Remodeling a DNA-binding protein as a specific *in vivo* inhibitor of bacterial secretin PulD

Barbara Mouratou†, Francis Schaeffer†, Ingrid Guilvout‡, Diana Tello-Manigne†, Anthony P. Pugsley‡, Pedro M. Alzari†, and Frédéric Pecorari†§

†Unité de Biochimie Structurale, Centre National de la Recherche Scientifique URA2185 and ‡Unité de Génétique Moléculaire, Centre National de la Recherche Scientifique URA2172, Institut Pasteur, 75724 Paris Cedex 15, France

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We engineered a class of proteins that binds selected polypeptides with high specificity and affinity. Use of the protein scaffold of Sac7d, belonging to a protein family that binds various ligands, overcomes limitations inherent in the use of antibodies as intracellular inhibitors: it lacks disulfide bridges, is small and stable, and can be produced in large amounts. An *in vitro* combinatorial/selection approach generated specific, high-affinity (up to 140 pM) binders against bacterial outer membrane secretin PulD. When exported to the *Escherichia coli* periplasm, they inhibited PulD oligomerization, thereby blocking the type II secretion pathway of which PulD is part. Thus, high-affinity inhibitors of protein function can be derived from Sac7d and can be exported to, and function in, a cell compartment other than that in which they are produced.

calorimetry | intrabody | ribosome display | Sac7d | type II secretion system

Molecular interference is invaluable in understanding biological processes and in drug design. Although extremely useful, small interfering RNAs (siRNAs) are unable to selectively block one activity in a multifunctional protein and are ineffective when mRNA turnover is fast, when target sequences are inaccessible, or when protein turnover is slow (1). Furthermore, off-target effects occur because of nonspecific innate responses and inhibition of endogenous microRNA, or when the siRNA shows unexpected complementarity to nontarget mRNAs (2).

Antibodies produced within cells (intrabodies) are attractive alternatives for modulating protein function. Unlike siRNAs, they can exert selective action on specific aspects of a multifunctional protein by recognizing a defined epitope in it. Intrabodies have been used in fundamental studies of signaling pathways, validation of targets in cellular assays, direct therapeutic intervention, and agricultural biotechnology (3). However, antibodies are critically dependent on intramolecular disulfide bond formation to maintain their functional conformation (4). This problem has hampered their exploitation as intracellular inhibitors. For these reasons, alternatives to antibodies/intrabodies have recently been developed by using scaffold proteins engineered via combinatorial mutation/selection (5).

Optimal scaffold proteins for generating intracellular inhibitors should be small and stable, lack disulfide bridges, and be produced at high levels. They should also belong to a family of proteins known to bind a wide range of structurally different ligands. One such family possesses the oligonucleotide/oligosaccharide binding fold, a five-stranded β-barrel capped by an amphiphilic α-helix. Members of this family recognize nucleic acids, oligosaccharides, proteins, and metallic ions (6). The same positions of the binding face of oligonucleotide/oligosaccharide binding folds from different proteins are always involved in ligand recognition. One such protein is Sac7d, a dsDNA-binding protein from the hyperthermophilic archaeon *Sulfolobus acidocaldarius* (7) (Fig. 1). Sac7d is chemically and thermally stable and is resistant to extreme pH. Its molecular organization is quite simple, being small (66 residues) and monomeric, lacking a disulfide bridge, and possessing only one structural domain (the oligonucleotide/oligosaccharide binding fold). These properties of Sac7d, together with its ability to bind large ligands, led us to explore the possibility of modifying its binding face to recognize proteins without changing its favorable biophysical properties.

Here, we report the generation of Sac7d derivatives that bind to secretin PulD, a component of the pullulanase type II secretion system (T2SS) (secreton) of the Gram-negative bacterium *Klebsiella oxytoca*. Dodecameric PulD forms a translocation channel in the outer membrane (8). Dodecamer formation is essential for secreton function and does not require the N-terminal half of the protein (PulD-N) (9), for which a role in substrate recognition has been proposed (10, 11). We generated a library of $\approx 3 \times 10^{12}$ variants of Sac7d and selected those that bind to PulD-N *in vitro* by ribosome display (12). Derivatives with subnanomolar affinities for PulD-N (hereafter called Sac7*) were analyzed for their physicochemical properties, their ability to bind full-length PulD and to be exported, and their effects on secreton function and secretin assembly. The results led us to propose the use of Sac7d-derived binders as a type of selective intracellular inhibitors.

Results

Generation of the Sac7d Library. The binding area in several three-dimensional structures of Sac7d–DNA complexes (13, 14) is mainly composed of 14 residues (K7, Y8, K9, K21, K22, W24, V26, M29, S31, T33, T40, R42, A44, and S46) (Fig. 1). Because this binding area is slightly concave and might match the spherical shape of globular proteins, we randomly substituted these 14 residues. The gene encoding Sac7d is short ($\approx$200 base pairs), and a DNA fragment with the corresponding random base substitutions in the 14 codons was obtained by a three-step PCR with a mixture of degenerate and standard oligonucleotides. The randomized codons were created from NNS triplets that encode all amino acids. According to the amount of the PCR product, the library contains up to 3.0×10^{12} variants. Sequencing of 70 random clones confirmed that the observed residue frequency was similar to that predicted (data not shown). The percentage of correct clones, without any frame shifts or deletions, was $\approx$65%. Hence, the

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Abbreviations: T2SS, type II secretion system; SPR, surface plasmon resonance; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry; IPTG, isopropyl β-D-thiogalactoside; PhoA, *E. coli* periplasmic alkaline phosphatase.

§To whom correspondence should be addressed at: Unité de Biotechnologie, Biocatalyse et Biorégulation, Centre National de la Recherche Scientifique UMR6204, Université de Nantes, 44322 Nantes Cedex 3, France. E-mail: frederic.pecorari@univ-nantes.fr.

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Fig. 1. Schematic representation of wild-type Sac7d (Protein Data Bank code 1AZP). Residues involved in DNA binding and that were substituted are shown in yellow.

"functional" diversity was considered satisfactory, and the library was used for selections.

Ribosome Display Selections. PulD cannot be maintained in solution without ionic detergents (9). Therefore, we used the soluble monomeric PulD-N fragment (8) to select Sac7d derivatives capable of binding to it. Selections were performed by using the library and immobilized PulD-N as a target protein. An RIA after the fifth round (Fig. 2*A*) indicated the expected enrichment for specific PulD-N binders. Binding could not be detected on immobilized BSA. Free PulD-N at 10 and 1 nM inhibited binding by 50% and 20%, respectively, suggesting that the pool contained specific binders for PulD-N with affinities in the nanomolar range.

Characterization of PulD-N Binders. ***Sequence analysis, expression, and purification of selected binders.*** The enriched pool was cloned into the expression plasmid pQE30 and the binders produced with His tags in the cytoplasm of *E. coli* strain DH5α. Crude extracts from 36 individual clones assayed by ELISA with immobilized PulD-N or BSA showed significant and specific PulD-N binding. Twenty-nine arbitrarily chosen clones were used for microexpression and immobilized metal ion affinity chromatography purification to identify those with highest affinities. The proteins were then screened by surface plasmon resonance (SPR) with a chip coated with PulD-N. Significant binding was not observed on the blank surface. The 29 clones represented a wide variety of sequences (see Fig. 2*B* for a subset of sequences), but only two pairs were identical, suggesting limited convergence. A clear preference for a particular residue could be observed at some positions. For example, position R42 was occupied by a tyrosine in approximately half of the clones.

The binders accumulated in large amounts in the *E. coli* cytoplasm at 30°C after overnight growth and could be purified to homogeneity in two steps by immobilized metal ion affinity chromatography and gel filtration with yields up to 200 mg per liter of shake-flask culture. The proteins ran on a 15% acrylamide SDS/PAGE gel at the position expected for their calculated molecular masses [supporting information (SI) Fig. 6]. Purified binders could be concentrated up to 60 mg/ml in a standard TBS buffer without precipitating and remained soluble over several months at 4°C. Thus, Sac7d is highly tolerant to substitution, and the selected variants retain satisfactory biophysical properties.

Binding properties of anti-PulD-N variants. According to analysis of the dissociation phases, five clones (Sac7*6, Sac7*33, Sac7*39, Sac7*40, and Sac7*41) with the slowest off-rates were chosen for detailed affinity determinations of the monovalent proteins by SPR. This analysis revealed that the dissociation constants of all PulD-N

Fig. 2. RIA and sequence analysis of selected binders. (*A*) RIA after five rounds of selection PulD-N. The selection pool was translated *in vitro* in the presence of ^{35}[S] methionine and tested for binding to PulD-N immobilized in an ELISA plate. After washing and elution with 0.1 M triethanolamine, amounts of binders were estimated with a β-radiation counter. Competitions were carried out in parallel with preincubation of translated pools with free PulD-N at 1 nM, 10 nM, 100 nM, and 1 μM. BSA was used as negative control (no competition). (*B*) Sequences of eight selected PulD-N-binding Sac7* derivatives. The sequence of Sac7d is shown at the top. Residues common to the Sac7d and Sac7* derivatives are indicated by a dot. Positions that were programmed in the substitution scheme are highlighted in gray.

binders were in the subnanomolar-to-low-nanomolar range (Table 1). Sac7*6 had the highest apparent affinity (K_D = 140 pM). K_D values of these five binders were confirmed by isothermal titration microcalorimetry (ITC) (Table 1 and Fig. 3*A*). Sac7* variants bound to PulD-N with a 1:1 stoichiometry and an enthalpy-driven reaction with large favorable binding enthalpies ($\Delta H = -17$ to -19

Table 1. Dissociation constants and thermal stability of binders

Clone	K_D, nM*	K_D, nM†	T_m, °C‡	ΔH, kcal.mol^{-1}‡
Sac7*6	0.14 ± 0.03	1.2 ± 0.5	66.4	17.6
Sac7*33	0.17 ± 0.03	10.4 ± 0.3	79.2	37.7
Sac7*39	1.1 ± 0.2	15.7 ± 0.2	89.6	41.7
Sac7*40	0.9 ± 0.2	3.9 ± 0.4	84.0	36.7
Sac7*41	1.1 ± 0.1	1.8 ± 0.5	87.8	33.5
Sac7d	–	–	89.6	42.5

–, no interaction detected (under detection limit).
*K_D values obtained from competition SPR experiments (29). Similar K_D values were determined by kinetic SPR experiments (data not shown).
†K_D values obtained from ITC experiments.
‡Thermal denaturation midpoints (± 0.1°C) and heats of denaturation of binders (± 0.2 kcal mol^{-1}) from DSC experiments.

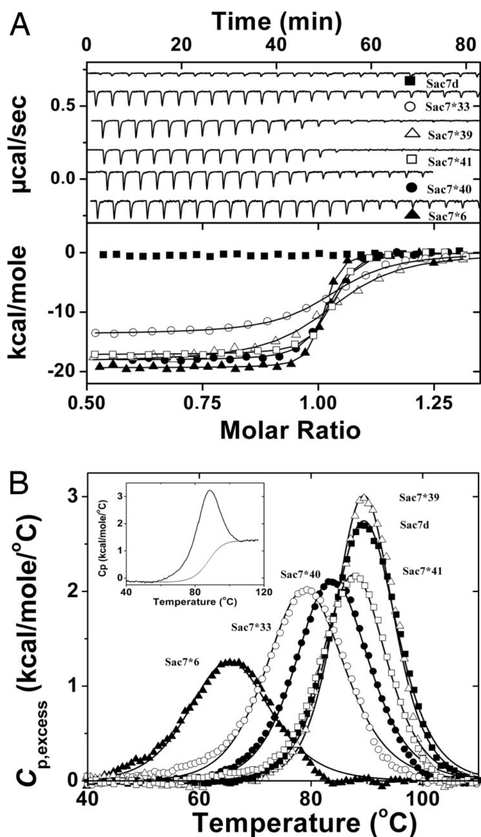

Fig. 3. Affinities and stabilities of binders determined by microcalorimetry. (*A*) ITC titrations of PulD-N with Sac7d* variants at 25°C using the protocol for high-affinity binding. (*Upper*) The raw heat signal for injections of solutions of Sac7* or Sac7d into a solution containing PulD-N and the corresponding Sac7* binder, respectively. (*Lower*) Transition curves for Sac7d and five Sac7* variants. Binding parameters are given in Table 1. (*B*) DSC characterization of the thermal stabilities of Sac7d and its derivatives. The experimental excess heat-capacity functions ($C_{p,excess}$) of Sac7d and of five Sac7* variants. Lines represent fits of the data to a non-two-state model. Stability parameters are given in Table 1. (*Inset*) Calculation of the $C_{p,excess}$ function for Sac7*41. The $C_{p,excess}$ function is obtained after subtraction of the chemical baseline from the C_p function. The chemical baseline (dotted line) is constructed from the experimental C_p function after subtraction of the instrument base line.

kal.mol^{-1}) and unfavorable binding entropies ($T\Delta S = -2.8$ to -7.7 kcal.mol^{-1}), consistent with the formation of rigid complexes. Sac7d–PulD-N interactions were not detected by SPR or ITC, demonstrating the specificity of interactions observed with Sac7* (SI Fig. 7).

Stability of PulD-N binders. Thermal stabilities of Sac7d and derivatives determined by differential scanning calorimetry (DSC) at pH 5.6 to ensure complete unfolding of the proteins below 130°C were between 66°C and 90°C (Fig. 3*B* and Table 1). Remarkably, Sac7*39 was as stable as Sac7d (89.6°C). Although assayed at an acidic pH, the T_m of Sac7*6 remained 8°C above the mean T_m value of proteins of the Protein Data Bank (15). DSC scans were characteristic of cooperative unfolding, indicating that Sac7* variants were well folded. Unfolding enthalpies were either similar (Sac7*39) or smaller than that of Sac7d by ≈20% (Sac7*33, Sac7*40, and Sac7*41) or 58% (Sac7*6) (Table 1). These data show that highly substituted (up to 23%) Sac7* variants adopt a native-like folded structure and retain their favorable thermal stability.

Sac7* Binding to Dodecameric PulD. Next, we investigated whether the three Sac7d derivatives with the highest affinities (Sac7*6, Sac7*33, and Sac7*40) and with different sequences recognize full-length dodecameric PulD. When increasing amounts of PulD-containing membranes from *E. coli* PAP105 (pCHAP3671 pCHAP580) were mixed with saturating amounts of GFP-tagged Sac7*40 or Sac7*33, the amount of binder remaining in the pellets after centrifugation was correspondingly increased (Fig. 4*A*). The GFP-tagged binders were not sedimented with membranes from PAP105 (pCHAP3711 pCHAP580) containing a PulD variant lacking the N-domain (9) (Fig. 4*A*). Thus, binding of these GFP-Sac7* binders to membranes is PulD-N-specific, and they bind to the native, dodecameric secretin complex despite the presence of the GFP tag. Sac7*6 bound only very weakly to membranes containing PulD (data not shown).

A far-Western immunoblot was used to validate binding of the Sac7* derivatives to PulD dodecamers. All three Sac7*-*E. coli* periplasmic alkaline phosphatase (PhoA) chimeras bound specifically to both monomeric and dodecameric PulD but not to PulD-CS (Fig. 4*B*). Although these three chimeras bound equally well to phenol-dissociated (monomeric; see ref. 16) PulD (data not shown), they consistently exhibited different apparent affinities for dodecameric PulD, ranging from high, Sac7*40, to low, Sac7*6.

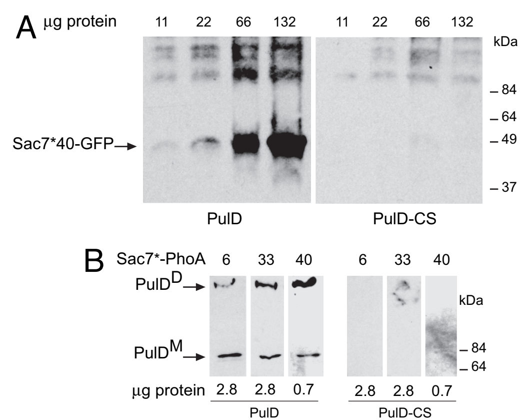

Fig. 4. Binding of Sac7*40, Sac7*33, and Sac7*6 to isolated cell envelopes and to PulD dodecamers (PulD^D) and PulD monomers (PulD^M). (*A*) Increasing amounts of cell envelope from strain PAP105 producing PulD and PulS or PulD-CS and PulS were incubated with Sac7*40-GFP, and the membrane fraction was then analyzed by SDS and immunoblotting with GFP antibodies. (*B*) Far-Western blotting of the indicated amounts of cell envelope proteins from the same strains using the three Sac7*-PhoA chimeras and antibodies against PhoA.

Sac7* Derivatives Inhibit Pullulanase Secretion and Prevent PulD Multimerization. The Sac7*-PhoA chimeras, in which Sac7d or its derivatives are sandwiched between the PhoA signal peptide and the catalytic part of PhoA, were efficiently exported to the periplasm, as monitored by the high PhoA activity and release upon periplasmic shock. Plasmids encoding the Sac7*-PhoA chimeras were transformed into *E. coli* strain PAP7232, in which the *pul* genes are integrated into the chromosome. All three chimeras were produced in similar amounts after isopropyl β-D-thiogalactoside (IPTG) induction and inhibited pullulanase secretion completely, whereas exported Sac7d-PhoA was without effect. Furthermore, neither PulD dodecamers nor monomers were detected in strains producing any of the Sac7*-PhoA chimeras (data not shown). To obtain more-precise information on these phenomena and to study the fate of PulD in strains producing the chimeras, they were produced in envelope protease-deficient strain PAP5198 carrying pCHAP231 (to increase T2SS production; see ref. 9). IPTG-induced levels of Sac7*-PhoA production (Fig. 5*A*) inhibited pullulanase secretion by >90%. Dodecameric PulD was much less abundant, and PulD monomers were correspondingly more abundant (arrows in Fig. 5*B*) than in controls without chimeras or with

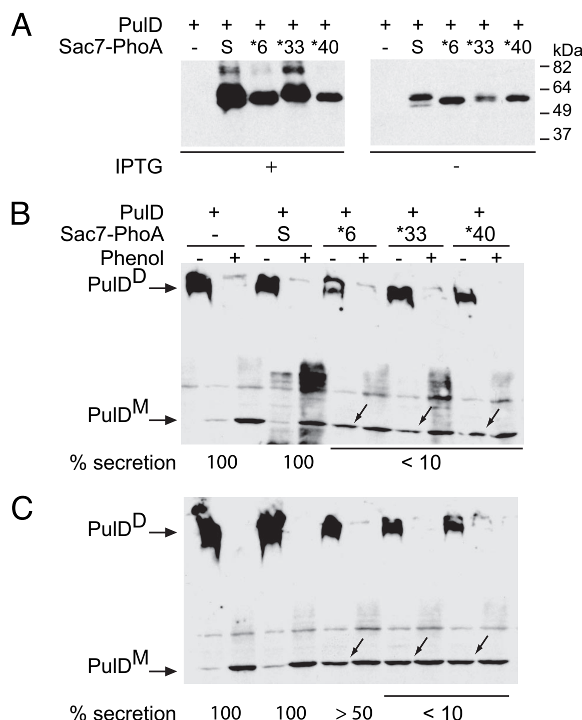

Fig. 5. Production of Sac7-PhoA chimeras with and without IPTG induction and their effects on secretion and PulD multimerization in cell envelope protease-deficient strain PAP5198 carrying pCHAP231. (*A*) Sac7-PhoA levels detected by immunoblotting (with PhoA antibodies) of the same amount of cell extract. (*B*) Levels of secretion (%) and presence of PulD dodecamers ($PulD^D$) and monomers ($PulD^M$) detected by immunoblotting of phenol-treated and nontreated cell extracts with PulD antibodies. Arrows indicate $PulD^M$ detected without phenol treatment. S indicates Sac7d-PhoA. (*C*) As *B*, but without IPTG induction.

Sac7d-PhoA, demonstrating that the chimeras prevent PulD multimerization and cause PulD monomer degradation by envelope proteases. Similar results were obtained with uninduced levels of Sac7*33-PhoA and Sac7*40-PhoA, but substantial pullulanase secretion (>50%) and PulD multimerization occurred when Sac7*6-PhoA was present at uninduced levels (Fig. 5*C*).

Discussion

Secretion pathways are attractive targets for antimicrobial agents (17) because exoproteins are often important bacterial virulence factors. We focused our attention on the T2SS, a secretion pathway essential for several important human bacterial pathogens such as *Vibrio cholerae*, in which it secretes proteases, chitinases, and cholera toxin (18). The pullulanase T2SS from *K. oxytoca*, of which secretin PulD is part, can be transferred in *E. coli* (16), and is thus a good model to study secretion mechanisms. Intracellular inhibitors of the T2SS would be useful in these studies and could provide openings for drug target discovery. Because PulD-N faces the periplasm (8) and might be involved in substrate recognition (11), we reasoned that binders directed against it might inhibit T2SS function.

Sac7d as an Alternative to Antibodies. Sac7d belongs to a family of proteins known to bind various ligands. Its scaffold properties overcome limitations in the use of antibodies as intracellular inhibitors (see the Introduction). The results demonstrate that Sac7d can be converted from a DNA-binding molecule to a protein-binding molecule without negating its favorable biophysical properties. Given the heavy mutational scheme used (>21% of Sac7d residues were changed), these results indicate minimal involvement of surface residues of Sac7d in its folding and/or stability and validate the use of an extremophile oligonucleotide/oligosaccharide binding fold protein as a scaffold.

Properties of Sac7d-Derived Binders. The PulD-N binding affinities of Sac7d derivatives characterized in detail were in the nanomolar range. The discrepancy observed between affinities determined by ITC and competition SPR probably result from basic physical differences between the two methods and assumptions underlying competition SPR experiments/treatments (compare to the discussion in ref. 19). Affinities determined by ITC were lower than by kinetic SPR, as often observed (20), because of the smaller entropy change upon binding a surface-immobilized protein in SPR compared with free-protein binding in solution in ITC (21). To our knowledge, these affinities are among the highest obtained with a scaffold protein without an affinity maturation step (ref. 22 and references therein). The high affinities obtained are associated with very high specificity, because none of the proteins in crude *E. coli* lysates react with the Sac7d derivatives. Sac7d* variants bind to PulD-N with enthalpy and entropy changes consistent with the formation of rigid complexes. Because Sac7d unfolds at high temperature and over a large temperature interval (Fig. 3*B*) with a small unfolding enthalpy (Table 1), the entropy change on unfolding must be small as a result of a high flexibility of Sac7d scaffold (23). This flexibility may enable Sac7d to adapt to selected protein targets in the combinatorial/selection approach and may explain the high affinities of Sac7* binders to PulD-N.

The yields of recombinant Sac7* protein obtained (see *Results*) were substantially higher than one can expect for recombinant antibody production. The variants were functional upon extraction from the reducing environment of the *E. coli* cytoplasm, consistent with the absence of a disulfide bridge. Functional cytoplasmic production is also an advantage compared with standard antibodies, which do not fold correctly in the strongly reducing environment of the *E. coli* cytoplasm, although this can be overcome by using a strain lacking glutathione oxidoreductase and thioredoxin reductase (24), or the antibody scaffold used is isolated or developed for this purpose (4).

The thermodynamic stability of the five variants studied compares well with several other proteins from thermophiles (23) and is close to that of native Sac7d (25). Sac7*39, with 15 residues substituted (23%), has the same stability and unfolding properties as Sac7d. Sac7*6, the least stable clone, still retained a T_m of 65°C at pH 5.6 and 76°C at pH 7.0. The biophysical properties of the binders also compare well with those of DARPins, another alternative to antibodies recently used as an intracellular protease inhibitor (26).

Binding *in Vivo* and Intracellular Inhibition of Pullulanase Secretion. All three Sac7*-PhoA chimeras that were tested bound to monomeric full-length PulD. Sac7*40-PhoA and Sac7*33-PhoA bound well to dodecameric PulD, indicating that their epitopes remain accessible. In contrast, Sac7*6-PhoA bound only weakly to PulD dodecamers but had the highest affinity for PulD-N *in vitro*, indicating that its epitope is partially masked upon multimerization and is different from those recognized by Sac7*40 and Sac7*33. ITC competition experiments indicated that epitopes recognized by Sac7*6 and Sac7*40 are identical or overlap (SI Fig. 8). The differences in the *in vivo* effects of these two binders, when fused to PhoA, suggest that their epitopes overlap.

All three Sac7*-PhoA variants prevented PulD multimerization and targeted the PulD monomers for degradation by envelope proteases, thereby blocking pullulanase secretion. Earlier evidence indicated that the N domain does not influence PulD multimerization (9). PhoA dimerization in Sac7*-PhoA might cause steric hindrance and consequent mispositioning of PulD monomers. Secretion levels in strains producing Sac7*-PhoA remained very low when the level of PulD produced was increased and envelope

proteases were inactivated (Fig. 5*B*). Low secretion could be due to the presence of only a few PulD dodecamers, channel occlusion, or masking of an essential interaction site with substrate (11) or another secreton component (27) by bound chimeras.

Reducing the level of Sac7*33 or Sac7*40 (by eliminating induction by IPTG) did not diminish their effect on secretion or PulD multimerization, but Sac7*6-PhoA was almost without effect under these conditions (Fig. 5*C*), even though it was at least as abundant as the other chimeras (Fig. 5*A*). Two different scenarios could explain this observation. First, the highly abundant Sac7*6-PhoA binds to almost all PulD monomers and prevents their multimerization. However, when Sac7*6-PhoA levels are lower, it cannot compete efficiently with PulD monomer–monomer interactions, and enough multimers assemble to allow efficient secretion. The apparently lower affinity of Sac7*6-PhoA for dodecameric PulD is insufficient to prevent secretion. Second, binding of the chaperone PulS to PulD monomers, a prerequisite for their correct targeting to the outer membrane (9, 16), prevents Sac7*6-PhoA binding and permits correct multimerization. In this scenario, the outcome depends on which protein binds first to PulD, PulS or Sac7*6-PhoA. Both scenarios are in agreement with the fact that the epitope recognized by Sac7*6 is strongly masked upon PulD multimerization, suggesting that it is at the interface between two monomers.

Conclusions

The data prove the concept of using Sac7d to generate binders able to recognize a target protein and having all of the properties needed for efficient intracellular inhibition of a given biological function. However, structural studies of binder-target complexes are required to validate the mode of binding. In addition to their potential use in functional knockout experiments, these binders could be used for affinity chromatography, detection, and *in vivo* localization experiments. Indeed, the favorable biophysical properties of these binders, along with their facile fusion to different reporter proteins and their small size (3 and 19 times smaller than scFv and IgG, respectively), might facilitate these applications. However, further experiments are required to test the range of macromolecules to which Sac7d can be adapted.

Experimental Procedures

General Molecular Biology. Enzymes and buffers were from New England Biolabs (Ipswich, MA) or Fermentas (Vilnius, Lithuania). Oligonucleotides were from MWG Biotech (Ebersberg, Germany). All PCRs were performed by using Vent polymerase unless otherwise indicated.

Synthesis of the DNA Encoding the Wild-Type Sac7d. The wild-type gene encoding Sac7d was synthesized by using eight oligonucleotides and was ligated into pQE30 expression vector by using BamHI and HindIII restriction enzymes (see *SI Materials and Methods*).

Generation of Combinatorial Libraries. The generation of the library in a format compatible with ribosome display is described in detail in *SI Materials and Methods*. In brief, the library was mainly constructed by gene synthesis and PCR assembly steps. The randomized gene for Sac7d, including the 5′-flanking region necessary for ribosome display, was obtained in a single-step PCR by using a combination of four standard and three degenerated oligonucleotides encoding NNS triplets (N = A, C, T or G and S = C or G). The 3′-flanking region and the TolA linker necessary to complete the ribosome display construct were added in a final PCR assembly step.

Ribosome Display Selection Rounds. Biotinylated PulD-N for selection experiments was prepared by incubating a 10 μM solution of PulD-N with a 20-fold molar excess of sulfosuccinimidyl-6-(biotinamido) hexanoate (Pierce, Rockford, IL) in PBS on ice for 1 h. The degree of biotinylation, determined by a 2-hydroxyazobenzen-4′-carboxylic acid (HABA) assay (Sigma, St. Louis, MO), was from two to three molecules of biotin per protein molecule. Biotinylated PulD-N was bound to immobilized neutravidin in a Maxisorp plate (Nunc, Naperville, IL), and selections by ribosome display were performed at 4°C as described in ref. 28. Up to five rounds of selection were performed to isolate high-affinity binders.

Analysis of Selected Pools and Isolated Clones. After four or five rounds, selected pools were tested by RIA as described in ref. 29 by using directly coated target protein in a Maxisorp plate and from 1 nM to 10 μM free target protein as competitor. The RT-PCR products from selected pools were cloned into the BamHI and HindIII restriction sites of pQE30 (Qiagen, Hilden, Germany), and the ligation mixture was transformed into *E. coli* DH5α. Clones were inoculated into a deep-well plate containing 1.5 ml of LB medium containing 100 μg/ml ampicillin and 1% glucose in each well. After overnight culture at 37°C with shaking at 250 rpm, 0.2 ml of each culture was used to inoculate another deep-well plate containing 1.3 ml 2xYT medium and 100 μg/ml ampicillin per well. The plate was incubated at 37°C for 1 h with shaking. Expression of the cloned gene was induced by 0.5 mM IPTG and incubation at 30°C for 4 h with shaking. Cells were pelleted by centrifugation (2,250 $\times$ *g*), and supernatants were discarded. Proteins were extracted with 50 μl of BugBuster (Novagen, Madison, WI) per well with shaking for 30 min, and 250 μl of TBS (20 mM Tris·HCl, pH 7.4/150 mM NaCl) were added. Cell debris were pelleted by centrifugation (2,500 $\times$ *g*). For ELISA screening, 100 μl of each supernatant were used to test the binding to target proteins coated into a Maxisorp plate by using the RGS His antibody HRP conjugate, which detects only the RGS-$(His)_6$-tag on binders and not the $(His)_6$-tag on targets (Qiagen), and BM-Blue substrate (Roche, Mannheim, Germany). All incubation steps were performed in TBS at pH 7.4 with 0.1% Tween 20.

Protein Production for Screening by SPR. To screen for binders based on their time of dissociation from PulD-N, clones were expressed in 5-ml cultures. An overnight preculture (500 μl in LB medium containing 100 μg/ml ampicillin and 1% glucose at 37°C) in deep wells was mixed with 4.5 ml of 2xYT + ampicillin in deep wells at 37°C. Expression was induced at OD_{600} = 1.0 by 0.5 mM IPTG, and cultures were shaken for 19 h at 30°C. Cells were harvested by centrifugation (2,500 $\times$ *g*), and proteins were extracted in deep wells by resuspending cells in 0.5 ml of TBS, pH 7.4, containing 25 mM imidazole, BugBuster, and Benzonase (Novagen). After 1 h of shaking at 4°C, the deep-well plate was centrifuged to pellet cell debris. Supernatants were purified on microspin columns containing 100 μl of Ni-Fast Flow Chelating Sepharose (GE Healthcare, Chalfont St. Giles, U.K.) equilibrated with TBS, pH 7.4, containing 20 mM imidazole. The resin was washed four times with the loading buffer, and purified proteins were eluted with 400 μl of TBS, pH 7.4, containing 250 mM imidazole.

Protein Production and Purification of Binders and Native Sac7d. For Biacore and microcalorimetry experiments, Sac7d and binders were purified as follows from recombinant DH5α. An overnight preculture (5 ml in LB + ampicillin + glucose at 37°C) was mixed with 1 liter of 2xYT + ampicillin and incubated and induced as described above. Cells were harvested by centrifugation and resuspended in 30 ml of TBS, pH 7.4, containing 25 mM imidazole at 30°C. Cells were lysed with an Avestin Emulsiflex homogenizer (Avestin, Ottawa, ON, Canada), and cell debris was removed by centrifugation. Proteins were purified on a 5-ml HiTrap column equilibrated with TBS, pH 7.4, containing 25 mM imidazole. Elution was performed with TBS, pH 7.4, containing 250 mM imidazole. Proteins were further purified by size-exclusion chromatography on a Superdex 75 26/60 gel filtration column (GE Healthcare) equilibrated with HBS (20 mM Hepes/150 mM NaCl), pH 7.0.

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Surface Plasmon Resonance. SPR was measured by using a BIAcore 2000 (BIAcore, Uppsala, Sweden) instrument at 25°C. Biotinylated PulD was immobilized on flow cells of an SA chip at densities of 200 RU (off-rate screening and kinetics) and 800 resonance units (RU) (competition SPR). The running buffer was HBST, pH 7.0 (20 mM Hepes/150 mM NaCl/0.05% Tween 20). The screening and ranking of binders according to their off-rates were performed by using microimmobilized metal ion affinity chromatography-purified proteins (see above) diluted 1:20 in running buffer before injection for kinetics measurements at a flow rate of 60 μl/min. Inhibition measurements were performed as described in ref. 19 with size-exclusion purified binders at 5 nM and different concentrations of PulD-N as a competitor ranging from 0.2 to 20 nM and a flow rate of 25 μl/min. The slope of the sensorgram for the binding phase was determined and plotted versus the concentration of PulD-N. BIAeval software (BIAcore) was used for data evaluation.

Microcalorimetry. ITC experiments were performed by using a VP-ITC (MicroCal, Northampton, MA) with the protocol for high-affinity measurements ($K_D \leq 10$ nM) that allowed reproducible measurements of K_D values down to 0.5 nM in a single titration experiment (*SI Materials and Methods*). Binding stoichiometries, enthalpy values, and K_D values were determined by fitting corrected data to a bimolecular model with Origin7 software (MicroCal). DSC experiments were performed with a VP-DSC (MicroCal) (*SI Materials and Methods*). Temperatures of half denaturation and unfolding enthalpy values were determined by fitting the $C_{p,excess}$ curves to a non-two-state model by using Origin7 software (MicroCal).

Construction and Production of GFP and PhoA Fusions. To construct GFP fusions, the genes encoding selected Sac7* variants were cloned via BamHI and HindIII sites into pQE30-derived pFP3000 containing the gene for EGFP and a region encoding a flexible peptide linker KLGSAGSAAGSGEF. This resulted in N-ter-binder-linker-EGFP-C-ter chimeras with an MRGS (His)6 tag at the N terminus. The sequences of individual clones were checked by DNA sequencing. Production and purification of Sac7*-GFP chimeras were as for Sac7* alone.

To construct PhoA fusions, genes encoding binders were PCR amplified with the primers SCPhoAF (5′-ATTAATGGTACCGGATCCGTGAAGGTGAAATTC-3′) and SCPhoAR (5′-ATAATTGAGCTCTAAGCTTTTTTTCACGCTCCGCAC-3′) and Phusion polymerase to introduce KpnI and SacI at 5′ and 3′ extremities, respectively. The PCR products were digested with KpnI and SacI and cloned into pQUANTagen (Qbiogene, Carlsbad, CA). Screening of PhoA$^+$ clones and periplasmic extractions were performed by using *E. coli* DH5α and instructions from the manufacturer. Briefly, positive clones were screened on LB agar containing 100 μg/ml ampicillin and 4 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate to detect PhoA. An overnight preculture (80 ml in LB medium containing 100 μg/ml ampicillin at 37°C) was inoculated into 400 ml of 2xYT medium containing 100 μg/ml ampicillin and 1 g dipotassium phosphate, pH 7.5. The *tac* promoter was induced with 0.5 mM IPTG at OD_{600} 0.7, and growth was allowed to continue for 4 h at 30°C. Cells were pelleted by centrifugation, resuspended in 40 ml of TSE buffer (30 mM Tris·HCl, pH 8.0, containing 20% sucrose, 0.5 mM EDTA, and 0.1 mg/ml lysozyme), and incubated for 20 min at 4°C with gentle agitation. Cell debris was removed by centrifugation for 30 min at 20,000 × *g* at 4°C, and supernatants were filtered through a 0.45-μm membrane before storage at −20°C.

Far-Western Blotting. Outer membranes of *E. coli* PAP105+/− PulD (pCHAP3671; see ref. 10) or PulD-CS (pCHAP3711; see ref. 9) together with PulS (pCHAP580; see ref. 30) were prepared as before. Membranes were resuspended and stored in 50 mM Tris·HCl, pH 7.5, containing 10% sucrose and 0.1 mg/ml of the protease inhibitor Pefabloc (Interchim, Montluçon, France) and were subjected to SDS/PAGE and transferred onto nitrocellulose sheets that were then blocked and incubated with periplasmic (osmotic shock) extracts of strains producing Sac7-PhoA chimeras. After washing, bound PhoA was detected by antibodies against PhoA, horseradish peroxidase-coupled secondary antibodies, and chemiluminescence.

Secretion. Strain PAP7232 (16) was transformed with the empty vector or with plasmids encoding Sac7-PhoA chimeras. Transformants were grown in medium containing 0.4% maltose (to induce production of pullulanase and its secretion system, including PulD) and 1 mM IPTG to induce Sac7-PhoA production. Secretion levels were measured as described in ref. 31 and are expressed as the amount of enzyme activity detected in whole cells compared with that detected in lysed cells (100%). Cell extracts were also examined by immunoblotting with antibodies against PulD and PhoA.

To analyze the effects of the Sac7-PhoA chimeras at higher (plasmid-encoded) levels of PulD production, a zeocin resistance gene was amplified with flanking PstI sites and inserted into the unique PstI site in the *blaM* gene of the corresponding plasmids. The recombinant plasmids were then transformed together with pCHAP231 (32) into the envelope protease-deficient strain PAP5198 (*degP*, *ompT*, *ptr*). Pullulanase secretion and levels of PulD (with or without prior treatment with phenol) were analyzed as above with or without induction by IPTG.

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1. Dove A (2002) *Nat Biotechnol* 20:121–124.
2. Cullen BR (2006) *Nat Methods* 3:677–681.
3. Stocks M (2005) *Curr Opin Chem Biol* 9:359–365.
4. Worn A, Pluckthun A (2001) *J Mol Biol* 305:989–1010.
5. Binz HK, Amstutz P, Pluckthun A (2005) *Nat Biotechnol* 23:1257–1268.
6. Arcus V (2002) *Curr Opin Struct Biol* 12:794–801.
7. Edmondson SP, Shriver JW (2001) *Methods Enzymol* 334:129–145.
8. Chami M, Guilvout I, Gregorini M, Remigy HW, Muller SA, Valerio M, Engel A, Pugsley AP, Bayan N (2005) *J Biol Chem* 280:37732–37741.
9. Guilvout I, Chami M, Engel A, Pugsley AP, Bayan N (2006) *EMBO J* 25:5241–5249.
10. Guilvout I, Hardie KR, Sauvonnet N, Pugsley AP (1999) *J Bacteriol* 181:7212–7220.
11. Shevchik VE, Robert-Badouy J, Condemine G (1997) *EMBO J* 16:3007–3016.
12. Hanes J, Pluckthun A (1997) *Proc Natl Acad Sci USA* 94:4937–4942.
13. Robinson H, Gao YG, McCrary BS, Edmondson SP, Shriver JW, Wang AH (1998) *Nature* 392:202–205.
14. Su S, Gao YG, Robinson H, Liaw YC, Edmondson SP, Shriver JW, Wang AH (2000) *J Mol Biol* 303:395–403.
15. Freire E (2001) *Methods Mol Biol* 168:37–68.
16. Hardie KR, Lory S, Pugsley AP (1996) *EMBO J* 15:978–988.
17. Walsh C (2000) *Nature* 406:775–781.
18. Sandkvist M (2001) *Infect Immun* 69:3523–3535.
19. Nieba L, Krebber A, Pluckthun A (1996) *Anal Biochem* 234:155–165.
20. Goldberg ME, Schaeffer F, Guillou Y, Djavadi-Ohaniance L (1999) *J Biol Chem* 274:16052–16061.
21. Mounier CM, Hackeng TM, Schaeffer F, Faure G, Bon C, Griffin JH (1998) *J Biol Chem* 273:23764–23772.
22. Hosse RJ, Rothe A, Power BE (2006) *Protein Sci* 15:14–27.
23. Kahsai MA, Martin E, Edmondson SP, Shriver JW (2005) *Biochemistry* 44:13500–13509.
24. Jurado P, Ritz D, Beckwith J, de Lorenzo V, Fernandez LA (2002) *J Mol Biol* 320:1–10.
25. McCrary BS, Edmondson SP, Shriver JW (1996) *J Mol Biol* 264:784–805.
26. Kawe M, Forrer P, Amstutz P, Pluckthun A (2006) *J Biol Chem* 281:40252–40263.
27. Possot O, Vignon G, Bomchil N, Ebel F, Pugsley AP (2000) *J Bacteriol* 182:2142–2152.
28. Binz HK, Amstutz P, Kohl A, Stumpp MT, Briand C, Forrer P, Grutter MG, Pluckthun A (2004) *Nat Biotechnol* 22:575–582.
29. Hanes J, Jermutus L, Weber-Bornhauser S, Bosshard HR, Plückthun A (1998) *Proc Natl Acad Sci USA* 95:14130–14135.
30. Daefler S, Guilvout I, Hardie KR, Pugsley AP, Russel M (1997) *Mol Microbiol* 24:465–475.
31. d'Enfert C, Reyss I, Wandersman C, Pugsley AP (1989) *J Biol Chem* 264:17462–17468.
32. d'Enfert C, Chapon C, Pugsley AP (1987) *Mol Microbiol* 1:107–116.