Peptide Aptamer-mediated Inhibition of Target Proteins by Sequestration into Aggresomes*

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Peptide aptamers (PAs) can be employed to block the intracellular function of target proteins. Little is known about the mechanism of PA-mediated protein inhibition. Here, we generated PAs that specifically bound to the duck hepatitis B virus (HBV) core protein. Among them, PA34 strongly blocked duck HBV replication by inhibiting viral capsid formation. We found that PA34 led to a dramatic intracellular redistribution of its target protein into perinuclear inclusion bodies, which exhibit the typical characteristics of aggresomes. As a result, the core protein is efficiently removed from the viral life cycle. The corresponding findings were obtained for bioactive PAs that bind to the HBV core protein or to the human papillomavirus-16 (HPV16) E6 protein, respectively. Observation that PAs induce the specific sequestration of bound proteins into aggresomes defines a novel mechanism as to how this new class of intracellular inhibitors blocks the function of their target proteins.

Peptide aptamers (PAs) are combinatorial protein reagents that are usually selected from randomized expression libraries in yeast, by virtue of their ability to bind to a given target protein under intracellular conditions. Typically, PAs consist of a short (e.g. 20 amino acids) variable binding domain that is presented as a conformationally restrained peptide in the context of a supporting protein scaffold (1–5). It has been shown that PAs can specifically bind to and block the intracellular activities of a wide range of proteins, including cell cycle regulators, oncoproteins, transcription factors, signal transducers, membrane receptors, and structural virus proteins (6–18). PAs have thus emerged as valuable new tools to study intracellular protein function, to delineate novel components of biochemical pathways, and to discover and validate new therapeutic targets. However, little is known about the molecular mechanism of PA-mediated inhibition of their target proteins.

To determine the molecular basis of the inhibitory action of PAs, we generated PAs that bind to the duck hepatitis B virus (DHBV) core protein. We found that PAs efficiently blocked viral replication by coaggregating and translocating the DHBV core protein into cellular aggresomes. As a result, the cytoplasm was cleared from soluble core protein. This shows that PAs can act as effective antiviral agents through depriving a virus of essential factors for life cycle progression. Further analyses of bioactive PAs binding to the hepatitis B virus (HBV) core or to the human papillomavirus (HPV) E6 protein indicate that the specific translocation of target proteins into cellular aggresomes represents a general mechanism for the inhibitory action of PAs.

MATERIALS AND METHODS

Peptide Aptamers and Plasmids—Peptide aptamer screening was performed as described previously, using yeast test strain KF-1 (11). Briefly, the N-terminal portion of the DHBV core protein (amino acids 1–180), fused to the Gal4 DNA-binding domain (Gal4BD), was expressed as bait from vector pPC97 (19). As prey, a peptide library encoding 20-mers of randomized sequence within the Escherichia coli thioredoxin A (TrxA) scaffold was expressed from pADtrx (11). Following selection for growth on adenine-deficient medium (activation of GAL1-HIS3 and SPO13-LRA3 markers of KF-1). Amino acid sequences of the variable peptide loops of the PAs used in this study are shown in Table I. HBV core-binding PA C1-1 (13) and HPV16 E6-binding PA E61-1 (11) have been described in detail before; PA 16E730 binds to the HPV16 E7 protein (not shown). pEGFP16E6 expresses the full-length HPV16 E6 protein, C-terminally linked to enhanced green fluorescent protein. Yeast two-hybrid analyses for binding specificity were performed as reported previously (11).

To analyze the aptamer/core interactions in higher eukaryotic cells, the CheckMate™ mammalian two-hybrid system (Promega, Mannheim, Germany) was employed. The DHBV core (amino acids 1–180) protein was expressed as a Gal4BD fusion protein from pBIND. Individual peptide aptamers were linked to the transcriptional activation domain of the HSV-1 VP16 protein, in pACT. Reporter plasmid pG5luc contains the firefly luciferase gene under the transcriptional control of five
Target Protein Inhibition by Peptide Aptamers

TABLE 1
Peptide aptamers employed in the study and their binding behavior
+; binding in yeast two hybrid analyses; —, no detectable interaction.

| Peptide aptamer | Amino acid sequence of peptide moiety | DBV core | HBV core | HPV16 E6 | GAL4 BD |
|-----------------|--------------------------------------|----------|----------|----------|--------|
| TrxA            | FLFVHPLLGLGVCSSGVC                  |          |          |          |        |
| PA13            | IAIQNFLLTLLGIGFPPGGGPL              |          |          |          |        |
| PA20            | AGLHLPQGWSRMMAMLR                   |          |          |          |        |
| PA34            | SFYLVFLFXTCGCDFHSHW                  |          |          |          |        |
| C1-1            | PPLRAFSCFCPWNLSTKRL                  |          |          |          |        |
| C2-5            | GALVHFLFQGSSGLCVCIS                  |          |          |          |        |
| E61-1           | FQYARQFPNGRVPGPVSVTM                 |          |          |          |        |
| 16E730          |                                     |          |          |          |        |

Gal4-binding sites. Cells were harvested 24 h after transfection, and luciferase activities were measured as described (13).

Analysis of DHBV Replication, Capsid Formation, and Virion Production—Plasmid pcD16 expresses a replication-competent DHBV RNA pregenome from a full size DHBV genome (20), and pcDNA3DHBVc codes for the full-length DHBV core protein. Exponentially growing LMH cells were transfected with 0.5 µg of pcD16 or 3 µg of pcDNA3DHBVc, respectively, together with 3 µg of pRC/ctymegamovirus (Invitrogen, Groningen, The Netherlands) expressing individual peptides in the context of the TrxA scaffold. The TrxA scaffold sequence was fused 3’ to the HSV VP22 gene and 5’ to a His₆ tag. In addition, 0.5 µg of β-galactosidase construct CMV-Gal was added, serving as internal control to account for possible variations in transfection efficiency. Analysis for nucleocapsid particles was performed essentially as described (13, 21). Cells were harvested 3 days after transfection and lysed in 50 mM Tris pH 8.0, 1 mM EDTA, and 1% Nonidet P-40 for 5 min at 37 °C. The lysate was centrifuged and, after adjustment of transfection efficiencies through the determination of β-galactosidase activities, the supernatant was analyzed on a non-denaturing 3% agarose gel (pH 7.5). Core particles were transferred onto a polyvinylidene difluoride membrane (PerkinElmer Life Sciences) and detected by enhanced chemiluminescence (ECL, Amersham Biosciences, Freiburg, Germany), using rabbit anti-DHBV-core antibody D103. Core particle-associated viral nucleic acids were released from the core particles in situ (13) and hybridized with radioactively labeled DHBV DNA (BamHI-EcoRI fragment from pcD16).

For detection of secreted DHBV virions, LMH cells were transfected on a 10-cm dish with 5 µg of pcD16, 10 µg of the respective aptamer expression vector, and 1 µg of pG5luc as internal standard. After 8 days, cell culture medium was collected and up to 2.5 ml (adjusted according to secreted alkaline phosphatase values) were loaded onto a CsCl/sucrose gradient, separated by centrifugation at 34,000 rpm for 4 h at 20 °C in an AG 650 rotor (Kendro, Hanau, Germany), 12 fractions of 200 µl each were collected from the bottom of the tube using a Beckman Fraction Recovery System (Beckman Coulter, Krefeld, Germany). Individual fractions were tested for the presence of DHBV DNA by a dot blot analysis.

Analysis of Soluble and Insoluble Protein Fractions—Extracts were prepared essentially as described by Johnston et al. (22). Briefly, for hepadnaviral core detection, Huh7 cells were transfected on a 10-cm dish with either 2 µg of DHBV vector pcD16 or 2 µg of HBV vector pd24 (13), together with 10 µg of individual PA expression plasmids and 0.5 µg of pCMV-Gal as internal control. Cells were lysed for 30 min on ice with 250 µl of radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 150 mM NaCl). Soluble and insoluble protein fractions were separated by centrifugation at 13,000 × g for 15 min. Pellets were resuspended in 50 µl of 10 mM Tris-HCl and 1% SDS for 10 min at room temperature. After the addition of 200 µl of radioimmune precipitation buffer, samples were sonicated for 20 s with a tip sonicator. For immunoblotting, cell fractions were normalized for β-galactosidase activities, separated by 12.5% SDS-PAGE, and analyzed by Western blot using rabbit anti-DHBV core antibody D103, anti-HBV core antibody H801, anti-His antibody (23), or anti-α-tubulin antibody CP06 (Calbiochem, Darmstadt, Germany).
Confocal and Electron Microscopy—For confocal microscopy, cells were grown on coverslips, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 5 min. The following primary antibodies were used for immunostaining: anti-vimentin V9 (Sigma, Taufkirchen, Germany), anti-\(/\)-tubulin GTU-88 (Sigma), anti-DHBV core antibody D103, and anti-His antibody (23). For nocodazole treatment, 10 μg/ml nocodazole (Sigma) was added to the culture medium 20 h before fixation.

For electron microscopy, cells were grown on glass coverslips and fixed for 1 h with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C. After washing in 50 mM sodium cacodylate buffer, the cells were post-fixed in 2% osmium tetroxide and, subsequently, 0.5% aqueous uranylacetate, dehydrated through ascending alcohol concentrations and propylene oxide, and embedded in Epon. Ultrathin sections of the fixed cells were cut with a Reichert-Jung microtome and examined with a Zeiss EM 10A transmission electron micro-

**FIGURE 2. PA34 induces the translocation of the DHBV core protein to large perinuclear inclusion bodies.** Shown are confocal microscopy of Huh-7 cells expressing the core protein from the complete DHBV pregenome (pcD16), together with control PA 16E730 (A–D), PA34 alone (E and F), and the DHBV core protein from pcD16, together with PA34 (G–J). K–N, formation of the perinuclear structures is prevented by nocodazole (NZ) treatment. anti-core, detection of DHBV core protein expression by anti-core antibody D103; anti-PA, detection of PA expression by anti-His antibody.
RESULTS

Isolation of Peptide Aptamers Specifically Binding to the DHBV Core Protein—To identify PAs that bind to the DHBV core protein, a yeast two-hybrid interaction screen was performed, using the N-terminal 180 amino acids of DHBV core as bait. As prey, a PA expression library with high complexity was employed in which variable 20-amino acid peptide domains were expressed in the context of the E. coli TrxA protein scaffold (11, 13). Screening was performed using yeast strain KF-1, which contains three selectable growth markers: GAL2-ADE2, GAL1-HIS3, and SPO13-URA3 (11). From 3.8 × 10^6 different transformants, we isolated 250 clones that activated both the ADE2 and the HIS3 markers. Among these, 64 also stimulated the URA3 marker, which is only activated by relatively strong protein-protein interactions (11). Binding of these 64 PAs was specific for DHBV core since all PAs did not interact with control proteins, with the exception of PA13, which also bound to the HBV core protein in addition to the DHBV core protein (Table 1).

Peptide Aptamer-induced Inhibition of DHBV Capsid Formation and Replication—The 64 PAs that also activated the URA3 marker in yeast were tested in viral replication assays (13, 24) for their ability to interfere with DHBV capsid formation. Since cellular expression levels of TrxA scaffolds presenting different peptide moieties can greatly vary (not shown), individual PAs were expressed as fusions to the HSV-1 VP22 protein (11, 13). This allows the expression of high intracellular PA amounts and results in comparable expression levels for different core-targeting and control PAs. Following transfection of pCD16, a plasmid expressing a replication-competent DHBV RNA pregenome (20), avian LMH hepatoma cells were lysed, and the amounts of newly formed DHBV capsids were determined (13, 21). Coexpression of most PAs did not significantly affect the concentrations of viral capsids (e.g. PA20). Notably, however, PA34 strongly reduced the amounts of newly formed DHBV capsids to almost undetectable levels (Fig. 1A). Corresponding results were obtained in human Huh-7 hepatoma cells (not shown). Viral nucleic acids were released from the capsids in situ and analyzed on the same filters with a DHBV DNA probe, showing that PA34 also strongly reduced the amounts of core particle-associated DHBV nucleic acids (Fig. 1B).

Hepadnaviral core proteins are able to undergo self-assembly into capsids in the absence of additional viral gene products (25). As shown in Fig. 1C, PA34 also efficiently reduced the amounts of self-assembled viral capsids to almost undetectable levels. Furthermore, in line with its strong inhibitory effects on capsid assembly, PA34 virtually completely extinguished the secretion of DHBV virions when compared with control aptamers (Fig. 1D). PA34 did not exert any detectable nonspecific toxic effect on the cells since neither the activities of cotransfected β-galactosidase reporter plasmids nor the core transcript levels were affected, and colony formation assays yielded equal colony numbers for PA34- and control-transfected LMH or Huh-7 cells (not shown).

To corroborate that PA34 can bind to DHBV core in higher eukaryotic cells, we coexpressed a GAL4BD-core fusion protein together with different peptide aptamers, linked to the
VP16 transcriptional activation domain of HSV-1. Under these conditions, the activity of a cotransfected luciferase reporter plasmid, which is under the transcriptional control of GAL4 recognition sites, depends on the interaction of the fusion proteins (13). In contrast to control PAs, PA34 clearly activated the luciferase reporter in LMH or HeLa cells (Fig. 1C). In accordance with this, PA34 clearly activated the luciferase reporter in LMH or HeLa cells (Fig. 1E), indicating that it can also interact with the DHBV core protein inside avian and mammalian cells.

**Detection of Intracellular Peptide Aptamer/Core Protein Aggregates**—To obtain closer insights into the mechanism of PA-mediated target protein inhibition, we investigated the effects of PAs at the cellular level by confocal microscopy. Coexpression of a control PA (such as 16E730, not binding to the core protein) with the DHBV core protein in Huh-7 cells resulted in a mainly cytoplasmic staining for both proteins, with no overlap of the signals (Fig. 2A–D). Expression of PA34 alone also led to a predominantly cytoplasmic staining (Fig. 2F). When compared with the core protein, both PAs were less homogeneously distributed in the cytoplasm when expressed alone, and some of the signals concentrated in small distinct spots (Fig. 2C and F). Strikingly, however, coexpression of core-binding PA34 together with DHBV core resulted in a dramatic relocalization of the core protein, changing from its diffuse cytoplasmic staining pattern (Fig. 2B) to the emergence of large and intensely stained perinuclear inclusion bodies (Fig. 2H). PA34 and the core protein colocalized within these structures (Fig. 2G–J). Corresponding experiments performed in LMH cells yielded identical results (not shown). Treatment of the cells with the microtubule-destabilizing agent nocodazole prohibited formation of the large perinuclear inclusion bodies and resulted in the emergence of small dispersed cytoplasmic foci in which PA34 and the core protein colocalized (Fig. 2K–N).

To extend these investigations to an unrelated PA binding to a different target, we analyzed E61-1, a previously isolated PA that specifically binds to the HPV16 E6 oncoprotein (11). Due to the lack of reliable E6 antibodies for intracellular detection, we expressed the HPV16 E6 protein as green fluorescent protein fusion, which, as assumed for E6, preferentially localizes to the nucleus due to nuclear localization signals within the HPV16 E6 protein (26). In line with this, expression of EGFP-16E6 in HeLa cells resulted in a predominantly nuclear signal in the presence of a coexpressed PA34, which served as negative control (Fig. 3A–D). In stark contrast, coexpression of the E6-binding PA E61-1 resulted in a relocalization of EGFP-16E6 to prominent perinuclear inclusions in which both interaction partners colocalized (Fig. 3E–H), as shown for the PA34/core interaction (Fig. 2G–J).

**PA34 Translocates the Viral Core Protein into Aggresomes**—Next, we aimed at elucidating the nature of the perinuclear structures in which the PA34/core complexes accumulated. In immunofluorescence studies, we did not detect coaining with a series of different endoplasmic reticulum or Golgi markers (data not shown). Electron microscopy revealed that the coexpression of PA34 and core induced massive perinuclear accumulations of closely packed electron-dense particles in the vicinity of mitochondria clusters (Fig. 4A). This picture is reminiscent of the ultrastructure of aggresomes, which are juxtanuclear inclusion bodies that accumulate protein aggregates (22, 27). Typically, aggresomes contain individual dense particles of ~40–90 nm in diameter and are surrounded by bundles of filamentous material (22, 27). Correspondingly, the perinuclear inclusion bodies formed upon PA34/core coexpression contained rodlike to spherical, membrane-free particles of ~45–65 nm in diameter. As shown at higher magnification, they were wrapped by roughly parallel bundles of filaments of 6–10 nm in diameter, in the periphery (Fig. 4B).

To further corroborate that PA34 translocates the viral core protein to aggresomes, we examined the expression pattern of vinculin by confocal microscopy. Typically, this intermediate
Filament is radically reorganized during aggresome formation, changing from long filamentous structures extending into the periphery of the cell to cage-like structures wrapped around the exterior of the aggresome (22, 27). We observed dramatic redistributions of the vimentin network upon PA34/core coexpression, forming a cage-like structure around the inclusion body (Fig. 5, A–D). Furthermore, costaining for the pericentriolar matrix protein α/β-tubulin (Figs. 5, E–H) revealed that the pA34/core-containing inclusion bodies typically localized to the microtubule-organizing center, consistent with the preferentially pericentriolar localization of aggresomes (22, 27).

Intracellular Depletion of Soluble Target Proteins by PAs—These findings indicate that PAs can block viral replication by specific sequestration of the core protein into aggresomes. Since aggresomal protein aggregates are detergent-insoluble (22), expression of PA34 should redistribute the DHBV core protein from the detergent-soluble to the detergent-insoluble protein fraction of the cell. When expressed in the absence of its target protein, PA34 accumulated in the detergent-insoluble protein fraction, in Huh-7 cells (Fig. 6A). We then coexpressed individual PAs together with the core protein from pCD16. The DHBV core protein was detected exclusively in the detergent-soluble protein fraction, when coexpressed with negative control peptide aptamers, such as 16E730 (Fig. 6A) or C1-1 (not shown). In contrast, when coexpressed with PA34, a substantial portion of the DHBV core protein shifted to the detergent-insoluble protein fraction after 1 day, and after 2 days, it was almost exclusively found in the insoluble protein fraction (Fig. 6A).

Next, we investigated whether this mechanism generally applies to PAs targeting the core protein of hepadnaviruses. To this end, we analyzed C1-1, an unrelated PA that specifically binds to the HBV core protein (13). As found for the PA34/DHBV core interaction, coexpression of C1-1 with the HBV core protein also induced a virtually complete sequestration of the HBV core protein into the insoluble cellular protein fraction (Fig. 6B). In contrast, HBV core remained in the soluble protein fraction upon coexpression of PA34, which served as negative control in this setting. These results show that core-binding PAs block replication of different hepadnaviruses by efficiently depleting the intracellular pool of soluble core protein in a target-specific manner.
with H1 but not with pRb (7). Similarly, a PA targeting the mediated phosphorylation of histone H1 but not of pRb, sug-

A 16E730  PA34  16E730  PA34  PA34  pcD16
   + + + + + +
   S P S P S P

24 h 48 h

DHBV Core PA

Tubulin

B PA34  C1-1  pd24
   + + + +
   S P S P

HBV Core PA

FIGURE 6. PAs shift their targets from the detergent-soluble to the detergent-insoluble cellular protein fraction. A, Huh-7 cells transfected with DHBV vector pcD16, together with expression vectors for either negative control PA 16E730 or DHBV core-binding PA34. Cell lysates were prepared 24 or 48 h after transfection, separated into detergent-soluble (soluble (S)) and -insoluble (pellet (P)), and analyzed by Western blot for the presence of PAs and DHBV core protein. B, Huh-7 cells transfected with HBV vector pd24, together with expression vectors for either negative control PA34 or HBV core-binding PA C1-1. Cell lysates were prepared 48 h after transfection, separated into detergent-soluble and -insoluble fractions, and investigated by Western blot for the presence of PAs and HBV core protein.

DISCUSSION

Although a series of studies have shown that PAs can interfere with the intracellular function of their target pro-
teins, little is known about the underlying biochemical mechanism. The present work shows that inhibitory PAs can block the activity of proteins by efficiently inducing their sequestration into aggresomes.

Aggresomes are large perinuclear inclusion bodies into which the cell collects aggregated proteins, possibly to avoid the accumulation of potentially toxic protein aggregates in the cytoplasm (28, 29). Several observations indicate that the large perinuclear structures, to which the PA34 and the core protein translocate, represent bona fide aggresomes. This includes their typical ultrastructural morphology, their pericentriolar localization, and their characteristic vimentin caging. Moreover, since aggresomes are formed by a pathway involving dynein/
dynactin-mediated retrograde transport on microtubules (27), their formation is prevented by treatment with the microtubule-destabilizing agent nocodazole, as found for the PA34/core containing inclusion bodies.

In principle, the screening method employed for the genera-
tion of PAs should result in a collection of molecules that bind to many different surface regions on the target protein, thereby potentially blocking the binding of natural interaction partners to distinct domains of the target protein. In indirect support of this idea, a Cdk2-binding PA has been shown to block Cdk2-mediated phosphorylation of histone H1 but not of pRb, suggesting that it binds to a region that is crucial for the interaction with H1 but not with pRb (7). Similarly, a PA targeting the intracellular domain of the epidermal growth factor receptor did not generally inhibit its tyrosine kinase activity but selectively interfered with the phosphorylation of certain tyrosine residues only (15). Thus, PAs should not only be useful as molecular tools for the intracellular inhibition of proteins but may also serve to map therapeutically relevant surface domains on the target protein for the development of non-peptide mimetics (5).

The present work defines a novel, but not mutually exclu-
sive, mechanism by which PAs block the activities of their target proteins. It is compatible with the following scenario. Expression of a PA alone, such as the core-binding PA34, leads to an accumulation of the PA in the detergent-insoluble cellular protein fraction, indicating an intrinsic tendency for PAs to aggregate. In contrast, the DHBV core protein is expressed as a soluble, largely cytoplasmic protein. Upon PA34/core coexpression, the interaction partners coaggregate and are transported on microtubules into aggresomes, accounting for the dramatic intracellular redistribution of the viral core protein. Notably, treatment with nocodazole resulted in dispersed foci in which PA34 and the core protein colocalized, indicating that PA34 and core can coaggregate at conditions where aggresome formation is suppressed. The aggresome formation induced by the PA34/core interaction was highly efficient and did not require treatment with proteasome inhibitors, which are widely used to increase the formation of cellular aggresomes (30). As a consequence, PA34 induced a virtually complete shift of the DHBV core protein from the detergent-soluble into the detergent-insoluble cellular protein fraction, where it is no longer available for viral capsid formation.

The PA34/core coaggregation could be supported by two features of PAs, namely their intrinsic tendency to aggregate, as shown here, and their ability to strongly bind to a specific target protein at intracellular conditions (6). The PA-mediated sequestration of the core protein into the detergent-insoluble protein fraction indicates that aggregated PAs do not necessarily adopt a completely non-native structure. Indeed, such partial aggregations have been proposed previously for other proteins (“native coaggregation”), indicating that binding partners of multidomain proteins can be sequestered into aggregates through their interaction with the native domain of aggregated proteins (31).

The efficacy of PA-mediated inhibition of DHBV replication is striking. We found that PA34 leads to an almost complete clearance of the soluble core protein from the cytoplasm and, consequently, to a dramatic reduction of the viral capsid concentration and virion production to virtually undetectable levels. The corresponding results obtained for a PA targeting the HBV core protein indicate that the PA-induced removal of soluble intracellular core protein can be employed as a general antiviral strategy against hepadnaviruses. Notably, the effects of individual PAs were highly selective for their respective target proteins in that PA34 specifically removed the DHBV core but not HBV core protein from the soluble intracellular protein fraction, whereas C1-1 cleared the HBV core but not DHBV core protein. Moreover, the PA-mediated translocation of bound proteins into aggresomes is not restricted to structural proteins of hepadnaviruses, as indicated by the analysis of a
bioactive PA targeting the HPV E6 protein, and should be useful as a general strategy for the targeted inhibition of intracellular proteins.

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