THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 52, pp. 40252–40263, December 29, 2006

Isolation of Intracellular Proteinase Inhibitors Derived from Designed Ankyrin Repeat Proteins by Genetic Screening*[S]

Received for publication, March 16, 2006, and in revised form, September 7, 2006 Published, JBC Papers in Press, October 18, 2006, DOI 10.1074/jbc.M602506200

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The specific intracellular inhibition of protein activity at the protein level is a highly valuable tool for the validation or modulation of cellular processes. We demonstrate here the use of designed ankyrin repeat proteins (DARPins) as tailor-made intracellular proteinase inhibitors. Site-specific proteolytic processing plays a critical role in the regulation of many biological processes, ranging from basic cellular functions to the propagation of viruses. The NIa^{pro} proteinase of tobacco etch virus, a major plant pathogen, can be functionally expressed in *Escherichia coli* without harming the bacterium. To identify inhibitors of this proteinase, we first selected binders to it from combinatorial libraries of DARPins and tested this pool with a novel *in vivo* screen for proteinase inhibition. For this purpose, a hybrid protein consisting of the ω subunit of *E. coli* RNA polymerase was covalently fused to a DNA-binding protein, the λcI repressor, containing an NIa^{pro} cleavage site in the linker between the two proteins. Thus, this transcriptional activator is inactivated by site-specific proteolytic cleavage, and inhibitors of this cleavage can be identified by the reconstitution of transcription of a reporter gene. Following this two-step approach of selection and screening, we could rapidly isolate NIa^{pro} proteinase inhibitors active inside the cell from highly diverse combinatorial DARPin libraries. These findings underline the great potential of DARPins for modulation of protein functionality in the intracellular space. In addition, our novel genetic screen can help to select and identify tailor-made proteinase inhibitors based on other protein scaffolds or even on low molecular weight compounds.

Selective inhibition of protein activity inside the cell is of fundamental importance for the investigation of biological processes as well as for the drug discovery process. Experimental approaches to achieve this goal have become increasingly available through the use of genetic knockouts and small interfering RNA-mediated knockdown of target proteins (1). However, these techniques knock out the expression of the entire gene of interest and are thus not able to discriminate, *e.g.* between the different functions of protein variants originating from the same gene (2). Moreover, the effect mediated by RNA interference is often only weak, especially if the cellular stability of the protein of interest is high, as only the *de novo* synthesis is (partially) inhibited. Recent studies have also demonstrated that RNA interference effects are not always specific for the targeted gene (3–5).

The use of inhibitory molecules directly acting at the protein level is thus a complementary approach. This strategy allows the targeting of single functions residing in different domains of multidomain protein complexes or those due to post-translational modifications. An important consideration is also that such proteinaceous inhibitors may be used in the subsequent characterization of the corresponding target protein *in vitro* or may even serve as first leads in the drug discovery process.

Intracellular proteinases are important regulators of signal transduction, RNA transcription, cell cycle progression, apoptosis, and development (6, 7) and many other processes, and they are also used by viruses in the processing of polyprotein precursors (8). To elucidate their function early in the discovery process, specific small molecule inhibitors will usually not be available, and thus a rapid approach to generate specific inhibitors that function inside the cell would be very valuable.

One way of approaching this challenge would be to use artificial proteinase inhibitors based on proteins. Although a large number of protein families have been used by nature for this purpose (10), the great majority of these inhibitors are secreted proteins and contain disulfide bonds. Thus, they work naturally on secreted proteinases and, consequently, have been re-engineered to target extracellular proteinases (9). Even though there are also natural intracellular proteinase inhibitors controlling many of the processes mentioned above (10), they have not been used as scaffolds for deriving new specificities up to now.

Another approach would be to use scaffolds that are not derived from proteinase inhibitors for this purpose. The generation of novel inhibitors is difficult, because polypeptides are first and foremost substrates of proteinases. The challenge is thus to achieve selective binding without cleavage or by maintaining a stable complex between proteinase and inhibitor even after cleavage of the latter.

An antibody scFv fragment that works in the reducing intracellular milieu (11) has been reported for this purpose. However, because these molecules also rely on disulfide bonds for stability (12–14), they may not provide a general solution for this kind of application. We therefore wished to investigate whether another class of proteins, repeat proteins, can be engineered to act as proteinase inhibitors.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Materials and Methods and Fig. S1.

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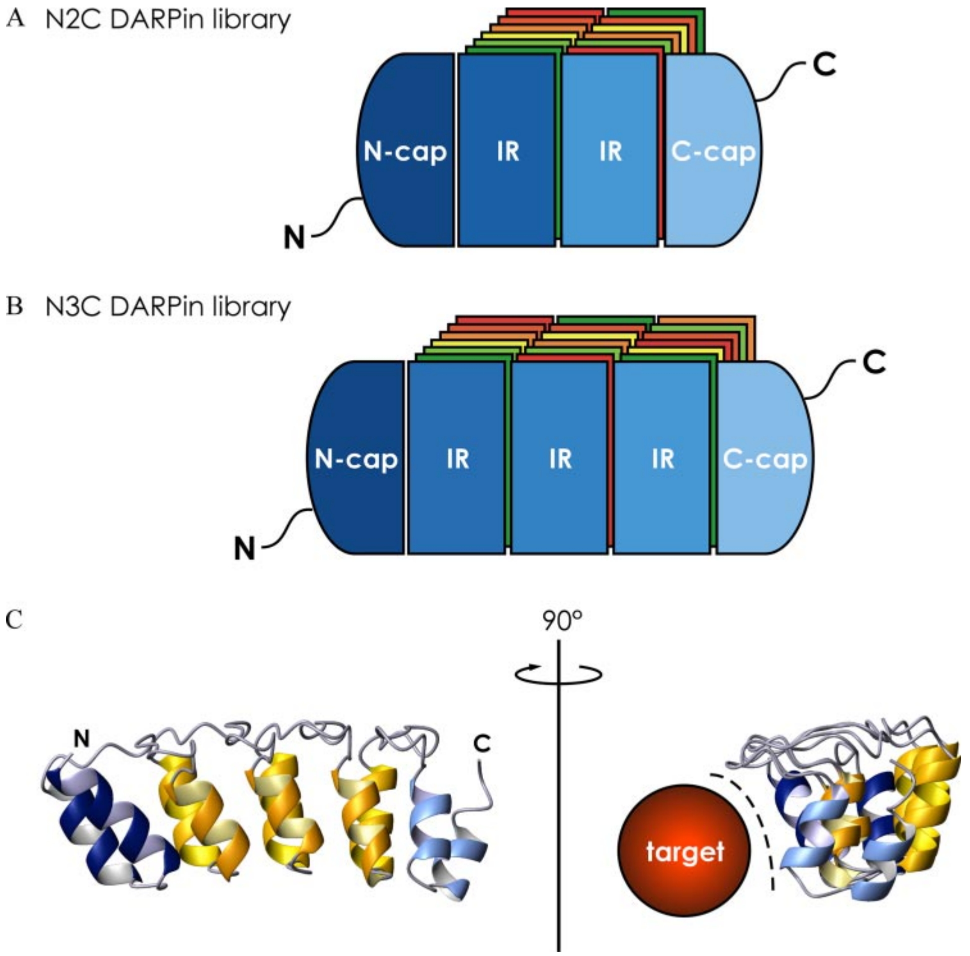

FIGURE 1. **Schematic illustration of combinatorial DARPin libraries.** *A* and *B*, members of combinatorial DARPin libraries consist of a single polypeptide chain, made up of a defined number of internal repeats (*IR*), here 2 or 3, each displaying a variable molecular surface and forming a continuous hydrophobic core. This hydrophobic core is sealed on both sides by capping repeats. DARPin libraries with 2 or 3 internal repeats are denoted N2C (*A*) and N3C (*B*). *C*, ribbon representation of the x-ray structure of the designed ankyrin repeat protein E3_5 in two perpendicular views (Protein Data Bank code 1MJ0 (23)). N- and C-terminal capping repeats are depicted in *dark* and *light blue*, respectively. Internal repeats are depicted in *yellow*. The *dotted line* in the side view representation denotes the interaction area with a potential target molecule (the figure was prepared with MOLMOL (63)).

We previously reported the generation of designed ankyrin repeat proteins (DARPins)[3] as specific binding molecules, and we also showed that they can be selected for intracellular enzyme inhibition, demonstrated for a bacterial kinase (15–17), but it was unclear whether they would contain the properties required for proteinase inhibition. Repeat proteins constitute the largest group of natural proteins specialized in binding. They can be found across all phyla, in the intra- and extracellular space, mediating a diverse set of biological functions (18–20). DARPins feature consecutive homologous structural units (repeats) of 33 amino acids, which stack to build up a single folded polypeptide. The elongated repeat domain can be of variable size, depending on the number of repeats, and it displays a rather rigid target-binding surface that can accommodate many different surface residues adaptable to specifically bind a wide range of targets.

By structural and sequence consensus analysis of this modular architecture of natural repeat proteins, we constructed highly diverse combinatorial DARPin libraries (15, 21, 22). These libraries consist of an N-terminal capping repeat, a defined number (typically 2 or 3) of engineered randomized internal repeats, and a C-terminal capping repeat (denoted an N2C and an N3C library; Fig. 1) in a single protein chain, and the molecules assume the ankyrin fold. The theoretical diversity exceeds 10^{14} for the N2C library and 10^{23} for the N3C library (22).

Unselected members of these libraries show very favorable biophysical properties (16, 22, 23), and selected members interact with their target molecules via their randomized positions in a highly specific manner (16, 24). DARPins do not rely on disulfide bonds for their stability, nor do they contain free cysteines. Furthermore, they do not show structural similarities to known naturally occurring proteinaceous inhibitors.

Here, we investigated whether DARPins can be selected to inhibit the main proteinase responsible for virus maturation of an agriculturally important plant virus, the NIa^{pro} proteinase of tobacco etch virus (TEV). NIa^{pro} is the main proteinase of potyvirus; it is responsible for two-thirds of all cleavage reactions occurring during the viral infection cycle, and its functionality is vital for successful virus propagation (25–27). Our aim to identify DARPin-based proteinase inhibitors was further encouraged by recent findings that naturally occurring proteinase inhibitors could mediate resistance against potyviruses in transgenic plants (28). In addition, NIa^{pro} is structurally highly homologous to the 3C proteinases of the picornavirus family, which are the major cause of numerous human diseases worldwide (29). Furthermore, this proteinase can be expressed in functional form in *Escherichia coli* without harm to the cell, as its highly specific cleavage reaction does not seem to destroy vital *E. coli* proteins.

To accomplish our task, we applied a two-step approach of *in vitro* selection for binding, followed by *in vivo* activity screening. Although many assays exist to study proteinase activity *in vitro* (30–32) and *in vivo* (6, 33, 34), these assays either need purified protein or they lack ease of handling. Therefore, we

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[3] The abbreviations used are: DARPin, designed ankyrin repeat protein; ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl-β-D-thiogalactopyranoside; NIa^{pro}, potyvirus nuclear inclusion-a proteinase (25 kDa, not containing the viral genome-linked protein domain VPg); RNAP, RNA polymerase; SPR, surface plasmon resonance; TEV, tobacco etch virus; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

adapted a known bacterial two-hybrid system (35) to serve as an *in vivo* proteinase activity screen. We were able to select and characterize *in vivo* active proteinaceous DARPin NIa^{pro} inhibitors. The potential of DARPins as a basis for proteinase inhibition and as a general intracellular target validation tool is discussed.

MATERIALS AND METHODS

Molecular Biology—Unless stated otherwise, all experiments were performed according to protocols of Sambrook *et al.* (36). Enzymes and buffers were from New England Biolabs (Beverly, MA) or Fermentas (Vilnius, Lithuania). All PCRs were performed using the proofreading Pfu^{Turbo} polymerase (Stratagene).

Plasmids—Plasmids used in this study are listed in Table 1, and their construction is described in detail in the Supplemental Material. The sequences of all inserts in plasmids that were generated by PCR were confirmed by DNA sequencing.

The vector for the expression of NIa^{pro} proteinase in all *in vivo* experiments, pZA55-TEV, was constructed by inserting the PCR-amplified *araC* gene plus the P$_{BAD}$ promoter sequence into pZA21-TEV. In turn, pZA21-TEV was constructed by inserting the PCR-amplified gene of the catalytic domain, NIa^{pro}, into pZA21 (37), thereby replacing its KpnI/BamHI fragment. The gene of the catalytic domain NIa^{pro} was amplified from pRK793 (38).

pBRcI-T-ω is a derivative of pBRcI-ω (35) containing a NIa^{pro} cleavage site. pMAKcI-T-ω-pD is a derivative of pQI-pD (39) and constitutively expresses the λcI-T-ω fusion protein under control of the β-lactamase promoter P$_{bla}$.

pMAKcI-T-ω-DL is a derivative of pMAKcI-T-ω-pD in which the pool of DARPins enriched by ribosome display, binding NIa^{pro}, replaces phage λ protein D (gpD). Expression of the DARPin pool is under control of the IPTG-inducible promoter P$_{T5/lac}$. It was used in all *in vivo* screening experiments. The open reading frames of the ribosome display-selected DARPins were digested with NcoI and HindIII and ligated into pMAKcI-T-ω-pD, yielding the selection plasmid ready for *in vivo* screening.

The pMAKcI-T-ω-DL tag is a derivative of pMAKcI-T-ω-DL and was used for the DARPins 9_1s, 13_1b, 20_2b, and E2_5 in the Western blot experiments. In this vector the DARPin genes lack their N-terminal RGS-His$_6$ tag. pAT223-TEV is a derivative of pAT223 and was used for the expression of His-tagged biotinylated and nonbiotinylated gpD-NIa^{pro} fusion protein.

Protein Production and Purification—The biotinylated fusion protein pD-NIa^{pro} and biotinylated pD alone (plasmids pAT223_TEV and pAT222) were produced by *in vivo* biotinylation of the N-terminal Avi tag (40) by co-expression of BirA from the plasmid pBirAcm in *E. coli* XL-1 Blue (Stratagene, La Jolla, CA) according to the protocols of Avidity (Denver) and Qiagen (Hilden, Germany). Efficient biotinylation was confirmed by ELISA and Western blotting using a streptavidin-alkaline phosphatase conjugate as detection agent (Roche Applied Science) and by mass spectrometry. Nonbiotinylated pD-NIa^{pro} for the ELISA analysis was produced in the same way as the DARPin proteins (22) using pAT223_TEV in *E. coli* XL-1 Blue. His tag purification of all proteins was carried out as described (22).

In Vitro Selection with Ribosome Display—The DARPin library generation has been described (22). In this study, an N2C and an N3C DARPin library were used, encoding DARPins consisting of a constant N-terminal capping repeat, two or three internal designed ankyrin repeats, respectively, containing randomized residues, and a constant C-terminal capping repeat as a continuous polypeptide chain. The PCR-amplified libraries in the ribosome display format were transcribed *in vitro*, and four standard ribosome display selection rounds were carried out as described (16, 41).

In Vivo Screening—The pools of NIa^{pro} binders selected by ribosome display from selection round three and four, starting from the N2C and the N3C library, were combined and ligated into pMAKcI-T-ω-pD, thereby replacing phage λ protein D (gpD) and generating pMAKcI-T-ω-DL, co-introduced with pZA55-TEV into *E. coli* KS1ΔZ (35) and plated on LB agar plates containing 1% glucose, 50 μg/ml ampicillin, 20 μg/ml tetracycline, 0.2% arabinose, 20 μg/ml X-gal, and 20–25 μM IPTG. Cells were grown at 30 °C overnight and checked for blue color development after various times. pMAKcI-T-ω-DL clones were isolated from different blue colonies and re-introduced together with pZA55-TEV into fresh *E. coli* KS1ΔZ cells, and the screening step was repeated to confirm the phenotype and to eliminate false positives. The DNA of those clones confirmed twice as positive was sequenced using standard DNA sequencing.

Size-exclusion Chromatography—Immobilized metal ion affinity chromatography-purified DARPins were analyzed on a Superdex 200 HR gel-filtration column (Amersham Biosciences) at room temperature using a SMART chromatography system (Amersham Biosciences) at a flow rate of 60 μl/min. TBS$_{150}$ (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) was used as running buffer.

ELISA—The biotinylated antigens (pD or pD-NIa^{pro}) were immobilized as follows: neutravidin (66 nM, 100 μl/well; Pierce) in TBS$_{150}$ was immobilized on a Maxisorp plate (Nunc, Roskilde, Denmark) by overnight incubation at 4 °C. The wells were then blocked with 300 μl of 0.5% bovine serum albumin (Fluka, Buchs, Switzerland) in TBS$_{150}$ for 1 h at room temperature. Biotinylated antigen (100 μl; 1 μM) in TBS$_{150}$ with 0.5% bovine serum albumin was allowed to bind for 1 h at 4 °C. To test whether the binding of the selected DARPins was specific for NIa^{pro}, 100 μl of purified DARPins (1 μM) were applied to wells with or without immobilized antigen for 1 h at room temperature. After extensive washing with TBS$_{150}$, binding was detected with an anti-RGS-His antibody (Qiagen; detects only the N-terminal RGS-His$_6$ tag of the DARPin but not the internal (pD-NIa^{pro}) or C-terminal (pD) His$_6$ tag of the antigen), an anti-mouse-IgG-alkaline phosphatase conjugate (Pierce), and *p*-nitrophenyl phosphate (Fluka). For competition ELISA, the purified DARPins were incubated with 5 μM of free NIa^{pro} prior to and during (4 °C, 100 min) the binding reaction.

Surface Plasmon Resonance—SPR was measured using a BIAcore 3000 instrument (BIAcore, Uppsala, Sweden). The running buffer was 20 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% Tween 20. A streptavidin SA chip (BIAcore) was used with 2000 response units of biotinylated pD-NIa^{pro} immobilized. The interactions were measured at a flow of 50 μl/min with a 5-min buffer flow, a 5-min injection of NIa^{pro} binding DARPins in varying concentrations (45 nM to 100 μM), and a dissociation step of 10 min with buffer flow. The signal of an uncoated reference cell was subtracted from the measurements. The equilibrium data of the interaction were evaluated with a global fit using BIAevaluation 3.0 (BIAcore), Scrubber (BioLogic software, Campbell, Australia), and Clamp (42).

Western Blot Analysis—Prior to Western blot analysis, antigenic samples were normalized to cell density (A_{600}), and proteins were separated by standard 15% SDS-PAGE. Western blot analysis was done following the protocol of Ref. 43. An Immobilon-P transfer membrane (Millipore, Billerica, MA) was used for sample transfer using semi-dry electroblotting. Sample detection was achieved using an anti-tetra-His antibody (Qiagen) and an anti-mouse-IgG-alkaline phosphatase conjugate (Pierce) or an anti-cI antibody (Invitrogen) and an anti-rabbit-IgG-horseradish peroxidase conjugate (Sigma). Blots were developed using nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate as substrates or chemiluminescent horseradish peroxidase substrate (Millipore).

In Vitro Inhibition Study—NIa^{pro} activity assays were performed essentially according to published procedures (44) with small modifications. Briefly, NIa^{pro} (3 μM) and selected DARPins or DARPin E2_5 (300 μM) were preincubated in NIa^{pro} reaction buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM dithiothreitol) at room temperature for 5 min prior to starting the measurement. The reaction was started by adding 7.5 μl of 200 μM substrate (Ac-TENLYFQ-amc, where amc is 7-amino-4-methyl-coumarin) to the reaction mixture (V_{final}, 100 μl; $c_{final\ substrate}$, 15 μM), and initial rates of substrate hydrolysis were immediately recorded by fluorometric measurement of the emission intensity. The assay was carried out at room temperature. The excitation wavelength was 360 nm, and the emission wavelength was 465 nm. Initial velocity data of substrate hydrolysis in the presence of the selected DARPins or E2_5 were normalized to initial velocity data obtained from measurements without DARPins; the value obtained here was arbitrarily set to 100%. All experiments were at least done in triplicate.

RESULTS

To obtain NIa^{pro} inhibitors from large combinatorial DARPin libraries (15), we chose to follow a two-step procedure consisting of an *in vitro* selection step to obtain pools of DARPins able to bind NIa^{pro}, followed by an *in vivo* screening step to identify those DARPins which not only bind but also inhibit the proteinase activity. This genetic screen is based on the well characterized transcriptional activation properties of fusion proteins consisting of the ω subunit of *E. coli* RNA polymerase (RNAP) connected covalently to a DNA-binding protein (bacteriophage λ repressor cI (35)).

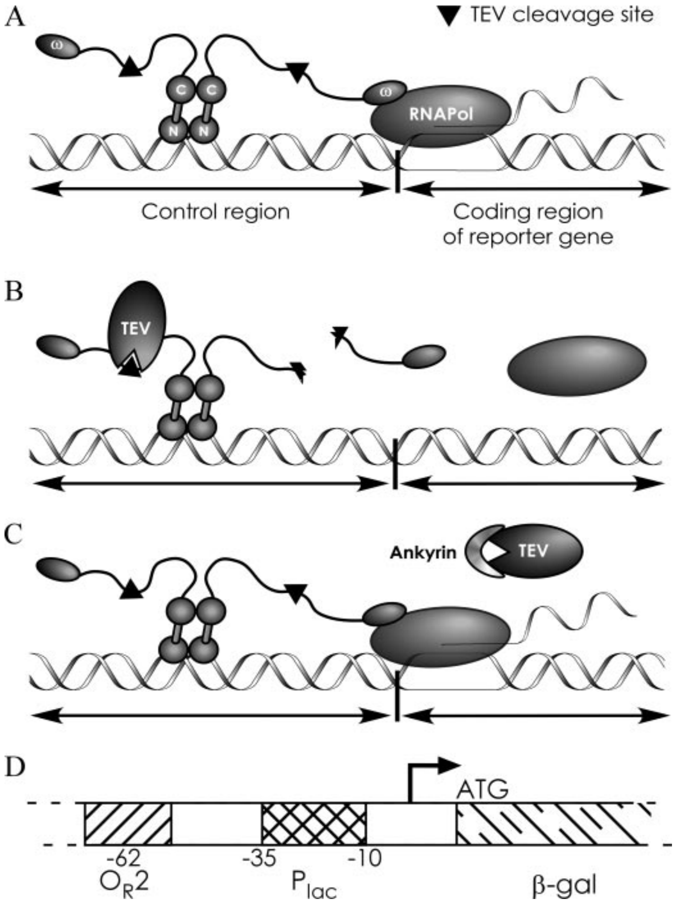

FIGURE 2. **Schematic illustration of the genetic screen, coupling intracellular proteinase activity to transcriptional activation of a reporter gene.** *A*, binding of the λcI part of a λcI-T-ω fusion protein to its cognate λ operator triggers transcriptional activation of the adjacent reporter gene via recruitment of RNAP by its ω part to a weak test promoter (note that this promoter does not function efficiently if the λcI-T-ω fusion protein fails to occupy the λ operator site). *B*, disruption of transcriptional activation because of proteolytic processing of the λcI-T-ω fusion protein by NIa^{pro}. *C*, restoration of transcriptional activation by λcI-T-ω fusion protein because of inhibition of NIa^{pro} by a co-expressed DARPin. *A–C*, *black triangle*, NIa^{pro} recognition sequence; *gray ellipse* ω, ω subunit of RNAP; *dumbbell*, DNA binding domain of bacteriophage λ inhibitor cI; *half-moon*, inhibitory DARPin. *D*, schematic representation of the artificial promoter derivative p*lac*O_R2–62 present on the chromosome of *E. coli* KS1ΔZ. This strain is defective in the *rpoZ* gene, which encodes the ω subunit of RNA polymerase. The λ operator O_R2 (depicted as a *hatched box*) is centered 62 bp upstream of the transcriptional start site (indicated by an *arrow*) of the *lac* promoter (depicted as a *cross-hatched box*). Note that the λcI-binding site is positioned too far away for λcI to activate transcription from p*lac*O_R2–62 by itself. Furthermore, the basal transcription of the β-galactosidase gene from this promoter by the *E. coli* RNA polymerase in the absence of the λcI-T-ω fusion protein is very weak.

A Genetic Activity Screen for Site-specific Proteolytic Enzymes—The basic strategy of our genetic screen is outlined in Fig. 2. The ω subunit of the RNA polymerase of *E. coli* can function as an activator of transcription when connected by a peptide linker to a DNA-binding protein that binds upstream near a promoter sequence (35). We reasoned that insertion of a defined proteinase cleavage site into this linker would allow its cleavage upon co-expression of a corresponding proteinase. This would destroy the activator, thereby abolishing transcription of a reporter gene (β-galactosidase) from an appropriately constructed promoter present in the chromosome of *E. coli* KS1ΔZ (35). Consequently, an inhibitor of that proteolytic enzyme, when additionally co-expressed, would restore the transcription of the reporter gene.

To verify that activation of transcription would only occur in the presence of the uncleaved transcriptional activator (*i.e.* the λcI-ω fusion protein), the gene encoding the λcI-ω fusion pro-

TABLE 1
Plasmids

The abbreviations used are as follows: Ap^R, ampicillin-resistant; Cm^R, chloramphenicol-resistant; Kan^R, kanamycin-resistant; Tc^R, tetracycline-resistant; gpD, bacteriophage λ coat protein D.

Plasmid	Relevant details	Source/Ref.
pBRcI-ω	Ap^R, ColE1, encodes λcI-wt(residues 1–236)-2Ala-ω(residues 1–90)	35
pBRcI-T-ω	Ap^R, ColE1, encodes λcI-wt(residues 1–236)-linker(NIa^{pro} recognition site plus His$_6$ tag)-ω(residues 1–90)	This work
pMAKcI-T-ω-pD	Ap^R, ColE1, encodes λcI-wt(residues 1–236)-linker(NIa^{pro} recognition site plus His$_6$ tag)-ω(residues 1–90) under control of P$_{bla,}$ gpD under control of P$_{T5/lac}$	This work
pMAKcI-T-ω-DL	Ap^R, ColE1, encodes λcI-wt(residues 1–236)-linker(NIa^{pro} recognition site plus His$_6$ tag)-ω(residues 1–90) under control of P$_{bla,}$ DARPin library under control of P$_{T5/lac}$	This work
pQI-pD	Ap^R, ColE1, RGS- His$_6$ tag-gpD under control of P$_{T5/lac}$	39
pZA55-TEV	Tc^R, p15A, encodes NIa^{pro} proteinase catalytic domain^a (S219N) under control of P$_{BAD}$	This work
pAT223_TEV	Ap^R, ColE1, encodes NIa^{pro} proteinase catalytic domain (S219N) as C-terminal fusion of gpD under control of P$_{T5/lac}$, N-terminal Avi tag for biotinylation	This work
pAT223	Ap^R, ColE1, encodes gpD under control of P$_{T5/lac}$, N-terminal Avi tag for biotinylation	This work
pRK793	Ap^R, ColE1, encodes NIa^{pro} proteinase catalytic domain (S219N)as C-terminal fusion of maltose-binding protein under control of P$_{tac}$	38
pBirAcm	Cm^R, p15A,used for *in vivo* biotinylation; contains *birA*	Avidity, Denver, CO
pMAKcI-T-ω-DL tag	pMAKcI-T-ω-DL analogue, DARPin library member devoid of its N-terminal RGS-His$_6$ tag	This work
pQE60	Ap^R, ColE1, P$_{T5/lac}$-controlled expression plasmid	Qiagen, Hilden, Germany
pBADGFPAC2	Ap^R, ColE1, encodes GFP under control of P$_{BAD}$	57
pZA21	Kan^R, p15A, PL$_{tet-O1}$-controlled expression plasmid	37
pTRG	Tc^R, ColE1; "dummy" plasmid to confer tetracycline resistance to cells during *in vivo* screening experiments (control)	45

[a] All experiments were performed with a mutant form (S219V) of the C-terminal proteolytic domain of the full-length NIa^{pro} protein of TEV. Thus, the proteinase used here lacks its N-terminal VPg domain and is resistant to autoinactivation by truncation of its C-terminal tail.

tein was placed downstream of an inducible promoter on a plasmid vector, generating plasmid pBRcI-ω (Table 1). This vector was introduced into an engineered *E. coli* reporter strain, KS1ΔZ (35). In this strain the chromosomal *rpoZ* gene, coding for the RNAP ω subunit, has been deleted. Furthermore, this strain harbors on its chromosome a single copy of a *lac* promoter derivative (named placO$_R$2–62) bearing a single λ operator site centered 62 bp upstream of the transcriptional start point (35). In this strain the basal transcription of the β-galactosidase reporter gene from this promoter is very weak (in the presence or absence of λcI alone) but can be stimulated more than ~70-fold in the presence of the λcI-ω fusion protein (35, 45), which results in blue colonies when grown on plates containing X-gal (Fig. 3*A*, *sector 5*).

We tested this screen with NIa^{pro}, which displays a high specificity for its 7-amino acid recognition sequence (E*XX*Y*X*Q↓(G/S), where ↓ indicates the cleavage point; *X* indicates any amino acid), at which the TEV polyprotein is cleaved (46). To examine whether the insertion of the recognition sequence would disrupt the transcriptional activation properties of the λcI-ω fusion protein, a 25-amino acid coding region, including the 7-amino acid recognition sequence (ENLYFQ↓S) flanked by a His$_6$ tag and a glycine-serine linker (see vector map in the Supplemental Material), was introduced into the linker region of the λcI-ω fusion protein, thus generating the λcI-T-ω fusion protein. This construct was assayed under the same conditions as the fusion protein without the inserted cleavage sequence, and no difference in transcriptional activation was observed (Fig. 3*A*, *sector 4*). Thus, *E. coli* proteinases do not cleave this λcI-T-ω fusion protein to the extent of abolishing activation.

We next asked whether co-expression of NIa^{pro} would affect the function of the λcI-ω or λcI-T-ω fusion protein *in vivo*. For this purpose, NIa^{pro} was expressed in *E. coli* under the control of an arabinose-inducible promoter (plasmid pZA55-TEV; Table 1) to regulate it independently of the λcI-ω or λcI-T-ω fusion protein and to ensure tight repression in the absence of inducer (37). pZA55-TEV was introduced together with the plasmid vectors encoding λcI-ω or λcI-T-ω fusion protein into *E. coli* KS1ΔZ. Cells were plated on LB agar plates containing X-gal, IPTG (inducing the activator proteins), and arabinose (inducing the proteinase) in various combinations. Although the λcI-ω fusion protein was still active as transcriptional activator (Fig. 3*A*, *sector 3, blue colonies*) in the presence of NIa^{pro}, the λcI-T-ω fusion protein was not (Fig. 3*A*, *sector 2, white colonies*). Thus, in the presence of NIa^{pro}, the λcI-T-ω fusion protein was indeed cleaved and can no longer procure transcriptional activation. In contrast, the λcI-ω fusion protein, which cannot be proteolytically processed, still activated transcription, also indicating that expression of NIa^{pro} itself had no influence on the transcriptional activation properties of the λcI-ω fusion protein.

These results show that the transcriptional activation properties of the λcI-ω or λcI-T-ω fusion protein are dependent on the integrity of the fusion proteins and that the λcI-T-ω fusion protein is thus suited to phenotypically monitor proteinase activity in the cytosol of *E. coli*. The same results were obtained when the λcI-T-ω fusion protein was constitutively expressed under the control of the β-lactamase promoter (Fig. 3*B*, pMAK vector series) instead of under the control of the IPTG-inducible P$_{lac\text{-}UV5}$ promoter (pBR vector series) as used in the experiments described above. The findings presented here are the bacterial counterpart to earlier experiments performed in yeast where the transcriptional activation properties of the GAL4 protein were used to also monitor proteinase inhibition *in vivo* (34).

In Vitro Enrichment of DARPins for NIa^{pro} Binding by Ribosome Display—Ribosome display (RD) selection rounds with both an N2C and an N3C DARPin library (22) on immobilized NIa^{pro} were performed as described (16). The DNA libraries used in the selection contained at least 10^{12} individual members each, as estimated from the initial amount of ligated library DNA (22), and further diversity is introduced by polymerase errors during the PCR cycles intrinsic to each ribosome display

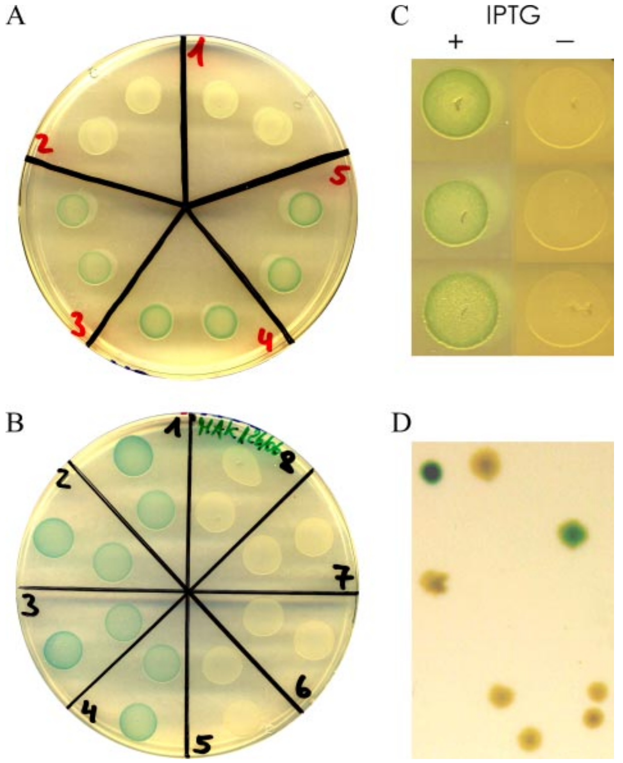

FIGURE 3. **Phenotypic monitoring of NIa^{pro} activity in *E. coli* KS1ΔZ.** Cells were grown at 30 °C overnight, followed by *blue color* development for various times. Media always contained 20 mg/ml X-gal. *A*, *E. coli* KS1ΔZ was transfected with pZA55-TEV (*sector 1*), pZA55-TEV and pBRcI-T-ω (*sector 2*), pZA55-TEV and pBRcI-ω (*sector 3*), pBRcI-T-ω (*sector 4*), and pBRcI-ω (*sector 5*). The plates contained 20–25 μM IPTG to induce the expression of the different DARPins encoded on the plasmids. In addition, 0.2% arabinose was added to ensure expression of NIa^{pro}. Positive transcriptional activation of β-galactosidase is visualized by blue colony formation (see text for details). *B*, *E. coli* KS1ΔZ was transfected with pMAKcI-T-ω (*sectors 1–4*) and pMAKcI-T-ω plus pZA55-TEV (*sectors 5–8*). Positive transcriptional activation is visualized by blue colony formation (refer to text for details; four independent colonies were tested each). *C*, *E. coli* KS1ΔZ was transfected with pMAKcI-T-ω-DL containing in each case selected DARPins 9_1s (*top*), 13_1b (*middle*), or 20_2b (*bottom*) and pZA55-TEV. Expression of the DARPins was accomplished by adding 20 μM IPTG to the medium (*left panel*); DARPins are not induced in the *right panel*. Positive transcriptional activation is visualized by *blue colony* formation (see text for details). *D*, close-up of a part of a screening plate.

selection round. An enrichment of binders was already observed after the second selection round at the level of reverse transcription-PCR (data not shown). The enriched pools of binders (potential inhibitors) from the third and fourth selection rounds from both libraries, N2C and N3C, were combined before screening for intracellularly active NIa^{pro} inhibitors.

In Vivo Identification of DARPins Intracellularly Inhibiting NIa^{pro}—To screen for inhibitory DARPins, the combined DARPin gene pool enriched by ribosome display was cloned into the plasmid vector pMAKcI-T-ω, which encodes the transcriptional activator under the control of a constitutive promoter, generating pMAKcI-T-ω-DL (Table 1). Thereby the DARPin genes were placed downstream of an IPTG-inducible T5/*lac* promoter (47) to ensure controlled and high level expression of the DARPins. The enriched pool was introduced together with pZA55-TEV into *E. coli* KS1ΔZ, and cells were plated on LB agar plates containing X-gal and the respective inducers. The chosen growth conditions were verified prior to the screening experiment to not influence cell growth, thereby securing both the expression of NIa^{pro} and the DARPin library members (data not shown). On average, 10^5 colony-forming units were plated per plate (24 × 24 cm), of which 0.4–0.6% colony-forming units displayed a "blue" phenotype (Fig. 3*D*). To test that the restoration of the blue phenotype was indeed due to inhibition of NIa^{pro} by a library member, and not because of endogenous mutations, a series of tests was carried out. First, 38 blue colonies were tested for DARPin library member expression and their binding to immobilized NIa^{pro} by crude cell extract ELISA. Nearly 80% of the analyzed colonies showed expression of DARPins of the expected size on SDS-polyacrylamide gels. The majority of the selected DARPins was of the N2C type (22), and only two N3C members were found. A positive ELISA for binders to immobilized NIa^{pro} was obtained in ~75% of the tested cases (data not shown).

To further verify and confirm the observed phenotype, 10 library members were chosen for a more detailed analysis. For this purpose, freshly retransformed *E. coli* KS1ΔZ cells harboring pZA55-TEV and one of the 10 selected library members were plated on LB agar plates under screening conditions with or without IPTG for induction of the inhibitory DARPins. As expected, colonies stayed white when IPTG was missing in the growth medium (proteinase cleaves the transcriptional activator) and turned blue when it was present (induction of DARPins which inhibit proteinase; Fig. 3*C*; cells harboring only pZA55-TEV stayed white in both cases (data not shown)). To ensure that the proteinase recognition site had not been lost during screening, the genes of three selected DARPins (9_1s, 13_1b, and 20_2b) were cut out from their respective plasmid vectors and freshly ligated into the pMAKcI-T-ω-pD backbone (Table 1); the same inhibition phenotype was observed when these clones were analyzed under screening conditions (data not shown). Thus, the phenotype observed in screening was reproducibly confirmed *in vivo*.

Sequence Analysis of Selected DARPins—We sequenced the 10 selected DARPins (Fig. 4) and found a high content of aromatic amino acids, Trp, Tyr, and Phe at the randomized positions, which are under-represented in the original library design (22). Most interesting in this respect is the high content of Tyr at the variable positions, which can mediate binding via hydrophobic and polar interactions as well as acting as an H donor (48). This is reminiscent of sequences obtained from selections against maltose-binding protein (16) or aminoglycoside phosphotransferase IIIa (17), where similar characteristics of the amino acid composition at the randomized positions, but of course completely different sequences, were found. Nevertheless, binding was entirely specific in each case. This finding also supports the idea that DARPins, similar to antibodies (2), prefer to use a number of aromatic amino acids in their binding site to achieve their binding function (16).

Size-exclusion Chromatography of Selected DARPins—The following experiments were all carried out with immobilized metal ion affinity chromatography-purified protein samples, and the correct molecular mass for all selected DARPins was confirmed by matrix-assisted laser desorption ionization mass spectrometry (Table 2). Size-exclusion chromatography showed that all 10 selected DARPins were predominantly monomeric, and only a single protein species was observed (Fig.

```
                          N-terminal cap                     repeat 1                         repeat 2
Residue_#       1...5...10...15...20...25...30...1...5...10...15...20...25...30...1...5...10...15...20...25...30...
Designed        GSDLGKKLLEAARAGQDDEVRILMANGADVNAxDxxGxTPLHLAAxxGHLEIVEVLLKzGADVNAxDxxGxTPLHLAAxxGHLEIVEVLLKzGADVNAx
                └───────────────────────────────┘└-------------------------------┘└───────────────────────────────┘

12_1b (N2C)     ................................N.FL.R.......YE...........Y......N.TF.M.......FD...........H......Q
7_1b  (N2C)     ................................L.FL.R.......YE...........Y......N.TF.M.......FD...........N......Q
17_2s (N2C)     ..N.............................V.ML.K.......YQ...........Y......F.TF.M.......FD...........Y......Q
15_3b (N2C)     ................................Q.DF.K.......YQ...........Y......N.LF.M.......LD...........H......Q
5_3s  (N2C)     ................................I.SL.K.S.....YQ...........Y......S.LF.L.......YD...........H......Q
2_2b  (N2C)     ..N.............G...............S.HF.K.......YV...........H......I.LF.M.......ID...........H......Q
20_2b (N2C)     ................................N.VI.K.......FF...........H......D.SF.R.......DY...........N......Q
9_1b  (N2C)     ................................Y.VI.L.......RN...........N......S.HF.R.......EI...........Y......Q
13_1b (N2C)     ................................D.HY.L.......NF...........Y......G.EE.S.......DW...........H......Q
9_1s  (N3C)     ................................N.FL.R.......YE...........Y......D.NH.K.......YK...........Y......I

                                                             repeat 3                   C-terminal cap
Residue_#                                        1...5...10...15...20...25...30...1...5...10...15...20...25..
Designed                                         DxxGxTPLHLAAxxGHLEIVEVLLKzGADVNAQDKFGKTAFDISIDNGNEDLAEILQKLN
                                                 └-------------------------------┘└─────────────────────────┘
12_1b (N2C)                                                                       ...........................
7_1b  (N2C)                                                                       ...........................
17_2s (N2C)                                                                       ...........................
15_3b (N2C)                                                                       ...........................
5_3s  (N2C)                                                                       ...........................
2_2b  (N2C)                                                                       ...........................
20_2b (N2C)                                                                       ...........................
9_1b  (N2C)                                                                       ...........................
13_1b (N2C)                                                                       ...........................
9_1s  (N3C)                                      .VF.M.....S.FD...........H.......Q..........................
```

FIGURE 4. **Sequences of the DARPins selected against NIa$^{\mathrm{pro}}$.** The designed sequences for the N3C and N2C libraries are given *above* the selected sequences (*x* represents a randomized potential interaction residue, where any amino acid was allowed except C, G, or P; *z* represents a randomized framework residue where the three amino acids N, H, or Y were allowed (22). The names of the clones and their length are given on the *left side* of the respective sequence. Note that in this representation only the N- and C-terminal caps and the internal repeats are shown and not the tags.

TABLE 2

Molecular masses of three selected DARPins

Protein	$M_{\mathrm{r}}^{\mathrm{calc}}$ [a]	$M_{\mathrm{r}}^{\mathrm{obs}}$ [b]	$M_{\mathrm{r}}^{\mathrm{obs}}$ [c]	$M_{\mathrm{r}}^{\mathrm{obs}}$ [d]
	kDa	*kDa*	*kDa*	*kDa*
13_1b	16.466	16.467	22.2	ND
20_2b	16.428	16.428	20.1	ND
9_1s	20.148	20.149	18.2	ND
N2C	14.369	14.369	18.6	14.9
N3C	17.895	17.901	23.1	17.4

[a] Values are as calculated from the sequence.

[b] Values are as determined by MALDI mass spectrometry.

[c] Values are as determined by gel filtration, using globular proteins as molecular weight standards.

[d] Values are as determined by multiangle light scattering.[4] Note that these proteins do not have a double His$_6$ tag preceding the DARPin open reading frame.

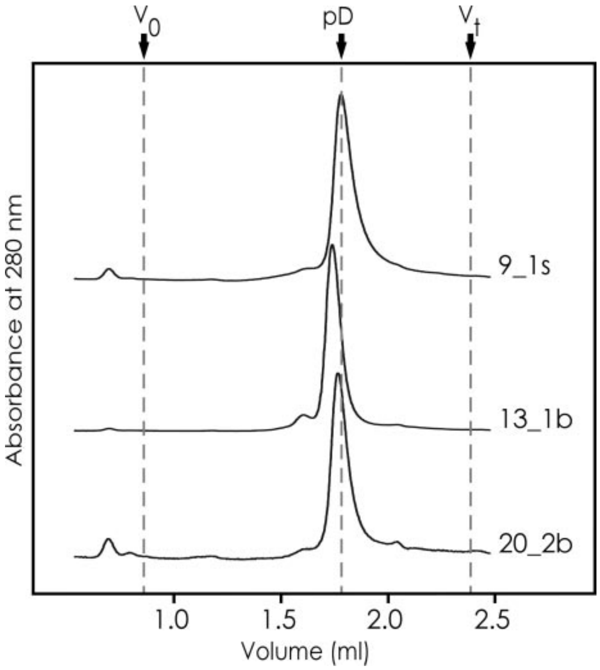

FIGURE 5. **Size-exclusion chromatography of selected DARPins.** The elution profiles of two selected N2C DARPins (13_1b; 20_2b) and one selected N3C DARPin (9_1s) are shown. All molecules are predominantly monomeric, as judged from the apparent molecular weight calculated from the elution volume. The void volume (V_0 = 0.9 ml), the total volume (V_t = 2.4 ml), and one of the molecular mass standards (bacteriophage λ coat protein D (*pD*) with an apparent mass of 17.6 kDa) are indicated by *dashed gray lines* in the chromatogram plots.

5; Table 2). Only two DARPins (13_1b and 17_2s) showed a small shoulder in the elution profile that could correspond to a dimer (*13_1b*, see Fig. 4; 17_2s; data not shown). The molecular size values obtained from the gel-filtration studies are given in Table 2. The observed molecular mass is, with the exception of the N3C DARPin 9_1s, always slightly higher than the value calculated from the sequence, which might reflect the elongated shape of ankyrin repeat domains, rather than the formation of higher oligomeric assemblies. These observations are corroborated by size-exclusion studies of full consensus ankyrin repeat proteins with different numbers of repeats in which the correct molecular size was verified by mass spectrometry, and the monomeric nature of the molecules was verified by multiangle light scattering (Table 2).[4] For the full consensus ankyrins, the apparent molecular size calculated from the elution volume was also always slightly higher than that expected for a globular protein of the same size (Table 2). The slightly delayed elution behavior of the N3C DARPin 9_1s might be caused by hydrophobic interactions with the column material (this library member has more hydrophobic residues compared with the other selected DARPins).

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[4] S. Wetzel, unpublished results.

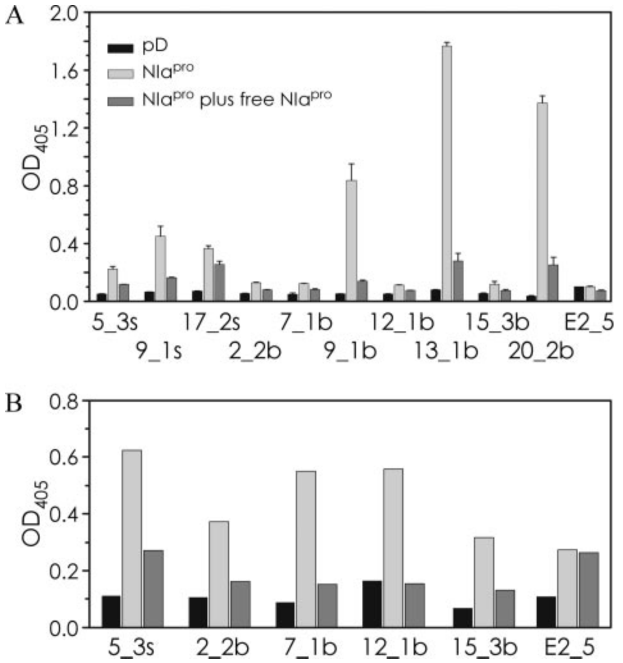

FIGURE 6. **Specific binding of NIa^{pro} inhibitors.** *A*, the interaction of the selected DARPins (5_3s, 9_1s, 17_2s, 2_2b, 7_1b, 9_1b, 12_1b, 13_1b, 15_3b, and 20_2b, all at a concentration of 1 μM) with immobilized NIa^{pro} (*light gray bars*) was compared with the interaction with noncognate control gpD (*black bars*). NIa^{pro} and gpD were immobilized via a biotinylated Avi tag on neutravidin-coated ELISA plates. The interactions of the selected DARPins with immobilized NIa^{pro} can be specifically inhibited by incubation with free NIa^{pro} (5 μM) prior to binding on immobilized NIa^{pro} (*dark gray bars*). As a control, an unselected library member (E2_5, 1 μM) was included. The background binding of the detection antibodies was not subtracted. *B*, same ELISA of the weakly interacting DARPins 5_3s, 2_2b, 7_1b, 12_1b, and 15_3b with reduced washing steps and shortened incubation times (see under "Materials and Methods").

In Vitro Target Binding and Binding Constants of the Inhibitory DARPins—The binding specifications of the selected DARPins were analyzed by ELISA. For a smaller subset of DARPins, the binding affinity was further determined by surface plasmon resonance (SPR) experiments. In an ELISA experiment with the 10 purified inhibitors, the binding to NIa^{pro} was compared with the binding to bacteriophage λ protein D (Fig. 6, *pD*) (49), which was present in the fusion protein used in the selection experiments. All selected DARPins bound their cognate target and did not bind to pD. The binding signal to immobilized NIa^{pro} could be suppressed by preincubation with free NIa^{pro}, demonstrating that the interaction is specific for the native protein (Fig. 6). The unselected N2C DARPin library member E2_5 (22), used as a control, did not interact with NIa^{pro}, indicating that the designed DARPin scaffolds *per se* do not bind NIa^{pro} (Fig. 6). It should be noted that the ELISA signal of some selected DARPins was very weak (2_2b, 7_1b, 12_1b, and 15_3b) and could be improved by reducing the washing time during the ELISA experiment (Fig. 6*B*). This finding suggests a very fast off-rate of these binders, which was confirmed by SPR experiments (Fig. 7). Thus, under our standard ELISA conditions, these DARPins are washed off before detection with an antibody.

To measure the affinity of the interaction of the *in vivo* selected DARPins specific for NIa^{pro}, equilibrium SPR experiments were performed with the DARPins 9_1s, 13_1b, and 20_2b. The K_D values of all three NIa^{pro} binders were found to be in the low micromolar range (Table 3). The SPR data were fitted with the assumption of a 1:1 interaction with one-site saturation (Fig. 7).

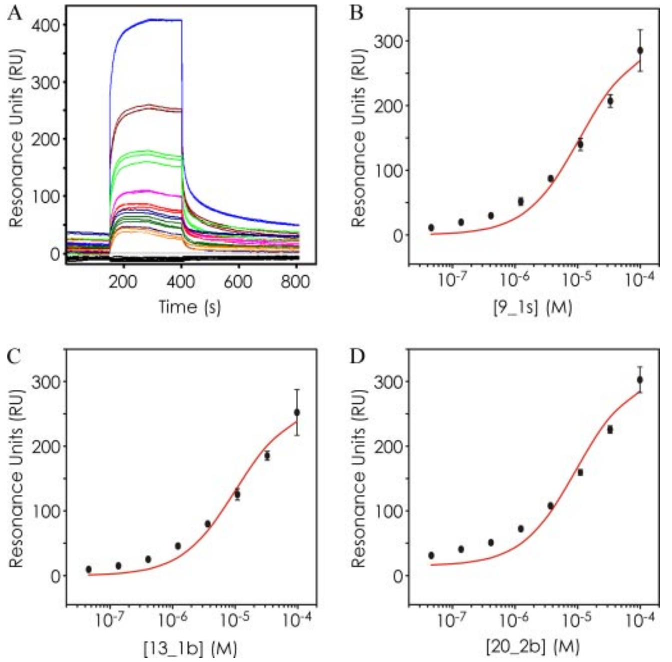

FIGURE 7. **SPR analysis of selected DARPins.** *A*, BIAcore analysis of 20_2b. Different concentrations of 20_2b (0.045, 0.137, 0.411, 1.2, 3.7, 11.1, 33.3, and 100 μM) were applied to a flow cell with immobilized NIa^{pro} (2000 response units (*RU*)) for 5 min, followed by washing with buffer flow for 10 min. *B–D*, fit of the experimental data obtained for DARPins 9_1s (b), 13_1b (c), and 20_2b (c); fits to the formula $\mathrm{RU} = \mathrm{RU}_{max} \times [\mathrm{I}]/([\mathrm{I}] + K_D)$ are indicated in the figure by *red lines*. Here, RU are the response units observed, RU_{max} is their plateau value at high DARPin concentration, and [I] is the concentration of inhibitory DARPin.

TABLE 3

Affinity data of selected clones determined by surface plasmon resonance

Target	Clone name (type)	K_D
		μM
NIa^{pro}	9_1s (N3C)	11 ± 4
NIa^{pro}	13_1b (N2C)	10 ± 4
NIa^{pro}	20_2b (N2C)	9 ± 4

Finally, we would like to stress that the DARPins 9_1s, 13_1b, and 20_2b did not bind to the immobilized transcriptional activator λcI-T-ω fusion protein, as tested by ELISA (data not shown). Protection of the NIa^{pro} cleavage site within the λcI-T-ω fusion protein by a DARPin might, in principle, result in the same phenotype. However, because we found no binding of the selected DARPins to the λcI-T-ω fusion protein, we can rule out the latter mechanism for transcriptional activation.

In Situ Inhibition by Selected DARPins—To confirm the direct inhibition of NIa^{pro}, we conducted co-expression tests of proteinase and DARPins in the presence of the λcI-T-ω fusion protein in *E. coli* KS1ΔZ in liquid culture, following the digestion of the transcriptional activator by Western blot analysis in crude cell extracts. For this experiment we used two different antibodies as follows: one was directed against the His$_6$ tag of the λcI-T-ω fusion protein, which is present in its linker sequence (Fig. 8*A*); and a second one was directed against the cI

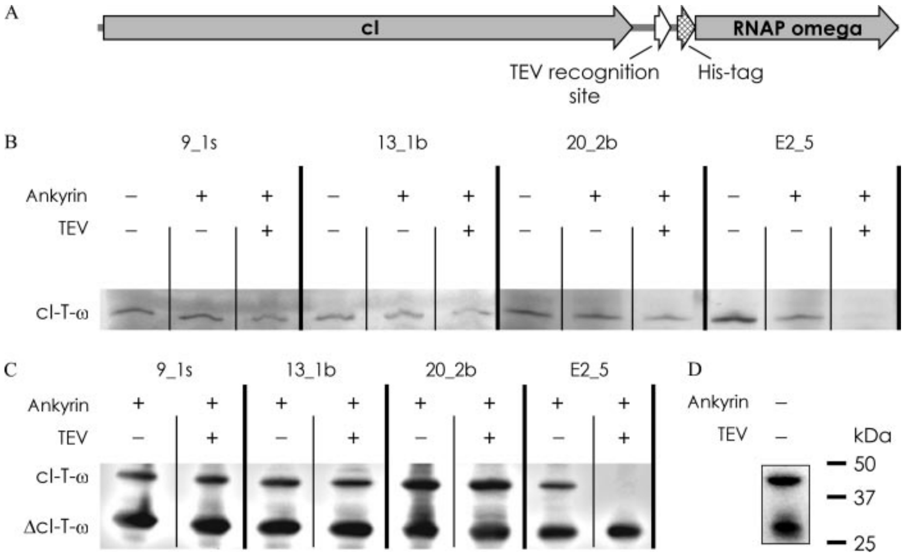

FIGURE 8. **Selected DARPins inhibit the cleavage of λcI-T-ω by NIa$^{\text{pro}}$.** *A*, schematic drawing of the λcI-T-ω fusion protein to scale. Open reading frames of the λcI protein and of the RNAP-ω subunit are indicated by *gray arrows*. The NIa$^{\text{pro}}$ recognition site is depicted by a *white arrow* and the His$_6$ tag by a *hatched arrow*, respectively. *B*, Western blot analysis of the expression profile of the λcI-T-ω fusion protein in the presence of the selected DARPins 9_1s, 13_1b, and 20_2b and NIa$^{\text{pro}}$. The λcI-T-ω fusion protein was monitored by detection of its His$_6$ tag with an α-tetra His-Ab following 15% SDS-PAGE and Western blotting. pMAKcI-T-ω-DL containing, in each case, DARPin 9_1s, 13_1b, or 20_2b was introduced alone or together with pZA55-TEV into *E. coli* KS1ΔZ. DARPin expression was induced with 20 μM IPTG 30 min before induction of NIa$^{\text{pro}}$ with 0.2% arabinose. Cells were collected before and 2 h after induction of proteinase, normalized, and lysed in loading buffer. As a control, the unselected library member E2_5 was included. *C*, same as in *B* but the λcI-T-ω fusion protein was monitored by detection of its cI part with an α-cI-Ab, following 15% SDS-PAGE and Western blotting. *D*, Western blot analysis of the expression profile of the λcI-T-ω fusion protein in the absence of the selected DARPins and in the absence of NIa$^{\text{pro}}$; λcI-T-ω fusion protein was monitored as in *C*.

domain of the λcI-T-ω fusion protein. Upon cleavage of the activator by NIa$^{\text{pro}}$, the band corresponding to the full-length activator disappears, whereas the two cleavage products, the His$_6$ tag-ω domain and the cI domain bands, should appear. However, when performing this experiment, we could not detect the His$_6$ tag-ω domain alone, as it might get further digested or its band might be too faint to be detected. The band of the full-length activator and the cleavage product could be detected with the anti-cI antibody. This experiment is the most direct means of analyzing the effect of the selected DARPins on NIa$^{\text{pro}}$ *in situ*, and in the presence of an inhibitor of NIa$^{\text{pro}}$ the full-length band of the activator is preserved.

For these experiments, plasmid vectors coding for the selected DARPins 9_1s, 13_1b, and 20_2b were co-introduced with pZA55-TEV into *E. coli* KS1ΔZ. Cells were grown nearly to the end of the log phase, and DARPin expression was induced by the addition of IPTG. After 30 min an aliquot was withdrawn, and NIa$^{\text{pro}}$ expression was started by the addition of arabinose. After 2 h another aliquot was withdrawn. The aliquots were normalized to A_{600}, and cells were disrupted by heating at 95 °C for 15 min in SDS-loading buffer, and proteins were subsequently separated by SDS-PAGE. The band of the λcI-T-ω fusion protein was monitored by Western blot analysis with an anti-tetra His-Ab or anti-cI-Ab, respectively. Only the selected DARPins can prevent the digestion of the λcI-T-ω fusion protein by NIa$^{\text{pro}}$ *in vivo* (Fig. 8). By contrast, the unselected library DARPin E2_5 (22) was unable to inhibit the digestion of the λcI-T-ω fusion protein by NIa$^{\text{pro}}$ (Fig. 8, *B* and *C*). Using the anti-cI-Ab for detection of the λcI-T-ω fusion protein, we also observed an unspecific degradation of the λcI-T-ω fusion protein by endogenous *E. coli* proteinases (Fig. 8*C*), which also occurs in the absence of any DARPins and in the absence of NIa$^{\text{pro}}$ (Fig. 8*D*). As expected, this cleavage by *E. coli* proteinases cannot be inhibited by the expression of our selected DARPins. Nevertheless, enough λcI-T-ω fusion protein is clearly remaining to induce the blue phenotype (Fig. 3). The results of these experiments indicated for the first time the inhibition of a site-specific proteinase by selected DARPins in the intracellular compartment of *E. coli.*

In Vitro Inhibition by Selected DARPins—To confirm that the *in vivo* effects observed for the selected DARPins were indeed due to direct inhibition of NIa$^{\text{pro}}$, *in vitro* enzyme assays were performed. Enzyme activity was monitored by the release of the fluorogenic leaving group (7-amino-4-methyl-coumarin (amc)) from a synthetic peptide substrate (Ac-TENLYFQ-amc) as described previously (44) with minor modifications (see under "Materials and Methods") to mimic the *in vivo* situation. *In vivo*, the DARPin concentration is much higher than the concentration of NIa$^{\text{pro}}$ and the substrate, *i.e.* the λcI-T-ω fusion protein. As estimated from SDS-PAGE analysis (see above) the ratio of DARPin to NIa$^{\text{pro}}$ to λcI-T-ω fusion protein (*i.e.* the *in vivo* substrate) is 800:8:1 μM (see under "Discussion"). Under these conditions, even considering the micromolar K_D value, the enzyme should be almost fully complexed with DARPin.

The enzymatic hydrolysis of our *in vitro* substrate (*i.e.* Ac-TENLYFQ-amc peptide) was found to be very slow ($K_m = 60$ μM; $k_{\text{cat}} = 1.54 \times 10^{-5}$ s^{-1}), but clearly distinguishable from samples containing no proteinase (background). For our *in vitro* experiments we used a ratio of DARPin to NIa$^{\text{pro}}$ to peptide of 100:1:5 (300:3:15 μM), which should mimic the *in vivo* situation quite closely.

For all DARPins, which were analyzed in more detail (9_1s, 13_1b, and 20_2b) and which show NIa$^{\text{pro}}$ inhibition *in vivo*, enzyme inhibition was detected, whereas the control protein (E2_5) showed no significant influence on NIa$^{\text{pro}}$ activity (Fig. 9). Under the assay conditions chosen, the inhibition was not complete for this synthetic substrate; some residual activity, ranging from 5 to 32%, depending on the individual inhibitor, was observed (Fig. 9). The different inhibition efficiencies of the selected DARPins do not correlate with differences in K_D values under the assay conditions.

There are several reasons why the *in vitro* inhibition assay may be only partial. First, the substrate and the inhibitor may compete at least partially for the same site. As we are forced to

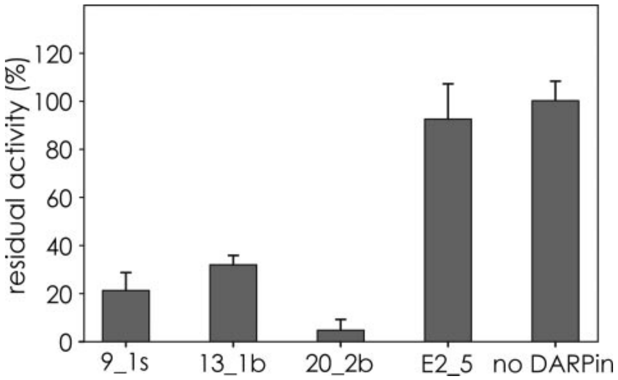

FIGURE 9. **Inhibition properties of selected DARPins *in vitro*.** The enzymatic activity of NIa^{pro} (3 μM) was determined fluorimetrically by following the fluorescence of the released leaving group after hydrolysis of a fluorogenic substrate (Ac-TENLYFQ-amc) in the presence and absence of an excess of DARPins (300 μM). The concentration of the fluorogenic substrate was 15 μM in all cases (for details, see under "Materials and Methods"). In the presence of the selected DARPin-based inhibitors, the NIa^{pro} activity was reduced to 5–32%. The control DARPin E2_5 had no significant influence on NIa^{pro} activity.

use higher substrate concentrations (to detect the reaction) *in vitro*, compared with the *in vivo* situation, the inhibitor may be partially displaced by the substrate. Conversely, the DARPins may not completely block the binding of the peptide substrate, but only decrease its affinity, as the DARPin-binding site may be adjacent and thus have a more dramatic effect only on (longer) protein substrates. Because of the extremely slow turnover of the peptide substrate with NIa^{pro}, it would be very difficult to conduct a full kinetic analysis of the inhibition mode (17). Nevertheless, the fact that K_D values and the extent of inhibition do not seem to correlate argues for a mixed inhibition as found previously (17).

In summary, all selected DARPins tested showed direct inhibition of NIa^{pro}, even though the inhibition was not complete under these conditions.

DISCUSSION

Targeting Site-specific Proteolytic Enzymes—Site-specific proteolysis plays an important role in the regulation of many biological processes as diverse as signal transduction, RNA transcription, cell cycle progression, apoptosis, and development (6, 7). This is reflected by the fact that about 2% of all genes encode proteolytic enzymes (10). Several distinct mechanisms exist for the control of proteinase activity, and the inhibition by proteinaceous inhibitors is one of the most frequently applied mechanisms to control proteinase activity within the living cell. Because the dysregulation of proteinases and proteinase inhibitors are the underlying reason for many diseases (50–52), it is not surprising that their interactions belong to the most intensively studied ones, and both small molecule inhibitors (53) and engineered variants of naturally occurring proteinase inhibitors serve as a basis for drug development (54). In the latter case, most proteinaceous proteinase inhibitors engineered for specificity and affinity have been derived from disulfide-containing scaffolds and been predominantly used for extracellular applications (for earlier work see Ref. 9).

Despite the great variety of natural proteinaceous inhibitors of proteolytic enzymes, they show some convergence of design and/or mechanism. The key challenge is how a protein can avoid being a substrate and instead become an inhibitor. A tight binding Michaelis complex, a tight binding product complex, or even a stable acyl-enzyme intermediate may be formed as a result of an inhibitor loop entering the active site of the proteinase (55). This tight binding efficiently prevents turnover. Alternatively, a loop binding nonproductively in the opposite orientation or a steric blockage by the whole inhibitor protein, without a peptide necessarily occupying the active site, are additional modes of inhibition (55).

Our DARPins are not proteinase inhibitors *per se*, and the input DARPin library of our experiments is completely unbiased for function. Nevertheless, we were able to selected specific NIa^{pro}-inhibiting DARPins. DARPins also lack pronounced extended flexible loops, which could reach into the active site of the proteinase, and thus it is more likely that inhibition is realized by steric blockage of the active site access or, alternatively, by an allosteric effect that arrests the proteinase in a nonproductive conformation, comparable with the mechanisms found for the inhibition of a prokaryotic kinase by a DARPin (24). The exact mechanism of action can only be elucidated from the crystal structure of the complex.

It should be mentioned that the identification of inhibiting DARPins within the obtained pools of NIa^{pro} binders was a rare event, because only $\sim$0.5% of all (by ribosome display) preselected binders screened gave rise to a blue phenotype. Nevertheless, the results presented here show that the fundamental structural requirements of proteinase inhibitors can be fulfilled by repeat proteins, whose versatility is thus further underlined. Although, to the best of our knowledge, no repeat protein has yet been ascertained as a natural proteinase inhibitor, the binding partners of most of the many members of the repeat protein families have not yet been identified. Thus, our results would make it not surprising if a natural repeat protein was found to act as a proteinase inhibitor. Their large, rigid interaction surface could clearly block an active site by avoiding all direct contacts with the catalytic center or even by an allosteric effect.

At first sight it may be surprising that the relatively low affinities of the DARPins identified as inhibitors could be sufficient for mediating such a profound *in vivo* effect. But these relatively low affinities of the inhibitors found in the present experiment can be explained, even though DARPin-based binders with K_D values in the low nanomolar range could be routinely obtained against a wide variety of targets from our combinatorial DARPin libraries (16, 17). The estimation of the intracellular DARPin concentrations during the screening experiment, calculated based on the densitometric analysis of protein bands from SDS-polyacrylamide gels of crude cell extracts, revealed an intracellular concentration between 800 and 900 μM for the selected DARPins (assuming an average *E. coli* cell volume of $\sim 10^{-15}$ liters and 1 A_{600} corresponding to 5×10^8 cells/ml (56)). This is far above the determined K_D values. Thus, these findings might demonstrate that in intracellular selections determinants other than affinity, such as activity, solubility, and expression (see below), govern the selection process of specific protein-protein interactions. Furthermore, only a very small subset of binders is expected to also be inhibitors. Therefore, these results have no bearing on the general high affinity of selected DARPins, which are in the nanomolar to picomolar range (16).[5]

Our findings presented here are corroborated by recent findings with the protein complementation assay selection system (58). In these studies, antibody scFv fragments were selected against various targets directly from a diverse library (59). In those cases where specific intracellular activity was obtained without prior *in vitro* selection, micromolar affinities were also observed (59, 60). Stability, expression level, solubility, and the monomeric state of the scFv fragments may be the main determinants for successful selection of these specific binders in this intracellular selection system (59). Thus, *in vivo* selections may not by themselves lead to high affinity binders without additional *in vitro* affinity maturation steps.

A Genetic Screen to Isolate Inhibitors of Site-specific Proteolytic Enzymes—Although the *in vitro* selection of binding molecules against almost any target from large combinatorial libraries using techniques such as ribosome or phage display (41, 61) is rapid and usually straightforward, the subsequently necessary assay to identify any additional desired functionality must be tailored to the specific requirement. Usually, only a small subset of the still large pool of binding molecules after the selection process will mediate the desired function (*e.g.* inhibition of an enzyme).

We modified a previously reported bacterial two-hybrid system (35), in which the transcriptional activation of a reporter gene is now coupled to the inhibition of a site-specific proteolytic enzyme. Our genetic screen allows the convenient screening of a large number of clones, and it tests them directly in the intracellular environment.

Nevertheless, as pointed out above, high affinity binders do not necessarily have an advantage in this screen. If high affinity binding is a requirement for later applications, either the amount of substrate has to be increased, the amount of inhibitor has to be decreased, or an affinity maturation step has to be added to the initial *in vitro* selection procedure. Only the latter step would be feasible for NIa^{pro} because of its relatively low turnover, which in consequence makes high cellular concentrations of this enzyme necessary to enable the assay to work.

This screen can in principle be adapted to any proteinase (as the cleavage site within the fusion protein can easily be exchanged), provided that such a proteinase is not toxic for *E. coli*. Also, the system is not limited to DARPins or even proteins, because cell-permeable compounds can be tested as well.

Conclusions and Perspectives—We report here for the first time DARPin-based proteinase inhibitors and chose the example of an agriculturally important plant virus fully active in the cell. The favorable biophysical properties of DARPins, in particular their high rigidity and stability, might have enabled us to generate inhibitors structurally distant from known naturally occurring inhibitors (10). Selected DARPins showing not only specific binding, but also providing a desired functionality for a given substrate, can be used as valuable tools in target validation experiments, extra- and intracellular (17). By arresting the protein in an inactive conformation and co-crystallization of such protein-protein complexes (24), the process of drug discovery even for small molecules may be accelerated.

Apart from the general applicability of DARPins as target validation tools, in plants DARPins might open a new general means to engineer resistance against those plant viruses or parasites possessing proteinase activity as part of their natural processing or insecticidal transmission mechanism (62). The selected DARPins presented here might potentially serve as leads to engineer resistance.

In human or animal health, the general applicability of DARPins as specific intracellular enzyme inhibitors in an organism is currently still hampered by the unsolved problem of delivering DARPins to the cytosol of the target cells, which has to await future progress in DNA/RNA or protein delivery strategies. Nevertheless, because of the ease of transfection of cell lines, protein knock-outs may become an important part of the drug discovery process.

Acknowledgments—We thank Eva Prenosil for performing the initial two rounds of ribosome display against NIa^{pro} and Dr. Hans Kaspar Binz for supervision of the work and members of the Plückthun laboratory for valuable discussions. We thank Drs. Ann Hochschild, Dave Waugh, and Hermann Bujard for kindly providing E. coli strains, plasmids, and helpful technical advice. We also thank Dr. Andreas Schweizer for helpful advice in establishing the in vitro inhibition experiments and Dr. Béatrice Luginbühl for help with BIAcore experiments.

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