/oS. Different clones, that expressed detectable β-gal activity within four hours, were used for further analysis. All assays of β-gal activity and histidine prototrophy were performed according to standard procedures [31].

4.3. GST pull-down assays

In vitro translation reaction was carried out with pGEM-ACT in the presence of [^{35}S]methionine using the TNT-T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI) according to the manufacturer's instructions. Overexpression and affinity purification of FKBP12 and FKBP13 was achieved in *E. coli* BL21pLysS and with the GST Purification Module (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Translated ACT was diluted in a sterile buffer containing 20 mM sodium phosphate (pH 7.3), 150 mM NaCl, and 0.5 mM EDTA and aliquots were incubated at 4 °C for 2 h with GST-FKBP12 or GST-FKBP13 bound to GSH-Sepharose beads in a final volume of 200 µl in the same buffer. Protein complexes were precipitated by centrifugation of the Sepharose beads. The supernatant was kept for analysis and after extensive washing with the same incubation buffer bound proteins were resolved with Laemmli sample buffer. Supernatant and the resolved proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); proteins were detected by coomassie blue staining or by autoradiography.

Acknowledgements: We thank Dr. B. Chambraud (INSERM U488, Bicêtre, France) for providing pBTM-FKBP12, pBTM-FKBP52, and pGEX-FKBP12 and Dr. S. Burakoff (Dana-Faber Cancer Institute, Boston, MA) for providing cDNA of FKBP13 and FKBP25. We thank Dr. C. Sorg (Institute of Experimental Dermatology, Münster, Germany) for the gift of the cDNA expression library and Dr. A. Friedrich (Fujisawa GmbH, München, Germany) for the gift of FK506.

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