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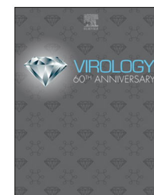

Ectromelia virus lacking the E3L ortholog is replication-defective and nonpathogenic but does induce protective immunity in a mouse strain susceptible to lethal mousepox

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ABSTRACT

All known orthopoxviruses, including ectromelia virus (ECTV), contain a gene in the E3L family. The protein product of this gene, E3, is a double-stranded RNA-binding protein. It can impact host range and is used by orthopoxviruses to combat cellular defense pathways, such as PKR and RNase L. In this work, we constructed an ECTV mutant with a targeted disruption of the E3L open reading frame (ECTVΔE3L). Infection with this virus resulted in an abortive replication cycle in all cell lines tested. We detected limited transcription of late genes but no significant translation of these mRNAs. Notably, the replication defects of ECTVΔE3L were rescued in human and mouse cells lacking PKR. ECTVΔE3L was nonpathogenic in BALB/c mice, a strain susceptible to lethal mousepox disease. However, infection with ECTVΔE3L induced protective immunity upon subsequent challenge with wild-type virus. In summary, E3L is an essential gene for ECTV.

1. Introduction

Ectromelia virus (ECTV; also referred to as "mousepox virus") is a double-stranded DNA virus in the *Poxviridae* family of the genus orthopoxvirus. ECTV is a natural pathogen of mice and primarily infects new hosts through abrasions in the skin (Fenner, 1947). Following initial replication at the site of infection, the virus spreads to multiple organs and eventually disseminates to the skin where characteristic pock lesions can manifest in certain mouse strains (Esteban and Buller, 2005; Fenner and Mortimer, 2006).

The formation of double-stranded RNA (dsRNA) occurs during the replication cycles of numerous viral infections (Weber et al., 2006). For this reason, dsRNA is an important pathogen-associated molecule that initiates an anti-viral immune response. With respect to poxviruses, it has been known for some time that vaccinia virus (VACV) forms abundant amounts of dsRNA, especially during intermediate and late time points of the infection cycle (i.e., after DNA replication; "post-replicative") (Boone et al., 1979; Colby and Duesberg, 1969; Colby et al., 1971; Duesberg and Colby, 1969). Other orthopoxviruses, such as cowpox virus, also form dsRNA albeit to a varying degree relative to VACV (Frey et al., 2017). The mechanism of dsRNA formation has been discussed elsewhere (Arndt et al., 2016; Frey et al., 2017). Briefly, it has been demonstrated using VACV as a model that dsRNA forms as a result of several factors: minimal intergenic space, convergent transcription from opposite DNA strands, and inefficient termination of transcription, particularly during post-replicative time points (Xiang et al., 2000, 1998). These events lead to the production of mRNAs with the capacity for complementary base pairing, thus allowing for dsRNA formation in the cytoplasm.

There are two important anti-viral pathways in cells that are induced by the presence of viral dsRNA: PKR and 2'5'-OAS/RNase L. Upon activation, both pathways can result in the termination of viral protein synthesis, thus reducing viral dissemination within the host organism. Upon recognition of dsRNA, PKR phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α), which results in translational termination (Proud, 2005). RNase L non-specifically

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cleaves cytoplasmic RNA molecules, including viral transcripts, which inhibits protein synthesis at the level of RNA stability (Chakrabarti et al., 2011). Because dsRNA is a potent trigger of anti-viral immunity, many viruses have evolved strategies to evade detection (Langland et al., 2006; Seet et al., 2003). This study focused on the immune evasion gene E3L, which produces a protein able to blunt anti-viral responses that become induced by viral dsRNA.

The E3L gene is conserved among a large fraction of the members of the subfamily *Chordopoxvirinae* (Bratke et al., 2013; Haller et al., 2014; Myskiw et al., 2011). It encodes a protein product (termed E3) with an amino-terminal Z-DNA-binding domain (Kim et al., 2003) and a carboxy-terminal dsRNA-binding domain (Chang and Jacobs, 1993). This protein was originally discovered from extracts of VACV-infected cells due to its ability to bind to Poly(I:C), a synthetic dsRNA analog (Chang et al., 1992; Watson et al., 1991). E3 has been shown to inhibit activation of PKR and RNase L, which is likely due in large part to its ability to sequester dsRNA (Chang et al., 1992; Frey et al., 2017; Langland et al., 2006; Rivas et al., 1998; Xiang et al., 2002; Zhang et al., 2008). Moreover, the dsRNA-binding domain is necessary for the virulence of VACV upon infection of mice (Brandt and Jacobs, 2001). E3L can be an important host range gene since its deletion renders some poxviruses unable to replicate in cells that are normally permissive for infection (Beattie et al., 1996; Rahman et al., 2013).

In this study, we demonstrate the critical importance of E3L to the ability of ECTV to replicate in cultured cells and cause disease in mice. An ECTV mutant (ECTVΔE3L) lacking an intact E3L open reading frame was replication-incompetent in all tested mammalian cell lines and avirulent in a mouse strain that is highly susceptible to lethal infection with wild-type ECTV. Interestingly, ECTVΔE3L induced protective immunity in these mousepox-susceptible mice when used as a vaccine prior to challenge with wild-type virus. This report is the first to characterize ECTVΔE3L and to define the role of E3L in the replication capacity and pathogenicity of mousepox virus.

2. Materials and methods

2.1. Cells and culture methods

The following cell lines were used in this study: BS-C-1 (ATCC# CCL-26), HeLa (ATCC# CCL-2), and L-929 (ATCC# CCL-1). These cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 5% fetal bovine serum (FBS; Gemini BioProducts), and penicillin-streptomycin (Gemini BioProducts). The DMEM contained 4 mM L-glutamine, 1 mM sodium pyruvate, and 4.5 mg/mL D-glucose from the manufacturer. All cells were maintained at 37 °C in a 5% CO_2 incubator and sub-cultured when they reached approximately 80–90% confluency. BS-C-1 cells constitutively expressing the E3 protein of VACV were also utilized (termed BS-C-1 + E3L cells; generously supplied by Dr. Stefan Rothenburg). The E3 protein produced in these cells has mCherry fused at the C-terminus to aid in visualization using fluorescence microscopy. The BS-C-1 + E3L cells were maintained in the continuous presence of 500 μg/mL Geneticin/G418 (Gemini BioProducts) to preserve high E3 levels, which were consistently ≥90% positive as determined by flow cytometry (Fig. 1A). These cells fully rescue the replication of VACVΔE3L to wild-type levels (data not shown). Wild-type and PKR knockout mouse embryonic fibroblasts (MEFs) transformed with SV40 large T antigen were kindly provided by Dr. Robert Silverman (Jha et al., 2011; Zhou et al., 1999). These cells were grown in RPMI medium (Invitrogen) supplemented with 10% FBS and penicillin-streptomycin. HeLa cells in which PKR expression was stably knocked-down by RNAi or cells expressing a control siRNA were kindly provided by Dr. Charles Samuel (Zhang et al., 2008; Zhang and Samuel, 2007). These cells were maintained in DMEM + 5% FBS containing 1 μg/mL puromycin (Invivogen) to maintain stable knockdown of PKR. We independently confirmed the absence of detectable PKR in the MEFs and HeLa cells via Western blotting before conducting experiments with these cells (data not shown).

2.2. Viruses

The following viruses were used during the course of this work: ECTV wild-type (Moscow strain; gift of Dr. Laurence Eisenlohr), ECTV expressing GFP [Moscow background; gift of Dr. Luis Sigal (Fang et al., 2008)], ECTVΔE3L, VACV wild-type (Western Reserve strain; gift of Dr. Laurence Eisenlohr), and VACVΔE3LΔK3L (Copenhagen background; gift of Dr. Stefan Rothenburg). VACVΔE3LΔK3L was constructed using standard homologous recombination techniques and expresses green fluorescent protein (GFP) (Brennan et al., 2014). ECTVΔE3L was constructed in a similar manner. Approximatley 90% of the E3L open reading frame was replaced with the coding sequence for GFP under the transcriptional control of the poxviral p7.5 early/late promoter. After the infection/transfection stage, a GFP-positive plaque was isolated and subsequently underwent six rounds of passage in BS-C-1 + E3L cells to ensure that 100% of plaques stably expressed GFP. As a control, we also constructed a revertant virus (ECTV E3L REV) in which the native E3L open reading frame was recombined back into ECTVΔE3L. To create the revertant virus, we amplified the region surrounding the E3L locus (~500 bp upstream and downstream) from w.t. ECTV. After purification of the PCR product, we performed a similar procedure as described above to generate the ECTV E3L REV. After the initial infection/transfection stage, a GFP-negative plaque was isolated that subsequently underwent several rounds of passaging on normal BS-C-1 cells. We then performed sequencing of the E3L gene to confirm that the wild-type sequence was present in the ECTV E3L REV (data not shown). VACVΔE3LΔK3L and ECTVΔE3L were titrated using BS-C-1 + E3L cells. All other viruses were titrated using normal BS-C-1 cells. Virus was released from infected cells by three cycles of freezing in liquid nitrogen followed by thawing in a 37 °C water bath. In some experiments, cytosine β-D-arabinofuranoside (AraC; Sigma-Aldrich) was used at a final concentration of 50 μg/mL to block viral DNA replication. This drug was added at the time of infection.

2.3. Mice

Female C57BL/6 and BALB/c mice aged 6–8 weeks were obtained from Jackson Laboratories. Immunizations with ECTVΔE3L or VACV were carried out using i.p. injection in 300 μL total volume of 1× PBS. Some infections, as described in the results, were carried out via injection of virus in 30 μL total volume of 1× PBS into the right hind footpad. Experimental procedures involving mice were carried out in accordance with regulations from the National Institutes of Health, the Association for Assessment and Accreditation of Laboratory Animal Care International, and the United States Department of Agriculture. Animal protocols were approved by the Institutional Animal Care and Use Committees (IACUC) at Thomas Jefferson University (Philadelphia, PA) [former institution of L.C. Eisenlohr] and the Children's Hospital of Philadelphia Research Institute (Philadelphia, PA) [current institution of L.C. Eisenlohr]. All mouse work was carried out in a humane manner and virus infections were performed under isoflurane anesthesia. Mice were sacrificed upon losing 20% of their pre-infection body weight.

2.4. Enzyme-linked immunospot (ELISPOT) assay

ELISPOT procedures were carried in a similar manner as described previously (Siciliano et al., 2014). On the indicated days post-infection, spleens were isolated and processed (including red blood cell lysis) to create single-cell suspensions. Three mice were used for each condition and splenocytes were pooled prior to plating and stimulation. T-cells were activated using bone marrow-derived dendritic cells (BMDCs) that had been incubated in the presence of the indicated peptides (2 μg/mL final concentration). BMDCs were grown and prepared in a similar

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