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Short communication

Protein A33 responsible for antibody-resistant spread of Vaccinia virus is homologous to C-type lectin-like proteins

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ABSTRACT

Protein A33 is a type II membrane protein present in the outer envelope of extracellular as well as cell-associated Vaccinia virus particles. A33 has been implicated in mediating cell-to-cell virus spread in an antibody-resistant manner. Here, using state-of-the-art structure prediction methods and structural modeling, we show that A33 has most likely evolved from a C-type lectin-like protein. Comparison of the three-dimensional A33 model to the X-ray structures of distant cellular homologues revealed that A33 retained the key residues required for adopting the C-type lectin-like fold. Our results provide insights into the structure and origin of protein A33.

Vaccinia virus (VACV), the most extensively studied member of the *Poxviridae* (Condit et al., 2006; Moss, 2007; Roberts and Smith, 2008), uses at least two mechanisms to move between cells in an antibody (Ab)-resistant way (Law et al., 2002). The first pathway is dependent on the VACV-induced polymerization of actin underneath the cell-associated enveloped virions (CEV). This leads to formation of long protrusions, actin tails, that propel virions away from the cell surface towards neighboring cells (Frischknecht et al., 1999). VACV can also utilize an actin-independent mechanism for cell-to-cell spread. It has been shown that in the presence of VACV-neutralizing Ab, all VACV mutants that are unable to make actin tails still form plaques, unless gene A33R is deleted (Law et al., 2002). This implicates protein A33 in mediating cell-to-cell spread in an Ab-resistant manner. While formation of actin tails has been studied intensively, understanding of the A33-dependent mechanism of virus spread remains scarce. A33 orthologs are encoded by all chordopoxviruses (Fig. S1) with a notable exception of Fowlpox virus (genus *Avipoxvirus*; Gubser et al., 2004). Importantly, the A33 ortholog in variola virus (VARV), the causative agent of smallpox, is 94.1% identical to that of VACV (Massung et al., 1994). This suggests that VARV might also rely on the A33-mediated Ab-resistant mechanism of cell-to-cell spread. The information on the function of A33 is therefore of great importance.

Previous analyses indicate that A33 of VACV is a 185 amino acid-long type II membrane protein residing in the outer envelope of extracellular as well as cell-associated VACV particles (Roper et al., 1996). A33 is expressed early as well as late in infection and is heavily post-translationally modified: N- and O-glycosylated (Payne, 1992; Roper et al., 1996), phosphorylated (Wolffe et al., 2001), and palmitoylated (Grosenbach et al., 2000). A33 has also been shown to form disulphide-linked homodimers (Roper et al., 1996) and to interact with VACV proteins A36 (Wolffe et al., 2001) and B5 (Perdiguero and Blasco, 2006). Bioinformatic investigations have previously yielded valuable information on VACV proteins (Da Silva et al., 2006; Da Silva and Upton, 2009). Therefore, to get further insights into the function and structure of VACV A33 we undertook a bioinformatic approach.

In order to identify distant homologues of VACV A33 we first used PSI-BLAST searches (Altschul et al., 1997) against non-redundant protein database at NCBI using A33 sequence (accession number: AAO89435) as a seed. The search reached convergence after two PSI-BLAST iterations. No non-viral A33 homologues were identified. To identify possible structural homologues, we submitted the VACV A33 sequence to the BioInfoBank MetaServer (Ginalski et al., 2003). A number of hits to C-type lectin-like domain (CTLD)-containing proteins were retrieved, albeit with the score just below the significance threshold (49.75; scores above 50 are considered significant; Ginalski et al., 2003). The predicted homology region was within the C-terminal part of A33 (residues 98–185; Fig. 1A). This region is relatively well conserved in all chordopoxvirus A33 orthologs (Fig. S1). The A33 sequence predicted to be homologous to CTLDs was then submitted to the BioInfoBank MetaServer. Again, hits to various lectin proteins were retrieved, this time with significant scores (ranging from 51.0 to 52.8). Notably, a similar strategy has recently allowed Wyrwicz et

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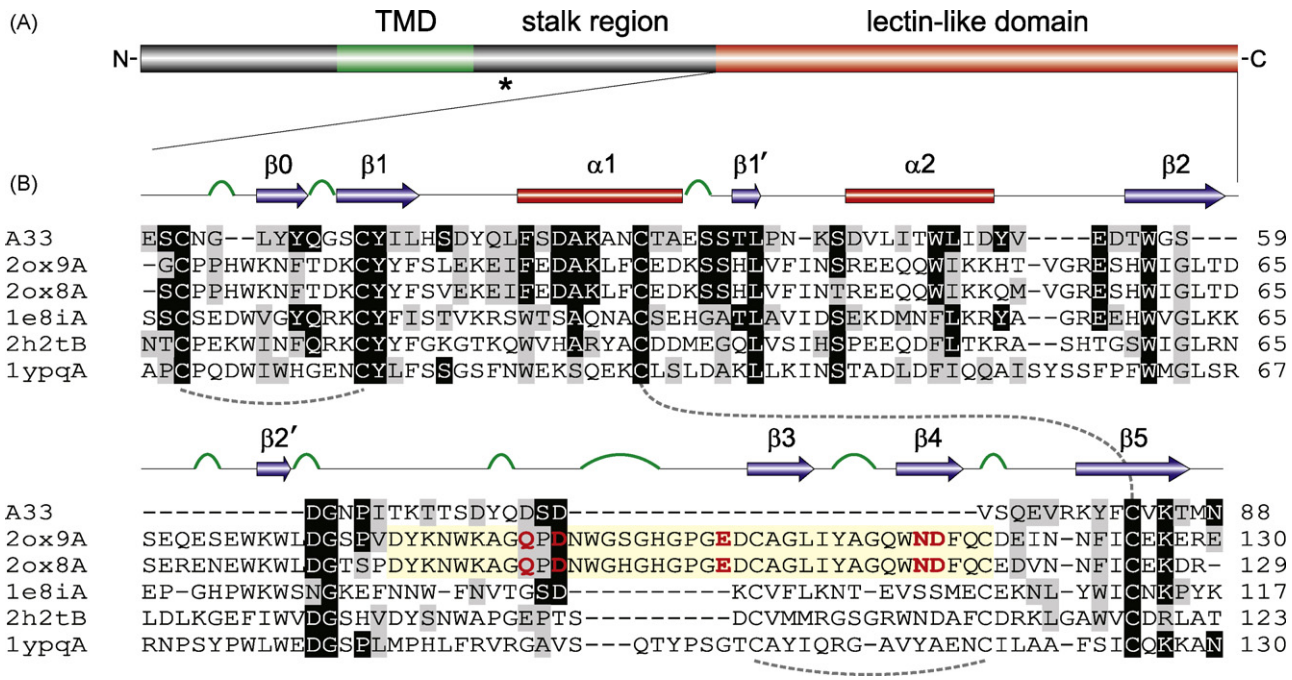

Fig. 1. Sequence analysis of VACV A33. (A) Domain organization of A33. Transmembrane domain (TMD; green), the C-type lectin-like domain (CTLD; red) and the stalk region are indicated. Position of the Cys62 which is likely to be involved in disulfide-linked A33 homodimer formation is indicated by an asterisk. (B) Alignment of the putative CTLD sequence of VACV A33 to those of cellular proteins solved by X-ray crystallography (sequences are named by their PDB identifiers). Residues that are identical or similar in the VACV A33 and in at least one cellular CTLD sequence are boxed in black or gray, respectively. Secondary structure elements calculated from the X-ray structure of mouse scavenger receptor (PDB id: 2OX9) are shown above the alignment with α helices, β strands, and turns represented by red rectangles, blue arrows, and green bulges, respectively. Regions forming the sugar-binding site of mouse (2OX9) and human (2OX8) scavenger receptors are shaded yellow with residues involved in coordination of calcium ions being in red. The three disulfide bonds are shown by dotted lines. (For interpretation of the references to color in the citation of this figure, the reader is referred to the web version of the article.)

al. (2008) to recognize the CTLD fold in Herpes Simplex Virus type I protein UL45.

In order to better understand the relationship of A33 with CTLD-containing proteins we employed homology-based structural modeling of the A33 protein. Based on the significance scores, no strong preference could be given to the identified structural homologues (see above). We therefore utilized multiple template-based modeling. For that, the top five structures (mouse scavenger receptor, PDB accession number 2OX9; human scavenger receptor, 2OX8; early activation antigen CD69, 1E8I; lymphocyte IgE receptor CD23, 2H2T; low density lipoprotein receptor, 1YPQ) predicted to be the closest structural homologues of A33 were aligned using version 9.6 of the MODELLER program (Marti-Renom et al., 2000) and the structure-based sequence alignment was extracted. The latter was used as a template against which the A33 sequence was aligned using MODELLER (Fig. 1B). Of the 88 amino acid residues which were predicted to constitute the CTLD of VACV A33, 68.2% had corresponding amino acids in at least one of the five structures (30 identical and 30 similar residues; Fig. 1B). Assuming that during the course of evolution the CTLD fold in eukaryotes originated once and that all CTLDs are true homologues, our observation indicates that VACV A33 and cellular C-type lectin domain-containing proteins are significantly similar also at the sequence level further supporting the relationship between these proteins.

The CTLD fold typically has two antiparallel β-sheets and two α-helices. The CTLD can be structurally divided into two parts, the upper and the lower one. The lower part is formed of the vertical antiparallel β-sheet (β1 and β5) and the two α-helices (α1 and α2), while the long loop region and the second antiparallel β-sheet (β2, β3, and β4) constitute the upper part of the structure (Zelensky and Gready, 2003, 2005). The characteristic feature of the CTLD fold is the close juxtaposition of the N- and C-termini at the bottom of the structure, which is stabilized by an absolutely conserved α1–β5 disulfide bridge and a set of hydrophobic interactions (Zelensky and Gready, 2003, 2005). The superfamily of extracellular CTLD-containing proteins is extremely diverse structurally and functionally (XVII groups have been proposed; Zelensky and Gready, 2005). The canonical members of the CTLD superfamily are carbohydrate binding type II membrane proteins, that bind substrates in a Ca-dependent manner (Zelensky and Gready, 2005). However, other members of the superfamily, such as CTLD NK cell receptors, interact with protein ligands and accordingly lack residues involved in coordination of calcium ions and carbohydrate binding (Natarajan et al., 2002).

The alignment shown in Fig. 1B was used as a guide for homology-based modeling of A33. One hundred models were generated and then rated based on their GA341 quality scores (generally GA341 scores range from 0.0 [worst] to 1.0 [native-like]) as calculated by MODELLER (Melo and Sali, 2007). Structural models with GA341 scores above 0.98 were selected for further stereochemical quality assessment with ProSA-web (Wiederstein and Sippl, 2007) and the model with the most optimal *Z*-score was selected (Figs. 2, S2). The quality (*Z*) score calculated by ProSA for a given structure is depicted in the context of quality scores computed for all experimentally determined X-ray and NMR structures available in the Protein Data Bank (Wiederstein and Sippl, 2007). Fig. S2 shows that the *Z*-score of the A33 structural model (Fig. 2) is well within the score range calculated for experimentally determined structures (Fig. S2) and is similar to those X-ray structures used to generate the three-dimensional model. This indicates that 88 C-terminal residues of VACV A33 can fold into a C-type lectin-like domain.

Inspection of the alignment (Fig. 1B) and comparison of the three-dimensional models of A33 and other CTLDs (Fig. 2) reveal that A33 lacks residues forming strands β2′, β3 and β4. This suggests that the ancestral A33R gene suffered two large deletions,

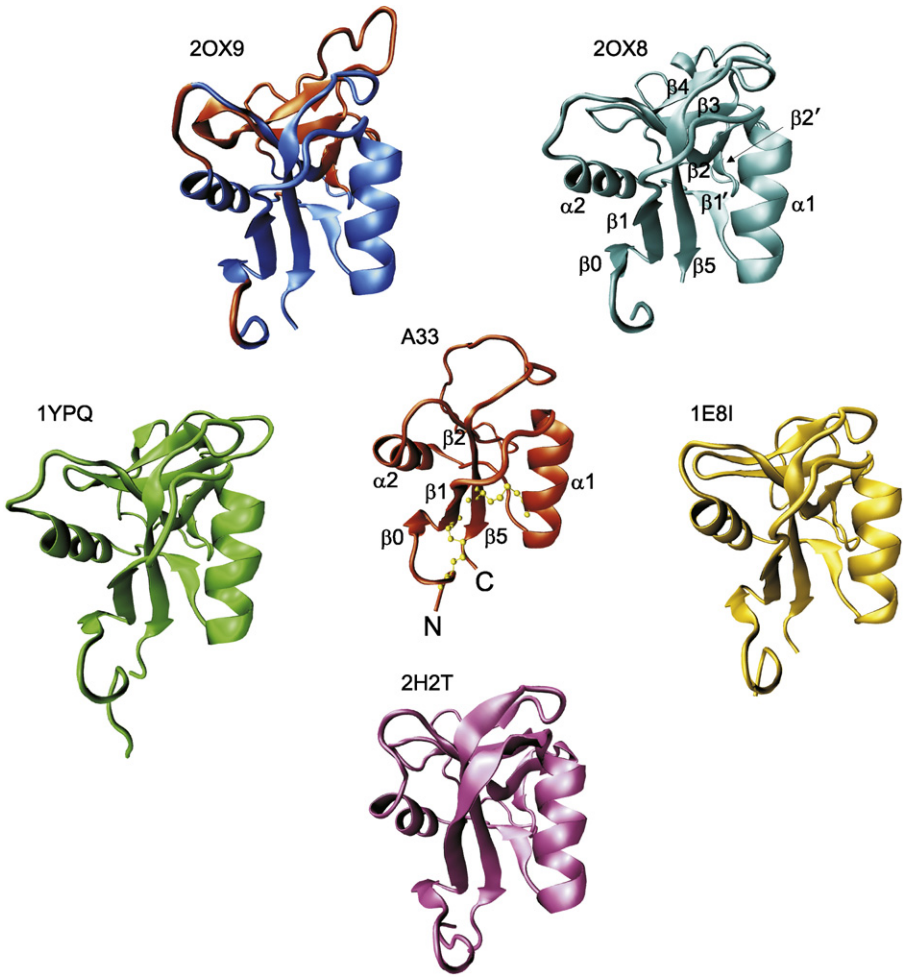

Fig. 2. Comparison of the pseudoatomic model of A33 (shown in red) and X-ray structures of cellular CTLDs (blue—mouse scavenger receptor, 2OX9; cyan—human scavenger receptor, 2OX8; yellow—early activation antigen CD69, 1E8I; mauve—lymphocyte IgE receptor CD23, 2H2T; green—low density lipoprotein receptor, 1YPQ). The two conserved disulfide bonds of A33 are indicated by ball-and-stick representation (yellow). Sequence regions that are absent in A33 but present in the cellular CTLDs are colored red on the structure of the mouse scavenger receptor (2OX9). The nomenclature for the secondary structure elements in (B) and (C) is also indicated (Zelensky and Gready, 2003).

where the first one resulted in the loss of the C-terminal part of the β2 and the entire β2′ strand, while the second one removed the segment encoding strands β3 and β4. Notably, strands β3 and β4 as well as the preceding loop region form the sugar-binding site in canonical C-type lectin proteins (Fig. 1B). For example, in human and mouse scavenger receptors carbohydrate binding is mediated by correctly positioned Asp, Glu, Asn and Gln residues that not only coordinate calcium ions but also act as hydrogen bond donors and acceptors for the galactose moiety of the substrate (Feinberg et al., 2007). Absence of the putative sugar-binding sites, characteristic to canonical CTLDs, suggests that the ligand for A33 is not a sugar moiety but perhaps a protein molecule (see below).

In most CTLDs at the end of the β1′ strand there are two non-polar residues (usually β1′L and β1′V; Zelensky and Gready, 2003). β1′L is conserved in most of the CTLDs and its side chain is paralleled to the β5 strand, thereby forming part of the hydrophobic core. Interestingly, in A33 the equivalent positions are occupied by Leu133 and Pro134, respectively (Fig. 1B). Structural modeling suggests that β1′ strand is not formed in A33 (Fig. 2), although the side chain of Leu133, like in other CTLDs, is parallel to the β5 strand (not shown). Notably, Leu–Pro sequence is also present at the equivalent position of the CTLD domain of bacterial protein intimin, structure of which has been solved by X-ray crystallography (PDB:1F00; Luo et al., 2000). Importantly, the β1′ strand is also missing in intimin. This indicates that β1′ is not essential for proper folding and stability of CTLDs. CTLDs are usually stabilized by two or three disulfide bonds (Fig. 1B). Two of these disulfide bridges (between β0–β1 and α1–β5) are conserved in A33, while the region encompassing the third one (between β3 and β4) is not present in A33. It should be noted that the disulfide bond between β3 and β4 is not absolutely conserved in CTLD-containing proteins (Zelensky and Gready, 2003), suggesting that it is also not essential. The Leu–Pro sequence and the two pairs of cysteins are invariably present in all chordopoxviral A33 orthologs (Fig. S1), pointing to their importance for correct A33 folding.

As mentioned above, A33 of VACV is a disulfide-linked homodimeric type II membrane protein (Roper et al., 1996). Interestingly, the same is true for CTLD NK receptors, such as Ly49, NKG2D, LOX-1 and CD69 (Natarajan et al., 2002; Ohki et al., 2005). The Cys residue mediating the dimerization in these NK cell receptors is situated in the stalk region (Bezouska et al., 1995; Ohki et al., 2005). There is only one Cys residue, Cys62, in the stalk region (residues 57–97) of VACV A33 (Fig. 1A). Consequently, we suggest that this Cys is involved in disulfide-linked A33 homodimer formation. This is in agreement with the observation that a mutant A33 protein, which had nearly half of the CTLD deleted (residues 143–185), was still capable of forming disulfide-linked dimers (Katz et al., 2003). Although the A33 CTLD domain is well conserved, the length of the stalk region in chordopoxvirus proteins varies from 41 residue in the VACV protein to only 17 in the ortholog from Mol-

luscum contagiosum virus (genus *Molluscipoxvirus*; Fig. S1). This suggests that functional differences might be expected between A33-like proteins in viruses from distinct genera. Furthermore, the Cys residue predicted here to be responsible for dimerization is not present in the A33 proteins of poxviruses belonging to the *Yatapoxvirus* genus (Fig. S1). Interestingly, it has been also shown that the truncation of A33 at the C-terminus incapacitated the virus of the actin tail formation as well as led to increased release of cell-associated enveloped virions (CEV) into the medium (Katz et al., 2002, 2003). This indicates that the C-terminal CTLD is essential for the A33 functionality, playing an important role in VACV virulence. Therefore, in order to understand the molecular mechanism behind actin-dependent as well as actin-independent cell-to-cell spread of VACV in an antibody-resistant manner, it is crucial to identify the natural ligand of the A33 CTLD. The characteristics shared between A33 and CTLD NK cell receptors (both are type II membrane proteins, form disulfide-linked homodimers, contain C-terminal CTLD domains and lack Ca^{2+}-binding motifs) suggest that the ligand for A33 is a protein molecule present on the surface of cells susceptible to VACV infection. From analogy with CTLD NK receptors (Natarajan et al., 2002), the long loop region between strands β2 and β5 (Figs. 1 and 2) in each of the two molecules of the A33 dimer is likely to create the ligand-binding surface. This prediction might be used as a guide for future experimental investigations.

A33 is not the only C-type lectin-like protein of VACV. Genes A34R (encoded immediately downstream of A33R) and A40R have also been shown to encode type II membrane proteins with C-terminal CTLDs (Duncan and Smith, 1992; Wilcock et al., 1999). The nonstructural protein A40 was shown to be nonessential for virus replication and virulence (Wilcock et al., 1999). Protein A34, on the contrary, was found to be incorporated into the viral envelope and play an important role in VACV infectivity (McIntosh and Smith, 1996). Interestingly, there are several features common to A33 and A34 (for reviews see Roberts and Smith (2008), Smith et al. (2002); and references therein): (i) both proteins are present in the envelope of the CEV as well as in the cytoplasmic membrane underneath the CEV; (ii) both are required for actin tail formation and interact with protein A36; (iii) disruption of either of the two genes leads to increased amount of extracellular virions, formation of tiny plaques as well as attenuated virulence of the mutant viruses *in vivo*. In addition, we show here that A33, like A34, has a C-terminal CTLD fold. Although A33 does not share detectable sequence similarity with A34 and A40 and cannot be linked to these two proteins by BLAST searches, it is tempting to speculate that genes encoding the three C-type lectin-like proteins of VACV are paralogues that diversified to fulfill different functions during the virus replication cycle. It is likely that deletion of strands β2′, β3 and β4 in the ancestral CTLD of A33 allowed the protein to acquire new substrate specificities and fulfill different/additional functions during the VACV infection cycle.

While this paper was under review, Su et al. (2010) published an X-ray structure of the A33 CTLD domain analyzed in this study. The experimentally determined structure of the A33 CTLD (PDB: 3K7B) is similar to our three-dimensional model of the same domain (see Fig. S3). This verifies the reliability of the state-of-the-art bioinformatic tools for distant homology detection and structural modeling, and highlights the usefulness of their application in virus research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2010.03.004.

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