



Development of orf virus as a bifunctional recombinant vaccine: Surface display of *Echinococcus granulosus* antigen EG95 by fusion to membrane structural proteins

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ABSTRACT

The parapoxvirus, orf virus (ORFV) causes superficial skin lesions in infected sheep. Unattenuated ORFV is used globally to vaccinate against orf. Recombinant poxviruses are proven delivery systems and we investigated strategies to express the immunogenic *Echinococcus granulosus* peptide EG95 from ORFV with the aim of developing a recombinant bivalent vaccine. EG95 is an oncosphere protein of the cestode *E. granulosus*, a parasite responsible for causing cystic hydatid disease in a wide range of hosts including humans and grazing animals such as sheep. Recombinant viruses were produced in which EG95 was expressed by itself or fused to ORFV envelope-associated structural proteins 10 kDa and F1L. Infection studies in sheep showed that specific antibodies were produced against ORFV and EG95 and that the antibody levels against EG95 were comparable to that of animals immunized with purified EG95 in Quil A adjuvant, an immunization regime that is known to afford protection. A single exposure to the dual vaccine has potential for protecting lambs against orf and for priming against EG95 so as to respond strongly to a later injection of EG95 protein.

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1. Introduction

Most monomeric proteins and peptides require chemical coupling to large molecular weight carriers or application with adjuvants in order to elicit a strong immune response. In more recent years, it has been shown using recombinant DNA technology, that the insertion of foreign epitopes into carrier proteins that have multimerisation capacity, such as particle-forming viral capsids or envelope proteins can potentiate their immunogenicity [1]. This approach has been explored further in the development of poxviruses as recombinant vaccines, in which the foreign antigen is fused to a viral structural protein such that the antigen is displayed on the surface of the viral particle. Studies have shown that fusing immunogenic epitopes of human immunodeficiency virus type 1 (HIV-1) with major antigenic envelope proteins of vaccinia virus (VACV) B5R, A27L, or the core protein A4L, elicits antibodies with higher specificity and stronger neutralizing activity than if the HIV-1 proteins were expressed alone [2–4]. Furthermore, the cellular immune response may also be enhanced as a consequence of the virions being taken up by antigen presenting cells [5].

The double-stranded DNA epitheliotropic parapoxvirus, orf virus (ORFV) induces acute superficial and localized skin lesions

in sheep, goats and man. Systemic infection from ORFV has never been demonstrated and its narrow host range, in conjunction with the above features, suggests that it has potential to be developed as a safe recombinant multivalent viral vaccine [6–11]. Furthermore, live unattenuated ORFV is used world-wide to vaccinate against natural ORFV infection and has a good safety profile.

Electron microscopy shows that ORFV has an oval shape and the viral particle appears to be wrapped by a continuous surface protein; the surface tubule protein. Little is known about the morphogenesis of ORFV or its structure at the molecular level, however the complete sequence of the ORFV genome has revealed that it has homologues of many VACV structural proteins despite its substantially smaller genome [12]. Recent studies have shown that the membranes that envelope the viral particle of ORFV are similar to VACV. VACV has predominantly two infectious forms. Mature virus (MV) is the predominant form in which the immature virus particle derives its membrane from the intermediate compartment [13–15]. In addition, wrapped virions are also produced in which additional membranes derived from the trans-Golgi network wraps the MV [16,17]. In VACV, specific structural proteins are associated with each form and that also appears to be the case with ORFV. The membrane-associated proteins of ORFV that have been most studied are the 10-kDa protein (ORF-104) [18] and F1L (ORF-059) with a predicted molecular mass of 36.7 kDa. These membrane proteins are specifically associated with the MV form of ORFV [19]. The 10-kDa protein is essential for the formation of an intact surface

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tubule that wraps the virus particle [20] but it is not known whether it is incorporated into the surface tubule. F1L is a major immunodominant antigen in ORFV. Strong immune responses have been detected in ORFV-infected sheep against F1L [21] and ORFV neutralizing antibodies have been produced against this protein in rabbits [22]. These observations, in conjunction with a previous observation in which it was shown that a small octapeptide FLAG can be displayed on the surface of ORFV by fusion to F1L and 10-kDa, suggested that these structural proteins are candidates for surface display of larger immunogenic peptides such as the well characterised *Echinococcus granulosus* antigen, EG95.

Cystic hydatid disease is a chronic disease in sheep caused by the cestode *E. granulosus*. The parasite has a ubiquitous distribution prevalent in pastoral areas where sheep and dogs, the intermediate and definitive hosts respectively, co-exist in close proximity. Humans may also unwittingly become infected, leading to the development of cysts in major organs. This occurs when the cestode eggs that are carried on dogs are accidentally ingested by humans. In such cases, the overall losses incurred from decreased livestock production together with the costs of treating the infections in humans pose a huge economic impact [23].

EG95, a native oncosphere protein of the parasite was identified as a protective antigen and may be involved in oncosphere attachment to the host intestinal mucosa [24,25]. This structure is surrounded by a membrane that can be ruptured by antibody-dependent, complement-mediated lysis [26]. Immunization studies in sheep using a recombinant protein GST-EG95, in which two doses of the antigen were administered, have shown that it is highly efficacious, affording between 96% and 98% protection in challenge experiments with *E. granulosus* eggs and protection is mediated by antibodies that function in the complement-mediated lysis of oncospheres *in vitro* [27,28].

In this study, we explored whether it was possible to display the 16.8 kDa EG95 peptide on the surface of ORFV as a strategy to develop a bivalent vaccine. Recombinants were constructed in which the EG95 peptide was fused to the membrane structural proteins 10-kDa or F1L and the fusion genes expressed from a non-essential region of the ORFV genome. In addition EG95 by itself was expressed from the same locus for comparison with surface display. We found that the fusion proteins were abundantly incorporated into viral particles. Experimental infection of sheep with the recombinant viruses showed that antibodies were produced against EG95.

2. Materials and methods

2.1. Cells and virus

ORFV strain NZ2 [29] was propagated in lamb testis (LT) cells using Eagle's minimal essential medium containing 10% foetal calf serum.

2.2. Antibodies

The antibodies used for Western blotting were 8D7, an anti-F1L monoclonal antibody (mAb) [21] (a gift from Dr. Peter Nettleton, Moredun Research Institute, UK), MAB6, an anti-EG95 mAb (a gift from Dr. David Heath, AgResearch, Palmerston North, NZ) and rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (HRP) (Dakocytomation). Immunogold labeling antibodies were MAB6, mouse anti-FLAG mAb (Sigma) and goat anti-mouse IgG conjugated to ultra-small gold particles (Aurion). The secondary antibody used in ELISA was rabbit anti-sheep Ig-HRP (Dakocytomation).

2.3. Construction of recombinant ORFVs

The intergenic locus between ORFV IL-10 and ORFV 128 of the ORFV NZ2 strain was used to insert the EG95 gene or the EG95 sequence fused to either the structural genes F1L or 10 kDa [18]. This locus is contained within the restriction endonuclease fragment KpnI-E [30]. Plasmid constructs (see Fig. 2) were made to insert the above genes into ORFV by homologous recombination. Initially a plasmid was made that contained the left arm and right arm sequences flanking the intergenic site inserted into pSP70 (Promega). The 1417 bp left arm spanned 823 bp upstream to 33 bp downstream of the viral IL-10 coding region. The right arm spanned 1474 bp, starting immediately where the left arm terminated, that is, 34 bp downstream of viral IL-10.

The full-length EG95 sequence [25] was inserted immediately upstream of a β -glucuronidase (GUS) reporter gene between the two arms derived from ORFV. The EG95 gene was directed by a synthetic early/late poxvirus promoter sequence (5'-TTTTTTTTTTTTTTTTTGGCATATAAATAAGTCGGGGCCCAAAATTGAAAACTATTCTAATTTATTGCACGG-3') [31] and the GUS gene was driven by the VACV H5 promoter, PH5 [32]. This plasmid (pV41) was used to produce recombinant ORFV ov2.EG1.

Constructs containing the ORFV structural genes fused to EG95 were made by utilizing the ov2.EG1 plasmid construct pV41. The 10-kDa and F1L genes were PCR amplified from ORFV NZ2 DNA [33]. The fusion of the F1L or 10-kDa gene sequences to the EG95 sequence was constructed in such a manner so as to delete the C-terminal hydrophobic domain of EG95. The above fusions were inserted into a utility plasmid that was derived from pV41 by deletion of the EG95 gene.

10-kDa (containing a FLAG tag at its N-terminus) was PCR amplified using primers 5'-CGCGGATCCGAATTCTCCGAGACTACAAGGACGACGATGACAAG-3' and 5'-CGCAGATCTCTAATA-TCTGCTGTATCTGT-3' and cloned into the BamHI and BglII sites of the utility plasmid, upstream of PH5/GUS to produce plasmid 699. The construct was used to make ORFV recombinant 699.

A second construct for the 10-kDa fusion was made that incorporated a flexible linker between the EG95 Δ TM and FLAG-10 kDa sequences. The linker sequence consisted of glycine and serine residues (GGGS)₃ [34]. The linker was made by annealing two oligonucleotides 5'-GATCCTGTGGTGGCGGTGCAGCGGCGGAGGTGGGTCCGG TGGCGGCGGATCTT-3' and 5'-CCGG-AAGATCCGCCGCCACCGGACCCACCTCC GCCGCTGCCACCGCCACAGAG-3' and cloning it into the BamHI and BspEI sites of plasmid 699 to generate pVU700. The construct was used to make ORFV recombinant 700.

The F1L fusion was constructed by amplifying F1L using the primers 5'-CGCGGATCCGAATTCTCCGAGACTACAAGGACGACGATGACAAG-3' and 5'-CTTAGATCTTACACGATGGCCGTGACCAG-3' and cloned into the BspEI and BglII sites of pVU700 following deletion of the 10-kDa fusion gene to produce pVU701. The construct was used to make ORFV recombinant 701.

Recombinant ORFVs were generated in LT cells using previously described methods [35]. Recombinants displaying blue-plaque phenotypes in the presence of 5-bromo-4-chloro-3-indolyl β -D-glucuronide (Glycosynth) were selected and purified through at least three rounds of plaque purification before virus stocks were prepared. Purified virus stocks were prepared as previously described [19]. The recombinant viruses were quantified by titration using a method described elsewhere [36].

2.4. Viral DNA preparation

The protocols for viral DNA preparation, restriction endonuclease digestion, gel electrophoresis and transfer of DNA to nylon membranes have been described [33].

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