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

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 NlaPro 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 αC repressor, containing an NlaPro 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 NlaPro 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.
We previously reported the generation of designed ankyrin repeat proteins (DARPins) 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 NIaproteinase of tobacco etch virus (TEV). NIaproteinase 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, NIaproteinase 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 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; NIaproteinase, 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 protease activity screen. We were able to select and characterize in vivo active proteinaceous DARPin Nla<sup>pro</sup> 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 PfuTurbo 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 Nla<sup>pro</sup> protease in all in vivo experiments, pZA55-TEV, was constructed by inserting the PCR-amplified araC gene plus the P<sub>bad</sub> promoter sequence into pZA21-TEV. In turn, pZA21-TEV was constructed by inserting the PCR-amplified gene of the catalytic domain, Nla<sup>pro</sup>, into pZA21 (37), thereby replacing its KpnI/BamHI fragment. The gene of the catalytic domain Nla<sup>pro</sup> was amplified from pRK793 (38).

pBRCl-T·ω is a derivative of pBRcl-ω (35) containing a Nla<sup>pro</sup> cleavage site. pMAKcl-T·ω-pD is a derivative of pQl-pD (39) and constitutively expresses the clc-T·ω fusion protein under control of the β-lactamase promoter P<sub>bla</sub>.

pMAKcl-T·ω-DL is a derivative of pMAKcl-T·ω-pD in which the pool of DARpins enriched by ribosome display, binding Nla<sup>pro</sup>, replaces phage λ protein D (gpD). Expression of the DARPin pool is under control of the IPTG-inducible promoter P<sub>ts</sub>/lac. It was used in all in vivo screening experiments. The open reading frames of the ribosome display-selected DARpins were digested with Ncol and HindIII and ligated into pMAKcl-T·ω-pD, yielding the selection plasmid ready for in vivo screening.

The pMAKcl-T·ω-DL tag is a derivative of pMAKcl-T·ω-DL and was used for the DARpin sequencing experiments. It was digested with Ncol and HindIII and ligated into pMAKcl-T·ω-pD, thereby replacing phage λ protein D (gpD) and generating pMAKcl-T·ω-DL, co-introduced with pZA55-TEV into E. coli K12ΔZ (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. pMAKcl-T·ω-DL clones were isolated from different blue colonies and re-introduced together with pZA55-TEV into fresh E. coli K12ΔZ 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<sub>150</sub> (50 mm Tris-HCl, pH 7.4, 150 mm NaCl) was used as running buffer.

**ELISA—** The biotinylated antigens (pD or pD-Nla<sup>pro</sup>) were immobilized as follows: neutravidin (66 nm, 100 μl/well; Pierce) in TBS<sub>150</sub> 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<sub>150</sub> for 1 h at room temperature. Biotinylated antigen (100 μl; 1 μM) in TBS<sub>150</sub> 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 Nla<sup>pro</sup>, 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<sub>150</sub>, the wells were dried and blocked with 300 μl of 0.5% bovine serum albumin (Fluka, Buchs, Switzerland) in TBS<sub>150</sub>. After extensive washing with TBS<sub>150</sub> and drying, the plate was analyzed on an ELISA reader (Bio-Tek, Winooski, VT) at 405 nm.

**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-Nla<sup>pro</sup> immobilized. The interactions were measured at a flow of 50 µl/min with a 5-min buffer flow, a 5-min injection of Nla<sup>pro</sup> binding DARPin 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 using the steady state model 

Western blot analysis—Prior to Western blot analysis, antigens were separated by size on an SDS-PAGE gel. The proteins were transferred to a nylon membrane (BA 85, Schleicher & Schuell). The membranes were probed with an anti-DARPins antibody (Abcam, Cambridge, MA) and an anti-RNAP antibody (Cell Signaling, Beverly, MA). Bands were detected using an enhanced chemiluminescence kit (Pierce). The relative expression levels were quantified using a densitometer (ImageQuant, Molecular Dynamics, Sunnyvale, CA).

In Vitro Inhibition Study—Nla<sup>pro</sup> activity assays were performed essentially according to published procedures (44) with small modifications. Briefly, Nla<sup>pro</sup> (3 µM) and selected DARPin libraries (15) were preincubated in Nla<sup>pro</sup> reaction buffer (50 mM Tris-Cl, 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<sub>final</sub> 100 µl; [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 DARPin or E2_5 were normalized to initial velocity data obtained from measurements without DARPin; the value obtained here was arbitrarily set to 100%. All experiments were at least done in triplicate.

RESULTS

To obtain Nla<sup>pro</sup> 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 DARPin libraries (15) able to bind Nla<sup>pro</sup>, followed by an in vivo screening step to identify those DARPin libraries which not only bind but also inhibit the proteasome 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 cI35).

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 protease cleavage site into this linker would allow its cleavage upon co-expression of a corresponding protease. 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. This strain is defective in the ρ<sup>+</sup> gene, which encodes the ω subunit of RNA polymerase. The ω operator O<sub>2</sub>(depicted as a boxed sequence) 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 ω-binding site is positioned too far away for ω to activate transcription from O<sub>2</sub> to ω fusion protein because of inhibition of Nla<sub>pro</sub> by a co-expressed DARPin. A, binding of the lac part of a lac-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 λ-T-ω fusion protein fails to occupy the λ operator site). B, disruption of transcriptional activation because of proteolytic processing of the Lac-T-ω fusion protein by Nla<sup>pro</sup>. C, restoration of transcriptional activity by λ-T-ω fusion protein because of inhibition of Nla<sup>pro</sup> by a co-expressed DARPin. D, schematic representation of the artificial promoter derivative placO<sub>2</sub>-62 present on the chromosome of E. coli KS1ΔZ. This strain is defective in the ρ<sup>+</sup> gene, which encodes the ω subunit of RNA polymerase. The λ operator O<sub>2</sub>(depicted as a boxed sequence) 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 λ-binding site is positioned too far away for λ to activate transcription from O<sub>2</sub> to ω 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 λ-T-ω fusion protein is very weak.
Ankyrin Repeat Protein Libraries as Source for Inhibitors

### TABLE 1

| Plasmid | Relevant details | Source/Ref. |
|---------|-----------------|-------------|
| pBRc1-ω | ApR, ColE1, encodes Actl-wt (residues 1–236): 2αla, act1 (residues 1–90) | This work |
| pBRc1-ω | ApR, ColE1, encodes Actl-wt (residues 1–236): linker (Nlaω recognition site plus His6 tag) (residues 1–90) | This work |
| pMACK1-ω-pD | ApR, ColE1, encodes Actl-wt (residues 1–236): linker (Nlaω recognition site plus His6 tag) (residues 1–90) | This work |
| pMACK1-ω-DL | ApR, ColE1, encodes Actl-wt (residues 1–236): linker (Nlaω recognition site plus His6 tag) (residues 1–90) | This work |
| pQl+pD | ApR, ColE1, RGS-His tag: pD under control of Ptac | This work |
| pZAS5-TEV | TcR, p15A, encodes Nlaω protease catalytic domain (S219N) under control of Pbad | This work |
| pAT223-TEV | ApR, ColE1, encodes Nlaω protease catalytic domain (S219N) as C-terminal fusion of gpD under control of Ptac | This work |
| pAT223 | ApR, ColE1, encodes gpD under control of Ptac | This work |
| pRK793 | ApR, ColE1, encodes Nlaω protease catalytic domain (S219N) as C-terminal fusion of maltose-binding protein under control of Pbad | This work |
| pBirAcm | CmR, p15A used for in vivo biotinylation; contains birA | Avidity, Denver, CO |
| pMACK1-ω-DL tag | pMACK1-ω-DL analogue, DARPin library member devoid of its N-terminal RGS-His6 tag | This work |
| pQE60 | ApR, ColE1, Pbad-controlled expression plasmid | Qiagen, Hilden, Germany |
| pBADGFPAC2 | ApR, ColE1, encodes GFP under control of Pbad | This work |
| pZA21 | KanR, p15A, P15A-controlled expression plasmid | This work |
| pTRG | TcR, ColE1; “dummy” plasmid to confer tetracycline resistance to cells during screening experiments (control) | This work |

* All experiments were performed with a mutant form (S219V) of the C-terminal proteolytic domain of the full-length Nlaω protein of TEV. Thus, the protease used here lacks its N-terminal VFG domain and is resistant to autoactivation by truncation of its C-terminal tail.

...tein was placed downstream of an inducible promoter on a plasmid vector, generating plasmid pBRc1-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 placoR2–62) 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 Actl alone) but can be stimulated more than ~70-fold in the presence of Actl-ω fusion protein (35, 45), which results in blue colonies when grown on plates containing X-gal (Fig. 3A, sector 5).

We tested this screen with Nlaω, which displays a high specificity for its 7-aminoc acid recognition sequence (EXXYXQ↓ (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 Actl-ω fusion protein, a 25-amino acid coding region, including the 7-amino acid recognition sequence (ENLYFQQ↓S) flanked by a His6 tag and a glycine-serine linker (see vector map in the Supplemental Material), was introduced into the linker region of the Actl-ω fusion protein, thus generating the Actl-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. 3A, sector 4). Thus, *E. coli* proteinases do not cleave this Actl-ω fusion protein to the extent of abolishing activation.

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

These results show that the transcriptional activation properties of the Actl-ω or Actl-ω fusion protein are dependent on the integrity of the fusion proteins and that the Actl-ω fusion protein is thus suited to phenotypically monitor protease activity in the cytosol of *E. coli*. The same results were obtained when the Actl-ω fusion protein was constitutively expressed under the control of the β-lactamase promoter (Fig. 3B, pMAK vector series) instead of under the control of the IPTG-inducible PlacUV5 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 protease inhibition in vivo (34).
Ankyrin Repeat Protein Libraries as Source for Inhibitors

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.

![Diagram](Image)

**FIGURE 3. Phenotypic monitoring of Nla<sup>pro</sup> 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 pBRcl-T-ω (sector 2), pZA55-TEV and pBRcl-ω (sector 3), pBRcl-T-ω (sector 4), and pBRcl-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 Nla<sup>pro</sup>. Positive transcriptional activation of β-galactosidase is visualized by blue colony formation (see text for details). B. *E. coli* KS1ΔZ was transfected with pMAKcl-T-ω (sectors 1–4) and pMAKcl-T-ω plus pZA55-TEV (sectors 5–8). Positive transcriptional activation is visualized by blue colony formation (see text for details). C. *E. coli* KS1ΔZ was transfected with pMAKcl-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.
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 always slightly higher than that expected for a globular protein of the same size (Table 2). The

TABLE 2
Molecular masses of three selected DARPins

| Protein | \(M_r\text{calc}^a\) | \(M_r\text{obs}^b\) | \(M_r\text{obs}^c\) | \(M_r\text{obs}^d\) |
|---------|--------------------|--------------------|--------------------|--------------------|
|         | 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               |

- Values are as calculated from the sequence.
- Values are as determined by MALDI mass spectrometry.
- Values are as determined by gel filtration, using globular proteins as molecular weight standards.
- Values are as determined by multiangle light scattering.

Note that these proteins do not have a double His6 tag preceding the DARPin open reading frame.

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). For the full consensus ankyrins, the apparent molecular size calculated from the elution volume was always slightly higher than that expected for a globular protein of the same size (Table 2). The

5 S. Wetzel, unpublished results.
Ankyrin Repeat Protein Libraries as Source for Inhibitors

In Vitro Target Binding and Binding Constants of the Inhibitory DARPin—The binding specifications of the selected DARPin were analyzed by ELISA. For a smaller subset of DARPin, the binding affinity was further determined by surface plasmon resonance (SPR) experiments. In an ELISA experiment with the 10 purified inhibitors, the binding to Nla<sup>prot</sup> 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 DARPin bound their cognate target and did not bind to pD. The binding signal to immobilized Nla<sup>prot</sup> could be suppressed by preincubation with free Nla<sup>prot</sup>, 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 Nla<sup>prot</sup>, indicating that the designed DARPin scaffolds per se do not bind Nla<sup>prot</sup> (Fig. 6). It should be noted that the ELISA signal of some selected DARPin 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. 6B). 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 DARPin are washed off before detection with an antibody.

To measure the affinity of the interaction of the in vivo selected DARPin specific for Nla<sup>prot</sup>, equilibrium SPR experiments were performed with the DARPin 9_1s, 13_1b, and 20_2b. The <i>K</i><sub>D</sub> of all three Nla<sup>prot</sup> 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).

Finally, we would like to stress that the DARPin 9_1s, 13_1b, and 20_2b did not bind to the immobilized transcriptional activator λcl-T-ω fusion protein, as tested by ELISA (data not shown). Protection of the Nla<sup>prot</sup> cleavage site within the fusion protein, as tested by ELISA (data not shown), suggests that these DARPin might, in principle, result in the same phenotype. However, because we found no binding of the selected DARPin to the λcl-T-ω fusion protein, we can rule out the latter mechanism for transcriptional activation.

**TABLE 3**

| Target     | Clone name (type) | <i>K</i><sub>D</sub> (μM) |
|------------|-------------------|--------------------------|
| Nla<sup>prot</sup> | 9_1s (N3C) | 11 ± 4                 |
| Nla<sup>prot</sup> | 13_1b (N2C) | 10 ± 4                 |
| Nla<sup>prot</sup> | 20_2b (N2C) | 9 ± 4                  |

In Situ Inhibition by Selected DARPin—To confirm the direct inhibition of Nla<sup>prot</sup>, we conducted co-expression tests of protease and DARPin in the presence of the λcl-T-ω fusion protein in <i>E. coli</i> KS1AZ 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<sub>6</sub> tag of the λcl-T-ω fusion protein, which is present in its linker sequence (Fig. 8A); and a second one was directed against the cl...
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FIGURE 8. Selected DARPin inhibit the cleavage of \( \lambda c I-T-\omega \) by Ni\(^{aa}\) pro. A, a schematic drawing of the \( \lambda c I-T-\omega \) fusion protein to scale. Open reading frames of the \( \lambda c I \) protein and of the RNAP-\( \omega \) subunit are indicated with gray arrows. The Ni\(^{aa}\) 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 \( \lambda c I-T-\omega \) fusion protein in the presence of the selected DARPin 9_1s, 13_1b, and 20_2b and Ni\(^{aa}\) pro. The \( \lambda c I-T-\omega \) fusion protein was monitored by detection of its His\(_6\) tag with an \( \alpha\)-tetra-His-Ab following 15% SDS-PAGE and Western blotting, pMakcI-T-\( \omega \)-DL containing, in each case, DARPin 9_1s, 13_1b, or 20_2b was introduced alone or together with pZA55-TEV into E. coli KSI1AZ. DARPin expression was induced with 20 \( \mu\)M IPTG 30 min before induction of Ni\(^{aa}\) 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 \( \lambda c I-T-\omega \) fusion protein was monitored by detection of its \( cI \) part with an \( \alpha-cI\)-Ab, following 15% SDS-PAGE and Western blotting. D, Western blot analysis of the expression profile of the \( \lambda c I-T-\omega \) fusion protein in the absence of the selected DARPin and in the absence of Ni\(^{aa}\) pro; \( \lambda c I-T-\omega \) fusion protein was monitored as in C.

For these experiments, plasmid vectors coding for the selected DARPin 9_1s, 13_1b, and 20_2b were co-introduced with pZA55-TEV into E. coli KSI1AZ. Cells were grown near 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 Ni\(^{aa}\) 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 \( \lambda c I-T-\omega \) fusion protein was monitored by Western blot analysis with an anti-tetra-His-Ab or anti-cI-Ab, respectively. Only the selected DARPin can prevent the digestion of the \( \lambda c I-T-\omega \) fusion protein by Ni\(^{aa}\) pro in vivo (Fig. 8). By contrast, the unselected library DARPin E2_5 (22) was unable to inhibit the digestion of the \( \lambda c I-T-\omega \) fusion protein by Ni\(^{aa}\) pro (Fig. 8, B and C). Using the anti-cI-Ab for detection of the \( \lambda c I-T-\omega \) fusion protein, we also observed an unspecific degradation of the \( \lambda c I-T-\omega \) fusion protein by endogenous E. coli proteinases (Fig. 8C), which also occurs in the absence of any DARPin and in the absence of Ni\(^{aa}\) pro (Fig. 8D). As expected, this cleavage by E. coli proteinases cannot be inhibited by the expression of our selected DARPins. Nevertheless, enough \( \lambda c I-T-\omega \) 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 \( \text{in vivo} \) effects observed for the selected DARPin were indeed due to direct inhibition of Ni\(^{aa}\) pro, \( \text{in vitro} \) 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 \( \text{in vivo} \) situation. \( \text{In vivo} \), the DARPin concentration is much higher than the concentration of Ni\(^{aa}\) pro and the substrate, i.e. the \( \lambda c I-T-\omega \) fusion protein. As estimated from SDS-PAGE analysis (see above) the ratio of DARPin to Ni\(^{aa}\) pro to \( \lambda c I-T-\omega \) fusion protein (i.e. the \( \text{in vivo} \) substrate) is 800:8:1 (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 \( \text{in vitro} \) substrate (i.e. Ac-TENLYFQ-amc peptide) was found to be very slow (\( K_{cat} = 60 \mu\text{M} \cdot \text{s}^{-1} \)), but clearly distinguishable from samples containing no proteinase (background). For our \( \text{in vitro} \) experiments we used a ratio of DARPin to Ni\(^{aa}\) pro to peptide of 100:1:5 (300:3:15 \( \mu\text{M} \)), which should mimic the \( \text{in vivo} \) situation quite closely.

For all DARPin, which were analyzed in more detail (9_1s, 13_1b, and 20_2b) and which show Ni\(^{aa}\) pro inhibition \( \text{in vivo} \), enzyme inhibition was detected, whereas the control protein (E2_5) showed no significant influence on Ni\(^{aa}\) 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 \( \text{in vitro} \) 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
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FIGURE 9. Inhibition properties of selected DARPins in vitro. The enzymatic activity of Nla\textsuperscript{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 Nla\textsuperscript{pro} activity was reduced to 5–32%. The control DARPin E\textsubscript{2.5} had no significant influence on Nla\textsuperscript{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 Nla\textsuperscript{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 argue for a mixed inhibition as found previously (17).

In summary, all selected DARPins tested showed direct inhibition of Nla\textsuperscript{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 protease activity, and the inhibition by proteinaceous inhibitors is one of the most frequently applied mechanisms to control protease 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 select specific Nla\textsuperscript{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 Nla\textsuperscript{pro} binders was a rare event, because only ~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 DARPin pools (assuming an average E. coli cell volume of $\sim 10^{-15}$ liters and $1 A_{600}$ corresponding to $5 \times 10^8$ 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, or an affinity maturation step has to be added to the initial in vitro selection procedure. Only the latter step would be feasible for Nla55 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 DARPin sequences 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 DARPin 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 DARPin sequences 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 DARPin 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 Nla55 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 Lugimbihü for help with BIAcore experiments.

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