The Cellular Antiviral Restriction Factor Tetherin Does Not Inhibit Poxviral Replication

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Interferon-stimulated genes fulfill innate antiviral effector functions. Among them, tetherin (THN) blocks the release of many enveloped viruses from infected cells. Vaccinia virus (VACV) encodes immune modulators interfering with antiviral host responses. Therefore, it was tempting to study a potential VACV-THN interaction. Remarkably, THN expression did not inhibit VACV release and replication. VACV infection did not diminish THN surface levels or impair its function on retroviral release. This suggests that THN is unable to restrict VACV replication.

Viral infections can induce a type I interferon (IFN) response in cells which stimulates the expression of numerous ISGs (interferon-stimulated genes) (30). In turn, viruses have evolved factors to counteract these defense mechanisms. One of these IFN-induced cellular proteins is tetherin (THN) (also referred to as BST-2, CD317, or HM1.24) (23, 30, 39), the expression of which inhibits the release of many enveloped viruses, including retroviruses (6, 9, 13, 23, 42, 46) and among them human endogenous retrovirus K (HERV-K) (10), filoviruses (10, 11, 31), herpesviruses (15), and arenaviruses (26). THN is a type II transmembrane glycoprotein with a short cytoplasmic N-terminal region, a transmembrane region, and an extracellular domain with a C-terminal glycosylphosphatidylinositol (GPI) anchor. THN localizes to budding sites of enveloped viruses and incorporates THN. These two membrane anchors into viral membranes and thereby tethers viral particles to the cell and to each other. Several viruses have evolved strategies to overcome this cellular virion tethering by expressing proteins that downregulate tetherin within the cell surface or interfere with its function. Seven viral proteins have been reported so far: human immunodeficiency virus type 1 (HIV-1) Vpu (viral protein U) (9, 23, 42, 44), HIV-2, and simian immunodeficiency virus type 2 (SIV-2) envelope protein (Env) (6, 13), SIV Nef (negative regulatory factor) (9, 46), Kaposi’s sarcoma-associated herpesvirus (KSHV) K5 (a membrane-associated, RING-CH-containing [MARCH] family E3 ubiquitin ligase) (15), the Ebola virus glycoprotein (GP) (11, 14), and neuraminidase from influenza A virus (45). These THN antagonists mediate their action by different mechanisms. The HIV-1 Vpu protein, the SIV Nef protein, and the KSHV K5 protein lead to the degradation of THN and diminish the protein concentration at its site of action, the plasma membrane. However, sequestration in intracellular compartments also interferes with THN action. This mode of action is used by the Ebola virus GP and the HIV-2 Env proteins, which downregulate and sequester THN within the trans-Golgi network (TGN) (4).

Poxviruses, like the prototype member vaccinia virus (VACV), are large double-stranded DNA viruses encoding over 200 gene products, many of them with still unknown function. Poxvirus replication is accompanied by a cascade-like viral gene expression in the infected cell and is divided into early, intermediate, and late genes and gene products (20). Although the molecular details of poxvirus assembly and maturation remain controversial, the most widely accepted mechanism involves the generation of at least three forms of infectious particles (21). The multiple infectious forms differ from each other by the number of membrane layers and the membrane protein composition. Poxviral assembly starts with the formation of previrion particles, which are crescent-shaped membranes, and ends with virions containing the DNA genome, virion enzymes, and structural proteins, referred to as intracellular mature virions (IMV) (20, 28). During the maturation process, a portion of the IMV become enveloped with additional membranes derived from the trans-Golgi apparatus (7, 33) or endosomal cisternae (40) and are referred to as intracellular enveloped virus (IEV) or wrapped virus (WV) (21). Following migration to the cell surface (22, 27, 38), the outer IEV membrane fuses with the plasma membrane, resulting in exocytosis, which gives rise to extracellular (enveloped) virus (EV) (25). The EV can either remain associated with the cell (cell-associated enveloped virus [CEV]) or detach and be released as extracellular enveloped virus (EEV) (2). The EEVs are mainly responsible for virus dissemination in vivo (24, 37, 38) and constitute only a small amount of all progeny virions (21, 24, 25). The associated form is usually predominant and primarily responsible for cell-to-cell spread by inducing membrane protrusions, so-called “actin tails” (2, 29, 32) (for detailed review, see references 36 to 38). Budding at the cell surface has also been described as an alternative release mechanism of EEV (17, 41). The process of assembly and maturation is also reviewed in reference 35. Poxviruses code for numerous proteins that interfere with the cellular innate immune responses, including the IFN system (reviewed in reference (34). Since THN expression is induced by IFN (42), VACV assembly and release involve membranes potentially containing THN, and cell-associated virus (CEV) is detectable, we were interested in determining whether VACV spread is affected by THN and whether VACV infection influences surface THN expression levels.

These questions were analyzed using three pairs of cell lines stably expressing high levels of a hemagglutinin (HA)-tagged THN or untagged THN and the corresponding cells without THN.
expression. We used HEK293 cells (293HA-THN or 293ev), NIH 3T3 cells (negative or expressing HA-THN), and HT1080 (negative or expressing THN) cells were stained with anti-human THN antibody (1:20 anti-BST2 phycoerythrin [PE] conjugate; eBioscience, Frankfurt am Main, Germany) and analyzed by FACS. We tested the three genetically modified cell lines and infected them with both VACV WR and MVA at an MOI of 0.05. THN expression levels at the surface of infected cells were not significantly altered (Fig. 2). Moreover, infection with an MOI of 5 did not alter THN expression (data not shown). We confirmed VACV infection of the cells by detection of the early protein K1 by Western blot analysis (data not shown).

Since VACV replicates to similar titers in THN-positive and -negative cells, we hypothesized that VACV may express proteins capable of antagonizing THN—for example, by downregulating THN protein. To identify a potential impact of VACV infection on THN cell surface levels, infected cells were stained at different time points after infection for THN expression and analyzed by fluorescence-activated cell sorter (FACS). Since poxvirus infection leads to inhibition of host cell protein synthesis starting at 5 to 6 h postinfection (20), we determined THN surface levels at time points up to 8 h postinfection. At later time points, reduced THN surface levels are likely a result of the general shutoff of host protein synthesis. We tested the three genetically modified cell lines and infected them with both VACV WR and Cop at an MOI of 0.05. THN expression levels at the surface of infected cells were not significantly altered (Fig. 2). Moreover, infection with an MOI of 5 did not alter THN expression (data not shown). We confirmed VACV infection of the cells by detection of the early protein K1 by Western blot analysis (data not shown).

The highly attenuated VACV strain modified vaccinia virus Ankara (MVA) has lost about 15% of the ancestral genome (1, 16, 18), including genes critically involved in virus-host interaction and immune modulation. As a result, MVA is, in contrast to
VACV, unable to repress the host cell IFN response (43). Consequently, MVA infection of host cells might stimulate THN expression. MVA does not replicate in 293 cells due to a defect in virus assembly, and as observed before (Fig. 2), MVA infection did not modulate THN surface expression in the context of a high or low MOI (MOI of 5 or 0.05, respectively) (data not shown). These data suggest that MVA does not require downmodulation of THN expression for survival.

Theoretically, VACV or MVA could still express proteins that efficiently counteract THN by a mechanism distinct from cell surface downregulation, as observed for HIV-2 or Ebola envelope proteins (3, 13, 14). To address this question, we made use of a recombinant MVA, MVA-HERV-K<sub>con</sub>, which expresses the human endogenous retrovirus K (HERV-K) Gag/Pol protein. Infection of cells with the recombinant MVA results in the release of HERV-K virus-like particles budding from the MVA-infected cell (12). HERV-K particles are inhibited in their release by THN (10). Consequently, budding of HERV-K particles should be decreased in THN-expressing cells in comparison to THN-negative cells. Potential THN antagonists encoded by MVA should restore the budding phenotype, as observed in THN-negative cells. 293HA-THN cells compared to THN-negative cells (Fig. 3, compare lanes 2 and 4). The experiment was performed three times, and a 4-fold inhibition of HERV-K release was found comparing supernatants with lysates. These observations indicate that poxviruses do not

![Image of graphs showing THN expression levels](image1.png)

**FIG 2** THN expression levels on the cell surface remain unaffected by VACV infection. Shown is THN surface expression of 293HA-THN, 3T3HA-THN, and HT1080THN cells infected with VACV WR or VACV Cop at an MOI of 0.05, determined by FACS-based measurement of the mean fluorescence intensity (MFI) at the depicted time points. Prior to measurement, cells were stained with a monoclonal anti-human THN antibody directly conjugated with PE (eBioscience, Frankfurt am Main, Germany, 1:20).

![Image of graphs showing Western blot analysis](image2.png)

**FIG 3** MVA infection does not alter THN function. Western blot analysis of MVA-HERV-K<sub>con</sub>-infected cells and concentrated cell culture supernatants. 293HA-THN cells (THN +) and 293ev cells (THN −) were infected with MVA-HERV-K<sub>con</sub> at MOI of 0.1 and 5. Cell lysates (L) and concentrated cell culture supernatants (S) of infected cells (24 h postinfection) are shown. HERV-K Gag was detected with a monoclonal antibody mixture directed against the capsid protein (α-HERV-K) (12). Loading was confirmed incubating the blots with an antibody directed against β-actin (1:10,000 anti-β-actin [α-β-Actin]; Sigma-Aldrich, Taufkirchen, Germany).
interfere with THN function and that the restriction factor is still functional to tether retroviral particles at the cell surface. In line with this observation, THN did not colocalize with poxviral proteins in infected 293 HA-THN cells when analyzed with a polyconal anti-vaccinia virus serum by immunofluorescence microscopy, nor could VACV proteins be coimmunoprecipitated with THN from HeLa cells (data not shown).

Our data demonstrate an unaltered coexistence of the cellular antiviral restriction factor THN and VACV replication in infected cells. Although poxviruses encode numerous proteins to circumvent the cellular antiviral defense mechanisms, they apparently do not influence surface expression levels or function of the cellular restriction factor THN, and concomitantly, THN seems incapable of restricting VACV replication.

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REFERENCES