Yaba-like disease virus protein Y144R, a member of the complement control protein family, is present on enveloped virions that are associated with virus-induced actin tails

Mansun Law,1,2 Michael Hollinshead,1 Han-Joo Lee2† and Geoffrey L. Smith1,2

Yaba-like disease virus (YLDV) is a yatapoxvirus, a group of slow-growing poxviruses from primates. Analysis of the growth cycle of YLDV in tissue culture showed that maximum virus titres were reached 3 days post-infection and at this time only 3-3% of infectious progeny was extracellular. The intracellular and extracellular virions have different buoyant densities and are separable on CsCl density gradients. They are also distinguishable by electron microscopy with the extracellular virions having an additional lipid envelope. In YLDV-infected cells, thick actin bundles with virions at their tips were seen protruding from the cell surface, despite the fact that YLDV lacks a protein comparable to Vaccinia virus A36R, which is required for VV-induced actin tail formation. In addition to these observations, the YLDV gene Y144R was characterized.

This gene is predicted to encode a transmembrane protein containing three short consensus repeat (SCR) motifs common to members of the complement control protein family. Antibody generated against recombinant Y144R recognized products of 36, 41 and 48–55 kDa in YLDV-infected cells and purified extracellular enveloped virus (EEV) but not intracellular mature virus (IMV). Y144R protein is a glycoprotein with type I membrane topology that is synthesized early and late during infection. By immunoblot, indirect immunofluorescence and immuno-cryoelectron microscopy the Y144R protein was detected on the intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and EEV. This represents the first study of a YLDV IEV, CEV and EEV protein at the molecular level.

INTRODUCTION

Yaba-like disease virus (YLDV), Tanapox virus and Yaba monkey tumour virus (YMTV) belong to the Yatapoxvirus genus of the Poxviridae (Knight et al., 1989). YLDV was isolated during epizootics in 1965 and 1966 in primate centres in USA. Yatapoxviruses infect monkeys but accidental infections of humans have been reported (España, 1971). Symptoms of yatapoxvirus infection are mild, with a brief fever followed by development of a few firm, elevated, round, necrotic maculopapular nodules at the site of infection. In vitro, YLDV replicates only in primate cell lines (Hu et al., 2001).

The YLDV genome has been sequenced (Lee et al., 2001) and is approximately 145 kb with 151 predicted open reading frames (ORFs). Several of these encode proteins with significant similarity to host immunomodulatory molecules and one example is gene 144R, which is predicted to encode a transmembrane protein with three short consensus repeat (SCR) motifs common to members of the complement control protein family. Antibody generated against recombinant Y144R recognized products of 36, 41 and 48–55 kDa in YLDV-infected cells and purified extracellular enveloped virus (EEV) but not intracellular mature virus (IMV). Y144R protein is a glycoprotein with type I membrane topology that is synthesized early and late during infection. By immunoblot, indirect immunofluorescence and immuno-cryoelectron microscopy the Y144R protein was detected on the intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and EEV. This represents the first study of a YLDV IEV, CEV and EEV protein at the molecular level.

In orthopoxviruses, such as Variola virus (VAR) and Vaccinia virus (VV), there are two SCR proteins (Fig. 2a). VV gene C21L encodes a 35 kDa soluble protein with four SCR domains that regulates complement activation (Kotwal & Moss, 1988). VV gene B5R encodes a 42 kDa transmembrane glycoprotein with four SCR domains that
is present on intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV) (Engelstad et al., 1992; Isaacs et al., 1992).

There is a minor 35 kDa soluble cleavage product of B5R of unknown function (Martinez-Pomares et al., 1993).

YLVD Y144R has several similarities to VV B5R: both genes are located towards the right end of the genome and are transcribed rightwards, the encoded proteins are known or predicted to have a type I membrane topology and each has several SCRs in the ectodomain.

The B5R protein is important for VV morphogenesis and is a major target for antibody-mediated neutralization of EEV (Galmiche et al., 1999; Law & Smith, 2001). The deletion of B5R inhibits the wrapping of the intracellular mature virus (IMV) by cellular membranes to form IEV, and hence the subsequent steps of virus morphogenesis and efficient virus spread (for review see Smith et al., 2002). B5R may also be involved in virus attachment because the deletion of any of the SCR domains from B5R caused more EEV to be released during infection (Herrera de Herrera et al., 2002).

In this report, we have characterized Y144R and show it encodes a type I membrane protein that is present on IEV, CEV and EEV. Like VV protein B5R, there is a smaller soluble form of Y144R. Lastly, we demonstrate that YLVD-infected cells induce the formation of virus-tipped actin tails, despite not encoding an orthologue of VV protein A36R that is required for this process in VV.

### METHODS

**Bioinformatics.** The sequences of Y144R and orthologues in other poxviruses were obtained from NCBI GenBank (www.ncbi.nlm.nih.gov) (Y144R, acc. no. CAC21382; Yaba monkey tumour virus YMTVCS5, acc. no. BAA88977; Shope fibroma virus SFV144R, acc. no. NP_052030; Myxoma virus M144R, acc. no. AAF15032; Swinepox virus SPV139, acc. no. AAL69878; Lumpy skin disease virus LSDV141 acc. no. AAK85102). These sequences were aligned using CLUSTAL W (Thompson et al., 1994). Since SPV139 and LSDV141 contain only two SCRs in contrast to other sequences that have three, the sequences of Y144R, YTMVCS5, SFV144R and MXV144 were aligned first using Gonnet Matrix (default parameter with a gap penalty of 5) and then SPV139 and LSDV141 sequences were added to the alignment using Gonnet Matrix default parameter (gap penalty = 10). The aligned sequences were edited using GeneDoc software (www.psc.edu/biomed/genedoc/) and the conserved amino acid residues were shaded using the default Conservation Mode.

**Viruses and cells.** YLVD was grown in owl monkey kidney (OMK) cells as described previously (Lee et al., 2001) and VV strain vAB5R was constructed in this laboratory (Engelstad & Smith, 1993). OMK, BS-C-1 and CV-1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco-BRL) containing 10% heat-inactivated fetal bovine serum (FBS). Dilution of virus and infection of cells were done using DMEM supplemented with 2% FBS. IMV and EEV of YLVD were prepared by sucrose or cesium chloride density gradient ultracentrifugation as described (Payne, 1978; Mackett et al., 1985) and quantified by spectrophotometry (1 OD₅₆₀ unit = 64 μg virus).

**Generation of anti-Y144R antibody.** The DNA encoding Y144R amino acid residues H19 to V196 was amplified from the YLVD genomic DNA by PCR using 5’-primer aacatatatgatttcagtttaa- caaaaatgatgc and 3’-primer taagatctcaaacaccatttgatgcc. The stop codon is shown in italics and NdeI and BamHI restriction sites (underlined) were used for cloning into pET16b (Novagen). The resultant plasmid was used for expression of an N-terminal His-tagged protein using E. coli strain BL-21 (Novagen) according to the manufacturer’s instructions. The bacteria were grown to an OD of 0.6 and protein expression was induced with 1 mM IPTG for 5 h at 37°C. The bacteria were collected by centrifugation and lysed by incubating with PBS containing 1% Triton X-100 and 0.5 mg lysisome ml⁻¹ followed by sonication. The inclusion bodies were solubilized in reducing and denaturing buffer (20 mM phosphate buffer, pH 7-0, 0-5 M NaCl, 10 mM imidazole, 6 M urea, 1 mM β-mercaptoethanol) and the His-tagged proteins in the soluble fraction were purified using a nickel-chelating column (HiTrap His column; Pharmacia). Bound proteins were eluted with increasing concentration of imidazole (10 mM–0–5 M) in the same buffer and were dialysed against PBS with decreasing urea concentration (4 M, 2 M then without urea) at 4°C. A soluble protein of 23 kDa representing SCR domains 1–3 (with >80% purity) was obtained. This protein was used to immunize rabbits by intramuscular injection of 75 μg of protein in Freund’s complete adjuvant followed by three booster injections of the same protein in Freund’s incomplete adjuvant (Sigma). IgG fractions were purified from the serum using a HiTrap Protein-G column (Pharmacia). The antibody was pre-absorbed with fixed OMK cells at 4°C overnight before use for microscopy.

**Immunoblotting.** Samples were resolved by reducing SDS-PAGE either using a Mini-Protean 3 (Bio-Rad) or Hoefer vertical gel electrophoresis system (Amersham) and were transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies followed by horseradish peroxidase-conjugated (HRP-) secondary antibodies and bound antibody was visualized using an ECL Western Blotting detection system (Amersham). The apparent molecular mass of specific products was calculated using Quantity One software (Bio-Rad). The primary antibodies for detecting Y144R, B5R and GFP were rabbit anti-Y144R (0-6 μg ml⁻¹), rat mAb 19C2 (tissue culture supernatant dialyzed 1/50) (Schmelz et al., 1994) and mouse mAb anti-GFP (diluted 1/1000, Clontech), respectively. The secondary antibodies used were HRP-goat anti-rabbit, anti-rat and anti-mouse IgG (diluted 1/2000, Sigma).

**Indirect immunofluorescent microscopy.** Immunofluorescent staining (including blocking, incubation and washes) were performed in PBS containing 10% FBS (PBSF). Live cells were stained on their surface by incubating cells growing on glass coverslips with anti-Y144R antibody (20 μg ml⁻¹) on ice for 1 h before washing and then fixation using 4% paraformaldehyde in 250 mM HEPES buffer (4% PFA). For intracellular labelling, cells were fixed and permeabilized with 0-1% saponin in PBSF for 30 min before incubating with anti-Y144R antibody (2 μg ml⁻¹). Anti-Y144R antibody and F-actin were detected by tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories; diluted 1/100) and fluorescein isothiocyanate (FITC)-conjugated phallolidin (Sigma; diluted to 5 ng ml⁻¹), respectively. The samples were mounted in Mowiol 4’,6-diamidino-2-phenylindole (DAPI) mounting medium (Sanderson et al., 1996).

**Electron microscopy.** CV-1 cells were infected with YLVD at 5 p.i.u per cell for 28 or 47 h. The methods for Epon embedding.
and conventional electron microscopy have been described elsewhere (Hollinshead et al., 1999). For immuno-cryoelectron microscopy, the cells were washed with ice-cold PBS and fixed in 4 % PFA on ice for 10 min, and then 8 % PFA at room temperature for 20 min. The cells were scraped and pelleted, fixed for an additional 30 min and washed in PBS. Samples were frozen in 2-1 M sucrose in PBS and stored under liquid nitrogen. Sections were cut using a Leica EMFC5 ultramicrotome and collected onto filmed EM grids and labelled with anti-Y144R antibody (10 μg ml⁻¹), followed by 6 nm gold-conjugated protein A. Images were collected using a transmission EM (FEI Tecnai G2) with a 953 MegaView III CCD camera and processed using Adobe Photoshop software.

**Actin tail rescue assay.** Six plasmids, pSE/L_EYFP, pSE/L_B5REYFP, pSE/L_Y144REYFP, pSE/L_ECFP, pSE/L_B5RECFP and pSE/L_Y144RECFP, were constructed. pSE/L_EYFP and pSE/L_ECFP were generated by subcloning the XhoI and NotI restriction enzyme-digested fragment of pEYFP-N1 and pECFP-N1 (Clontech), which contained the enhanced yellow fluorescent protein (EYFP) or enhanced cyan fluorescent protein (ECFP) sequences, behind the VV synthetic early and late (SE/L) promoter (Chakrabarti et al., 1997) of the pSE/L vector. BSR was amplified by PCR using the 5′-primer ccgtaacctggccacctgaataacagttcggtt (HindIII site underlined) and 3′-primer tttagcgtactgatcatggatagat (BgII site underlined). Y144R was amplified using 5′-primer ccgtaacctggccacctgaataacagttc and 3′-primer ggttcatagacggtagcaatttatggaac (HindIII site underlined). The cells were scraped and pelleted, fixed for an additional 30 min and washed in PBS. Samples were frozen in 2-1 M sucrose in PBS and stored under liquid nitrogen. Sections were cut using a Leica EMFC5 ultramicrotome and collected onto filmed EM grids and labelled with anti-Y144R antibody (10 μg ml⁻¹), followed by 6 nm gold-conjugated protein A. Images were collected using a transmission EM (FEI Tecnai G2) with a 953 MegaView III CCD camera and processed using Adobe Photoshop software.

**RESULTS**

**Growth properties of YLDV in cell culture**

The growth of YLDV was investigated in OMK cells (Fig. 1). Following infection at 5 p.f.u. per cell, the infectious virus present in infected cells and the clarified supernatant was measured from 1 to 7 days post-infection (p.i.). Maximum levels of total virus and intracellular virus occurred 3 days p.i., while the titre of extracellular virus continued to increase gradually up to 7 days p.i. However, at all time-points the extracellular virus represented only a small fraction of total infectivity (3-3 % at 3 days p.i.). The gradual increase in extracellular virus after 3 days was probably partly attributable to release of intracellular virus following cell lysis. A similar situation has been reported with VV, in which with the Western Reserve strain less than 1 % of infectivity is extracellular when the peak intracellular titre is reached (12-15 h p.i.).

To determine if the YLDV extracellular and intracellular virion densities were physically different, IMV and EEV preparations were analysed by CsCl density-gradient centrifugation. IMV was prepared from lysate of infected cells by centrifugation through a 36 % (w/v) sucrose cushion (Mackett et al., 1985) and EEV was collected from the supernatant of infected cells by centrifugation. Like VV, the YLDV extracellular virus had a lower buoyant density (1:23 g ml⁻¹) than intracellular virus (1:26 g ml⁻¹), although IMV of VV has a density of 1:27 g ml⁻¹ (Boulter & Appleyard, 1973). An additional viral band of aggregated virions that had lower density was also observed in the IMV preparation, and a similar observation had been made previously with *Tanapoxvirus* (Knight et al., 1989).

**Analysis of YLDV ORF 144**

The YLDV ORF 144 (Y144R) is predicted to encode a polypeptide of 267 amino acid residues and 30-8 kDa with six potential N-glycosylation sites (Fig. 2b). The protein has hydrophobic sequences near the N (M1–I13) and C termini (I229–L252) that may represent the signal peptide and transmembrane anchor region of a type I membrane protein, respectively. Three SCR domains and a stalk region are predicted between these hydrophobic sequences. The first SCR domain (SCR1) lacks several conserved amino acids such as a proline and a glycine residue after the first cysteine and a proline before the fourth cysteine that are characteristic of SCR domains (Hourcade et al., 1989), and consequently was not detected as an SCR here. Other features of SCR domains such as four conserved cysteines, a tryptophan before the fourth cysteine and a size of about 60 amino acids are present. This domain is considered an SCR here.
In contrast to orthopoxviruses, only a single gene encoding an SCR protein is present in viruses of the Yatapoxvirus, Leporipoxvirus (Cameron et al., 1999; Willer et al., 1999), Suipoxvirus (Afonso et al., 2002) and Capripoxvirus (Tulman et al., 2001, 2002) genera. In a BLAST search, Y144R was most similar to gene C5R of YMTV of the same genus (57 % amino acid identity and 69 % similarity over 266 amino acid residues), followed by leporipoxvirus proteins (Shope fibroma virus S144R and Myxoma virus M144R), SPV139 of Swinepox virus and LSDV141 of Lumpy skin disease virus. Interestingly, the SCR proteins of Swinepox virus (SPV139) and Lumpy skin disease virus (LSDV141) have only two SCR domains. Fig. 2(b) shows the amino acid alignment of the SCR proteins of these poxviruses.

The genomic location of YLDV 144R resembles B5R. Y144R shares 28 % amino acid identity and 49 % similarity over 312 amino acid residues with B5R, but only 24 % identity and 44 % similarity over 256 amino acid residues to the second VV SCR protein C21L. Y144R also shares 34 % identity and 51 % similarity over 172 amino acid residues with human CD46, the membrane cofactor protein involved in complement regulation. Notably, SCR3 of Y144R has 50 % amino acid identity to SCR4 of CD46. In CD46, SCR3 and SCR4 are important for binding complement factors C3b and C4b and therefore cofactor activity (Adams et al., 1991). The similarities of Y144R to B5R and CD46 might suggest that YLDV encodes a single gene that functions in both virus morphogenesis and complement regulation.

**Characterization of Y144R gene product**

To identify the Y144R protein, YLDV-infected cells and purified YLDV virus particles were analysed by immuno blotting. An antibody, anti-Y144R, was generated by immunizing a rabbit with recombinant Y144R SCR1–3 produced in E. coli (see Methods). This antibody recognized specific products between 36 and 41 kDa by 4 h p.i. After 8 h p.i., two dominant products of 36 and 41 kDa and a faint band of 39 kDa were observed. This polyclonal antibody also reacted with several host proteins (see mock-infected sample). After 12 h p.i., specific higher molecular mass products of 48–55 kDa appeared as a smear on the blot.

The nucleotide sequence of gene Y144R (Lee et al., 2001) shows a TAAAT motif 6 nucleotides upstream of the ATG codon. This motif is present at the transcriptional start site of VV late genes (Davison & Moss, 1989). There are also early transcriptional terminator signals (T2NT) 170 nucleotides downstream of the ATG codon and immediately after the stop codon. A prediction from these sequences was that Y144R would be expressed late during infection (Lee et al., 2001). To address this experimentally, the effect of cytosine arabinoside (AraC), which blocks DNA replication and poxvirus late gene expression, was investigated. This drug did not affect Y144R expression before 12 h p.i. but reduced expression after 24 h p.i. (Fig. 3a). The early expression of Y144R (4 h p.i.) and reduced level of expression in the presence of AraC suggested that Y144R is expressed early and late during infection, similar to VV B5R (Engelstad et al., 1992). The fact that AraC did not affect Y144R levels before 24 h p.i. is consistent with the slower replication cycle of YLDV compared to VV (Fig. 1).

The pattern of multiple diffuse proteins suggested that Y144R is a glycoprotein, consistent with the presence of six potential N-glycosylation sites. However, only four of these might be used because one is within the signal peptide and one is located immediately after the transmembrane region (Fig. 2b). In the presence of tunicamycin, a drug that inhibits N-glycosylation, a single product of 28 kDa was detected (Fig. 3b). This is slightly smaller than the predicted polypeptide size (30–8 kDa), possibly due to cleavage of the signal peptide. In contrast monensin, a drug that blocks O-glycosylation, reduced the level of expression rather than the size of Y144R. The results suggested that Y144R is N- but not O-glycosylated.

VV B5R is present in EEV particles, and so the presence of Y144R in virus particles was also investigated. By immunoblot, Y144R was present on purified EEV but not IMV (Fig. 3c) and the higher molecular mass products (48–55 kDa) were more abundant in EEV than the smaller forms (36 and 41 kDa). Smaller proteins (~32–36 kDa) were also found in the tissue culture supernatant, suggesting Y144R might be cleaved and released into the supernatant. In summary, Y144R is similar to B5R because it is an EEV glycoprotein that is synthesized early and late during infection and is also released from cells as smaller cleavage products.

**Indirect immunofluorescent staining of YLDV-infected cells**

The localization of Y144R in infected cells was investigated. YLDV-infected CV-1 cells were stained with the anti-Y144R antibody before or after membrane permeabilization. Bound antibody was detected with TRITC-conjugated goat anti-rabbit IgG (red stain). Fig. 4(b–f) shows the

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**Fig. 2.** (a) Genomic location of VV and YLDV genes encoding SCR proteins. (b) Amino acid alignment of poxvirus SCR proteins excluding those from orthopoxviruses. The SCR proteins of yatapoxvirus (Y144R and YMTV C5R), leporipoxvirus (SPV144R and M144R), suipoxvirus (SPV139) and capripoxvirus (LSDV141) were aligned using CLUSTAL W. The four conserved cysteines of each SCR domain are marked by arrowheads. For Y144R, the putative signal and transmembrane peptides are boxed and the potential N-glycosylation sites are marked with stars. The conserved (identical or similar) amino acid residues were shaded from black (conserved in all aligned proteins) to light grey (conserved in four aligned proteins), according to the levels of conservation among the aligned sequence.
intracellular labelling of YLDV-infected cells at 28 h p.i. The antibody reacted non-specifically with the nucleus and surrounding area in both infected (Fig. 4b) and uninfected cells (Fig. 4a), but also detected many punctate structures in infected cells in areas not masked by the non-specific staining (Fig. 4b–d). Many of these structures were DAPI-positive (Fig. 4c, e), indicating that they contained DNA and were likely to be virus particles. Moreover, some were associated with the tips of thick actin bundles resembling VV-induced actin tails (Fig. 4c, f). These data suggested that Y144R is present on enveloped virions (IEV, CEV or EEV) and showed that actin tails are induced by YLDV. Actin tails are important for efficient virus cell-to-cell spread and are present on the surface of orthopoxvirus-infected cells. However, they have not been demonstrated in other poxviruses and their presence in YLDV is surprising because YLVD lacks a counterpart of VV protein A36R, which is required for VV-induced actin tail (Frischknecht et al., 1999). Further examination of the location of actin tails by optical sectioning revealed that all actin tails (n = 50) formed by YLDV were extending from the cell surface.

To determine if Y144R was expressed on the cell surface, non-permeabilized YLDV-infected cells were stained at 30 h p.i. with anti-Y144R antibody. Specific labelling at the cell surface was seen (compared to anti-Y144R antibody on mock-infected cells or non-immune rabbit IgG on YLDV-infected cells; data not shown), but nuclear staining was not seen, indicating that the cell membrane was intact. The antibody detected many punctate particles on the cell surface and some of them were associated with the tips of actin tails (Fig. 4g–i). This demonstrated that (i) Y144R is present on CEV and (ii) the SCR domains of Y144R are extracellular because the antibody was generated against these domains. This suggests that Y144R adopts a type I membrane topology.

**Electron microscopy**

The above data indicated that CEV-tipped actin tails were formed in YLDV-infected cells and Y144R was associated with IEV/CEV/EEV. This was investigated further by electron microscopy. Fig. 5(a) shows a fully wrapped IEV particle (asterisked) and an IMV particle undergoing wrapping by host membranes. Fig. 5(b) shows a CEV-tipped actin tail protruding away from cell surface. Note that the electron-dense ribosomes and cell organelles were excluded in the protrusion underneath the CEV and the outer CEV membrane is seen clearly in the expanded inset.

The association of Y144R with the enveloped virions required further investigation because the IEV-specific proteins A36R and F12L of VV co-purified in sucrose gradient purified EEV preparations and were detected by immunoblotting (van Eijl et al., 2000, 2002; Zhang et al., 2000). Fig. 5(c–f) shows the labelling of different virus structures during morphogenesis using the anti-Y144R antibody and immuno-electron microscopy. Strong colloidal gold labelling was seen on the membrane of IEV and CEV but not immature virions (IV) and IMV. These data confirm that Y144R is an IEV/CEV/EEV glycoprotein, similar to B5R.

**Anti-comet assay**

Since B5R is a major target for antibody neutralization of EEV in VV (Galmiche et al., 1999; Law & Smith, 2001), an
antibody against Y144R might inhibit YLDV EEV. To measure the neutralizing activity of an antibody against EEV using a standard virus neutralization assay, an IMV neutralizing antibody is required to remove contaminating IMV in an EEV preparation (Law & Smith, 2001). However, no such antibody was available and therefore an anti-comet assay that measures the ability of an antibody to inhibit the formation of secondary plaques (comet tails) mediated by EEV spread was used (Appleyard et al., 1971). The anti-comet activity of the anti-Y144R antibody (antiserum diluted 50- and 200-fold) was tested and an antiserum against live VV-infection (Law et al., 2002) and preimmune serum were used as controls. The results showed that none of the antisera inhibited the formation

Fig. 4. Indirect immunofluorescent microscopy of Y144R-infected cells. CV-1 cells were infected with YLDV at 5 p.f.u. per cell or mock infected. Y144R, actin and DNA were detected with anti-Y144R antibody (red), phalloidin (green) and DAPI (blue), respectively. Panels (a)–(f). Intracellular labelling of Y144R at 28 h p.i. using 2 μg anti-Y144R antibody ml⁻¹. (a) Merged image of actin, Y144R and DNA labelling of mock-infected cells. (b) Merged image of infected cells. (c) Inset of (b) expanded. (d) Y144R. (e) DNA. (f) Actin. Bar, 10 μm. Panels (g)–(i). Cell-surface labelling of Y144R at 30 h p.i. using 20 μg anti-Y144R antibody ml⁻¹. (g) Y144R. (h) Merged image of (g) and actin staining. (i) Inset of (h) expanded. Bar, 5 μm.
of YLDV comet-shaped plaques at day 8 p.i. (data not shown). Therefore the anti-Y144R antibody generated in this study did not neutralize the EEV of YLDV.

**Actin tail rescue assay**

Because Y144R is similar to B5R in terms of gene structure, genomic location, temporal expression pattern and presence on IEV/CEV/EEV, it was possible that like B5R it is necessary for the efficient envelopment of IMV by cellular membranes and subsequent stages of virus dissemination including the induction of actin tails by CEV (for review, see Smith et al., 2002). It would be time consuming to knock-out a gene important in morphogenesis in YLDV because of its slow growth rate in tissue culture. As an alternative, we investigated whether Y144R could replace B5R in a VV mutant lacking B5R gene (vDB5R) (Engelstad & Smith, 1993). If Y144R is an orthologue of B5R, it might rescue the defects of vDB5R, such as IEV and actin tail formation.

Plasmids in which B5R and Y144R were fused with EYFP or ECFP under the control of a VV synthetic early/late promoter were constructed (see Methods). These plasmids were transfected into vDB5R-infected cells and the distribution of IEV/CEV particles and the formation of actin tails were detected using anti-F13L antibody and TRITC-conjugated phalloidin, respectively. The results from the EYFP and ECFP constructs were identical and only data from the EYFP constructs are shown.

The infected and transfected cells were first analysed by immunoblot to investigate if the correct fusion proteins were synthesized (Fig. 6). As expected, products of 27, 64–71 and 60–64 kDa were detected with an anti-GFP antibody in cells transfected with pSE/L_EYFP, pSE/L_B5REYFP and pSE/L_Y144REYFP corresponding to the predicted size of GFP (27 kDa), B5REYFP (63 kDa plus three N-glycosylation sites) and Y144REYFP (58 kDa plus four N-glycosylation sites on SCR domains and stalk region), respectively. With the anti-B5R antibody, only pSE/L_B5REYFP-transfected cells gave a product of similar size to that detected by the anti-GFP antibody. The anti-Y144R antibody detected products of similar size to those detected by anti-GFP, but with anti-Y144R these were found in only pSE/L_Y144REYFP-transfected cells. A non-specific product was also detected in other transfected and non-transfected cells.

The transfection of pSE/L_B5REYFP restored the dispersal of virions to the cell periphery and the formation of actin tails at 8 h p.i. as shown by the appearance of B5REYFP and F13L-positive virions on the tips of actin tails (Fig. 7a–d).

However, virus-tipped actin tails were not formed in pSE/L_Y144REYFP-transfected and vAB5R-infected cells (n > 30) at 8 h p.i. (data not shown) and even at 24 h p.i. (n > 50) (Fig. 7e–h). Y144REYFP was expressed strongly in ER and the Golgi apparatus and in some areas colocalized with F13L (Fig. 7h). However, the distribution of F13L in cells expressing Y144REYFP was not different from non-expressing cells, suggesting that Y144R did not replace the function of B5R in promoting the wrapping of IMV particles into IEV and the migration of IEV to the cell surface.

In summary, the failure to observe redistribution of virions to the cell surface suggests that Y144R cannot replace B5R for the formation of IEV.

**DISCUSSION**

YLDV is a member of the Yatapoxvirus genus of the Chordopoxvirinae and infects primates. Here we present an analysis of the growth of YLDV in OMK cells, provide electron micrographs of morphologically distinct virions that are produced from each infected cell and show that YLDV induces the formation of virus-tipped actin tails at the cell surface. Thereafter, a single YLDV gene (Y144R) was...
Fig. 7. Actin tail rescue assay. CV-1 cells were infected with vΔB5R for 4 h and then were transfected with plasmids pSE/L_B5REFYP for 4 h or pSE/L_Y144REFYP for 20 h (green). The infected and transfected cells were fixed and labelled with mAb 15B6 (anti-F13L) (blue) and phalloidin (red). Bar, 10 μm.
characterized. This gene encodes a protein with similarity to VV protein B5R. Like B5R, the Y144R protein is a member of the complement control protein superfamily and is a glycosylated protein that is expressed throughout the infectious cycle. Also like B5R, Y144R becomes part of the envelope of IEV, CEV and EEV particles, and has a smaller form that is released into the culture medium. Despite these similarities between Y144R and B5R, Y144R was unable to functionally replace VV B5R when transfected into vΔB5R-infected cells.

Analysis of the one-step growth curve of YLDV showed that this virus grows much more slowly than orthopoxviruses such as VV. Whereas maximum titres of VV are produced by 12–15 h p.i., the maximum titres of YLDV were not reached until 3 days p.i. However, like VV strain WR, the great majority of infectious progeny remained cell associated and at 3 days p.i. only 3-3% of infectious progeny was present in the supernatant. Analysis of the intracellular and extracellular virus by CsCl density-gradient centrifugation showed that the latter virions had a lower buoyant density compared with the intracellular form. This is consistent with additional lipid in the extracellular virions. In accord with this, electron microscopy showed IMV, IEV and CEV forms (as found with VV), with the CEV form having one more lipid envelope that IMV. Therefore, the morphogenesis of YLDV is very similar to VV but develops more slowly.

Confocal microscopy showed that YLDV-infected cells developed virus-tipped actin bundles on the cell surface. This is reminiscent of the situation with VV. However, unlike VV where the A36R protein is needed for the formation of actin tails (Sanderson et al., 1998; Wolfe et al., 1998; Frischknecht et al., 1999), there is no A36R-like protein encoded in the YLDV genome (Lee et al., 2001), suggesting other YLDV protein(s) may be involved. In VV, these actin tails are formed beneath CEV particles at the cell surface and are needed for the efficient cell-to-cell dissemination of virus particles (reviewed by Smith et al., 2002). The plaques formed by YLDV develop very slowly (8–10 days for a plaque equivalent in size to that formed by VV in 2 days). The fact that YLDV induces actin tail formation suggests that its small plaque phenotype is more likely due to slow replication of the virus (3 days rather than 12–15 h for VV) than to inefficient virus transmission from cell to cell.

A characterization of the Y144R gene shows that it resembles VV gene B5R in several respects. Y144R is expressed early and late during infection, and it encodes a transmembrane glycoprotein with type I membrane topology that is found in IEV, CEV and EEV but not IMV. A smaller form of Y144R is also present in the supernatant. All these properties are shared with B5R.

B5R is a major target for antibody neutralization of VV EEV. However, the anti-Y144R antibody was unable to inhibit the formation of comet-shaped plaques that are formed by the unidirectional spread of EEV from the primary plaque (Law et al., 2002). Although the Y144R antibody raised against antigen made in E. coli did not prevent comet formation, it remains possible that Y144R is a target for antibody neutralization and that other sera raised against more natural Y144R protein might induce neutralizing antibody. Similar non-neutralizing antibody had been reported for VV when the antibody was generated against recombinant B5R produced in bacteria (Law & Smith, 2001) although neutralizing antibody resulted when the antigen was expressed by baculovirus (Galmiche et al., 1999; Law & Smith, 2001).

In view of the similarities between the Y144R and B5R proteins and the requirement for B5R in VV-induced actin tail formation, we tested whether the Y144R protein could restore actin tail formation by a VV mutant engineered to lack the B5R protein. However, no complementation was observed. The role of Y144R in the formation of actin tails therefore needs investigation by the construction and analysis of a YLDV deletion mutant lacking the Y144R gene.

In summary, we show that YLDV gene Y144R encodes a transmembrane glycoprotein that is present on YLDV IEV, CEV and EEV particles. This is the first YLDV structural protein to be analysed. In addition, we show that YLDV induces the formation of actin tails despite not encoding a protein closely related to the VV A36R protein. Y144R is the only SCR protein found in YLDV whereas two SCR proteins are present in VV, one for morphogenesis and the other for counteracting complement. Perhaps YLDV encodes a single protein for both functions to reduce its genome size. The interaction of Y144R with complement needs investigation.

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