The alternate triad motif of the poly(ADP-ribose) polymerase-like domain of the human zinc finger antiviral protein is essential for its antiviral activity

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The human zinc finger antiviral protein (hZAP) gene is spliced to yield a short (hZAP-S) and a long (hZAP-L) isoform. The long isoform possesses a poly(ADP-ribose) polymerase (PARP)-like domain in its C-terminus predicted to be inactive due to alterations in its triad motif compared with bona fide PARPs. Using Sindbis virus as prototype member of alphaviruses we confirmed that hZAP-L is a more potent inhibitor of alphaviruses than hZAP-S. Specific small interfering RNA knockdown of hZAP-L but not hZAP-S demonstrated a role of endogenous hZAP-L in restriction of alphavirus replication. Whilst single amino-acid substitutions in the triad motif of hZAP-L’s PARP-like domain reduced the antiviral activity, exchange of all three triad motif residues to alanine or to the amino acids of active PARPs virtually abolished the antiviral effect. Contrary to previous assumptions, these results indicate an essential function of the PARP-like domain in hZAP-L’s antiviral activity.

Rat (r) and human (h) zinc finger antiviral proteins (ZAPs) have been shown to exhibit antiviral activity against retroviruses (Moloney murine leukemia virus, human immunodeficiency virus type 1 and xenotropic murine leukemia virus-related virus), RNA viruses (alphaviruses and filoviruses) and DNA viruses (murine gammaherpesvirus 68 and hepatitis B virus) in vitro (Bick et al., 2003; Gao et al., 2002; Kerns et al., 2008; Mao et al., 2013; Müller et al., 2007; Wang et al., 2012; Xuan et al., 2012; Zhu et al., 2011). However, this ZAP-associated viral restriction does not appear to result from the induction of a general antiviral state as ZAP failed to inhibit replication of other RNA and DNA viruses (vesicular stomatitis virus, yellow fever virus and herpes simplex virus) (Bick et al., 2003).

The N-terminal part of ZAP is thought to be the major functional domain with regard to antiviral activity. It comprises four CCCH-type zinc finger motifs and mutations within the motifs abolish ZAP’s antiviral activity (Guo et al., 2004; Mao et al., 2013; Müller et al., 2007). It was shown that the zinc finger motifs bind to so-called ZAP-responsive elements (ZREs) (Guo et al., 2004). These ZREs have been mapped to certain regions within the genomes of different ZAP-sensitive viruses; however, the features that determine a sequence to be a ZRE are still unknown. Interaction of ZAP with the ZRE results in degradation of the viral RNA by recruiting different host RNA helicases and the RNA exosome (Chen et al., 2008; Guo et al., 2007; Ye et al., 2010; Zhu et al., 2011; Zhu & Gao, 2008). Additionally, ZAP has been shown to also mediate translational repression of target mRNAs (Zhu et al., 2012).

The hZAP gene is spliced to yield a short (hZAP-S) and a long (hZAP-L) isoform. Unlike IFN-α-induced hZAP-S, the long isoform is expressed constitutively and fails to potentiate signalling of the RNA helicase RIG-I (Hayakawa et al., 2011). Whilst both isoforms contain the N-terminal zinc finger motifs, hZAP-L contains an additional C-terminal poly(ADP-ribose) polymerase (PARP)-like domain (Kerns et al., 2008). The latter exhibits three characteristic motifs each composed of an amino acid triplet. From each triplet, a single residue contributes to a conserved triad motif. hZAP-L exhibits a stronger antiviral activity than hZAP-S against Semliki Forest virus infection and Moloney murine leukemia virus expression (Kerns et al., 2008). The enhanced antiviral activity is in line with the positive selection found in the PARP-like domain of primate orthologues of ZAP-L (Kerns et al., 2008). For murine (m) ZAP-L, it was shown recently that the increased antiviral activity is attributed to S-farnesylation, as mutation of the S-farnesylation motif localized at the C-terminus of mZAP-L reduced the inhibitory effect against Sindbis virus (SINV) to the level of mZAP-S (Charroin et al., 2013). However, the three residues affected mainly by the positive selection of ZAP-L’s PARP-like domain were close to the first two residues of the triad motif typical of proteins of the PARP-like family. Therefore, it was hypothesized that the triad motif region might play an important role in hZAP-L’s antiviral activity (Kerns et al., 2008). In general, proteins of
the PARP family can be divided into three subfamilies: (i) PARPs characterized by the typical H-Y-E triad motif possessing PARP activity; (ii) PARPs lacking the glutamate in the triad motif and only possessing mono-ADP-ribosyltransferase activity; and (iii) PARPs lacking the glutamate as well as the histidine residue important for $\beta$-NAD$^+$ cofactor binding, and therefore thought to be catalytically inactive (Kleine et al., 2008). hZAP-L, as well as PARP9, belongs to the third subfamily of PARP-like proteins as it encodes a Y-Y-V motif, or Q-Y-T motif, respectively. PARP9 has been reported to be catalytically inactive (Aguiar et al., 2005) and the PARP-like domain of hZAP also lacked catalytic activity when assayed in vitro (Kleine et al., 2008). This raised the question whether a presumably inactive domain can influence the antiviral activity of hZAP-L.

As a prerequisite to study hZAP isoforms, including the role of the alternate triad motif in the PARP-like domain, we cloned hZAP-L and hZAP-S with a C-terminal hemagglutinin (HA)-tag into the vector pcDNA5/FRT/TO (Invitrogen) (Supplementary Methods, available in the online Supplementary Material). Using the Flp-In T-REx system (Invitrogen), T-REx-293 cells inducibly expressing C-terminally HA-tagged hZAP-L or hZAP-S were established according to the manufacturer’s instructions using 200 $\mu$g hygromycin B ml$^{-1}$ (PAA) for selection. Expression after induction with 1.5 $\mu$g doxycycline ml$^{-1}$ (PAA) was confirmed by Western blot analysis using an anti-HA antibody (1 : 3000; Sigma) (Fig. 1a). Comparable levels of both isoforms were expressed without affecting cellular viability as assayed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay 24 and 96 h post-induction (Fig. 1b; data not shown) (Mosmann, 1983).

It has been proposed that the viral antagonist(s) that drove the evolution of ZAP in primates resulting in positive selection of the PARP-like domain of ZAP-L was a member of the family Togaviridae (Kerns et al., 2008). Hence, we performed our experiments with members of this virus family. Induced and non-induced Flp-In T-REx hZAP-L and hZAP-S cells were infected with SINV derived from pToto1101 as prototype alphavirus (m.o.i. 0.1) (Rice et al., 1987). At defined time points post-infection (p.i.), viral titres were determined by plaque assay on baby hamster kidney (BHK) cells stained with crystal violet (Gläsker et al., 2013). Whilst hZAP-L inhibited SINV by ~4 log units when compared with non-induced cells, inhibition by hZAP-S was only of the order of 2 log units (Fig. 1c). At a lower m.o.i. (0.01), SINV was even inhibited ~6 log units at

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**Fig. 1.** (a) Inducible hZAP expression. Lysates of stably transfected Flp-In T-REx-293 cells were examined 24 h post-induction for inducible expression of HA-tagged hZAP-L or hZAP-S. Expression was detected by Western blot analysis using anti-HA antibody. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a loading control. (b) Cell viability of overexpressing hZAP cells. The quantity of viable cells was assessed 24 h after induction of hZAP expression by MTT reduction assay. The percentage viability was normalized to the respective non-induced cells. Data represent means ± SD of experiments performed in quadruplicate. Black bars, + doxycycline; white bars, −doxycycline. (c) Growth kinetics of SINV on induced (+ doxycycline , ▲) and non-induced (−doxycycline , ○) T-REx-293-hZAP-L or T-REx-293-hZAP-S cells. After infection at m.o.i. 0.1, viral titres were determined by plaque assay on BHK cells with cell culture supernatants collected at defined time points p.i. Data represent means and ranges of duplicate infection experiments; error bars for some points are obscured by the symbols.
72 h.p.i. by hZAP-L and in the range of 3-4 log units by hZAP-S (data not shown). The increased inhibition by hZAP-L is consistent with that observed for another alphavirus, Semliki Forest virus (Kerns et al., 2008). Similarly, hZAP-L also inhibited Chikungunya virus more than hZAP-S, albeit the level of inhibition by hZAP-L was only ~1 log unit even at the lower m.o.i. of 0.01 (data not shown). Together, this corroborates that hZAP-L is a more potent inhibitor of alphaviruses than hZAP-S.

To further assess the antiviral potential of endogenous hZAP-L on alphavirus growth, we performed a small interfering (si) RNA knockdown experiment. In previously described hZAP knockdown studies the target region was localized in the N-terminal part of hZAP, thereby resulting in knockdown of both hZAP-L and hZAP-S (MacDonald et al., 2007; Mao et al., 2013; Wang et al., 2012; Zhu et al., 2011). However, we aimed to selectively knockdown endogenous hZAP-L without affecting hZAP-S expression. To this end, a reverse-forward transfection at a 48 h interval (Yi et al., 2011) was carried out in six-well format with 12.5 pmol siRNA (Ambion) targeting the PARP-like domain region of the hZAP-L mRNA not present in the hZAP-S mRNA (5′-GAAUCUAUCUGUUCCGAAUA-3′) using 4.0 μl Lipofectamine RNAiMAX (Invitrogen) reagent for 2.5 x 10^5 T-REx-293 cells. Transfection with a scrambled siRNA (Ambion), as well as untreated cells, was used as control. At 48 h after the forward transfection, knockdown of hZAP-L was confirmed by Western blot analysis using an anti-hZAP antibody (anti-ZCCHV, 1:2500; Abcam) (Fig. 2a).

Although the anti-hZAP antibody has been proven to detect overexpressed hZAP-S via Western blotting, this method was not sensitive enough to detect endogenous hZAP-L in the T-REx-293 cells (data not shown). Hence, we analysed the specificity of the knockdown by real-time PCR detecting specifically either hZAP-L or hZAP-S mRNA. Total cellular RNA was isolated using PeqGOLD TriFast (Peqlab) 48 h after knockdown, of which 1500 ng was applied to real-time PCR analysis. Reactions for both hZAP-L and hZAP-S were carried out in a single tube using the SuperScript III One-Step reverse transcription (RT)-PCR system with Platinum Taq DNA polymerase (Invitrogen), and primers and probes (Biomers) targeting specifically the respective hZAP mRNA. Each 12.5 μl reaction mix contained 0.4 μM primer hZAP-L-F (5′-TCCGACGCTGTTGATCAGCA-3′), 0.4 μM hZAP-L-R (5′-TCACATATGTTGGGTTAACAATGATC-3′) and 0.2 μM probe hZAP-L-P (5′-FAM-TCCAGATCCCTCGTTTGTGCATCTTGCAT-3′) plus 0.4 μM hZAP-S-F (5′-GAGCTTCTTCTGCTAGATAA-3′), 0.4 μM hZAP-S-R (5′-TCAGGGAAACAGGCTTGCAACATGATC-3′) and 0.2 μM probe hZAP-S-P (5′-YAKCATGCCTGGGCTCATGTAAGTC-CAT-3′). Thermocycling was performed on a LightCycler 480 (Roche) programmed for: 20 min at 55 °C, 3 min at 95 °C, and 45 PCR cycles of 15 s at 95 °C and 20 s at 58 °C. Samples were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels also determined via real-time PCR (Pfefferle et al., 2009). Photometrically quantified in vitro transcripts of the target regions were used in the PCR to generate a standard curve for RNA quantification. Quantification revealed that hZAP-S mRNA was unaffected by the knockdown, whereas the hZAP-L mRNA...
concentration was reduced significantly (P<0.05, one-way ANOVA followed by Tukey’s test) (Fig. 2b). To analyse the effect of endogenous hZAP-L reduction on alphavirus replication, hZAP-L knockdown and control cells (control siRNA cells or untreated cells) were infected with SINV (m.o.i. 0.01). Viral titres determined at 24 h p.i. revealed a 2 log increased growth of SINV after hZAP-L knockdown, demonstrating that endogenous hZAP-L plays a distinct and important role in alphavirus restriction in human cells (Fig. 2c).

We next wanted to analyse whether the alternate triad motif in the PARP-like domain of hZAP-L is important for its antiviral activity despite being assumed to be inactive. Mutations were made in the pcDNA5/FRT/TO-hZAP-L plasmid leading to either single- or multiple-amino-acid exchanges in the alternate triad motif Y$_{87}$-Y$_{119}$-V$_{876}$ (Fig. 3a). The mutations were introduced by fusion PCR technology using specific mutant primers and standard cloning methodology (Supplementary Methods and Table S1). First, amino acids of the triad motif were exchanged to alanine. Using the Flp-In T-REx-293 system, cell lines inducibly expressing the HA-tagged hZAP-L mutants were established as described above. Western blot analysis of induced mutant cell lines confirmed comparable expression levels for the hZAP-L variants (Fig. 3b).

To analyse the effect of the hZAP-L mutations on the antiviral activity of hZAP-L, the respective induced and non-induced cell lines were infected with SINV (m.o.i. 0.1). In comparison with Fig. 1(c), the level of SINV inhibition was increased slightly for WT hZAP-L (Fig. 3c). As the level of SINV inhibition by hZAP-L varied to a certain extent when different m.o.i. levels were used, this indicates a slightly lower actual infectivity here compared with Fig. 1(c). Those slight variations might result from the use of a new virus stock. Hence, after all mutants had been tested in subsets, all infections shown in Fig. 3(c) were performed in parallel using the same premixed virus inoculum to ensure comparability. At defined time points p.i., viral titres were determined by plaque assay on BHK cells as described previously. As can be seen in Fig. 3(c), exchange of the first or second amino acid in the triad motif to alanine resulted in only a slight (A-Y-V) or no reduction (Y-A-V) of hZAP-L antiviral activity, whilst exchange of the third amino acid in the motif (Y-Y-A) clearly diminished the antiviral effect of hZAP-L. Exchange of all amino acids of the triad motif to alanine (A-A-A) virtually completely knocked down the antiviral activity of hZAP-L and to a level far below that seen for hZAP-S (Figs 3c and 1c).

As our results for the alanine PARP-like domain mutants were unexpected, we further tested whether exchanging the Y-Y-V motif of hZAP-L to a corresponding motif found in bona fide PARPs (H-Y-E) would retain or even enhance the antiviral activity of hZAP-L. Therefore, the Y-Y-V motif of hZAP-L was either exchanged to H-Y-V, Y-Y-E or H-Y-E, with the latter variant representing the triad motif of catalytically active PARPs (Hottiger et al., 2010; Kleine et al., 2008). These PARPs all possess a conserved glycine next to the histidine of the first triplet motif (Hottiger et al., 2010). Hence, for the H-Y-V and H-Y-E mutants, the corresponding alanine of the first triplet motif in hZAP-L was also exchanged to glycine (Fig. 3a, Table S1). Monitoring the levels of the induced hZAP-L variants in the Flp-In T-REx-293 system by Western blot analysis proved comparable expression levels (Fig. 3b). Again, viral titres were determined for infected induced and non-induced Flp-In T-REx cells expressing the hZAP-L mutants. Interestingly, whilst the exchange of the third amino acid in the triad motif to glutamate (Y-Y-E) did not affect the antiviral capacity of hZAP-L, exchange of the first amino acid to histidine (H-Y-V) noticeably diminished the antiviral activity (Fig. 3c). Strikingly, introduction of the typical H-Y-E motif abolished the antiviral effect of hZAP-L similar to what was seen for the A-A-A mutant (Fig. 3c).

Both multiple mutants also no longer inhibited Chikungunya virus (data not shown). Together, this indicates that the alternate triad motif of hZAP-L is essential for the antiviral activity.

Notably, all hZAP-L mutants still contain the unaltered four CCCH-type zinc finger motifs, which are thought to have the major functional role with regard to antiviral activity (Guo et al., 2004), and which are also present in hZAP-S. Nevertheless, the inhibitory activities for the hZAP-L triad motif mutants A-A-A and H-Y-E were virtually completely abrogated (Fig. 3c). Thus, these mutations rendered the hZAP-L antiviral activity less effective than that of hZAP-S, which completely lacks a PARP-like domain (Figs 1c and 3a). Hence, the loss of antiviral activity observed for the multiple triad motif mutants might be linked to structural rearrangements affecting the accessibility of the zinc finger motifs rather than a change of subcellular localization since preliminary data indicate that WT and A-A-A mutant HA-tagged hZAP-L possess comparable subcellular localization (data not shown). Interestingly, mutation of the S-farnesylation motif of mZAP-L reduced the antiviral activity to the level observed for mZAP-S (Charron et al., 2013). hZAP-L also possesses a CaaX S-farnesylation motif at its C-terminus. However, in our case an HA-tag was fused to the C-terminus, which most likely would prevent a possible S-farnesylation since it is the cysteine residue at precisely the −4 position from the C-terminus that is modified (Winter-Vann & Casey, 2005). Hence, hZAP-L seems to be more active than hZAP-S independent of S-farnesylation. In general, it will be of interest to perform a more detailed comparison between mZAP and hZAP also, as mZAP in contrast to hZAP possesses a H-Y-V triad motif in its PARP-like domain and therefore still should possess mono-ADP-ribosyltransferase activity (Hottiger et al., 2010).

It was especially interesting that the exchange of the Y-Y-V motif to the triad motif of catalytically active PARPs also negatively affected the antiviral activity of hZAP-L. A similar observation was obtained for PARP10,
Fig. 3. (a) Schematic representation of hZAP-S and hZAP-L protein domains. Positions of the triad motif amino acids in hZAP-L’s PARP-like domain are marked by arrows. The table indicates the three triplet motifs with the resulting triad motif of hZAP-L and bona fide PARPs. The asterisks mark the alanine–glycine exchange also performed in H-Y-V and H-Y-E mutants. (b)
a mono-ADP-ribosyltransferase comprising an H-Y-I triad motif (Kleine et al., 2008). Exchanging the triad motif to H-Y-E resulted in loss of mono-ADP-ribosyltransferase activity rather than in acquiring polymerase activity. However, in contrast to PARP10, hZAP-L is supposed to not even possess mono-ADP-ribosyltransferase activity and the detailed function of the presumably catalytically inactive PARP-like domain still remains obscure. Nevertheless, the fact that mutations in the alternate triad motif affect or even abolish the antiviral activity of hZAP-L demonstrates the importance of this domain in antiviral activity, particularly in restricting alphaviruses in humans.

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References


