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Inhibition of vaccinia virus replication by peptide aptamers

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ABSTRACT

A20 protein is a major component of the vaccinia virus replication complex. It binds to the DNA polymerase E9, the uracil DNA glycosylase D4 and the primase/helicase D5, three proteins that are essential for viral DNA synthesis. The identification of molecules able to interact with the replication complex and inhibit its activity is a promising strategy for the design of new anti-orthopoxvirus drugs. In this study, we used a yeast genetic approach to select, from combinatorial libraries, 8-mers peptide aptamers that specifically interact with A20. From this screen, we isolated five peptide aptamers whose binding to A20 was confirmed by a glutathione S-transferase (GST) pull-down assay. Among those, we determined that peptide aptamer 72 binds to a central domain on A20. Interestingly, this region of A20 was previously shown to be important for its function in DNA replication. We next showed that vaccinia virus DNA synthesis was impaired in cells constitutively expressing peptide aptamer 72 and that virus production was inhibited in those cells. Thus, peptide aptamer 72 may be a useful tool for the development of new compounds specifically targeting poxvirus replication.

1. Introduction

Smallpox was eradicated 30 years ago after a worldwide vaccination campaign coordinated by the World Health Organization (WHO) (Geddes, 2006). Since then, vaccination has been stopped (except for some military and health care personnel), which results in a high proportion of unprotected people in today's population. This situation raises concerns about the potential use of variola virus (VARV) as a biological weapon. Indeed, variola virus is listed as a category A agent for bioterrorism by the Centers for Disease Control (CDC) (Lane et al., 2001). The fear of an attack using VARV has stimulated the development of new anti-orthopoxviral compounds that would be effective in response to a smallpox outbreak.

Up to date, there is no marketed antiviral molecule to treat poxvirus infection. The food and drug administration (FDA) has only approved the use of cidofovir (CDV), but solely as investigational new drug application. This nucleoside analogue is an inhibitor of a broad range of DNA viruses in cell culture (De Clercq, 2003). Unfortunately, CDV exhibits a number of disadvantages that would prevent its massive use in case of an orthopoxvirus epidemic: first, it has a poor oral bioavailability and therefore must be administered by the intravenous route; second it has a significant nephrotoxicity and, finally, cidofovir-resistant poxviruses have been isolated (Andrei et al., 2006; Kornbluth et al., 2006; Smee et al., 2002). Recently, ST-246, a promising new therapeutic against orthopoxvirus infection has been identified. This molecule, which can be administered by the oral route, is active against several orthopoxviruses such as vaccinia virus (VACV), cowpox virus (CPXV) and camelpox virus (CMLV) (Yang et al., 2005). ST-246 was shown to target the VACV F13 protein, which is involved in the formation of extracellular enveloped viruses (Blasco and Moss, 1991). This compound is currently in phase II clinical trials (Parker et al., 2008). There is still a need to enlarge the armamentarium of antiviral molecules active against poxviruses with different and original mechanism of action. The selection of new compounds acting against different targets could be useful in case of emergence of drug resistance to the reference anti-poxvirus agents and will be crucial to rapidly and efficiently respond to a potential smallpox outbreak.

Orthopoxviruses, for which VACV is the prototypical member, are unique among DNA viruses in that they carry out their replication exclusively in the cytoplasm of the host cell. Thus, the virus does not depend on nuclear enzymes, and consequently encodes most if not all the proteins necessary for genome replication (Moss, 2007). Among these proteins, five were shown to be absolutely essential for the synthesis of the ~200 kbp viral genome. These

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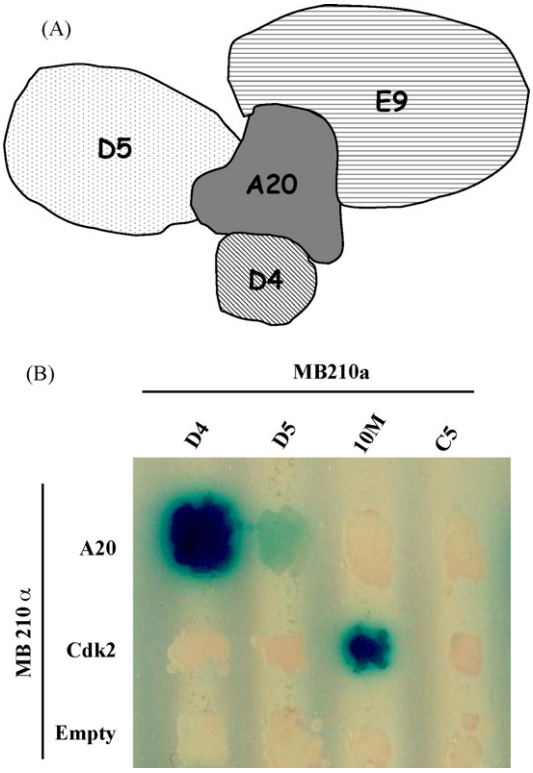

Fig. 1. Protein–protein interactions within the VACV replication complex. (A) Schematic representation of the VACV replication complex. E9: DNA polymerase, D4: uracil DNA glycosylase, D5: Primase/Helicase. A20 which has no known enzymatic activity interacts with E9, D4 and D5. (B) A20 interaction with D4 and D5 in yeast two-hybrid. MB210α yeast cells were co-transformed with pSH18-34 and pEG202-A20. MB210*a* yeast cells were transformed with pWP2 vectors expressing D4-B112 and D5-B112. After mating, the diploid yeasts were plated onto Ura$^-$, His$^-$, Trp$^-$, galactose/raffinose plate containing X-gal. After 3 days incubation, the plate was scanned. 10 M: peptide aptamer that interacts with Cdk2 (Colas et al., 2000). C5: peptide aptamer randomly picked from a library. Empty: pEG202 vector (i.e. without A20R gene).

are E9, the catalytic subunit of the DNA polymerase, D5, a DNA-independent nucleoside triphosphatase (NTPase) that contains a helicase domain (Boyle et al., 2007) and a primase activity (De Silva et al., 2007), the uracil DNA glycosylase D4, the protein kinase B1 (Traktman et al., 1989) and the A20 protein. The latter protein has no known enzymatic activity but plays a major role in poxvirus replication. A20 in complex with D4 was recently shown to form a heterodimeric processivity factor for the viral DNA polymerase (Stanitsa et al., 2006). Through its direct interaction with E9 (Klemperer et al., 2001), it probably serves as a bridge between D4 and the DNA polymerase. Yeast two-hybrid experiments have also revealed that A20 interacts with D5 and H5, a protein involved in transcription elongation (Cresawn and Condit, 2007; Ishii and Moss, 2002; McCraith et al., 2000). Thus, A20 is a central component whose role is likely to form and/or stabilize the DNA replication complex (Fig. 1A). The discovery of molecules able to inhibit the function of A20 within the replication complex could represent a relevant strategy to generate new compounds against orthopoxviruses.

Peptide aptamers are combinatorial protein molecules designed to inhibit the function of target proteins in living cells (Colas et al., 1996). They consist of conformationally constrained random sequence peptide loops displayed by a platform protein like the bacterial Thioredoxin A (TrxA) protein (Colas, 2008; Hoppe-Seyler et al., 2004). Once expressed intracellularly, peptide aptamers can inhibit the function of their target proteins by interfering with protein interactions or by inhibiting their enzymatic activity. Peptide aptamers are selected, against the protein of interest, from combinatorial expression libraries using screening techniques like the yeast two-hybrid system (Geyer and Brent, 2000). A number of reports have described the selection of peptide aptamers targeting viral proteins such as the hepatitis B virus core protein (Butz et al., 2001), the tomato golden mosaic virus AL1 protein (Lopez-Ochoa et al., 2006) and human papillomavirus E6 and E7 oncoproteins (Butz et al., 2000; Nauenburg et al., 2001). In all these studies, the selected peptides were able to interfere with the viral life cycle. Therefore, the peptide aptamer technology is a useful tool to validate therapeutic targets (Baines and Colas, 2006).

In this study we report the selection of peptide aptamers directed against VACV A20 protein. We show that one of the aptamers is a strong inhibitor of viral DNA synthesis and is able to inhibit virus production in cell culture. Interestingly, this peptide interacts with a domain of A20 that was previously shown to be critical for its function in DNA synthesis (Ishii and Moss, 2001; Punjabi et al., 2001).

2. Materials and methods

2.1. Materials

The yeast strains and yeast two-hybrid plasmids used in this study were supplied by APTANOMICS (Lyon, France) and described in detail elsewhere (Bickle et al., 2006).

2.2. Plasmid constructions

pEG202-A20 plasmid was constructed by PCR amplification of the A20R gene from VACV (Copenhagen strain) DNA using the primers 5′-CTG GAA TTC ATG ACT TCT AGC GCT GAT TTA AC-3′ and 5′-CGA CTC GAG TCA CTC GAA TAA TCT TTT TTT GAC ATC-3′ that contain an EcoRI and a XhoI site, respectively. The PCR fragment was cleaved and ligated into the EcoRI/XhoI-cleaved pEG202 plasmid.

pWP2-D4 and pWP2-D5 vectors were constructed in the same way using the primers 5′-TCT GAA TTC ACC ATG AAC TCA GTG ACT GTA TCA CAC G-3′ and 5′-CTC CTC GAG ATA AAT AAA CCC TTG AGC CCA ATT TAT A-3′, 5′-TCT GAA TTC ACC ATG GAT GCG GCT ATT AGA GGT AAT G-3′ and 5′-CTC CTC GAG CGG AGA TGA AAT ATC CTC TAT GAA TAT-3′, respectively. After digestion, the PCR products were cloned into the EcoRI/XhoI-cleaved pWP2 vector.

pEG202-A20 1–150, pEG202-A20 1–176, pEG202-A20 1–203, pEG202-A20 1–227, pEG202-A20 1–327 vectors containing truncations of the A20R gene were constructed using the forward primer 5′-CTG GAA TTC ATG ACT TCT AGC GCT GAT TTA AC-3′ and reverse primers 5′-CGA CTC GAG TCA ATT AAC TGG AGA CTG ATA TTT TG-3′ (A20 1–150); 5′-CGA CTC GAG TCA AAA TAA TGT GTC TTC CTC TAT TTC-3′ (A20 1–176), 5′-CGA CTC GAG TCA TCC CAA CTT AAT ATA CGA TAT GG-3′ (A20 1–203), 5′-CGA CTC GAG TCA ACG ATC TAC CTT GAT GGA CTC-3′ (A20 1–227), 5′-CGA CTC GAG TCA CTC ATT AAT ATC TAA TTT ATT AG-3′ (A20 1–327). The cleaved PCR fragments were ligated into the EcoRI/XhoI-cleaved pEG202 plasmid.

pGST/TrxA/aptamers vectors were obtained by PCR amplification of the TrxA/aptamer sequence from pWP2 plasmid using the primers 5′-CGA CAC CGA CGT GCT GAA GGC C-3′ and 5′-GCT TGG CCA CGG TCA GCT TGC C-3′. The amplified product was introduced into a RsrII-cut pGST2/TrxA plasmid by homologous recombination in yeast as previously described (Abed et al., 2007).

2.3. Two-hybrid screening of the peptide aptamer library

The randomized eight amino-acid peptide aptamer library constructed in the pWP2 vector was supplied by APTANOMICS. 800 ml of MB210*a* yeasts were transformed with 100 μg of peptide aptamer library and 1.5×10^8 transformants were obtained.

MB226α yeasts were co-transformed with pEG202-A20 and pSH18-34 (bearing a *lacZ* reporter gene). The yeast two-hybrid selection was performed essentially as previously described (Bickle et al., 2006); 2×10^7 diploid exconjugants were obtained (i.e. 33% mating efficiency). Diploids were plated onto $Ura^-His^-Trp^-Leu^-Ade^-$ galactose/raffinose plates and incubated for 7 days at 30 °C. Plates were then replica-plated onto $Ura^-His^-Trp^-$ X-gal galactose/raffinose. Clones that grew in the absence of leucine and adenine, which possessed a β-galactosidase activity, were picked. Library plasmids were recovered and re-transformed into EGY48α yeasts. The interaction phenotypes were confirmed by mating assays with EGY42a yeast strains co-transformed with pEG202-A20 and pSH18-34. The aptamer genes obtained from positive clones were sequenced.

2.4. Interaction mating assay in yeast

MB210α yeasts were co-transformed with pSH18-34 and pEG202-A20 (or pEG202-ΔA20 constructs). MB210*a* yeasts were transformed with the pWP2 vectors (coding for D4, D5 or peptide aptamers). The two haploid yeast strains were streaked across plates as horizontal or vertical lines on yeast rich solid medium (YAPD). The diploid exconjugants formed by mating at the intersections were replicated onto $Ura^-His^-Trp^-$ galactose/raffinose plates containing X-gal (to detect activation of the *lacZ* reporter gene) and incubated for 3 days at 30 °C.

2.5. Expression of A20 in insect cells

To produce the A20 protein in insect cells, a recombinant baculovirus carrying the A20R gene was built according to the manufacturer's instructions (Bac-to-Bac Baculovirus Expression System, Invitrogen, Carlsbad, CA). Briefly, the VACV A20R gene (Copenhagen strain) was cloned into the pFastBac™HT A vector. The construct was used to transform competent DH 10Bac™ *E. coli* cells (Invitrogen, Carlsbad, CA). A recombinant bacmid DNA containing one copy of the A20R gene was recovered and transfected into Sf9 cells (Invitrogen, Cat. No. 11496-015). The recombinant viruses produced in the cell culture medium were used to obtain a high titer viral stock. For A20 expression, 7.5×10^7 Sf21 cells (Invitrogen, Cat. No. 11497-013) in suspension were infected at an MOI of 5 PFU/cell for 72 h in Sf-900 II SFM medium (GIBCO, Auckland, NZ). Infected cells were resuspended in 10 ml of lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM beta-Mercaptoethanol, 0.01% Tween20, protease inhibitors (Complete EDTA-free, Roche Diagnostics GmbH, Mannheim, Germany), and 2.5 μg ml^{-1} DNase I (Sigma Chemical Co., St. Louis, MO)], sonicated 3 times 1 min on ice and centrifuged at 15,000 × *g* for 15 min. Protein extract was then resolved on a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The expression of A20 (that carries an N terminal 6 His-tag) was detected by Western blot using the Penta-His Antibody (Qiagen GmbH, Hilden, Germany) at a 1:2000 dilution.

2.6. GST pull-down

TrxA/aptamers were produced as glutathione S-transferase (GST) fusion proteins in BL21 (DE3) *E. coli* strain. Protein expression (from a 20 ml culture) was induced with 0.5 mM of isopropyl thiogalagtopyranoside (IPTG) for 16 h at room temperature. Bacteria were pelleted, resuspended in 1 ml PBS 1× and sonicated three times 1 min on ice. One per cent Triton X-100 was added and after a 30 min incubation on ice, the lysates were clarified by centrifugation at 12,000 × *g* for 10 min. Extracts were then incubated with 45 μl glutathione sepharose 4B (Amersham Biosciences, Uppsala, Sweden) at room temperature for 30 min on a rotating wheel. The beads were washed three times with 1 ml of PBS 1× and incubated with 800 μl of cell extracts from Sf21 cells overexpressing the His-tagged A20 (see above) for 2 h at 4 °C on a rotating wheel. The beads were washed three times with 1 ml of wash buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 2 mM beta-Mercaptoethanol, 0.01% Tween20) and resuspended in 45 μl of Laemmli buffer 2×. The retained material was loaded on a 12% SDS-PAGE and analyzed by Western blot using the Penta-His Antibody for A20 detection and anti-glutathione S-transferase antibody (Sigma Chemical Co., St. Louis, MO) at a 1:1000 dilution for TrxA/aptamers detection. Anti-mouse or anti-rabbit HRP-conjugated immunoglobulin was used as secondary antibody followed by chemoluminescent revelation (Amersham GE Healthcare, Buckinghamshire, UK).

2.7. Production of stable cell lines

Stable integration of CAT and TrxA/aptamers in 293 cells was accomplished according to the manufacturer's *Flp-In System* protocol (Invitrogen, Carlsbad, CA). pcDNA5/FRT/TrxA–aptamers vectors were constructed, first, by subcloning the TrxA–aptamer genes from pGST/TrxA/aptamer constructs into the XhoI/NotI-cleaved pPERh-MCS vector (Pamonsinlapatham et al., 2008). TrxA–aptamer genes were then subcloned into the XhoI/NheI cleaved pcDNA5/FRT vector. Flp-In™-293 cells (Invitrogen, Carlsbad, CA) containing stably integrated pFRT/lacZeo (with the yeast FRT recombination site) were co-transfected with pcDNA5/FRT/TrxA–aptamers (or pcDNA5/FRT/CAT) and pOG44, a plasmid expressing the *S. cerevisiae* Flp recombinase. Stable cell lines were then selected by growth in 100 μg ml^{-1} hygromycin (Sigma Chemical Co., St. Louis, MO).

2.8. Cell lysis and detection of TrxA/aptamers

Confluent 293 cell cultures expressing the peptide aptamers were washed twice in cold PBS 1× and lysed in 1 ml cold lysis buffer [20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% (v/v) NP40 and protease inhibitors cocktail (Complete EDTA-free, Roche Diagnostics GmbH, Mannheim, Germany)] for 10 min on ice. Insoluble debris were removed by centrifugation at 12,000 × *g* for 10 min. For Western blot analysis, protein extracts were boiled for 5 min in Laemmli buffer and separated on a 15% SDS-PAGE. The proteins were electrotransferred onto a 0.2 μm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The TrxA/aptamer fusion proteins were detected using the Thio-probe (K-20): sc-801 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

2.9. Virus and infections

Twenty-four-well plate containing 293 cells expressing the peptide aptamers (or CAT) were infected with VACV (WR strain, ATCC VR-119) at an MOI of 0.001 PFU/cell in DMEM supplemented with 0.4% (v/v) fetal calf serum at 37 °C in a 5% CO_2 atmosphere. Cells were harvested at the indicated time points (7, 24, 30, 48, 54, and 72 hpi). The virus yield was determined by virus titration in 96-well plates of Vero cells (ATCC CCL-81). Titration was performed with serial fourfold dilutions of the samples and the viral titer was expressed in $CCID_{50}$ ml^{-1} (Ferrier et al., 2004). Three separate experiments were performed, and each sample was titrated in quadruplicate.

2.10. Quantification of viral DNA synthesis

293 cells expressing the peptide aptamers (or CAT) were infected with VACV (WR strain) at an MOI of 3 PFU/cell. At 2 hpi, cells were washed twice and supplemented with fresh media. Cells were harvested at the indicated time points and total DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) according to the Manufacturer's instructions. Amplification

of the VACV A27L gene by real-time PCR was performed using the primers 5′-CAA CGA CTA ACT AAT TTG GAA AAA AAG AT-3′ and 5′-GCC AGA GAT ATC ATA GCC GCT C-3′ and the A27L gene specific probe 5′-TTT TCC AAC CTA AAT AGA ACT TCA TCG TTG CGT T-3′ in an Applied Biosystems Prism 7000 SDS instrument with v2.1.1 software (Scaramozzino et al., 2007). Reactions were carried out in 20 μl using the TaqMan qPCR Mastermix Plus (Eurogentec, Seraing, Belgium), 0.3 μM of each primer, 0.2 μM of TaqMan probe and 5 μl of extracted viral DNA (or 5 μl of serial 10-fold dilutions of pVACV-A27L standard plasmid). Thermal cycling was performed as follows: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles each of 95 °C for 15 s, followed by 60 °C for 60 s. All PCR runs included negative and positive controls and all samples were assayed in triplicate. Normalization of the DNA quantity to GAPDH was performed using specific GAPDH primers and probe from the TaqMan GAPDH control reagents kit (Applied Biosystems, Foster City, CA) according to the Manufacturer's instructions. Correlation values of standard curves were always >99%.

2.11. Statistical analysis

Significant variations of quantitative data were calculated using the two-tailed Student's *t*-test ($P < 0.05$: statistical significance).

3. Results

3.1. Protein interactions within the VACV replication complex

A20 is a major component of the VACV replication complex that physically interacts with three other essential proteins involved in viral DNA synthesis (Fig. 1A). In order to isolate peptide aptamers targeting A20 we used an improved yeast two-hybrid peptide aptamer screen (Bickle et al., 2006). As a preliminary experiment, we wanted to verify that A20 fused to the DNA binding domain of LexA was well expressed in yeast. A mating interaction assay shows that A20 exhibits a strong interaction phenotype with D4 and to a lesser extent with D5, confirming that A20 is indeed expressed in its native conformation in this yeast two-hybrid system (Fig. 1B). Both A20 interaction phenotypes with D4 and D5 are specific, since no interaction is detected with peptide aptamer 10 M, selected to bind to the cell cycle regulator Cdk2 (Colas et al., 2000), or with peptide aptamer C5, randomly taken from a library. The differential strength of the interaction phenotypes observed between A20/D4 and A20/D5 is in agreement with previous data obtained in yeast using the *GAL4* two-hybrid system (Ishii and Moss, 2002).

3.2. Two-hybrid screening of the peptide aptamer library

Peptide aptamers targeting A20 were selected from a randomized library of eight amino acid long peptides displayed at the surface of a modified *E. coli* thioredoxin A protein (Bickle et al., 2006). Haploid MB226α yeasts, co-transformed with pEG202-A20 and the *lacZ* reporter plasmid pSH18-34, were mated with MB210*a* yeasts transformed with the library in which TrxA/peptide aptamers are fused to the B112 transcriptional module (Bickle et al., 2006). From 2×10^7 transformants, 11 clones expressed a peptide aptamer showing an interaction phenotype with A20. The interactions between the 11 peptides and A20 were reconfirmed using different yeast strains (i.e. by mating EGY48α yeasts expressing peptide aptamers with EGY42*a* yeasts expressing A20) (data not shown).

3.3. Interactions between A20 and peptide aptamers in vitro

The interaction between the selected peptide aptamers and A20 was then tested, in vitro, in glutathione S-transferase (GST) pull-down experiments. Bacterially expressed TrxA/aptamer fused to GST were immobilized on glutathione beads and incubated with an extract from insect cells expressing A20. The retained material was loaded on a 12% SDS-PAGE and analyzed by Western blot (Fig. 2). This experiment was repeated several times and aptamers 15, 38, 60, 72 and 79 reproducibly pulled down A20 from the cell extract. We also noticed that peptide aptamer 72 (and to a lesser extent peptide aptamer 60) pulled down A20 with a better efficiency than peptide aptamers 79, 38 and 15. The remaining six peptide aptamers did not interact with A20 expressed from insect cells in this assay (data not shown). Overall, the results obtained with the pull-down assay and with the yeast two-hybrid analysis led us to conclude that the relative interaction strength between A20 and peptide aptamers are as follows: A20–Apt72 > A20–Apt60 > A20–Apt79 > A20–Apt38 and A20–Apt15 (Fig. 2 and Table 1).

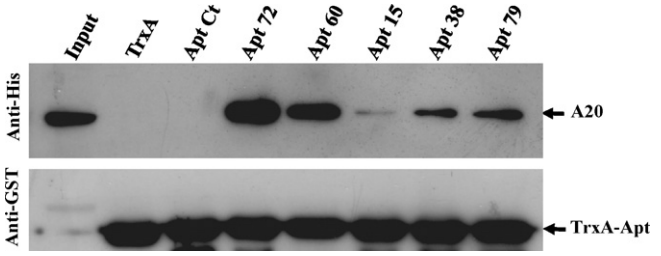

Fig. 2. In vitro interactions between A20 and peptide aptamers. TrxA/Aptamers (TrxA–Apt) were fused to GST and expressed in bacteria. Fusion proteins were immobilized on glutathione sepharose 4B beads and incubated with cell extracts from Sf21 cells overexpressing the His-tagged A20. After washes, the retained complexes were analyzed by Western blot using anti-His and anti-GST antibodies for A20 and TrxA/aptamers detection, respectively. TrxA: thioredoxin A without peptide. Apt Ct: unrelated peptide aptamer used as negative control. Input: cell lysate from Sf21 cells expressing His-A20.

The sequences of the variable regions of peptide aptamers 72, 60, 79, 38 and 15 (inserted in the active-site loop of TrxA) are shown in Table 1. While peptides 15 and 38 do not show any similarities with the other peptides, peptides 72, 60 and 79 share common features. Peptides 60 and 72 are enriched in positively charged amino acids (50% and 37%, respectively). A BLAST analysis against available viral databases revealed that peptide 72 exhibits sequence similarity with VACV A47 protein (see Section 4).

3.4. Mapping of peptide aptamer binding sites on A20

In order to determine the A20 domains that are recognized by peptide aptamers, a yeast interaction mating assay was performed between peptide aptamers and various C-terminal deletion constructs of A20 (Fig. 3A). In this experiment, D4 protein was used to validate the expression of A20 truncations. Indeed, D4, known to interact with the first 25 amino acid of A20 (Ishii and Moss, 2002), shows a strong interaction phenotype with all A20 deletion constructs tested (Fig. 3A). Since the interaction phenotypes observed with construct AA 1–227 are at least as strong as those observed with the full length A20 protein, we conclude that the A20 C-terminal region comprised between AA 227 and AA 426 is

Table 1
Sequences of peptide aptamer variable regions.

Aptamer	A20 interaction	Variable regions
72	+++	TrxA-RQLVLRTR-TrxA
60	++	TrxA-RHMMLARR-TrxA
79	++	TrxA-GALILRQV-TrxA
38	+	TrxA-EALMWEGF-TrxA
15	+	TrxA-GPWRVWSP-TrxA

TrxA: thioredoxin A

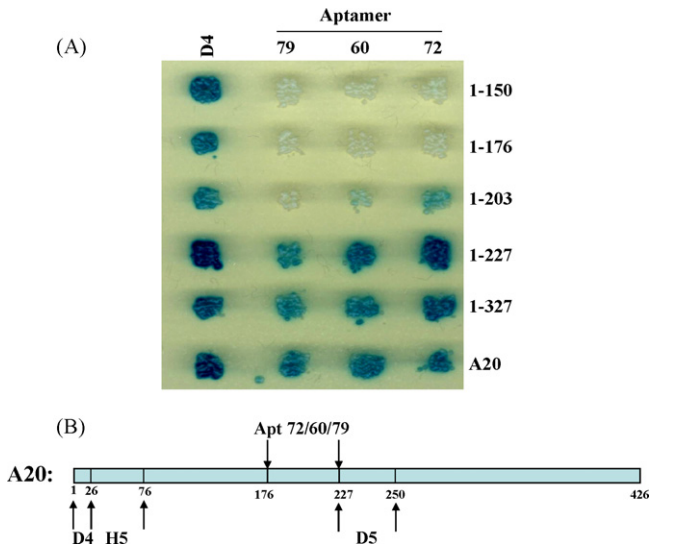

Fig. 3. Mapping of peptide aptamer binding sites on A20. (A) A yeast two-hybrid mating assay was performed using full length A20 and C-term truncated mutants (numbers on the right indicated the region of A20 that is expressed) as baits and peptide aptamers 72, 60 and 79 as preys. D4, which interacts with the first 25 AA of A20 (Ishii and Moss, 2002) was used as a positive interaction control. (B) Location of binding sites on A20. The arrows and amino-acid numbers indicate the binding site of D4, H5, D5 (Ishii and Moss, 2002) and peptide aptamers on A20.

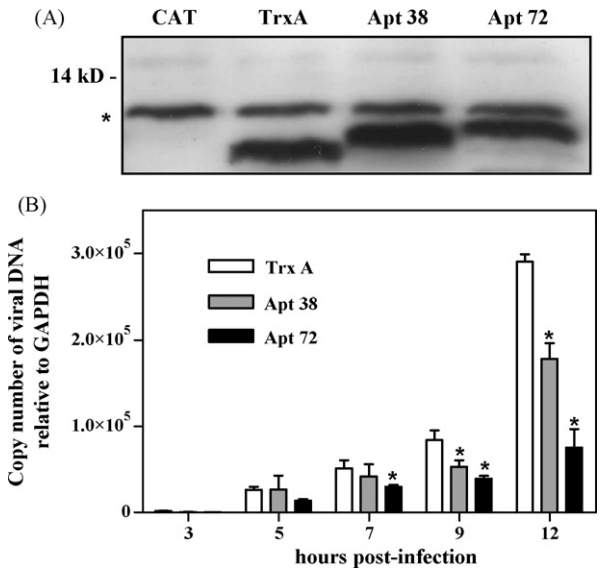

Fig. 4. Inhibition of VACV DNA synthesis by peptide aptamers. (A) Lysates from stable 293 cells lines expressing TrxA, TrxA/aptamers (38 and 72) and CAT were loaded on a 15% SDS-PAGE and electrotransferred on a nitrocellulose membrane. Expression of TrxA/aptamers was detected with an anti-TrxA antibody. A molecular weight marker is shown on the left. * indicates a cellular protein that cross-reacts with the anti TrxA antibody. (B) 293 cells stably expressing TrxA, and TrxA/aptamers 38 and 72 were infected with VACV (WR strain) at an MOI of 3 PFU/cell. Cells were harvested at the indicated time points; VACV DNA was extracted and quantified by real-time PCR using primers specific to VACV A27L gene. Results are expressed as copy number of viral DNA relative to GAPDH and reported as mean ± S.D. from three samples. *Significant differences compared to the TrxA control: Student's *t*-test; $P < 0.05$. These experiments were performed twice with comparable results.

not required for binding of peptide aptamers 72, 60 and 79. No interaction phenotypes are observed with the construct AA 1–176, therefore we conclude that the interaction site of peptide aptamers involve key residues located between AA 176 and 227. However, we cannot rule out that region 1–176 contains residues that also contribute to the interaction. Although peptide aptamers 15 and 38 bind to full length A20, we did not detect any interaction phenotype between these aptamers and the A20 deletion mutants (data not shown). This is probably due to a weaker interaction between these aptamers and A20 or to the fact that they recognize a region located in the last 100 AA of the protein. The interaction sites of D4, D5 and H5 on A20 have been previously mapped (Ishii and Moss, 2002). D4 and H5 interact with the very N-terminus of A20, while D5 binds to a central domain, between amino acids 227 and 250. Thus, our results indicate that peptide aptamers 72, 60 and 79 interact with a domain that is adjacent (but not overlapping) to the D5 binding site (Fig. 3B).

3.5. Inhibition of VACV DNA synthesis by peptide aptamers

We then wanted to determine whether VACV DNA synthesis was inhibited in cells expressing peptide aptamers. We decided to test the potential inhibitory effect of two peptide aptamers: peptide aptamer 72, which exhibits the highest apparent binding affinity for A20, and peptide aptamer 38 whose A20 interacting domain remains unknown. Stable 293 cell lines that constitutively express these peptide aptamers were obtained together with a control cell line producing the scaffold TrxA protein without inserted peptide. Fig. 4A shows that the three cell lines produce similar amounts of the expected proteins with an apparent molecular weight of ~10–12 kDa. Peptide aptamer expressing cells were infected with VACV at an MOI of 3 PFU/cell. Viral DNA synthesis was then quantified by real-time PCR at 3, 5, 7, 9 and 12 h post-infection (hpi). Fig. 4B shows that in cells expressing the control TrxA, the level of viral DNA produced over time is higher than in cells expressing the peptide aptamers. A notable reduction in DNA synthesis was observed as early as 7 hpi for peptide aptamer 72 and 9 hpi for peptide aptamer 38. The inhibition is then maintained up to 12 hpi, the last time point tested. We reproducibly noticed that the inhibitory activity is statistically stronger (at 9 and 12 hpi) in cells expressing peptide aptamer 72 than in cells expressing peptide aptamers 38 (Student's *t*-test; $P < 0.05$). Thus, the expression of peptide aptamers 72 and 38 induced a significant inhibition of VACV DNA synthesis (75% and 40%, respectively, at 12 hpi), suggesting an efficient intracellular targeting of A20 by these peptide aptamers and confirming the essential role played by this protein in viral DNA replication.

3.6. Inhibition of VACV growth by peptide aptamers

We next investigated whether viral DNA synthesis inhibition was correlated with a reduction of virus production in peptide aptamer expressing cells infected with VACV. Stable 293 cell lines were infected at an MOI of 0.001 PFU/cell and virus yield was quantified at different time points up to 72 hpi. The results, shown in Fig. 5, indicate that from 24 hpi virus replication in cells expressing peptide aptamer 72 is significantly reduced (>75% from 32 hpi) in comparison with the virus yield obtained in TrxA-producing cells (Student's *t*-test; $P < 0.05$). However, in cells expressing peptide aptamer 38, production of infectious virus is less inhibited and only significantly at 48 and 72 hpi.

4. Discussion

The A20 protein plays a major role in VACV DNA replication. It is a central component that interacts with the DNA polymerase E9 and two others partners, D5 and D4 with which it forms the heterodimeric processivity factor for the viral DNA polymerase (Stanitsa et al., 2006). The four above-mentioned proteins are essential for DNA synthesis and share 98% sequence identity with the corresponding variola proteins. Thus, the identification of molecules that specifically target the VACV replication complex

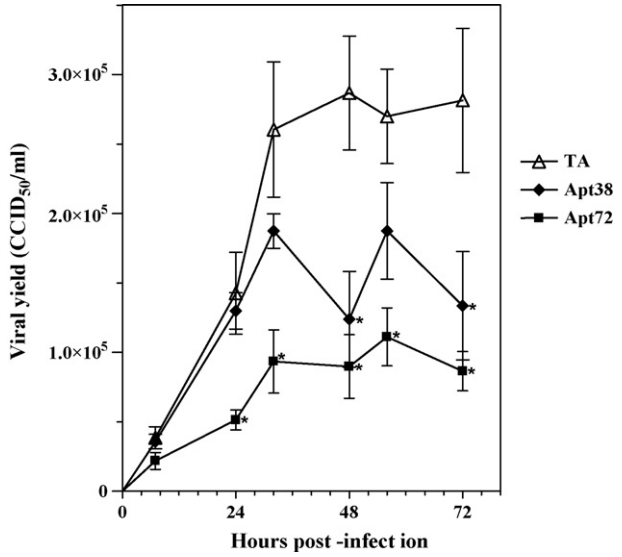

Fig. 5. Inhibition of VACV growth by peptide aptamers. 293 cells stably expressing TrxA, TrxA/aptamers 38 and 72 were infected with VACV (WR strain) at an MOI of 0.001 PFU/cell. Infections were stopped at the indicated time points. Virus yields were determined by virus titration and expressed as $CCID_{50}$ ml^{-1} and reported as mean ± S.D. For peptide aptamers 38 and 72 significant differences from control TrxA are indicated by $^{*}P < 0.05$ (Student's t-test). These experiments were performed three times with comparable results.

could be relevant for the design of new anti-orthopoxvirus drugs in case of a smallpox outbreak. In this study we have used a yeast two-hybrid screen to select 8-mer peptide aptamers that specifically interact with A20. From yeast two-hybrid interaction assays against different truncated forms of A20, we demonstrated that three peptide aptamers (peptides 72, 60, and 79) interact with a central domain of this protein between AA 176 and AA 227. This region does not correspond to the D4 and H5 binding domain (located at the A20 N-terminus extremity) (Ishii and Moss, 2002) and is adjacent to (but does not overlap with) the D5 interacting domain. Since the E9-binding site(s) on A20 has not been mapped yet, we do not know whether these peptide aptamers compete with the DNA polymerase for a common binding site on A20.

When the sequence of peptide aptamer 72 (R Q L *V* L R T R) was aligned against available viral databases, it appeared to share a certain degree of identity with the sequence of VACV A47 protein: ^{190}R Q L *I* L R^{195} (*V* and *I* being two close aliphatic residues). Furthermore, both sequences are flanked by Proline residues, suggesting that the A47 sequence may be exposed in a loop at the surface of the viral protein. A47 (28 kDa), as A20, is expressed early after VACV infection (as predicted from its gene promoter sequence) but its function in the virus life cycle remains unknown. Recently, a $CD8^{+}$ T cell epitope on A47 was identified and characterized (Mathew et al., 2005). The sequence similarity between A47 and peptide aptamer 72 suggests that A47 may interact with A20. Unfortunately, we were not able to detect an interaction phenotype between A47 and A20 in a yeast two-hybrid assay. However, this negative result could be explained by a poor expression level of the A47 two-hybrid construct and/or by a misfolding or mislocalisation of the fusion protein. Moreover, in our hands, A47 was poorly expressed in bacteria as a GST fusion protein.

Viral DNA synthesis was impaired in cells constitutively expressing peptide aptamer 72. This inhibition correlates with a decrease in virus production in those cells (Figs. 4 and 5). Although the inhibitory activity of Apt 72 in the virus yield assay is weak, it was significant and reproducible. One reason that may explain the low inhibitory effect of this aptamer is its limited expression in the stable cell line. Furthermore, the level of peptide aptamer expression may be decreased upon virus infection since VACV is known to shut off host protein synthesis (Moss, 2007).

Our results suggest that peptide aptamer 72 binds to a domain of A20 that is critical for its function in viral DNA polymerase activity. Interestingly, two independent reports highlighted the importance of this domain of A20 (Ishii and Moss, 2001; Punjabi et al., 2001). Using an approach in which clusters of three or four charged residues are changed to alanine (i.e. clustered charge-to-alanine mutagenesis), mutants of A20 were generated. Recombinant VACV viruses carrying the mutated A20R gene were then recovered and characterized. Strikingly, several recombinant viruses containing mutations in the region between AA 167 and AA 227 of A20 could not be rescued suggesting that these particular mutations are lethal to the virus (Ishii and Moss, 2001; Punjabi et al., 2001). In contrast, other recombinant viruses with mutations either at the N- or C-terminus of A20 were viable. Overall, these results and our data delineate a region of A20 that is critical for its function in DNA polymerase activity.

Peptide aptamer 72 is enriched with positively charged arginine residues suggesting that its interaction with the highly (mainly) negatively charged domain of A20 may be electrostatic. Further experiments will focus on the precise mapping of the aptamer binding surface of A20, by an AptaPrint approach (Pamonsinlapatham et al., 2008). Peptide aptamer 72 provides a promising tool for the discovery of new compounds targeting specifically the poxvirus replication complex. It could itself be developed as a therapeutic molecule. Indeed, by fusing the thioredoxin scaffold to a protein transduction domain, consisting of nine arginine residues, peptide aptamers can be introduced into cultured cells and can inhibit their protein targets (Borghouts et al., 2008). Alternatively, peptide aptamer 72 could be used as guide for the discovery of small molecules through a yeast two-hybrid peptide aptamer displacement screening assay (Baines and Colas, 2006). Developing inhibitors that bind to a precise domain of a protein involved in DNA synthesis may represent an alternative to the more classical screen for inhibitors of the viral DNA polymerase activity.

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References

Abed, N., Bickle, M., Mari, B., Schapira, M., Sanjuan-Espana, R., Robbe Sermesant, K., Moncorgé, O., Mouradian-Garcia, S., Barbry, P., Rudkin, B.B., Fauvarque, M.O., Michaud-Soret, I., Colas, P., 2007. A comparative analysis of perturbations caused by a gene knock-out, a dominant negative allele, and a set of peptide aptamers. Mol. Cell. Proteomics 6, 2110–2121.

Andrei, G., Gammon, D.B., Fiten, P., De Clercq, E., Opdenakker, G., Snoeck, R., Evans, D.H., 2006. Cidofovir resistance in vaccinia virus is linked to diminished virulence in mice. J. Virol. 80, 9391–9401.

Baines, I.C., Colas, P., 2006. Peptide aptamers as guides for small-molecule drug discovery. Drug Discov. Today 11, 334–341.

Bickle, M.B., Dusserre, E., Moncorgé, O., Bottin, H., Colas, P., 2006. Selection and characterization of large collections of peptide aptamers through optimized yeast two-hybrid procedures. Nat. Protoc. 1, 1066–1091.

Blasco, R., Moss, B., 1991. Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-Dalton outer envelope protein. J. Virol. 65, 5910–5920.

Borghouts, C., Kunz, C., Delis, N., Groner, B., 2008. Monomeric recombinant peptide aptamers are required for efficient intracellular uptake and target inhibition. Mol. Cancer Res. 6, 267–281.

Boyle, K.A., Arps, L., Traktman, P., 2007. Biochemical and genetic analysis of the vaccinia virus d5 protein: multimerization-dependent ATPase activity is required to support viral DNA replication. J. Virol. 81, 844–859.

Butz, K., Denk, C., Fitscher, B., Crnkovic-Mertens, I., Ullmann, A., Schröder, C.H., Hoppe-Seyler, F., 2001. Peptide aptamers targeting the hepatitis B virus core protein: a new class of molecules with antiviral activity. Oncogene 20, 6579–6586.

Butz, K., Denk, C., Ullmann, A., Scheffner, M., Hoppe-Seyler, F., 2000. Induction of apoptosis in human papillomaviruspositive cancer cells by peptide aptamers targeting the viral E6 oncoprotein. Proc. Natl. Acad. Sci. U.S.A. 97, 6693–6697.

Colas, P., 2008. The eleven-year switch of peptide aptamers. J. Biol. 7, 2.

Colas, P., Cohen, B., Jessen, T., Grishina, I., McCoy, J., Brent, R., 1996. Genetic selection of peptide aptamers that recognize and inhibit cyclin-dependent kinase 2. Nature 380, 548–550.

Colas, P., Cohen, B., Ko Ferrigno, P., Silver, P.A., Brent, R., 2000. Targeted modification and transportation of cellular proteins. Proc. Natl. Acad. Sci. U.S.A. 97, 13720–13725.

Cresawn, S.G., Condit, R.C., 2007. A targeted approach to identification of vaccinia virus postreplicative transcription elongation factors: genetic evidence for a role of the H5R gene in vaccinia transcription. Virology 363, 333–341.

De Clercq, E., 2003. Clinical potential of the acyclic nucleoside phosphonates cidofovir, adefovir, and tenofovir in treatment of DNA virus and retrovirus infections. Clin. Microbiol. Rev. 16, 569–596.

De Silva, F.S., Lewis, W., Berglund, P., Koonin, E.V., Moss, B., 2007. Poxvirus DNA primase. Proc. Natl. Acad. Sci. U.S.A. 104, 18724–18729.

Ferrier, A., Garin, D., Crance, J.M., 2004. Rapid inactivation of vaccinia virus in suspension and dried on surfaces. J. Hosp. Infect. 57, 73–79.

Geddes, A.M., 2006. The history of smallpox. Clin. Dermatol. 24, 152–157.

Geyer, C.R., Brent, R., 2000. Selection of genetic agents from random peptide aptamer expression libraries. Methods Enzymol. 328, 171–208.

Hoppe-Seyler, F., Crnkovic-Mertens, I., Tomai, E., Butz, K., 2004. Peptide aptamers: specific inhibitors of protein function. Curr. Mol. Med. 4, 529–538.

Ishii, K., Moss, B., 2001. Role of vaccinia virus A20R protein in DNA replication: construction and characterization of temperature-sensitive mutants. J. Virol. 75, 1656–1663.

Ishii, K., Moss, B., 2002. Mapping interaction sites of the A20R protein component of the vaccinia virus DNA replication complex. Virology 303, 232–239.

Klemperer, N., McDonald, W., Boyle, K., Unger, B., Traktman, P., 2001. The A20R protein is a stoichiometric component of the processive form of vaccinia virus DNA polymerase. J. Virol. 75, 12298–12307.

Kornbluth, R.S., Smee, D.F., Sidwell, R.W., Snarsky, V., Evans, D.H., Hostetler, K.Y., 2006. Mutations in the E9L polymerase gene of cidofovir-resistant vaccinia virus strain WR are associated with the drug resistance phenotype. Antimicrob. Agents Chemother. 50, 4038–4043.

Lane, H.C., Montagne, J.L., Fauci, A.S., 2001. Bioterrorism: a clear and present danger. Nat. Med. 7, 1271–1273.

Lopez-Ochoa, L., Ramirez-Prado, J., Hanley-Bowdoin, L., 2006. Peptide aptamers that bind to a geminivirus replication protein interfere with viral replication in plant cells. J. Virol. 80, 5841–5853.

Mathew, A., Terajima, M., West, K., Green, S., Rothman, A.L., Ennis, F.A., Kennedy, J.S., 2005. Identification of murine poxvirus-specific CD8+ CTL epitopes with distinct functional profiles. J. Immunol. 174, 2212–2219.

McCraith, S., Holtzman, T., Moss, B., Fields, S., 2000. Genome-wide analysis of vaccinia virus protein–protein interactions. Proc. Natl. Acad. Sci. U.S.A. 97, 4879–4884.

Moss, B., 2007. Poxviridae: The viruses and their replication. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, vol. 2. Lippincott, Williams, & Wilkins, Philadelphia, pp. 2905–2945 (2 vols.).

Nauenburg, S., Zwerschke, W., Jansen-Durr, P., 2001. Induction of apoptosis in cervical carcinoma cells by peptide aptamers that bind to the HPV-16 E7 oncoprotein. FASEB J. 15, 592–594.

Pamonsinlapatham, P., Hadj-Slimane, R., Raynaud, F., Bickle, M., Corneloup, C., Barthelaix, A., Lepelletier, Y., Mercier, P., Schapira, M., Samson, J., Mathieu, A.L., Hugo, N., Moncorgé, O., Mikaelian, I., Dufour, S., Garbay, C., Colas, P., 2008. A RasGAP SH3 peptide aptamer inhibits RasGAP–Aurora interaction and induces caspase-independent tumor cell death. PLoS ONE 3, e2902.

Parker, S., Touchette, E., Oberle, C., Almond, M., Robertson, A., Trost, L.C., Lampert, B., Painter, G., Buller, R.M., 2008. Efficacy of therapeutic intervention with an oral ether-lipid analogue of cidofovir (CMX001) in a lethal mousepox model. Antivir. Res. 77, 39–49.

Punjabi, A., Boyle, K., DeMasi, J., Grubisha, O., Unger, B., Khanna, M., Traktman, P., 2001. Clustered charge-to-alanine mutagenesis of the vaccinia virus A20 gene: temperature-sensitive mutants have a DNA-minus phenotype and are defective in the production of processive DNA polymerase activity. J. Virol. 75, 12308–12318.

Scaramozzino, N., Ferrier-Rembert, A., Favier, A.L., Rothlisberger, C., Richard, S., Crance, J.M., Meyer, H., Garin, D., 2007. Real-time PCR to identify variola virus or other human pathogenic orthopox viruses. Clin. Chem. 53, 606–613.

Smee, D.F., Sidwell, R.W., Kefauver, D., Bray, M., Huggins, J.W., 2002. Characterization of wild-type and cidofovir-resistant strains of camelpox, cowpox, monkeypox, and vaccinia viruses. Antimicrob. Agents Chemother. 46, 1329–1335.

Stanitsa, E.S., Arps, L., Traktman, P., 2006. Vaccinia virus uracil DNA glycosylase interacts with the A20 protein to form a heterodimeric processivity factor for the viral DNA polymerase. J. Biol. Chem. 281, 3439–3451.

Traktman, P., Anderson, M.K., Rempel, R.E., 1989. Vaccinia virus encodes an essential gene with strong homology to protein kinases. J. Biol. Chem. 264, 21458–21461.

Yang, G., Pevear, D.C., Davies, M.H., Collett, M.S., Bailey, T., Rippen, S., Barone, L., Burns, C., Rhodes, G., Tohan, S., Huggins, J.W., Baker, R.O., Buller, R.L., Touchette, E., Waller, K., Schriewer, J., Neyts, J., De Clercq, E., Jones, K., Hruby, D., Jordan, R., 2005. An orally bioavailable antipoxvirus compound (ST-246) inhibits extracellular virus formation and protects mice from lethal orthopoxvirus challenge. J. Virol. 79, 13139–13149.