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Research Article

Peptide aptamers: exchange of the thioredoxin-A scaffold by alternative platform proteins and its influence on target protein binding

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Abstract. Peptide aptamers have emerged as powerful new tools for molecular medicine. They can specifically bind to and functionally inactivate a given target molecule under intracellular conditions. Typically, peptide aptamers are generated by screening a randomized peptide expression library, displayed from the *Escherichia coli* thioredoxin A (TrxA) protein. Here, we transferred peptide moieties from defined TrxA-based peptide aptamers to alternative scaffold proteins, such as the green fluorescent protein and staphylococcal nuclease. Yeast and mammalian two-hybrid assays as well as in vitro binding analyses show that the TrxA scaffold can be a major determinant for the binding of peptide aptamers. In addition, we demonstrate that TrxA can correctly display peptide sequences that correspond to the binding domains of natural interaction partners. Therefore, sequence analyses of TrxA-based peptide aptamers, isolated by two-hybrid screening from randomized expression libraries, should also be useful to find cellular binding partners for a given target protein, by homology.

Key words. Peptide aptamer; human papillomavirus; hepatitis B virus; protein scaffold; protein therapy.

Peptide aptamers are a novel class of molecules in which conformationally constrained peptides of variable sequence are displayed from a protein scaffold [reviewed in refs 1–3]. They are isolated from randomized peptide expression libraries in yeast, by virtue of their ability to bind specifically to a given target protein, under intracellular conditions. Importantly, these molecules can inhibit the function of their target protein at the intracellular level, with high specificity [4–11]. Therefore, peptide aptamers represent novel tools for functional studies of proteins within living cells. In addition, peptide aptamers possess important therapeutic potential for various aspects of molecular medicine, because they can be utilized to validate proteins as therapeutic targets, should be useful to provide structures for drug design, and may be developed into protein drugs [1–3].

In currently available peptide aptamer expression libraries, the *Escherichia coli* thioredoxin A protein (TrxA) has been commonly used as a scaffold for the display of the variable peptide moiety [4–12]. In this setting, peptides of randomized sequence are introduced into the active-site loop of TrxA, thereby destroying its enzymatic activity and exposing the peptide moiety to the outside of the molecule [4]. The constrained conformation of the peptides has several theoretical advantages for interaction screening: (i) constrained peptides (CPs) can expose amino acid residues that are commonly buried inside flexible peptides, such as hydrophobic amino acid residues at intracellular conditions, (ii) CPs often have substantially higher binding affinities than corresponding flexible peptides, (iii) CPs are often more stable than un-

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structured peptides under biological conditions, and (iv) the preferred conformation of CPs should be helpful in determining their binding structure, as a prerequisite for drug design [13].

Little is known about the molecular determinants for the interaction between peptide aptamers and their target proteins, such as the contribution of the TrxA scaffold. Here, we investigated whether the peptide moieties from defined TrxA-based peptide aptamers can bind to their targets when displayed from alternative platform proteins, such as a catalytically inactive derivative of the staphylococcal nuclease [14] or the green fluorescent protein [15]. In addition, compiled data from the literature suggested that TrxA-based peptide aptamers may not be suitable to display correctly binding peptides with significant sequence homology to natural interaction partners, in two-hybrid assays [3]. To investigate this issue, we tested the 18-amino-acid (aa) E6-binding domain of the E6-associated protein (E6AP) [16, 17] in the context of different scaffold proteins.

Materials and methods

Yeast two-hybrid assays

Reporter strain KF1 (*MAT**a** trp1-901 leu2-3,112 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ SPAL10-URA3*) contains three selectable marker genes under the transcriptional control of GAL4-binding sites: *GAL2-ADE2*, *GAL1-HIS3*, and *SPO13-URA3* [10]. The URA3 marker in KF1 contains a negative regulatory element in its promoter and is activated only by strong protein-protein interactions [10, 18]. Synthetic oligonucleotides, encoding the peptide moieties E61-1 (GALVHKLFSQTSGSCLVCIS), C1-1 (SFYSVLFLWGTCGGFSHSWY), and E6APp (IPESSELTLQELLGEERR), were introduced into the unique *Rsr*II site within TrxA [10], or into *Bgl*II/*Xho*I restriction sites created by PCR-mediated mutagenesis between green fluorescent protein (GFP) aa positions 157/158 and staphylococcal nuncleage (SNase) aa 19/27 (derived from pTCN23 [14]), respectively. The correct sequences of all constructs were verified by DNA sequencing. pAD-Trx-, pAD-GFP-, and pAD-SNase-based constructs use vector backbone pRS424 [19] and express individual peptide aptamers in fusion with GAL4AD. Control peptide aptamer TrxA-CoPep was arbitrarily chosen from the library [10]. In vectors pAD-E61-1, pAD-C1-1, and pAD-E6APp, the peptide moieties were directly fused to GAL4AD. Human papillomavirus-16 (HPV) E6 or hepatitis B virus (HBV) core coding sequences were expressed in frame with the GAL4BD from pPC97 [20]. Following transformation of KF1 cells by the indicated combinations of GAL4BD and GAL4AD vectors, yeast transformants were analyzed by replica plating for activation of the selectable growth markers, as described elsewhere [10].

Western blot analyses

Yeast protein extracts were prepared from pelleted overnight cultures, after resuspension in lysis buffer (100 mM Tris-HCl, 20% glycerol, 1 mM EDTA, 0.1% TritonX-100, 5 mM $MgCl_2$), supplemented with protease inhibitors, and vortexing in the presence of glass beads. Proteins (30 µg/lane) were separated by SDS-PAGE and transferred to a PVDF membrane (Immobilon-P; Millipore, Bedford, Mass.). Peptide aptamers were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia, Freiburg, Germany), using rabbit polyclonal anti-GFP antibody GFP(FL) (Santa Cruz, Heidelberg, Germany) or monoclonal anti-HA-Tag antibody HA-(F7) (Santa Cruz), directed against an HA-Tag linked to SNase-based peptide aptamers.

Mammalian two-hybrid assays

Mammalian two-hybrid analyses were performed in HeLa cells using the 'Checkmate' (Promega, Mannheim, Germany) system [12]. E6 deletion mutant E6(1-84) or the complete HBV core protein were expressed as GAL4BD fusion proteins from pBIND. Individual peptide aptamers were expressed in fusion with the HSV-1 VP16AD from pACT. Reporter plasmid pG5luc contains the *Photinus pyralis* luciferase gene under transcriptional control of GAL4-binding sites. Cells were harvested 48 h after transfection, and luciferase activities were measured as described previously [12].

In vitro binding assays

Peptide aptamer sequences from pAD-Trx, pAD-GFP, and pAD-SNase were subcloned, in frame with glutathione S-transferase (GST), into pGEX4T3 or pGEX4T1 (Amersham Pharmacia). The GST/peptide-aptamer fusion proteins were purified from *E. coli* and immobilized on glutathione Sepharose beads. In vitro-translated (Wheat Germ Extract System; Promega), ^{35}S-radiolabeled HPV16 E6 protein was added, washed and boiled in SDS-containing loading buffer. Bound E6 protein was detected by SDS/PAGE and subsequent fluorography.

Results

Yeast two-hybrid analyses of peptide aptamers

TrxA-based peptide aptamers TrxA-E61-1 and TrxA-C1-1 specifically bind to the HPV16 E6 oncoprotein and the HBV core protein, respectively [10, 12]. A third peptide aptamer, TrxA-E6APp, was generated; it displays the 18-aa interaction domain of the cellular HPV16 E6 interaction partner E6-AP [17] from the TrxA scaffold (see Materials and methods for peptide sequences). Individual

peptide aptamers were expressed in fusion to the GAL4 transcriptional activation domain (GAL4AD), together with the complete HPV16 E6 protein, linked to the GAL4 DNA-binding domain (GAL4BD).

As expected, TrxA-E61-1 bound to the HPV16 E6 protein, leading to the activation of all three selectable markers in yeast reporter strain KF1, whereas no interaction was detected using the HBV core-binding peptide aptamer TrxA-C1-1 (fig. 1A). Interestingly, TrxA-E6APp also clearly activated the *ADE2* and *HIS3* marker, indicating that TrxA aptamers can also display natural interaction domains in a binding active form, in two-hybrid analyses. Moreover, TrxA-E6APp showed the same binding specificity as the wild-type, full-length E6-AP protein [16, 17], being competent to bind to the HPV16 and HPV18 E6 proteins, but not to HPV6 or HPV11 E6 (fig. 1B).

Next, the TrxA scaffold of the peptide aptamers was substituted by other platform proteins. The different peptide moieties were introduced into GFP between amino acid positions Glu157 and Lys158, a region that has been demonstrated to be particularly suitable for the display of CPs [15], leading to peptide aptamers GFP-E61-1, GFP-E6APp, and GFP-C1-1. In addition, the peptide moieties were introduced into the surface loop of a catalytically inactive derivative of the staphylococcal nuclease (SNase) [14], yielding aptamers SNase-E61-1, SNase-E6APp, and SNase-C1-1. As shown in figure 1A, peptide moiety E61-1 lost its ability to bind to E6 when displayed from the GFP or SNase scaffold. In contrast, peptide moiety E6APp retained the ability to bind to E6, in both the GFP and SNase context.

Corresponding two-hybrid analyses using the HBV core protein as target showed that peptide moiety C1-1 bound only when displayed from the TrxA scaffold, but not after replacement of the TrxA scaffold by GFP or SNase (fig. 1C).

Since negative yeast two-hybrid data can be due to protein instability or decreased protein expression, we performed Western blot experiments. Both the group of GFP- and SNase-based peptide aptamers exhibited comparable steady-state protein levels in yeast (fig. 1D), yet only E6APp-containing aptamers retained the ability to bind to their target within these alternative scaffold proteins.

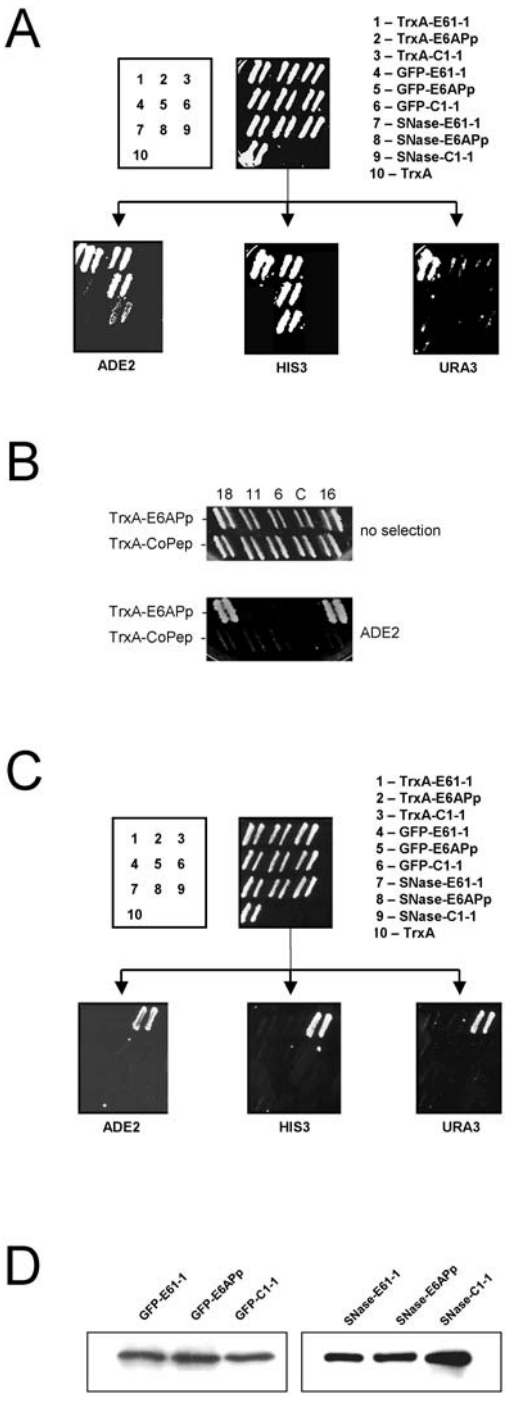

Figure 1. Yeast two-hybrid analyses of peptide moieties E61-1, E6APp, and C1-1, displayed from different scaffold proteins. (*A*) Binding to HPV16 E6. Upper plate: masterplate (no selection). Lower plates: Replica platings on adenine-, histidine-, or uracil-free growth media. TrxA, pAD-TrxA without peptide insert (negative control). (*B*) Binding specificity of TrxA-E6APp. Yeast two-hybrid assays with the E6 proteins from HPV18 (18), HPV11 (11), HPV6 (6), and the HBV core protein (C). TrxA-CoPep, negative control. Above: master plate (no selection). Below: growth on adenine-free medium. (*C*) Binding of peptide aptamers to the HBV core protein. (*D*) Western blot analyses of yeast cells expressing GFP- and SNase-based peptide aptamers.

In Vitro binding analyses

To obtain additional biochemical evidence for the differential binding behavior of the peptide aptamers, independent from two-hybrid analyses, we tested their binding to the HPV16 E6 protein by GST-pull-down assays in vitro. In agreement with the yeast two-hybrid data, we observed that the E61-1 peptide moiety confers binding only when displayed from the TrxA scaffold, but not when expressed from a GFP or SNase platform (fig. 2). In contrast, peptide moiety E6APp was competent to bind to its target protein in the context of all three scaffold proteins tested.

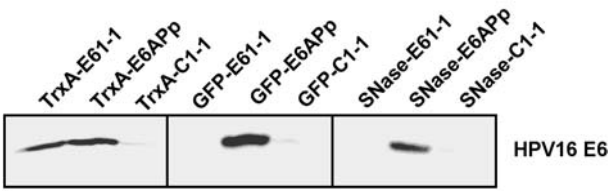

Figure 2. In vitro binding of peptide moieties E61-1, E6APp, and C1-1, displayed from different scaffold proteins, to in vitro-translated and S^{35}-labeled HPV16 E6 protein. Peptide aptamers were expressed as GST-fusion proteins.

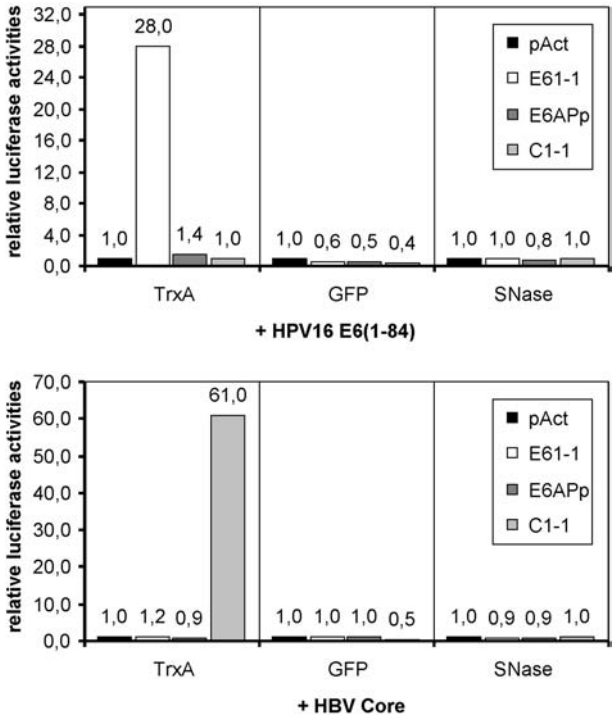

Figure 3. Mammalian two-hybrid analyses of peptide aptamers, displaying peptide moieties E61-1, E6APp, and C1-1 from TrxA, GFP, or SNase. Above: interaction with HPV16 E6 deletion mutant E6(1-84). Below: interaction with HBV core protein. Values represent relative luciferase activities above pAct (AD-domain vector devoid of peptide aptamer sequences, arbitrarily set at 1.0).

Mammalian two-hybrid analyses

In view of the biomedical potential of peptide aptamers [1–3], they must also be able to bind to their targets in mammalian cells. To investigate this issue, we employed a mammalian version of the yeast two-hybrid system, in which the two putative interaction partners are expressed in fusion with GAL4BD and with the transcriptional activation domain of the herpes simplex virus-1 VP16 protein (VP16AD), respectively. In this scenario, activation of a cotransfected luciferase reporter construct, under the transcriptional control of GAL4 recognition sites, depends on the interaction of the GAL4BD- and VP16-fusion proteins.

The 151-aa full-length HPV16 E6 protein exhibits significant intrinsic transactivating activity in human cells when linked to a heterologous DNA-binding domain [21; B. Klevenz, K. Butz and F. Hoppe-Seyler, unpublished data]. Since this can interfere with the interpretation of the two-hybrid assay, we removed the C-terminal 67 aa of HPV16 E6. This deletion mutant E6(1-84) has a strongly reduced intrinsic transactivation potential but is still bound by TrxA-E61-1, in yeast two-hybrid analyses (data not shown). Binding of the E61-1 peptide moiety to E6(1-84) was also detected in mammalian cells when displayed from the TrxA scaffold (fig. 3A), but was undetectable following replacement of TrxA by GFP or SNase. None of the E6APp-containing aptamers detectably interacted with the E6(1-84) deletion mutant in yeast (not shown) and mammalian two-hybrid assays (fig. 3A), indicating that the C-terminal 67 aa of HPV16 E6 are required for binding of the E6APp peptide moiety. In line with the data from yeast two-hybrid analyses, binding of peptide moiety C1-1 to the HBV core protein was observed in mammalian cells only when displayed from the TrxA scaffold, but was undetectable within the context of either the GFP or SNase platform (fig. 3B).

Binding of peptide moieties in the absence of scaffold proteins

To investigate the binding properties of the peptide moieties in the absence of their respective protein scaffolds, peptide moieties E61-1, E6APp, and C1-1 were directly fused to GAL4AD and analyzed by yeast two-hybrid studies. As shown in figure 4, peptide moiety E6APp was able to bind to the HPV16 E6 protein, leading to the activation of both the *ADE2* and *HIS3* marker in yeast test strain KF1. In contrast, neither E61-1 nor C1-1 were able to detectably interact with their targets, in the absence of a scaffold protein (fig. 4).

Discussion

Currently available libraries for peptide aptamer screening commonly display randomized peptide moieties from a TrxA scaffold. To investigate the contribution of the TrxA scaffold to the binding activity of peptide aptamers, we displayed the peptide moieties of three defined peptide aptamers from alternative platform proteins. Binding analyses were performed both in vitro and at the intracellular level, in yeast and mammalian cells. They consistently showed that two out of three peptide moieties, namely E61-1 and C1-1, lost detectable binding to their target proteins when transferred from TrxA to either GFP or SNase. Molecular explanations for this differential binding behavior include the possibility that the peptide moieties of E61-1 and C1-1 may adapt different conformations, in the

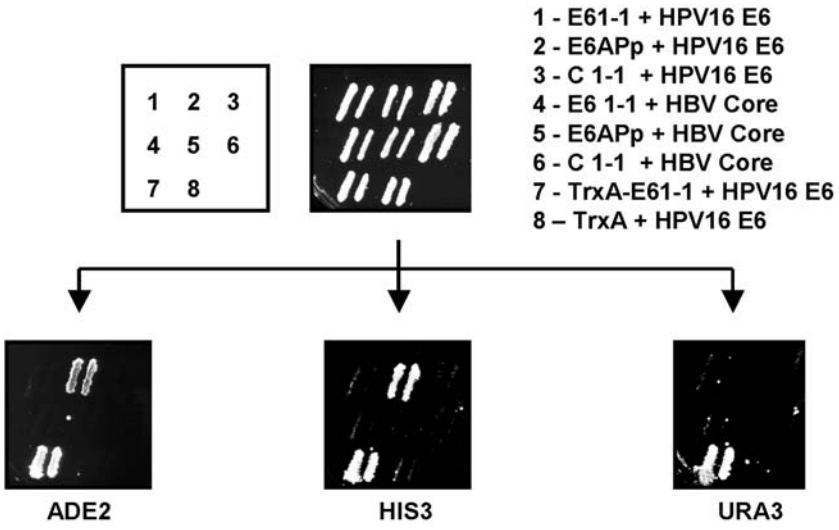

Figure 4. Binding analyses of peptide moieties E61-1, E6APp, and C1-1, devoid of scaffold proteins, to the HPV16 E6 and HBV core proteins. TrxA-E61-1 + HPV16 E6, positive control; TrxA + HPV16 E6, negative control.

context of different scaffolds. This could be theoretically due, for example, to differences in the distances between the constraining points of the different scaffold loops, which may not allow peptides to adopt the same conformation. Nevertheless, the 18-aa peptide moiety E6APp bound to E6 in the context of all three scaffolds, indicating that all three investigated scaffolds can in principle display the same short peptide moiety in a binding-active conformation. On the other hand, the TrxA scaffold may directly contribute to the binding of some peptide aptamers to their target proteins. Whereas these possibilities should be examined in detail in further investigations, they do not interfere with the potential applications of peptide aptamers discussed in the Introduction.

Under certain experimental aspects, transfer of the peptide moieties of TrxA-based peptide aptamers to other scaffold proteins could be theoretically advantageous. For example, in some cases, GFP-based scaffolds have been reported to retain their autofluorescence, thereby allowing direct monitoring of the fate of peptide aptamers in mammalian cells [15]. However, the results of this study show that the exchange of scaffold proteins can abolish binding of the peptide aptamers, indicating that the same scaffold protein should be used for initial peptide aptamer screening in yeast and subsequent biochemical analyses in vitro and in human cells.

In contrast to E61-1 and C1-1, peptide moiety E6APp bound to its target protein in the context of all three scaffold proteins investigated. These robust binding characteristics suggest that the E6APp peptide contains a relatively high intrinsic conformational stability. In line with this interpretation, only E6APp detectably bound to its target protein when expressed as a 'linear' peptide, i.e., devoid of a supporting scaffold protein.

Thus far, the peptide moieties that mediate binding of TrxA-based aptamers to a given target exhibited no [4, 11, 12, 22, 23], or limited [6, 10], sequence homologies to known natural interaction partners. This led to the suggestion that the sequences of TrxA-based peptide aptamers, isolated by two-hybrid screening of randomized expression libraries, may not be informative for the identification of natural interaction partners [3]. However, the possibility exists that sequences more closely related to natural binding partners still reside in TrxA-based peptide aptamer expression libraries, since only a very low percentage of the theoretically enormous number of different peptides in these libraries have been investigated. We show here that the 18-aa interaction domain of the cellular E6-binding protein, E6AP, clearly bound to E6, when displayed from TrxA. Moreover, the aptamer retained the binding specificity of the wild-type E6AP protein [16, 17], since it bound to the E6 proteins of the oncogenic HPV types HPV16 and HPV18, but did not interact with the E6 proteins of HPV types which usually are not associated with cancers, such as HPV6 or HPV11. These results show that TrxA-based peptide aptamers can display binding motifs that correspond to the contact sites of natural interaction partners, in two-hybrid assays. Thus, computer-aided homology searches of Trx-A-based peptide aptamers derived from randomized expression libraries should, in principle, also be informative for identifying cellular binding partners for a given target protein.

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