

Designed Ankyrin Repeat Proteins as Anti-Idiotypic-Binding Molecules

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ABSTRACT: According to the network theory antibodies may act as antigens thus generating anti-idiotypic antibodies that can function as regulators of immune responses. Designed ankyrin repeat proteins (DARPs) are a new class of binding proteins and may serve as an alternative to antibodies. Selections from large DARPin libraries against the variable regions of a murine monoclonal anti-human IgE antibody, termed BSW17, yielded two highly specific anti-idiotypic DARPs both with high affinity. Their binding characteristics were comparable with these of a previously selected anti-idiotypic antibody. *In vitro* cell assays showed that the anti-idiotypic DARPs were able to inhibit the binding of BSW17 to cell-bound IgE and prevented BSW17 functional activity. These experiments demonstrate the possibility to isolate anti-idiotypic DARPs recognizing idiotypic determinants analogous to antibodies. In the future these DARPs may be further analyzed for their potential as putative vaccine candidates.

KEYWORDS: designed ankyrin repeat proteins; DARPs; anti-idiotypic; allergy

INTRODUCTION

The immune network theory states that antibodies can be induced against the variable regions of other antibody molecules.¹ A set of these antibodies termed anti-idiotypic antibodies, bearing the internal image of the antigen,² may serve to regulate the immune responses associated with autoimmunity, cancer, and allergy.^{3,4}

In allergy there is evidence that anti-idiotypic antibodies as well as antibodies against the constant regions play a regulatory role in the synthesis

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of IgE.⁵⁻⁸ We have previously isolated a murine monoclonal anti-IgE antibody, termed BSW17, that is non-anaphylactogenic and prevents the binding of IgE to the high-affinity receptors for IgE (Fc ϵ RI) on mast cells and basophils.⁹ Recently, a humanized monoclonal anti-IgE antibody (Omalizumab, Xolair[®]) has been developed that possesses similar characteristics as BSW17 and is currently used to treat atopic individuals.¹⁰⁻¹² In a previous study using a human Fab phage display library, we have isolated two anti-idiotypic antibodies that are specific to BSW17.¹³ When used for immunization experiments, these antibodies were able to induce a BSW17-like anti-IgE response, indicating that they were successfully mimicking the original epitope. These findings suggested that anti-idiotypic antibodies can be used as a vaccine to induce an anti-IgE response preventing the binding of IgE to Fc ϵ RI and thereby the degranulation of effector cells implicating in allergic disease.¹⁴

While antibodies are being widely used for therapy, they suffer from many drawbacks including low expression yields, a tendency to aggregate, and dependence on disulfide bonds for stability. To solve the problems of antibodies, while maintaining their ideal target-binding characteristics, designed ankyrin repeat proteins (DARPs) were developed, based on natural repeat proteins.^{15,16} Repeat proteins were evolved by nature, similar to antibodies, to mediate, protein-protein interactions.^{17,18} They are built from repeat units that stack together forming a hydrophobic core and a large solvent accessible surface involved in target binding. In fact, the adaptive immune system of jawless vertebrates is based on leucine-rich repeat proteins and not on antibodies.^{19,20} DARPs were developed by a consensus design approach, relying on several thousands of known ankyrin repeat proteins.^{21,22} The number of repeat modules in a DARP can be adapted, allowing the potential target interaction surface to vary in its dimension. Libraries with different numbers of repeat modules have been created and DARPs were isolated, which bound their target with great specificity, selectivity, and affinity.²³⁻²⁵ Further, the DARPs are well expressed in the bacterial cytoplasm, are devoid of cysteines, and are highly stable in the intra- and extracellular space.

This study investigated the potential of DARP libraries to be used as a source for anti-idiotypic binders having the same characteristics as anti-idiotypic antibodies. To address this question we used DARP libraries to isolate binders against the variable region of the murine monoclonal anti-human IgE antibody, BSW17. The specificity of two of the selected binders was determined by enzyme-linked immunosorbent assay (ELISA) experiments using different monoclonal antibodies and *in vitro* using functional cell assays. The high specificity of the selected DARPs and their capacity to prevent functional activity of BSW17 are encouraging results. Further investigations will show their potential use as vaccine candidates to induce a BSW17-like antibody response *in vivo* that might prevent allergic reactions.

MATERIALS AND METHODS

Selection of Anti-Idiotypic DARPins

The selection of DARPins against the mouse monoclonal anti-human IgE, BSW17 was performed using two DARPin libraries containing two or three randomized internal ankyrin repeats (AR) between an N- and a C-terminal capping AR, thus named N2C and N3C, respectively.²² The libraries were subjected to four selection rounds against the target molecule BSW17 using ribosome display as described.^{25–27} To prevent elution of binders against the constant part of the mouse IgG1, preselection rounds were performed on a mouse monoclonal anti-human IgE antibody with another IgE epitope specificity, termed Le27.

After the fourth round of panning, DNA of the selected pools was cloned into pQE30 and transformed into *E. coli* XL1-Blue cells. For the ELISA screening the crude extract of 1 mL overnight cultures in LB containing 50 µg/mL carbenicillin was used and prepared as described previously.²⁵ For the inhibition ELISA and cell assay selected anti-idiotypic DARPins were produced in larger expression culture volumes and then purified over a metal affinity column according to the manufacturer's instructions (Clontech, TALON™ Metal Affinity Resins User Manual; Mountain View, CA).

Inhibition ELISA

For the inhibition assay Maxisorp plate wells (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 5 µg/mL IgE (Sus11) and blocked with 200 µL phosphate-buffered saline (PBS) containing 0.5% BSA for 1 h at RT and 1 h at 4°C. BSW17 at a concentration of 0.2 µg/mL was preincubated with different concentrations of anti-idiotypic DARPins (D35 and D40) and as control of anti-maltose-binding protein (MBP) DARPin (off7) for 30 min at RT and then added to the wells for 1 h at 4°C. After washing with PBS and PBS/Tween 0.01% binding of BSW17 was detected with horseradish peroxidase-conjugated anti-mouse IgG antibody diluted in 1:1000 in PBS/0.5% BSA. ELISA was developed with 3,3',5,5'-tetramethylbenzidine as substrate for 5 min and then stopped with 1M sulfuric acid. The absorbance was measured after 10 min at 450 nm with a standard ELISA reader (BIO-TEK, EL808; Winooski, VT).

Sulfdoleukotriene (sLT) Release from Rat Basophilic Leukemia Cells

The sLT release from RBL-2H3-huα cells²⁸ (rat basophilic leukemia cell line stably transfected with the α-chain of FcεRI) was measured using the Cellular

Antigen Stimulation Test (CAST®-2000 ELISA) from Bühlmann Laboratories (Allschwil, Switzerland) following a slightly modified assay protocol.²⁹ Briefly, cells were seeded in 24-well tissue culture plate (5×10^4 cells in 0.5 mL of antibiotic-free RPMI 1640, 10% FCS) and incubated with either 50 ng of human IgE (Sus 11) alone or in combination with 5 nM BSW17 or Omalizumab (Xolair®; Novartis, Basel, Switzerland) and anti-idiotypic DARPins (D35 and D40) at different concentrations overnight in a 5% CO₂ incubator. The cells were then washed and triggered with the mouse monoclonal anti-IgE antibody, Le27 (500 ng/well) for 60 min at 37°C. Secreted sLT was quantified by competition ELISA using a constant amount of alkaline phosphatase-labeled sLT (sLT-AP) to compete with cellular sLT. Samples were briefly centrifuged (1000 g) and 200 µL supernatant aliquots mixed with 200 µL of a 1:1 mixture of sLT-AP solution and ELISA buffer. Anti-mouse IgG precoated microtiter plates were incubated with 50 µL of the mouse anti-sLT mAb (kit component) at 25°C for 2 h. The wells were washed twice with PBS and 195 µL of the sLT competition mixture was added and allowed to incubate for 90 min at 25°C. The wells were washed twice with 300 µL PBS and substrate was added for 40 min at 25°C. The OD at 405 nm was measured and the amount of secreted sLT was determined according to the CAST-ELISA evaluation protocol.

RESULTS

Selection of Specific High-Affinity Binders against BSW17

Two different DARPin libraries with either two or three randomized internal repeats, termed N2C and N3C, were applied to select binders against the murine monoclonal anti-human IgE antibody, BSW17. Four panning rounds were performed to enrich for specific anti-BSW17 DARPins, which bind to the antibody's variable region. Two DARPins, one from each library, were isolated showing BSW17 specificity. They were produced at a high level in *E. coli* for further characterization. BIAcore measurement showed affinities in the nanomolar range for both DARPins (D35 and D40 with K_D of 18 nM and 12 nM, respectively). To test the epitope specificity of the two binders, they were applied as inhibitors for the binding of BSW17 to IgE in an ELISA. Both anti-idiotypic DARPins (D35 and D40, FIG. 1) prevent the binding of BSW17 to IgE in a concentration-dependent manner, indicating that they recognized the variable antigen binding site of BSW17. No inhibition was observed when a control DARPin (off7) with specificity to MBP was tested, indicating that the inhibitory activity was due to a non-specific DARPin effect.

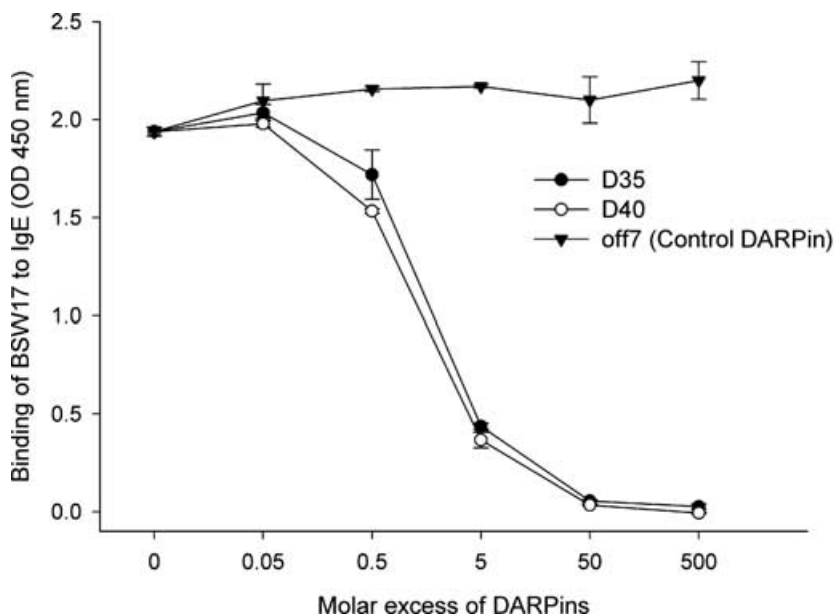


FIGURE 1. Inhibition of BSW17 binding to IgE by selected DARPins. Human IgE (antigen) was coated at a concentration of 5 $\mu\text{g/mL}$. BSW17, at a concentration of 0.2 $\mu\text{g/mL}$, was preincubated with different concentrations of anti-idiotypic DARPins (D35 and D40) and as control of an MBP-specific DARPIn (off7) for 30 min at RT and then added to the wells. The amount of bound BSW17 was detected with a horseradish peroxidase-conjugated anti-mouse IgG.

Selected DARPins Restore Mediator Release from Human $\text{Fc}\epsilon\text{RI}\alpha$ -Transfected Rat RBL-2H3 Cells

It has been demonstrated that BSW17 interferes with the binding of IgE to the α -chain of cell surface $\text{Fc}\epsilon\text{RI}$ ($\text{Fc}\epsilon\text{RI}\alpha$) and prevents mediator release from basophils.⁹ To test whether the anti-idiotypic DARPins could modulate the functional activity of BSW17, a rat basophilic cell line expressing human $\text{Fc}\epsilon\text{RI}\alpha$ (RBL-2H3-hu α) was incubated with IgE and with BSW17 and Omalizumab, either alone or in combination with different concentrations of anti-idiotypic DARPins overnight at 37°C. The cells were tested for mediator release after triggering them with an optimal amount of an anaphylactogenic mouse monoclonal anti-human IgE antibody, Le27. To measure the capacity of cells to degranulate they were incubated with both IgE and Le27. Under these conditions the maximum release of (sLT) was determined to be up to 180 $\text{pg}/5 \times 10^4$ cells. Addition of either BSW17 or Omalizumab produced a marked inhibition (over 90%) of release compared to the maximal release obtained with IgE and Le27 alone (FIG. 2). Incubation of anti-idiotypic DARPins with

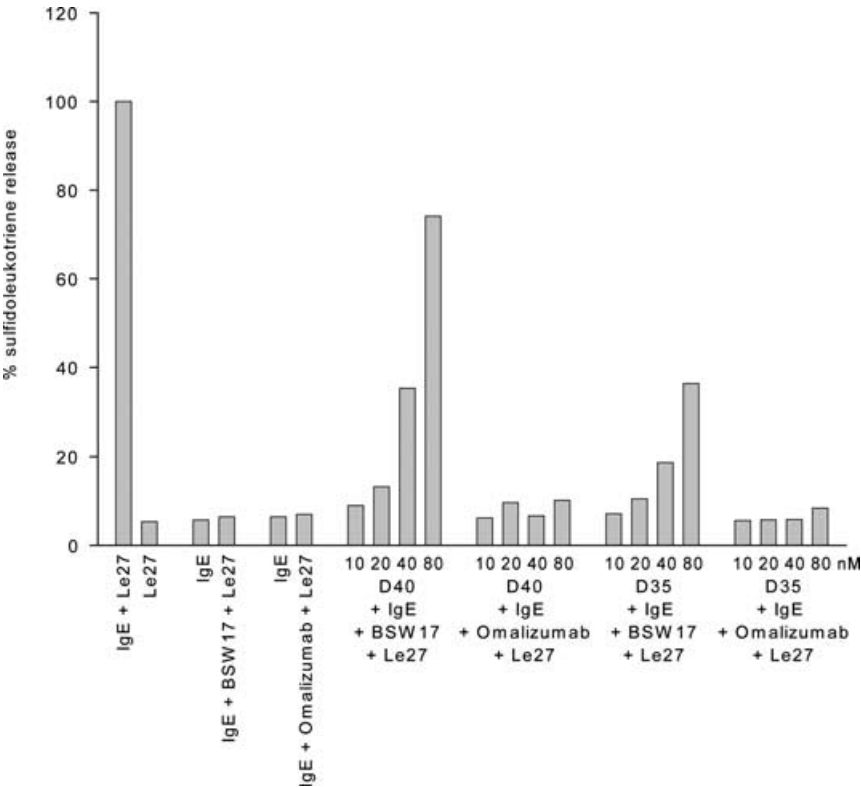


FIGURE 2. Anti-idiotypic DARPin inhibiting BSW17 activity on RBL 2H3α cells. Cells (5×10^4 /well) were incubated with either 50 ng human IgE alone or in combination with either 5 nM BSW17 or Omalizumab and DARPin (D35 and D40) at different concentrations (10, 20, 40, and 80 nM) overnight at 37°C. Subsequently, the cells were triggered with mouse mAb Le27 (500 ng/well). Secreted sulfidoleukotriene was measured by CAST-ELISA as described in “Materials and Methods” section. Pg sLT represents the mean of duplicate assays of sLT release from 150 μL cell supernatant aliquots. Non-cell activation was detected after incubating the cells with IgE plus BSW17 and Le27. As negative controls cells were incubated with either IgE or Le27 alone.

BSW17 restored release in a concentration-dependent manner. DARPin D40, applied in an 80-fold molar excess over BSW17, showed an inhibition of release to 60%. In contrast, incubation of Omalizumab with increasing concentrations of DARPin D35 or D40 did not prevent the inhibitory effect of Omalizumab indicating that the selected DARPin do not compete for the IgE-binding site of Omalizumab. These results indicated that anti-idiotypic DARPin only mimic functional epitope regions on IgE recognized by BSW17.

DISCUSSION

DARPin libraries have previously been successfully used to isolate binding molecules with high specificity and affinity to a variety of different targets including kinases, proteases, tumor markers, and membrane proteins.^{23–25,30–32} In this study we reported for the first time the successful isolation of anti-idiotypic DARPins against a murine monoclonal anti-human IgE antibody, termed BSW17. The selected DARPins bind to BSW17 with high affinity but not to another monoclonal mouse antibody of the same isotype with other epitope specificity. Furthermore, the selected DARPins were able to compete with the cognate antigen of BSW17 for binding, suggesting that similar epitopes are present on the DARPin and on the antigen.

The variable regions of antibodies carry immunogenic determinants, known as idiotopes to which specific anti-idiotypic antibodies can be generated. Previously, we have demonstrated the isolation of two anti-idiotypic antibodies specific for BSW17 from a human Fab phage display library.¹³ These antibodies mimic an IgE epitope involved in the IgE-binding site of FcεRIα and prevent the binding of BSW17 to IgE primed cells. In this study we have isolated two anti-idiotypic DARPins that showed the same activities as these antibodies, that is, they prevent the functional activity of BSW17. Preincubation of BSW17 with DARPins prevents the binding of BSW17 to IgE and thus restores the mediator release capacity of the cells upon challenge with an anaphylactogenic anti-IgE antibody. It has been shown that private idiotypes are unique and specific for each antibody and monoclonal antibodies have been raised that recognize specifically one particular private idiotypic.³³ Interestingly, the same specific recognition was shown for the selected DARPins, as they do not react with other monoclonal anti-IgE antibodies carrying a different idiotypic. These results indicate that the DARPins display an interaction interface that should be comparable to the one found in anti-idiotypic antibody–antibody complexes.

The selection of the anti-idiotypic DARPins was carried out with ribosome display, a method that works entirely *in vitro* by linking genotype (mRNA) and phenotype (nascent protein) on the ribosome.²⁶ Interestingly, the affinity of the selected DARPins were in the nanomolar range, which compares well with affinities obtained for the previously isolated anti-idiotypic antibodies.¹³ Affinity maturation protocols, such as off rate selection, might be applied to further improve the binding characteristics.^{34,35}

While antibodies suffer from limited and expensive production capacities, this is not the case for DARPins. DARPins are well expressed in large amounts in *E. coli*,^{22,25} making the selected anti-idiotypic DARPins interesting candidates for therapeutic applications, for example, as anti-idiotypic vaccines. A number of studies have shown that selected anti-idiotypic antibodies that bind to the antigen-binding sites of antibodies can effectively mimic the antigen structure and thus can be used as surrogate antigens for inducing specific

and protective immunity to bacterial, viral, and parasitic infections as well as tumors.^{3,36,37} Our previous results with the anti-idiotypic antibodies have demonstrated that these molecules can act as internal image of the epitope and are able to elicit anti-idiotypic antibodies, which again react with IgE and show inhibitory effects.¹³ The selected DARPins might be ideal tools for this approach. From structural investigations of DARPin target protein complexes it is known that the binding interface is comparable to the one of antibody–antigen interaction.^{25,30,38} Naturally, a functional epitope mimicry by the selected DARPins, which is able to induce a “BSW17-like anti-IgE” response *in vivo* has not yet been shown and still needs further investigations.

REFERENCES

1. JERNE, N.K. 1974. Towards a network theory of the immune system. *Ann. Immunol. (Paris)* **125C**: 373–389.
2. NISONOFF, A. 1991. Idiotypes: concepts and applications. *J. Immunol.* **147**: 2429–2438.
3. UEMURA, H., E. OKAJIMA, F.M. DEBRUYNE, *et al.* 1994. Immunization with anti-idiotypic monoclonal antibodies bearing the internal image of the renal-cell carcinoma-associated antigen G250 induces specific cellular immune responses. *Int. J. Cancer* **59**: 802–807.
4. MCCARTHY, H., C.H. OTTENSMEIER, T.J. HAMBLIN, *et al.* 2003. Anti-idiotypic vaccines. *Br. J. Haematol.* **123**: 770–781.
5. JONKERS, R.E. & J.S. VAN DER ZEE. 2005. Anti-IgE and other new immunomodulation-based therapies for allergic asthma. *Neth. J. Med.* **63**: 121–128.
6. SHAKIB, F., S.J. SMITH & D.I. PRITCHARD. 1995. Do autoantibodies to IgE play a role in IgE-mediated events? *Immunol. Cell Biol.* **73**: 109–112.
7. MIESCHER, S.M. & M. VOGEL. 2002. Molecular aspects of allergy. *Mol. Aspects Med.* **23**: 413–462.
8. HEBERT, J. & Y. BOUTIN. 1996. Anti-idiotypic antibodies in the treatment of allergies. *Adv. Exp. Med. Biol.* **409**: 431–437.
9. KNUTTI-MULLER, J.M., B.M. STADLER, C.M. MAGNUSSON, *et al.* 1986. Human IgE synthesis *in vitro*. Detection with monoclonal antibodies. *Allergy* **41**: 457–467.
10. ADELROTH, E., S. RAK, T. HAAHTELA, *et al.* 2000. Recombinant humanized mAb-E25, an anti-IgE mAb, in birch pollen-induced seasonal allergic rhinitis. *J. Allergy Clin. Immunol.* **106**: 253–259.
11. HEUSSER, C. & P. JARDIEU. 1997. Therapeutic potential of anti-IgE antibodies. *Curr. Opin. Immunol.* **9**: 805–813.
12. MILGROM, H., R.B. FICK, JR., J.Q. SU, *et al.* 1999. Treatment of allergic asthma with monoclonal anti-IgE antibody. RhuMAB-E25 Study Group. *N. Engl. J. Med.* **341**: 1966–1973.
13. VOGEL, M., S. MIESCHER, S. KUHN, *et al.* 2000. Mimicry of human IgE epitopes by anti-idiotypic antibodies. *J. Mol. Biol.* **298**: 729–735.
14. RIVERA, J. & A.M. GILFILLAN. 2006. Molecular regulation of mast cell activation. *J. Allergy Clin. Immunol.* **117**: 1214–1225; quiz 1226.
15. FORRER, P., M.T. STUMPP, H.K. BINZ, *et al.* 2003. A novel strategy to design binding molecules harnessing the modular nature of repeat proteins. *FEBS Lett.* **539**: 2–6.

16. FORRER, P., H.K. BINZ, M.T. STUMPP, *et al.* 2004. Consensus design of repeat proteins. *Chembiochem.* **5**: 183–189.
17. MOSAVI, L.K., T.J. CAMMETT, D.C. DESROSIERS, *et al.* 2004. The ankyrin repeat as molecular architecture for protein recognition. *Protein Sci.* **13**: 1435–1448.
18. SEDGWICK, S.G. & S.J. SMERDON 1999. The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem. Sci.* **24**: 311–316.
19. ALDER, M.N., I.B. ROGOZIN, L.M. IYER, *et al.* 2005. Diversity and function of adaptive immune receptors in a jawless vertebrate. *Science* **310**: 1970–1973.
20. PANCER, Z., C.T. AMEMIYA, G.R. EHRHARDT, *et al.* 2004. Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. *Nature* **430**: 174–180.
21. BINZ, H.K., P. AMSTUTZ & A. PLUCKTHUN. 2005. Engineering novel binding proteins from nonimmunoglobulin domains. *Nat. Biotechnol.* **23**: 1257–1268.
22. BINZ, H.K., M.T. STUMPP, P. FORRER, *et al.* 2003. Designing repeat proteins: well-expressed, soluble and stable proteins from combinatorial libraries of consensus ankyrin repeat proteins. *J. Mol. Biol.* **332**: 489–503.
23. AMSTUTZ, P., H. KOCH, H.K. BINZ, *et al.* 2006. Rapid selection of specific MAP kinase-binders from designed ankyrin repeat protein libraries. *Protein Eng. Des. Sel.* **19**: 219–229.
24. ZAHND, C., F. PECORARI, N. STRAUMANN, *et al.* 2006. Selection and characterization of Her2 binding-designed ankyrin repeat proteins. *J. Biol. Chem.* **281**: 35167–35175.
25. BINZ, H.K., P. AMSTUTZ, A. KOHL, *et al.* 2004. High-affinity binders selected from designed ankyrin repeat protein libraries. *Nat. Biotechnol.* **22**: 575–582.
26. HANES, J. & A. PLUCKTHUN. 1997. *In vitro* selection and evolution of functional proteins by using ribosome display. *Proc. Natl. Acad. Sci. USA* **94**: 4937–4942.
27. AMSTUTZ, P., H.K. BINZ, C. ZAHND, *et al.* 2006. A Laboratory Handbook: Ribosome Display: *in Vitro* Selection of Protein–Protein Interactions. Vol. **1**, 497–509. Elsevier Academic Press.
28. WILSON, A.P., C.E. PULLAR, A.M. CAMP, *et al.* 1993. Human IgE mediates stimulus secretion coupling in rat basophilic leukemia cells transfected with the alpha chain of the human high-affinity receptor. *Eur. J. Immunol.* **23**: 240–244.
29. NECHANSKY, A., M.W. ROBERTSON, B.A. ALBRECHT, *et al.* 2001. Inhibition of antigen-induced mediator release from IgE-sensitized cells by a monoclonal anti-Fc epsilon RI alpha-chain receptor antibody: implications for the involvement of the membrane-proximal alpha-chain region in Fc epsilon RI-mediated cell activation. *J. Immunol.* **166**: 5979–5990.
30. SENNHAUSER, G., P. AMSTUTZ, C. BRIAND, *et al.* 2006. Drug export pathway of multidrug exporter AcrB revealed by DARPIn inhibitors. *PLoS Biol.* **5**: e7.
31. KAWA, M., P. FORRER, P. AMSTUTZ, *et al.* 2006. Isolation of intracellular proteinase inhibitors derived from designed ankyrin repeat proteins by genetic screening. *J. Biol. Chem.* **281**: 40252–40263.
32. AMSTUTZ, P., H.K. BINZ, P. PARIZEK, *et al.* 2005. Intracellular kinase inhibitors selected from combinatorial libraries of designed ankyrin repeat proteins. *J. Biol. Chem.* **280**: 24715–24722.
33. HAJIGHASEMI, F., S. GHARAGOZLOU, R. GHODS, *et al.* 2006. Private idiotypes located on light and heavy chains of human myeloma proteins characterized by monoclonal antibodies. *Hybridoma (Larchmt.)* **25**: 329–335.

34. JERMUTUS, L., A. HONEGGER, F. SCHWESINGER, *et al.* 2001. Tailoring *in vitro* evolution for protein affinity or stability. *Proc. Natl. Acad. Sci. USA* **98**: 75–80.
35. ZAHND, C., S. SPINELLI, B. LUGINBUHL, *et al.* 2004. Directed *in vitro* evolution and crystallographic analysis of a peptide-binding single chain antibody fragment (scFv) with low picomolar affinity. *J. Biol. Chem.* **279**: 18870–18877.
36. FENG, Z., Z. QIU, Y. LI, *et al.* 2002. Protective immunity induced by the anti-idiotypic monoclonal antibody NP30 of *Schistosoma japonicum*. *Chin. Med. J. (Engl.)* **115**: 576–579.
37. BENINATI, C., M. OGGIONI, G. MANCUSO, *et al.* 2001. Anti-idiotypic vaccination against group B streptococci. *Int. Rev. Immunol.* **20**: 263–273.
38. KOHL, A., H.K. BINZ, P. FORRER, *et al.* 2003. Designed to be stable: crystal structure of a consensus ankyrin repeat protein. *Proc. Natl. Acad. Sci. USA* **100**: 1700–1705.