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The Orthopoxvirus 68-Kilodalton Ankyrin-Like Protein Is Essential for DNA Replication and Complete Gene Expression of Modified Vaccinia Virus Ankara in Nonpermissive Human and Murine Cells▽

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Modified vaccinia virus Ankara (MVA) is a highly attenuated and replication-deficient vaccinia virus (VACV) that is being evaluated as replacement smallpox vaccine and candidate viral vector. MVA lacks many genes associated with virulence and/or regulation of virus tropism. The 68-kDa ankyrin-like protein (68k-ank) is the only ankyrin repeat-containing protein that is encoded by the MVA genome and is highly conserved throughout the *Orthopoxvirus* genus. We showed previously that 68k-ank is composed of ankyrin repeats and an F-box-like domain and forms an SCF ubiquitin ligase complex together with the cellular proteins Skp1a and Cullin-1. We now report that 68k-ank (MVA open reading frame 186R) is an essential factor for completion of the MVA intracellular life cycle in nonpermissive human and murine cells. Infection of mouse NIH 3T3 and human HaCaT cells with MVA with a deletion of the 68k-ank gene (MVA-Δ68k-ank) was characterized by an extensive reduction of viral intermediate RNA and protein, as well as late transcripts and drastically impaired late protein synthesis. Furthermore, infections with MVA-Δ68k-ank failed to induce the host protein shutoff that is characteristic of VACV infections. Although we demonstrated that proteasome function in general is essential for the completion of the MVA molecular life cycle, we found that a mutant 68k-ank protein with a deletion of the F-box-like domain was able to fully complement the deficiency of MVA-Δ68k-ank to express all classes of viral genes. Thus, our data demonstrate that the 68k-ank protein contains another critical domain that may function independently of SCF ubiquitin ligase complex formation, suggesting multiple activities of this interesting regulatory protein.

Poxviruses encode more than 100 different viral proteins including many enzymes and cofactors that enable the virus to autonomously replicate and express its genetic information in the host cytoplasm, leading to the synthesis of translatable mRNAs with typical eukaryotic features (27). In addition, poxviruses employ numerous proteins to regulate their interaction with the host cell for interference with antiviral defense mechanisms (reviewed in reference 36) and to create a favorable environment for viral replication. These genes determine the pathogenicity and host range of poxviruses, which can be very diverse. The host range of vaccinia virus (VACV) is very broad in vivo as well as in cultured cell lines. Modified vaccinia virus Ankara (MVA) is an attenuated VACV that is growth restricted in human and most other mammalian tissue culture cell lines (10, 25). It was derived from its ancestor VACV Ankara by serial passages on chicken embryo fibroblasts (CEF) and thereby lost substantial genetic information (23). The MVA genome seems to be reduced to the minimal essential information for the virus; it is still able to infect most mammalian cells and express the complete genetic information but does not produce progeny virus (44). During attenuation many host-interacting genes, including immunomodulatory factors or essential host range genes, were lost in MVA (1). Among those is the rather well-known K1L host range gene, a crucial factor for VACV replication in RK13 cells (45) and, together with C7L, also a regulator of VACV growth in human cell lines (15, 16, 32). K1L is a member of the ankyrin repeat (ANK) superfamiliy of proteins. The ANK is a 33-amino-acid motif described to be important in many protein-protein interactions and found in many cellular processes (26). Surprisingly, poxvirus proteins that exert host range function frequently belong to this particular superfamily. Cowpox virus, CP77, or CHOhr was found to confer replication capacity to VACV in Chinese hamster ovary (CHO) cells that are naturally nonpermissive for VACV (39). Furthermore CP77 was shown to be able to rescue the K1L/C7L host range defect of VACV in human cells (32, 33). In addition to ANKs, CP77 harbors an F-box-like PRANC (pox protein repeats of ankyrin C-terminal) domain (24) that is closely related to the cellular F box. More interesting, this is also the case for another well-described host range factor, the M-T5 protein of myxoma virus (MV), a member of the genus *Leporipoxvirus.* An M-T5 deletion from the MV genome resulted in a host range defect in rabbit T lymphocytes in cell culture, as well as attenuated myxomatosis in European rabbits (29).

This common composition of ANK and F-box is shared by the orthopoxviral 68-kDa ankyrin-like (68k-ank) protein, which is conserved throughout the genus and is notably the only ANK-containing protein that was retained during the

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attenuation of MVA (1), suggesting its pivotal role. We previously reported that MVA 68k-ank (encoded by open reading frame [ORF] 186R) interacts with cellular Skp1a and forms a Cullin-1-based SCF complex together with these host factors in an F-box domain-dependent manner (40). To further analyze the function of 68k-ank, we chose to delete 68k-ank (ORF 186R) from the MVA genome and analyze the role of 68k-ank in the intracellular molecular life cycle of MVA. We selected the human keratinocyte cell line HaCaT and the murine fibroblast cell line NIH 3T3 as target cells because these are also considered relevant cell lines for preclinical evaluation of MVA candidate vaccines. A 68k-ank deletion mutant of MVA (MVA-Δ68k-ank) was constructed together with a revertant virus (MVA-68k-Rev). Comparative analysis with these viruses together with wild-type MVA revealed a mutant phenotype for MVA-Δ68k-ank. This phenotype manifested with highly reduced amounts of viral intermediate and late RNA transcripts, as well as delayed and significantly reduced expression (NIH 3T3) or absence (HaCaT) of intermediate and late viral proteins. The assessment of polypeptide synthesis showed that the absence of 68k-ank negatively impacted host protein shutoff. Furthermore, proteasome inhibition blocked MVA intermediate and late gene expression in nonpermissive and also permissive cells. Moreover, we demonstrate that the F-box domain of 68k-ank is dispensable for rescue of viral gene expression in the mutant virus MVA-Δ68k-ank. Thus, our data indicate that 68k-ank is a viral factor with multiple functions and, taking into account the high conservation of 68k-ank, an important regulatory protein for orthopoxviruses in general.

MATERIALS AND METHODS

Cells and viruses. Monolayers of murine embryonic fibroblasts NIH 3T3 were maintained in Dulbecco's modified Eagle's medium, and human adult skin keratinocytes, HaCaT cells (4), were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. CEF were prepared freshly from 10-day-old embryos, cultured in Earl's minimum essential medium, and used in the second passage. MVA (clonal isolate F6) (25, 43) and MVA-Δ68k-ank and MVA-68k-Rev were routinely propagated and titers were determined by VACV-specific immunostaining on CEF.

Plasmid constructs. MVA DNA sequences flanking the 186R gene (MVA nucleotides 164209 to 165933; GenBank accession no. U94848) were amplified by PCR using genomic MVA DNA as a template. Primers of the upstream flanking region of ORF 186R were 5′-GGCCGGGCCCGGATTAAT TACAA TAGCATGATCCGCGC-3′ (ApaI site underlined) and 5′-GGCCGCGGCCG CACGACTCATTTTTATGATGCTTTGTGG-3′ (NotI site underlined). Primers for the downstream region were 5′-GGCCTTCGAAAAAGTATAGT TGTATTTTTCTCATGCG-3′ (StuI site underlined) and 5′-GGCCACGCGTG GAATGCATGAAGGAGG-3′ (MluI site underlined). The amplified DNA fragments were treated with the respective restriction endonucleases and cloned into the corresponding sites of plasmid pΔK1L (41) to obtain the 186R deletion plasmid pΔK1L-186R. To generate rescue plasmid p186R-rev, we amplified the complete ORF 186R together with its authentic promoter region by use of the following primers: 5′-ATATATGGATCCTAACTTGGAGTCTAACGCGC-3′ (BamHI site underlined) and 5′-ATATATCTGCAGACACATCGCATGAGA AAAATAC-3′ (PstI site underlined). The PCR fragment was restricted with the indicated enzymes and ligated into the corresponding sites of pIII-dHR-P7.5 (41) following removal of promoter P7.5 sequences. For construction of MVA-68kΔFbox, MVA DNA sequences flanking the C-terminal sequences encoding the F-box-like domain within ORF 186R were amplified by PCR using genomic MVA DNA as template. Primers of the upstream flanking region were 5′-ATA AAGCATGCAATGTGATAAGATCGTTGGTG-3′ (SphI site underlined) and 5′-TAATAGTTTAAACTACCTTTGACACGTACATATC-3′ (PmeI site underlined). Downstream primers were 5′-ATAAAGGATCCTAGTTGTATTTT TCTCATGCGATGTGTG-3′ (BamHI site underlined) and 5′-TAATAACGC GTTGAGATCTAACTATACCCTGAACAC-3′ (MluI site underlined). The amplified DNA fragments were treated with the respective restriction endonucleases and cloned into the corresponding sites of plasmid pΔK1L (41) to obtain the 186R deletion plasmid pΔK1L-186RΔFbox.

Recombinant MVA construction. Mutant MVA was generated following the transient K1L-based host range selection protocol as described previously (41, 43). Briefly, for the generation of MVA-Δ68k-ank, monolayers of confluent CEF cells in six-well plates were infected with MVA at a multiplicity of infection (MOI) of 0.01. Ninety minutes after infection, cells were transfected with 1.5 μg of plasmid pΔK1L-186R DNA using Fugene-6 (Roche Diagnostics) as recommended by the manufacturer. At 48 h after infection, transfected cells were harvested and plated on RK13 cell monolayers for growth selection. Mutant viruses were isolated through plaque cloning on RK13 cells and then passaged on CEF cells to remove the selectable marker gene K1L. For control purposes, we additionally constructed a revertant virus, MVA-68k-Rev, by inserting 186R together with its authentic promoter region into deletion III of the MVA-Δ68k-ank genome using the same method. MVA-68kΔFbox was constructed by deleting the C-terminal 135 nucleotides of ORF 186R encoding the F-box-like domain. Viral DNA from cloned isolates was routinely analyzed by PCR as described previously (41). To monitor for deletion III, we used the primer pair 5′-TGA CGA GCT TCC GAG TTC C-3′ and 5′-GTA CCG GCA TCT CTA GCA GT-3′, and for ORF 186R we used the pair 5′-CCT GTA ACT GGG TAT ACT GC-3′ and 5′-AGT GTA GTG TCT AGA ACA GTG-3′.

Viral growth analysis. To determine growth properties of recombinant and nonrecombinant MVA, we infected CEF cells grown in six-well plates at an MOI of 0.01 for multiple-step growth analysis or at an MOI of 10 for one-step growth analysis. After virus adsorption for 60 min at 37°C, inocula were removed, and cells were washed twice with medium. Then cells were incubated with fresh medium at 37°C. At multiple time points post infection (p.i.), cells were harvested, and virus was released by three freeze-thaw cycles and brief sonication. Virus titers were determined following standard procedures, as described previously (17).

Western blot analysis. Cells were mock infected or infected with MVA, MVA-Δ68k-ank, or MVA-68k-Rev at an MOI of 5. For analysis in the presence of proteasomal inhibitors, cells were preincubated for 1 h with 10 μM MG-132 (Calbiochem), and infections were carried out with medium containing the inhibitors. At multiple time points p.i., cells were washed with phosphate-buffered saline (PBS) and lysed either with 1× sodium dodecyl sulfate (SDS) lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 3.2% ß-mercaptoethanol, 0.01% bromphenol blue, and 10% glycerol) or NET-N (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 10% glycerol) supplemented with protease inhibitor cocktail (Complete; Roche Diagnostics), respectively. Lysates obtained with 1× SDS lysis buffer were cleared with Qiashredder columns (Qiagen), and for NET-N lysates the cell debris was removed by centrifugation. Lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. After a blocking step, membranes were incubated with the specific antiserum overnight at 4°C, and bound primary antibody was detected with species-specific secondary peroxidase-coupled antibodies (Dianova) and ECL substrate (Amersham). Rabbit polyclonal antisera specific for VACV E3, A1, B5, and 68k-ank protein were applied in 1:1,000, 1:250, 1:3,000, and 1:500 dilutions, respectively.

Northern blot analysis. Cells were mock infected or infected with MVA, MVA-Δ68k-ank, or MVA-68k-Rev at an MOI of 5. Total RNA was isolated with TRIzol reagent (Invitrogen) following the manufacturer's instructions. Total RNA was separated by electrophoresis in 1% agarose formaldehyde gels. Subsequently, RNA was transferred onto positively charged nylon membranes (Roche Diagnostics). Riboprobes for detection of MVA-encoded mRNAs 005R, 078R, and 047R were synthesized by in vitro transcription using PCR products generated from viral DNA templates; primer sequences were published previously (22). Reverse primers contained a T7 RNA polymerase promoter recognition sequence. Digoxigenin (DIG)-labeled riboprobes were obtained by in vitro transcription with T7 RNA polymerase (Roche Diagnostics) using PCR-generated DNA fragments as templates. In vitro RNA labeling, hybridization, and signal detection were carried out according to the manufacturer's instructions (DIG RNA labeling kit and anti-DIG detection chemicals; Roche Diagnostics), applying 68°C for hybridization and a high stringency wash in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS buffer.

Metabolic labeling. Confluent monolayers of cells in 12-well plates were mock infected or infected with MVA, MVA-Δ68k-ank, or MVA-68k-Rev at an MOI of 10 or 25. Following 45 min of adsorption at 4°C, virus inocula were replaced by prewarmed medium, and the cells were incubated at 37°C. At the desired time p.i., cells were washed once with cysteine- and methionine-free medium and were

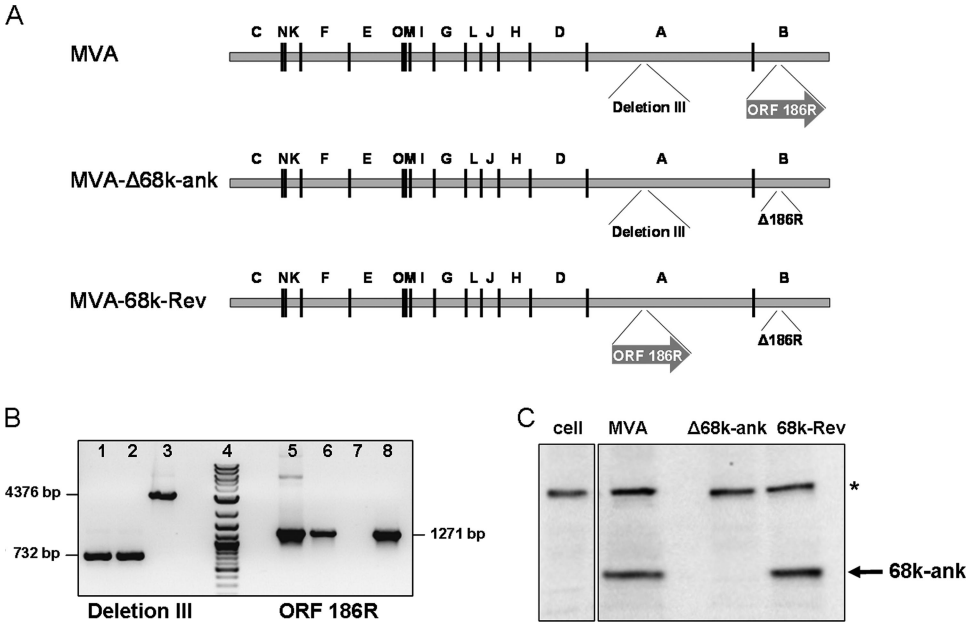

FIG. 1. Construction of 68k-ank deletion mutant MVA and a revertant virus. (A) Schematic overview of HindIII restriction map of MVA and mutants generated in this study. ORF 186R encoding the 68k-ank protein resides in the HindIII restriction B fragment and was deleted to generate MVA-Δ68k-ank. For construction of the revertant MVA-68k-Rev, ORF 186R together with its authentic promoter was inserted into deletion III, which maps to the A fragment. (B) PCR analysis of viral DNA. MVA (lanes 1 and 6), MVA-Δ68k-ank (lanes 2 and 7), and MVA-68k-Rev (lanes 3 and 8) were analyzed with specific primers for deletion III and ORF 186R. A 2-log DNA ladder (New England BioLabs) served as molecular weight marker (lane 4); lane 5 shows analysis of the transfer plasmid p186R-rev. (C) Western blot analysis of CEF lysates from mock or MVA, MVA-Δ68k-ank (Δ68k-ank), or MVA-68k-Rev (68k-Rev) infection at 3 h p.i. Cell lysates were separated by SDS-PAGE, and proteins were transferred to a PVDF membrane. 68k-ank expression was detected with a specific rabbit antiserum; an asterisk marks an unspecific band recognized by anti-68k-ank rabbit polyclonal antibody.

then incubated for 30 min at 37°C with 50 μCi of [^{35}S]cysteine-methionine (Perkin-Elmer) per well. Afterwards, cells were washed once with PBS and lysed directly in 1× SDS lysis buffer. Samples were separated by 10% SDS-PAGE, and gels were fixed in 7% acetic acid and incubated with Amplify (Amersham). Vacuum-dried gels were then analyzed by autoradiography.

Viral DNA replication. Cells were infected with MVA, MVA-Δ68k-ank, or MVA-68k-Rev at an MOI of 5. Genomic viral DNA was isolated from infected cells as described previously (12). Total DNA was transferred by a dot blot procedure to a positively charged nylon membrane (Roche Diagnostics) and hybridized at 42°C to a randomly DIG-labeled MVA-specific probe, generated as described for Northern blot analysis. Buffers including 2× SSC with 0.1% SDS (at room temperature) and 0.5× SSC with 0.1% SDS (at 65°C) were used for low- and high-stringency washes, respectively.

Active caspase-3 FACS analysis. Subconfluent NIH 3T3 cells in six-well plates were infected with MVA, MVA-Δ68k-ank, or MVA-68k-Rev at an MOI of 5. As a positive control, cells were stimulated with 1 μM staurosporine in dimethyl sulfoxide (DMSO); mock-infected cells were given equal amounts of DMSO as a control. At 24 h p.i. supernatants together with the trypsinized cells were transferred to fluorescence-activated cell sorting (FACS) tubes. Subsequently, cells were washed twice with PBS with low-spin centrifugation. Resulting cell pellets were then incubated with 200 μl of Cytofix-Cytoperm (BD Biosciences) for 20 min at 4°C. Following fixation/permeabilization, cells were washed two times with 1 ml of 1× Perm/Wash solution (BD Biosciences). For staining of active caspase-3, cells were incubated with 50 μl of 1× Perm/Wash solution containing 20 μl of phycoerythrin (PE)-labeled anti-active caspase-3 antibody (BD Biosciences) for 30 min at room temperature in the dark. Again, cells were washed with 1× Perm/Wash solution and afterwards analyzed with a BDLSR II flow cytometer (BD Biosciences).

RESULTS

Generation and characterization of MVA-Δ68k-ank and its revertant virus. To study the function of 68k-ank during the MVA life cycle, we deleted ORF 186R (encoding 68k-ank) from the MVA genome to generate MVA-Δ68k-ank. For mutant virus generation, we chose the K1L-specific growth selection method in RK13 cells (41, 43) and constructed a plasmid with the K1L selection marker cloned in between flanking sequences of ORF 186R, which maps within the HindIII restriction fragment B of the MVA genome (Fig. 1A). MVA-infected cells were transfected with the knockout plasmid, and recombinant plaque isolates were selected on RK13 cells. Thereafter, the isolated viruses were passaged on CEF to eliminate the K1L marker gene. A revertant virus, MVA-68k-Rev harboring ORF 186R together with its authentic promoter inserted into the site of deletion III, the HindIII restriction fragment A (Fig. 1A), was generated accordingly. Successful deletion and reinsertion of 186R gene sequences were confirmed by PCR analysis of viral DNA. As shown in Fig. 1B, a DNA fragment corresponding to the ORF 186R-specific band of 1,271 bp is amplified from DNA of cells infected with MVA (lane 6) and MVA-68k-Rev (lane 8) but not MVA-Δ68k-ank (lane 7). Additionally, PCR analysis of MVA-68k-Rev DNA demonstrated the correct reinsertion of ORF 186R into deletion III (Fig. 1B, lane 3), resulting in the amplification of a larger (4,376 bp) deletion III-specific PCR fragment than the 732-bp PCR products of MVA and MVA-Δ68k-ank (lanes 1 and 2) DNA.

Furthermore, we tested the inactivation of the 186R gene by Western blot analysis of whole-cell CEF lysates using antibodies directed against 68k-ank. As shown in Fig. 1C, 68k-ank is readily detectable at early times of 2 to 3 h p.i. (see also Fig.

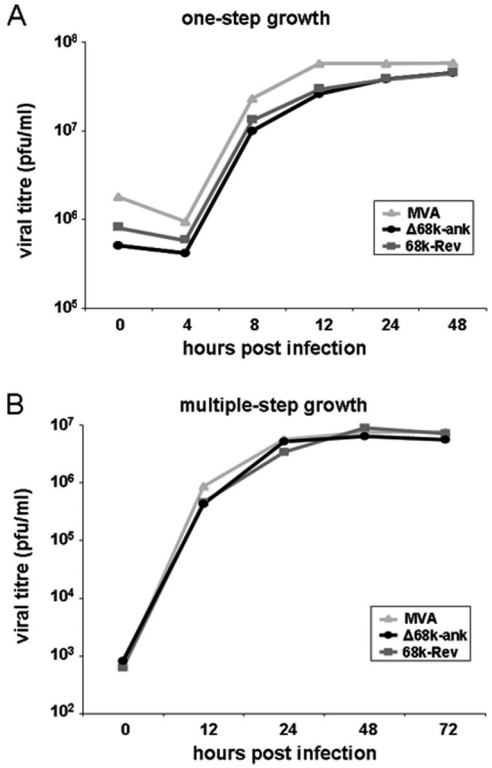

FIG. 2. Analysis of virus growth in CEF cells. Cells were infected at an MOI of 10 for one-step growth analysis (A) and at an MOI of 0.01 for multiple-step growth analysis (B) of MVA, MVA-Δ68k-ank (Δ68k-ank), or MVA-68k-Rev (68k-Rev). Growth curves are representative of two independent experiments with double titrations.

9A) in MVA- and MVA-68k-Rev-infected cells but not in MVA-Δ68k-ank-infected cells.

To test the replicative capacity of MVA in CEF in the absence of 68k-ank, we performed one-step and multiple-step growth analyses comparing MVA, MVA-Δ68k-ank, and MVA-68k-Rev. All three viruses replicated with comparable kinetics and produced very similar amounts of infectious progeny (Fig. 2).

Defect of viral transcription in MVA-Δ68k-ank-infected NIH 3T3 and HaCaT cells. Since we were able to construct MVA-Δ68k-ank and since its replication in permissive CEF cells was unimpaired, 68k-ank function is not essential in these permissive cells. As MVA is a prime candidate vaccine for humans, we were interested to analyze how the virus lacking 68k-ank would behave in nonpermissive cells, especially in those of human origin. Because MVA maturation is blocked in these cells, we monitored the intracellular molecular life cycle, which is tightly regulated as in all poxvirus infections (27).

We first tested viral gene expression on the transcriptional level. Northern blot analysis was performed, and transcription of well-characterized vaccinia genes of all three classes, early ORF 005R (epidermal growth factor), intermediate ORF 078R (viral late gene transcription factor 1), and late ORF 047R (11-kDa DNA binding phosphoprotein), was monitored as described previously (21). As shown in Fig. 3A, we found drastically reduced levels of viral intermediate and late transcripts in murine fibroblasts (NIH 3T3) infected with mutant virus MVA-Δ68k-ank, with a more profound effect on late transcription. In contrast, wild-type MVA- and MVA-68k-Rev-infected cells produced nearly identical amounts of all three transcripts, and, thus, the deficiency in transcription could be evidently ascribed to the lack of 68k-ank gene function in mutant virus-infected cells. Similar results were obtained by Northern blot analysis of infected human keratinocytes (HaCaT). The probes for 078R (intermediate) and 047R (late) transcripts detected only very faint signals, and even the amount of early transcripts appeared to be slightly reduced in HaCaT cells infected with MVA lacking 68k-ank (Fig. 3B). Thus, deletion of ORF 186R strongly reduced the amount of viral transcripts, suggesting a general inhibition of viral intermediate and late gene expression in murine and human cells.

Impairment of viral intermediate A1 and late B5 protein synthesis in MVA-Δ68k-ank-infected NIH 3T3 and HaCaT cells. As a consequence of the observation that deleting 68k-

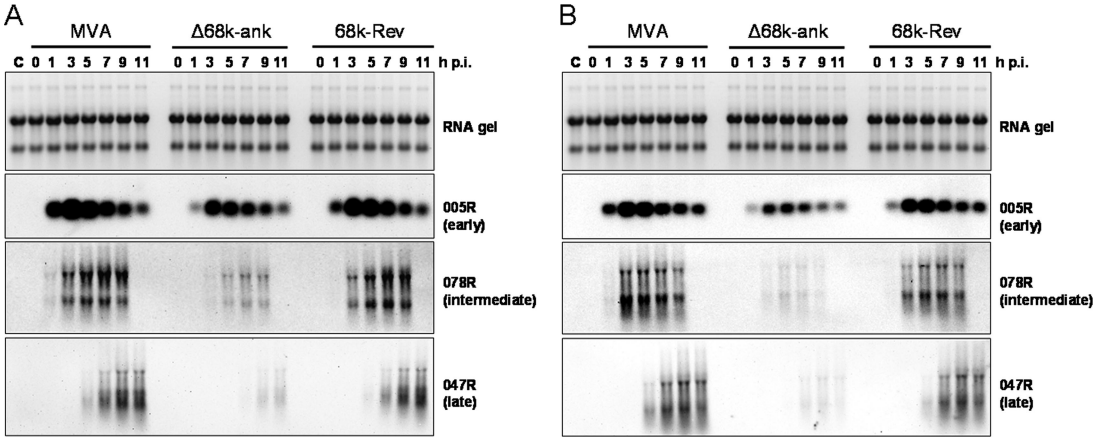

FIG. 3. Northern blot analysis of viral early 005R, intermediate 078R, and late 047R transcripts. NIH 3T3 cells (A) or HaCaT cells (B) were mock infected (C) or infected with MVA, MVA-Δ68k-ank (Δ68k-ank), or MVA-68k-Rev (68k-Rev) at an MOI of 5. Total RNA was isolated at 0, 1, 3, 5, 7, 9, and 11 h p.i., and 1.5 μg of each sample was electrophoretically separated in 1% agarose formaldehyde gels. RNA was subsequently transferred onto a positively charged nylon membrane (Roche Diagnostics) by vacuum blotting and probed with riboprobes specific for 005R, 078R, and 047R. Ethidium bromide-stained RNA gel is shown as a loading control.

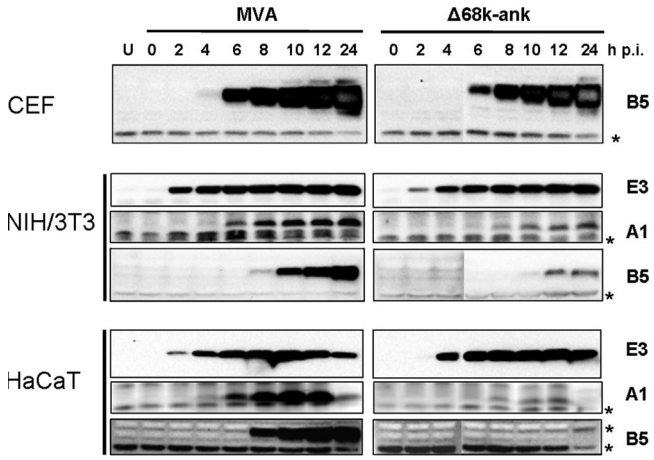

FIG. 4. Western blot analysis of early E3, intermediate A1, and late B5 protein expression. CEF, NIH 3T3, or HaCaT cells were mock infected (U) or infected with MVA or MVA-Δ68k-ank (Δ68k-ank) at an MOI of 5. Whole-cell lysates were prepared at 0, 2, 4, 6, 8, 10, 12, and 24 h p.i. and separated by SDS-PAGE. After transfer of proteins to PVDF membranes, E3, A1, and B5 proteins were detected with specific rabbit polyclonal antiserum. Unspecific bands recognized by the respective polyclonal antibodies are indicated (*).

ank from the MVA genome had such a great impact on viral RNA transcription, we monitored for intermediate and late protein production by Western blot analysis. The viral late gene transcription factor 2 A1 protein and envelope protein B5, representing well-characterized intermediate and late gene products, respectively (13, 20), served as model antigens for newly synthesized intermediate and late viral polypeptides. Infections of CEF defined the optimal conditions for high-level generation of A1 and B5 proteins, which were detectable at 4 h and 6 h of infection, respectively, and thereafter (Fig. 4; see also Fig. 8). In comparison to these permissive cells, in nonpermissive NIH 3T3 cells, MVA-produced A1 protein was first detectable at 6 h p.i., and B5 protein was first detected at 8 h p.i. B5 was made at reduced levels but with similar expression kinetics and increasing amounts over time. In contrast, we found substantially lower amounts of both A1 and B5 proteins in mutant MVA-Δ68k-ank virus-infected murine fibroblasts. A1 proteins were detectable at delayed time points p.i. compared to wild-type infection. A first faint band appeared at 8 h after infection with increasing amounts over time, but overall amounts were reduced in comparison with MVA. B5 protein was present only at delayed time points at 12 h and 24 h after infection, and there appeared to be no increase in B5 protein synthesis between these two time points. Of note, the impairment in protein production as a mutant phenotype was even more distinct in human HaCaT cells. A1 protein was barely detectable, with only a very faint signal at 8 to 12 h p.i., and we were not able to detect late B5 protein at any time point of infection with MVA-Δ68k-ank. In contrast, detection of the early E3 gene product clearly confirmed comparable infections of wild-type and mutant viruses in HaCaT cells (Fig. 4, bottom panel).

Impairment of viral late protein synthesis and delayed shutoff of host protein synthesis in the absence of 68k-ank. Poxviral infection is typically accompanied by a rapid shutoff of host protein synthesis (28). We therefore studied polypeptide synthesis by metabolic labeling of infected cells. NIH 3T3 and HaCaT cells were either mock infected or infected with MVA, MVA-Δ68k-ank, or MVA-68k-Rev and labeled with [^{35}S]methionine-cysteine for 30 min at several time points postinfection. Subsequently, total cell lysates were resolved by SDS-PAGE and analyzed by autoradiography, allowing the visualization of synthesis of new viral and cellular proteins (Fig. 5). At 3 h after infection of NIH 3T3 cells with wild-type, knockout, or revertant virus, comparable early protein synthesis was detectable, and typical viral late polypeptides were clearly visible at 6 h and 9 h after infection with MVA and MVA-68k-Rev viruses (Fig. 5A). In sharp contrast, these prominent viral late proteins were not made in NIH 3T3 cells infected with the 68k-ank deletion mutant. This result sug-

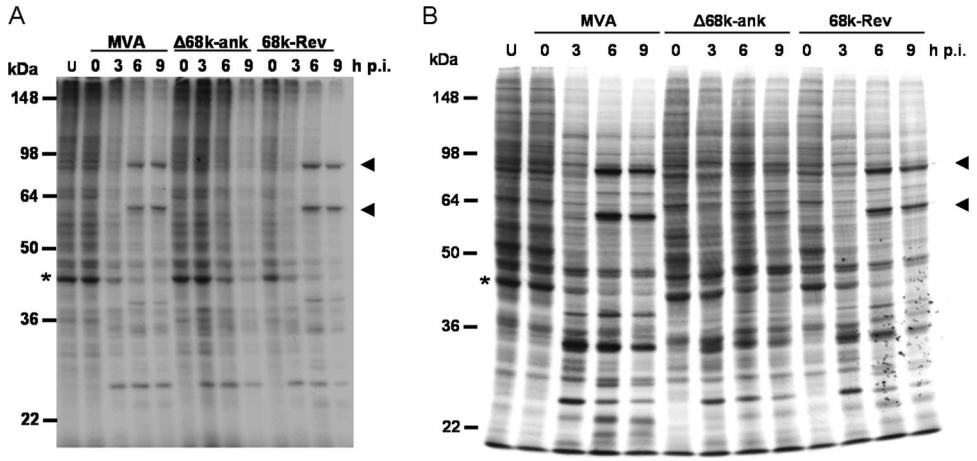

FIG. 5. Analysis of viral polypeptide synthesis. NIH 3T3 cells (A) and HaCaT cells (B) were either mock infected (U) or infected at an MOI of 10 (A) or 25 (B) with either MVA, MVA-Δ68k-ank (Δ68k-ank), or MVA-68k-Rev (68k-Rev). At 0, 3, 6, and 9 h p.i., cells were labeled with [^{35}S]methionine-cysteine for 30 min. Whole-cell lysates were prepared and separated by 10% SDS-PAGE and analyzed by autoradiography. Typical viral late polypeptides are indicated by arrowheads, and a prominent cellular protein is indicated by an asterisk. Marker protein sizes (kDa) are indicated to the left of each panel.

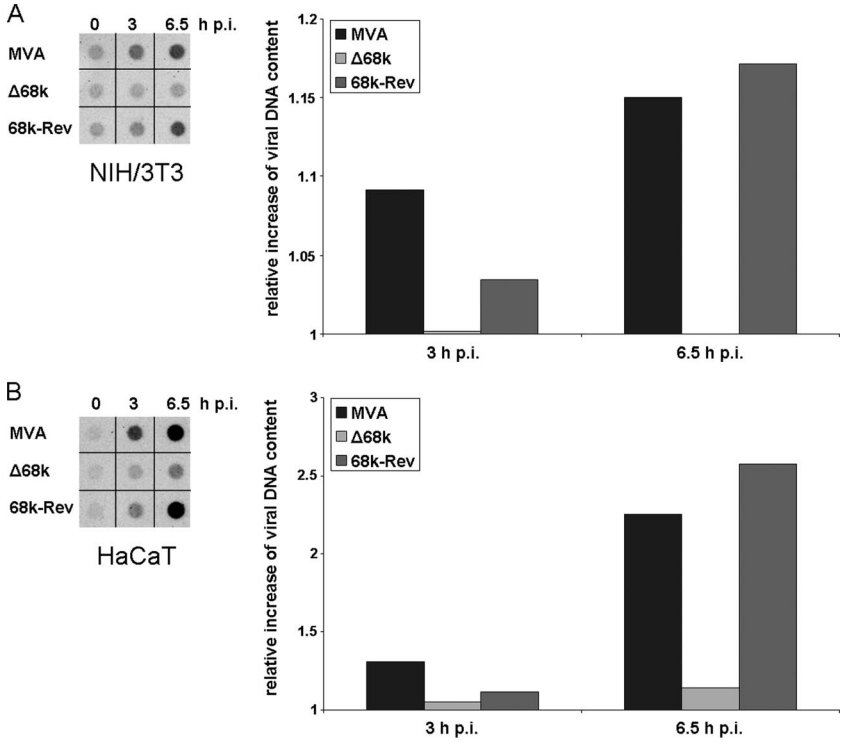

FIG. 6. Analysis of viral DNA replication. NIH 3T3 cells (A) or HaCaT (B) cells were infected with MVA, MVA-Δ68k-ank (Δ68k-ank), or MVA-68k-Rev (68k-Rev) at an MOI of 3. At 0, 3, and 6.5 h p.i., DNA was prepared from cell lysates. DNA (0.5 μg) was transferred onto positively charged nylon membranes and hybridized with an MVA-specific DIG-labeled DNA probe. Bar graphs show increase in viral DNA content relative to time point zero p.i.

gested a general inhibition of viral late protein synthesis and confirmed the data obtained from our analysis of B5 late protein expression (Fig. 4). In addition, we observed that the shutoff of host protein synthesis seemed to be delayed in MVA-Δ68k-ank-infected NIH 3T3 cells. In MVA- and MVA-68k-Rev-infected cells, there was an early cessation of host protein synthesis detectable by the absence of labeled cellular proteins, as seen by comparison of the polypeptide patterns at time points 0 h p.i. and 3 h p.i. Cellular protein synthesis was better maintained in MVA-Δ68k-ank-infected cells, with substantial levels of shutoff visible only late in infection. Also in HaCaT cells we corroborated the striking impairment of the mutant virus to mediate late viral polypeptide production (Fig. 5B). Moreover, in this human cell line, the failure of host shutoff was even more pronounced. In contrast, in CEF cells, which were also permissive for MVA-Δ68k-ank replication (Fig. 2), this influence on host protein synthesis was not detected (Fig. 8B). Thus, deletion of the 68k-ank gene not only impaired viral late protein synthesis but also seemed to interfere with the capability of the virus to efficiently interrupt the host cell protein synthesis in nonpermissive cells.

Lack of intermediate and late gene expression in MVA-Δ68k-ank-infected cells is accompanied by lack of viral DNA replication. The poxvirus replication cycle within the host cytoplasm is tightly regulated, and one distinct feature is the fact that DNA replication is a prerequisite (19, 47) for intermediate gene expression. As the mutant phenotype described so far was characterized by impaired intermediate transcription and translation, we wanted to additionally test for viral DNA replication. Indeed, the mutant MVA-Δ68k-ank failed to efficiently amplify its DNA in infected NIH 3T3 (Fig. 6A) and HaCaT (Fig. 6B) cells, whereas MVA and MVA-68k-Rev demonstrated the typical increase of viral DNA over time. Therefore, the impairment of intermediate and late gene expression in mutant virus infections is obviously a consequence of insufficient DNA replication.

Mutant phenotype is apoptosis independent. Host range function is in some cases related to the inhibition of apoptosis, as was shown for VACV E3 protein or the ANK-F-box protein of MV, M-T5, as well as for CP77 (reviewed in reference 37). Thus, we were interested in whether the disturbance of the intracellular life cycle observed in MVA-Δ68k-ank-infected cells would correlate with an increased induction of apoptosis compared to MVA infection. As a positive control for the assay, we treated the cells with 1 μM staurosporine, which is an unspecific inducer of apoptosis. The F1 protein of VACV is a fairly well-characterized inhibitor of apoptosis, and it was shown previously that MVA with a deletion of the F1L gene (MVA-ΔF1L) induced increased levels of apoptosis (14). Consequently, MVA-ΔF1L infection was included as a positive control for virally mediated apoptosis. Hence, we infected NIH 3T3 cells with MVA, MVA-Δ68k-ank, MVA-68k-Rev, or MVA-ΔF1L at an MOI of 5 and analyzed active caspase-3 as a marker of apoptosis induction at 24 h p.i. This was done by staining exclusively the active form of caspase-3 with a PE-labeled specific antibody, followed by flow cytometric analysis. Figure 7 depicts the percentage of active caspase-3-positive cells measured in three independent assays, and it clearly

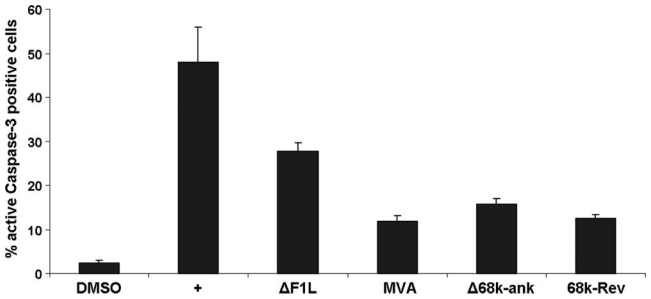

FIG. 7. Analysis of apoptosis induction by active caspase-3 staining. NIH 3T3 cells were either mock infected (DMSO), treated with 1 μM staurosporine (+), or infected with MVA, MVA-ΔF1L (ΔF1L), MVA-Δ68k-ank (Δ68k-ank), or MVA-68k-Rev (68k-Rev) at an MOI of 5. At 24 h p.i., cells were fixed, permeabilized, and subsequently stained with a PE-labeled anti-active caspase-3 antibody. Mean results from FACS analysis of three independent experiments are displayed.

shows that infection with MVA-Δ68k-ank resulted in background levels of apoptosis equal to the level seen with wild-type or revertant virus. In contrast, infection with MVA-ΔF1L led to increased amounts of active caspase-3. These data clearly suggested that the newly established mutant phenotype of MVA-Δ68k-ank is independent from the induction of apoptosis.

Inhibition of the cellular proteasome abrogates MVA intermediate and late gene expression. The cellular ubiquitin-proteasome system (UPS) plays a major role in controlling the abundance of cellular proteins by proteolytic degradation (9). Various cellular pathways are steered by selective regulator protein turnover, as, for example, the timely regulation of cell-cycle progression (34). Polyubiquitinylation of proteins is a prerequisite for the degradation of proteins via the 26S proteasome. The Skp1a-Cullin-1-based SCF complex is a multisubunit RING finger E3 ligase that ubiquitinates proteins of various cellular pathways (6). Previously, we reported that 68k-ank via its F-box-like domain binds Skp1a and associates with Cullin-1 to form an SCF complex (40), whose targets for ubiquitinylation are still unknown. Nevertheless, we were interested in whether the cellular UPS is important to MVA infections. Therefore, we blocked the 26S proteasome with the inhibitor MG-132. Cells were treated with this compound for 1 h and subsequently either mock infected or infected with MVA at an MOI of 5. At different time points after infection, we tested for the early E3, intermediate A1, and late B5 proteins by Western blotting. Treatment of nonpermissive NIH 3T3 and HaCaT cells, as well as permissive CEF cells, with a 10 μM concentration of the reversible proteasome inhibitor MG-132 completely abrogated intermediate A1 and late B5 production upon MVA infection (Fig. 8A). In contrast, early protein E3 was detectable from 2 h p.i. and throughout all time points analyzed, irrespective of treatment with MG-132. We further analyzed viral protein synthesis upon proteasome inhibition by metabolic labeling, confirming that major viral polypeptides of the intermediate/late class were not made in CEF cells treated with MG-132 (Fig. 8B). Consistently, proteasome inhibition also blocked MVA replication in CEF cells (data not shown). Interestingly, the phenotype we established for MVA-Δ68k-ank in nonpermissive cells resembles that of MVA in the presence of proteasomal inhibition. Thus, we wondered whether host protein shutoff was also affected by proteasome inhibition. As Fig. 8B clearly shows, this was not the case; shutoff of host protein synthesis is readily obvious at 3 h p.i., irrespective of MG-132 treatment (Fig. 8B).

F-box-like domain of 68k-ank is not essential to maintain intermediate and late gene expression. The outcome that pro-

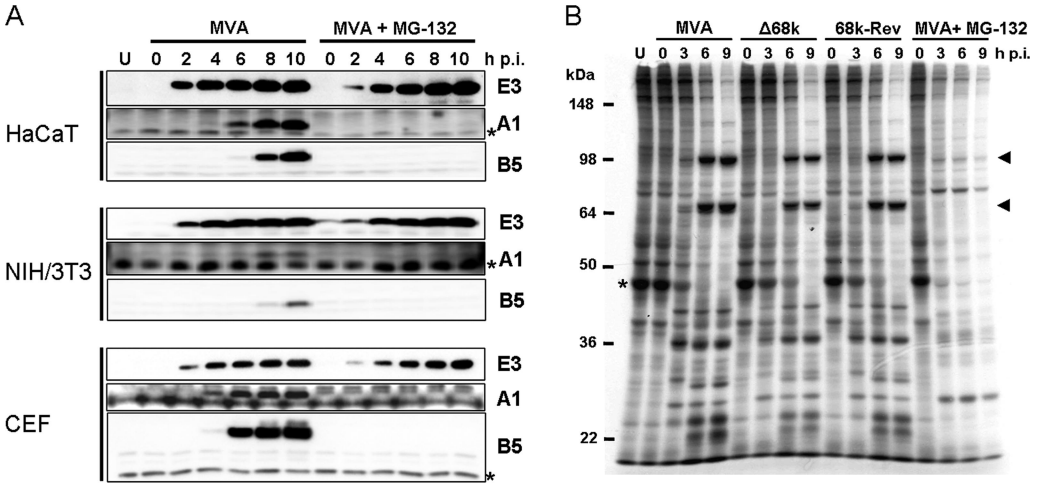

FIG. 8. Influence of proteasome inhibition on viral protein expression. (A) Western blot analysis of early E3, intermediate A1, and late B5 expression. CEF, HaCaT, or NIH 3T3 cells were mock treated or incubated with 10 μM MG-132 for 1 h. Cells were then mock infected or infected with MVA at an MOI of 5 in infection medium containing 10 μM MG-132. Whole-cell lysates were prepared at 0, 2, 4, 6, 8, and 10 h p.i. and separated by SDS-PAGE. After transfer of proteins to PVDF membranes, E3, A1, and B5 proteins were detected with specific rabbit polyclonal antisera. Unspecific bands recognized by the respective polyclonal antibodies are indicated by an asterisk. (B) Metabolic labeling of infected CEF cells. Cells were either mock infected (U) or infected at an MOI of 10 with either MVA, MVA-Δ68k-ank (Δ68k-ank), or MVA-68k-Rev (68k-Rev) or with MVA in the presence of 10 μM MG-132. At 0, 3, 6, and 9 h p.i., cells were labeled with [^{35}S]methionine-cysteine for 30 min. Whole-cell lysates were prepared and separated by 10% SDS-PAGE and analyzed by autoradiography. Typical viral late polypeptides are indicated by arrowheads, and a prominent cellular protein is indicated by an asterisk. Marker protein sizes (kDa) are indicated to the left.

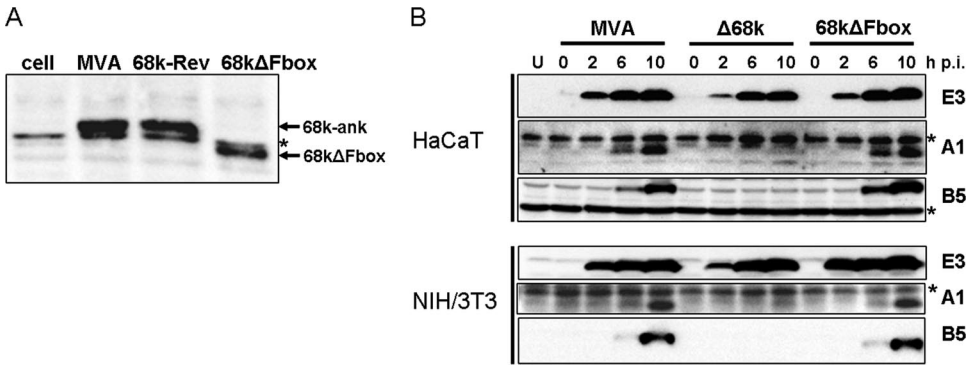

FIG. 9. Effect of F-box-like domain deletion in 68k-ank. (A) Western blot analysis of 68k-ank or 68k-ankΔFbox expression. CEF cells were mock infected (cell) or infected with MVA, MVA-68k-Rev (68k-Rev), or MVA-68kΔFbox (68kΔFbox) at an MOI of 5 for 2 h. Cell lysates were separated by SDS-PAGE, and proteins were transferred to a PVDF membrane. 68k-ank expression was detected with a specific rabbit antiserum; an asterisk marks an unspecific band recognized by anti-68k-ank rabbit polyclonal antibody. (B) Western blot analysis of early E3, intermediate A1, and late B5 protein expression. HaCaT or NIH 3T3 cells were mock infected (U) or infected with MVA, MVA-Δ68k-ank (Δ68k-ank), or MVA-68kΔFbox (68kΔFbox) at an MOI of 5. Whole-cell lysates were prepared at 0, 2, 6, and 10 h p.i. and separated by SDS-PAGE. After transfer of proteins to PVDF membranes, E3, A1, and B5 proteins were detected with specific rabbit polyclonal antisera. Unspecific bands recognized by the respective polyclonal antibody are indicated by an asterisk.

teasome inhibition efficiently blocked MVA intermediate and late gene expression encouraged us to test the hypothesis that a lack of 68k-ank, possibly predominantly functioning in an SCF complex, could be associated with the very similar phenotype of the knockout virus in murine and human cells. To directly address this question, we constructed an MVA mutant in which we specifically removed the C-terminal F-box-like domain from the 68k-ank protein. This additional recombinant virus was successfully constructed and amplified in CEF cells to levels very comparable to wild-type MVA (data not shown). Western blot analysis verified expression of the truncated 68k-ank (68kΔFbox) in infected CEF cells as early as 2 h p.i. (Fig. 9A). To test the relevance of the F-box-like domain of 68k-ank for MVA protein synthesis, we infected HaCaT and NIH 3T3 cells with MVA, MVA-Δ68k-ank, and MVA-68kΔFbox, and at various times p.i. we again monitored for the presence of the early, intermediate, and late viral proteins E3, A1, and B5, respectively (Fig. 9B). Early E3 protein was, as in the previous analyses, expressed at comparable levels in both cell lines infected with all of the three viruses. Surprisingly, in sharp contrast to the infections with the full-length 68k-ank deletion mutant MVA-Δ68k-ank, the F-box mutant virus MVA-68kΔFbox was able to express viral intermediate A1 and late B5 as efficiently as wild-type MVA in these nonpermissive cells. Importantly, these data clearly demonstrated that the functional activity of 68k-ank in supporting the virus to express its complete genetic information is independent of the F-box-like domain of 68k-ank and thus likely unconnected to its role in SCF complex formation.

DISCUSSION

Virus-host interactions are likely to play a major role in defining the desirable phenotype of MVA as a (vector) vaccine, and their study is indispensable to understanding the poxviral life cycle in general. MVA vaccines are characterized by the attractive combination of well-established safety and high efficiency. MVA is replication deficient in most mammalian cells, especially in those of human origin, but has not lost potency for high-level antigen delivery due to its ability to complete the whole cascade of viral gene expression in nonpermissive cells. Particularly, the loss of many viral virulence and immune evasion factors is believed to account for the attenuation of MVA and for its particular immunostimulatory capacity. Hence, there is also need to understand the function of nonessential genes preserved in the genome of MVA, how these regulatory proteins contribute to the viral life cycle, and if modulations of these functions may allow the derivation of optimized MVA-based vaccines or therapeutics.

The 68k-ank protein is the only ANK-containing protein that was preserved during the attenuation of MVA, and in addition it has a high degree of conservation throughout the *Orthopoxvirus* genus (40). This implies a crucial role for 68k-ank for the MVA molecular life cycle and for orthopoxviruses in general. Therefore, we constructed a mutant MVA deficient for 68k-ank expression (MVA-Δ68k-ank), as well as a revertant virus (MVA-68k-Rev). Despite the high degree of conservation, growth in permissive CEF cells, routinely used to propagate MVA, remains unaffected by the deletion of 68k-ank. Similarly, an MV mutant that had the ANK-F-box protein (M-T5) gene deleted was able to replicate with wild-type kinetics in MV-permissive rabbit fibroblasts (29). The apparent paradox that a gene that is nonessential for MVA growth in CEF cells is nevertheless retained in the MVA genome is something that is found for several gene products encoded by MVA. For instance, inactivation of the MVA genes encoding the viral interleukin-1ß receptor (42), the apoptosis inhibitor F1 (14), or the host-range factor C7 (G. Sutter, K. Sperling, C. Staib, et al., unpublished data) does not affect MVA replication in CEF cells. How the extensive passage on CEF cells led to the evolution of MVA and what selective mechanisms drove the loss of certain but not all so-called nonessential genes are still unknown.

Analysis of the molecular life cycle of MVA-Δ68k-ank in nonpermissive cells, human keratinocytes, HaCaT, and murine fibroblast (NIH 3T3) cells revealed that, in contrast to permis-

sive conditions, 68k-ank is indeed an essential factor that is expressed early during infection and contributes to the completion of the intracellular molecular life cycle of MVA. We established a mutant phenotype that is characterized by greatly impaired viral RNA production, possibly starting at the level of early transcription but with a very minor impact, increasing at intermediate stages, and finally resulting in severely impaired late RNA synthesis and consequently in reduced intermediate protein production and impaired late protein synthesis. Consistently, we could show that 68k-ank is essential for DNA replication, thus corroborating the need for early synthesis of 68k-ank. The phenotype of impaired intermediate and late gene expression found in murine NIH 3T3 cells was more pronounced in human HaCaT cells and was similar to observations made for infection of the rabbit $CD4^+$ T-cell line (RL5) with the MV M-T5 deletion mutant (29), which is characterized by rapid and complete cessation of both host and viral protein synthesis accompanied by the induction of programmed cell death. In contrast, absence of 68k-ank did not induce apoptosis in infected cells but affected mainly intermediate and consequently late protein synthesis. In addition, shutoff of host protein synthesis following infection of a cell with VACV was strongly delayed by the deletion of the 68k-ank gene. Again, this effect was more pronounced in HaCaT cells. Regulation of selective host protein synthesis shutoff is still not well understood. A class of short polyadenylated virus-directed RNAs that are synthesized in VACV-infected cells during the early phase of transcription was correlated with host shutoff (5). This observation indicates that slightly lower amounts of early RNA in MVA-Δ68k-ank-infected cells might negatively influence host protein shutoff and lead to a delay. This effect is more prominent in HaCaT cells and might be overcome by larger amounts of virus in NIH 3T3 cells (data not shown). Moreover, earlier studies on host protein shutoff with UV-irradiated VACV that exhibited decreased transcription (3, 31) also suggested a general relation between viral mRNA levels and host protein synthesis (2). Thus, the total mRNA reduction in MVA-Δ68k-ank-infected cells observed in comparison to wild-type or revertant virus might be the cause for a dampened impact on host protein synthesis. Studies on VACV infections in CHO cells showed a significant host shutoff accompanied by the induction of apoptosis that was reversed by expression of the cowpox host range factor CP77 (CHOhr) (11, 18). In view of these data, our finding that ablation of 68k-ank from MVA rather prolongs host protein synthesis seems to fit well with our failure to observe induction of apoptosis upon mutant virus infection.

Previously, we showed that 68k-ank forms an SCF ubiquitin ligase complex together with cellular Skp1a and Cullin-1; however, the target of proteasomal degradation is still unknown. Due to the connection of 68k-ank with the UPS, we wondered whether the cellular proteasome is important for MVA in general. Indeed, the interaction of poxviruses with the UPS has come into focus of recent research with the demonstration that UPS function is relevant for productive replication of orthopoxviruses (35, 46). Upon treatment with proteasome inhibitors, the orthopoxvirus life cycle is blocked at a stage succeeding early gene expression. Our finding that nonreplicating MVA was also affected by MG-132 treatment is consistent with these recently published data. For MVA infection, we could also show that viral intermediate and late gene expression depends on a functional UPS not only in permissive CEF cells but also in HaCaT and NIH 3T3 cells that do not allow for production of MVA progeny. There have also been reports demonstrating that a functional UPS is essential to other viruses. Replication of coxsackievirus B3 was shown to be reduced in HeLa cells treated with proteasome inhibitors, and similar results were obtained for vesicular stomatitis virus (30, 38); moreover, avian reovirus protein expression was markedly reduced in BHK-21 cells treated with MG-132 (8). Therefore, the UPS seems to be a cellular mechanism frequently targeted or used by many different viruses. When we first established the phenotype of the mutant virus MVA-Δ68k-ank, it was very tempting to speculate that the lack of an interaction of 68k-ank in an SCF complex could be the dominant mechanism underlying the severe deficiency of this mutant in gene expression. This idea was first supported by the fact that use of the proteasome inhibitor MG-132 in cells nonpermissive to MVA replication abolished viral intermediate and late gene expression and resulted in a block of the virus molecular life cycle very similar to the one observed with the knockout virus. Yet a functional UPS was also required for MVA infection of CEF cells, which were permissive in the absence of 68k-ank. Moreover, we found that production of a mutant 68k-ank protein lacking the F-box-like domain was sufficient to allow for the complete pattern of MVA gene expression. Thus, it seems as if 68k-ank may be a multifunctional protein, probably as a result of the distinct functions of the different domains of the protein. Actually, 68k-ank might work in a way similar to the cowpox virus host range protein CP77 that, independent of its host range function, can form an SCF complex with Skp1a and Cullin-1 to target NF-kB subunit p65 (7). What we describe here for the role of 68k-ank in MVA infection is, strictly speaking, not a host range function because MVA cannot produce infectious progeny in HaCaT and NIH 3T3 cells due to a late block in morphogenesis. However, MVA does express all three classes of viral genes in these typical nonpermissive mammalian cells, and a failure to do so, as in the absence of 68k-ank, can be ascribed to a host range-like defect. Therefore, 68k-ank is an essential determinant for MVA to express its genetic information in nonpermissive cells of human and murine origin, and this function of 68k-ank is independent of the F-box-like domain. Further studies to define this 68k-ank function should likely include the analysis of the ANK domains because these motifs are also found in other poxvirus host range factors, such as VACV K1, cowpox virus CP77, or MV M-T5.

In summary, our data clearly define the MVA 68k-ank protein encoded by the 186R gene as an essential factor necessary for completion of the viral life cycle in nonpermissive NIH 3T3 and HaCaT cells. Since these cells must be considered representative for in vivo target cells of MVA infection during vaccination, the 186R gene should be retained in the MVA genome to secure high-level expression of heterologous proteins. The 68k-ank protein is highly conserved among orthopoxviruses, and we believe that it has also a significant function for replication-competent orthopoxviruses in vitro and maybe in vivo.

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