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BRIEF REPORT

Vaccinia virus G1 protein: absence of autocatalytic self-processing

F. G. G. Leite[1,3] · A. Bergthaler[2] · T. Skern[1]

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Abstract Vaccinia virus relies on a series of proteolytic cleavage events involving two viral proteins, I7 and G1, to complete its life cycle. Furthermore, G1 itself is cleaved during vaccinia virus infection. However, convincing evidence is lacking to show whether G1 participates in autoproteolysis or is a substrate of another protease. We employed both biochemical and cell-based approaches to investigate G1 cleavage. G1, when expressed in bacteria, rabbit reticulocyte lysates or HeLa cells, was not processed. Moreover, G1 was cleaved in infected cells, but only in the presence of virus late gene expression; cleavage was strongly inhibited by proteasome inhibitors. Thus, these results imply a more complex G1 cleavage reaction than previously envisaged.

The *Poxviridae* comprise a family of complex cytoplasmic replicating DNA viruses, which includes a number of important human and animal pathogens such as variola virus and vaccinia virus [19]. Poxviruses have a complex life cycle including distinct morphogenetic stages in which a spherical, immature particle is transformed into a brick-shaped, infectious virion. An essential step for this maturation is a series of proteolytic cleavage events involving the viral proteins I7 viral core cysteine proteinase and metalloendopeptidase G1 [11]. In the past decade, attention has focused on these two proteins as interesting targets for development of anti-poxvirus drugs [7, 9, 18]. I7 has been shown to be inhibited by classical cysteine protease inhibitors (such as iodoacetic acid and N-ethyl maleimide) as well as an active-site inhibitor (termed TTP-6171) that is specific for I7 and unrelated to any canonical protease inhibitors [1, 4, 6–10, 12, 18].

In contrast, biochemical knowledge about G1 is still limited. Mutational analysis has shown that G1 is required for conversion of immature virions into infectious mature particles. As G1 mutants arrest replication subsequent to core protein cleavage, the role of G1 in the conversion of the immature virus remains obscure [3, 6, 15]. However, G1, a 68-kDa protein of 591 amino acids, has been predicted by bioinformatics to be a metalloproteinase [16]. This conclusion is based on the presence of a conserved zinc-binding motif, HXXEH [5]; this sequence is a direct inversion of the established HEXXH motif found in many matrix metalloendopeptidases (MMPs). The HXXEH motif is 100% conserved among members of the family *Poxviridae*, implying that it is vital for the enzymés active site. Interestingly, in all poxvirus family members, both X residues are always leucine. Additionally, G1 contains a region 65 residues downstream of the HXXEH sequence consisting of ELENEXXXXXE. This sequence is also very highly conserved among poxviruses and is proposed to be involved in the coordination of a zinc ion [16]. In the infected cell, G1 exists initially as a 68-kDa entity, which can be cleaved into N-terminal (46 kDa) and C-terminal (22 kDa) products [3, 16]. However, the significance of this cleavage remains unclear. It is also unclear whether G1 participates in autoproteolysis or is a substrate of another

✉ F. G. G. Leite
flavia.leite@crick.ac.uk

[1] Max F. Perutz Laboratories, Medical University of Vienna, Dr. Bohr-Gasse 9/3, 1030 Vienna, Austria

[2] CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Lazarettgasse 14, 1090 Vienna, Austria

[3] Present Address: Cellular Signalling and Cytoskeletal Function, The Francis Crick Institute, 1 Midland Road, London NW1 1AT, UK

protease [3, 16]. Indeed, direct evidence that G1 can act as a proteinase or that it binds zinc is lacking.

To investigate whether VV G1 is involved in autoproteolysis, we first expressed VV G1 as a fusion protein with an N-terminal thioredoxin tag. To this end, we cloned the chemically synthesised *E. coli* codon-optimized gene (GeneArt) into a pET vector (Novagen). The *E. coli* Rosetta 2 (DE3) pLysS strain (Novagen) was transformed with pET-HisTRXG1L, and expression was induced using 0.1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) for 16 h at 16 °C. Cells were collected by centrifugation, resuspended in 50 mM Tris pH 8.5 buffer containing 5% glycerol, 100 mM NaCl and 10 mM β-mercaptoethanol and lysed using a French press. The pellet was removed by centrifugation (38,000 *g* for 30 min) and the soluble fraction was probed by western blot with anti-His antibody (GE Healthcare). Only full-length HisTRX-G1 was observed; the putative cleavage products were not visible (Fig. 1A).

Since it was possible that the bacterial system did not provide the correct environment for protein processing, we expressed G1 in rabbit reticulocyte lysates (RRLs) from an *in vitro*-synthesised mRNA. To this end, we cloned VV G1 into pCITE vector (Novagen) downstream of the encephalomyocarditis virus internal ribosome entry site. After plasmid linearization with BamH1, *in vitro* transcription reactions of G1 were performed as described previously [20]. *In vitro* translation reactions were performed using the rabbit reticulocyte lysate (RRL) system from Promega as described previously [27]. Proteins were separated using SDS-PAGE gels containing 17.5% acrylamide [13] and then analysed by fluorography to detect the radiolabeled translation products. Again, we observed no processing of G1 in this assay (Fig. 1B, lane 2), not even when the translated products were incubated for a longer time at different temperatures (Fig. 1B, lanes 3–5) or when $ZnCl_2$ was added to the translation mixture at different concentrations (5 and 10 μM; Fig. 1C, lanes 2-3).

We examined whether G1 would be cleaved when expressed ectopically in HeLa cells. HeLa cells in a 10-cm dish were transiently transfected with pCDNA3.1G1HA (expressing a human-codon-optimized G1 with a C-terminal HA tag) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. After 48 hours, cells were lysed with RIPA buffer (Sigma), and G1-HA present in the soluble fraction was immunoprecipitated using Ezview Red Anti-HA Affinity Gel (Sigma) according to the manufacturer's instructions. G1-HA was probed with anti-HA (3F10) from Roche. The insoluble fraction was also loaded on the gel for analytical purposes. Again, we saw no evidence of G1 cleavage in the absence of viral infection (Fig. 1D, lanes 3-4), even when we analysed the cellular fraction containing membranes and debris (Fig. 1D, lanes 2 and 4). This observation indicated that G1 cleavage, whether mediated by a cellular protein or by G1 itself, is dependent on other viral proteins. This agrees with previous studies in which G1 cleavage was always assessed in the context of viral infection [3, 16].

To investigate this, we took advantage of the conditional-lethal mutant virus vG1Li (obtained from Dr. B. Moss, NIH/USA), in which the expression of G1 with a C-terminal HA tag is controlled by IPTG under the control of a T7 promoter [3]. Viruses were propagated in BSC40 cells in the presence of IPTG, and the titre was determined by plaque assay as described [21]. After infection, cells were lysed with CelLytic M buffer (Sigma) containing protease inhibitor cocktail (Sigma P8340). Forty micrograms of whole protein extract was separated by electrophoresis, transferred to a nitrocellulose membrane, and subjected to western blot analysis with anti-HA antibody (6E2) (Cell Signaling). In a time-course experiment in infected BSC40 cells induced with 50 μM IPTG using vG1Li at a multiplicity of infection (MOI) of 1, we could detect G1-HA expression at 6 hours postinfection (hpi). The G1 cleavage product was only observed after 10 hpi (Fig. 2B); however, we cannot rule out that cleavage occurs simultaneously with expression, as the detection methods may not be sufficiently sensitive to detect low amounts of cleavage product.

To investigate which cellular or viral proteins are required for G1 cleavage, we screened infected cells with protease inhibitors such as batimastat (metalloproteases), E64d (papain family), lactacystin and MG132 (proteasome), and zVAD.fmk (caspase family) (Table 1 lists all inhibitors and the concentrations at which they were tested). We also tested various inhibitors known to affect the life cycle of the virus to identify a viral process that would affect G1 cleavage. To this end, we tested AraC (VV DNA replication), cidofovir (VV DNA synthesis), leptomycin B (nuclear transport), LY294002 (PI3K pathway), PD98059 (MEK pathway), rifampicin (virus assembly) and SP600125 (JNK pathway) [2, 14, 17, 22, 23, 25, 26, 28, 29]. BSC40 cells were pre-treated with different concentrations of the listed inhibitors (Table 1) for 30 min and then infected with vG1Li at an MOI of 1 in the presence of 50 μM IPTG. Cells were incubated in the presence of the inhibitors, and after 30 hours, cells were lysed with Cell LyticM buffer plus protease inhibitor cocktail (Sigma P8340). Twenty micrograms of whole protein extract was separated by electrophoresis and transferred to a nitrocellulose membrane. Cleavage was judged to have occurred when the C-terminal (20-kDa) band was detected using an HA antibody in a Western blot as described previously [3]. Blockage of virus assembly, as seen with rifampicin, did not impair G1 cleavage. In contrast, inhibition of DNA replication with AraC did prevent cleavage (Table 1 and

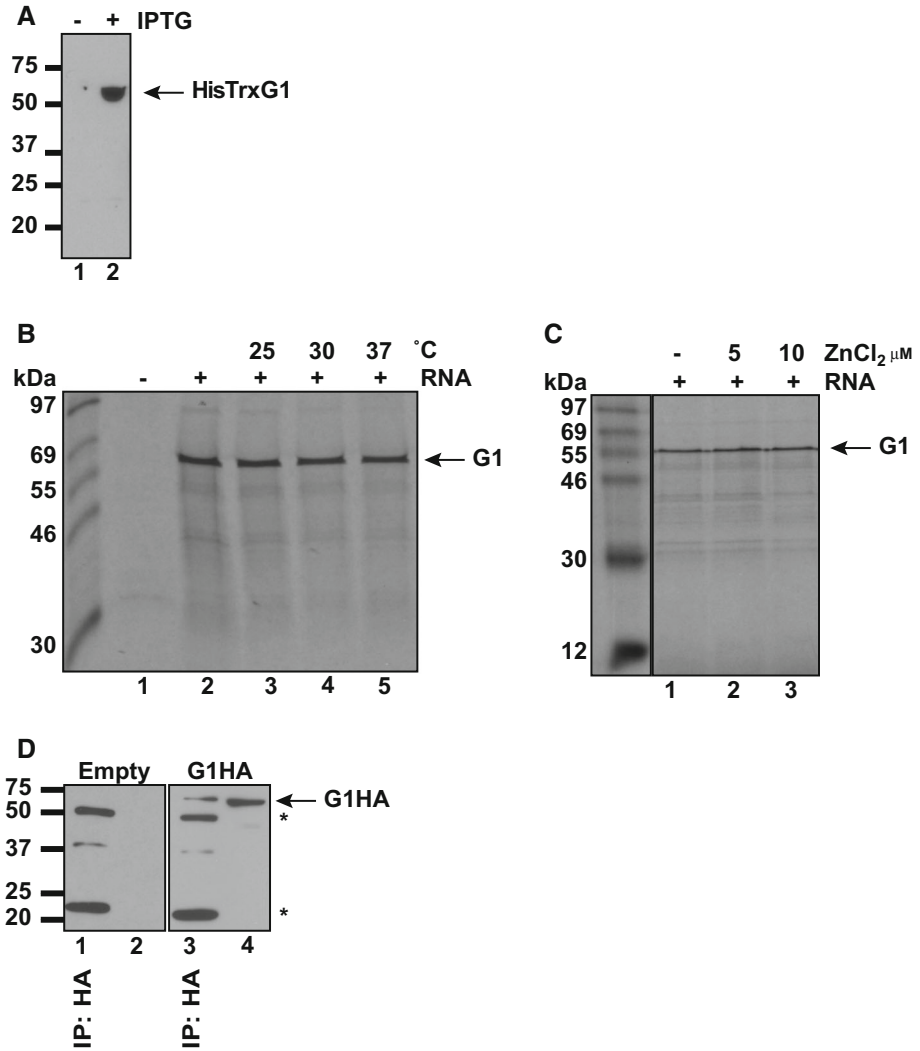

Fig. 1 Cleavage of G1 is not observed when expressed in *E. coli*, rabbit reticulocytes or mammalian cells. (**a**) Western blot analysis of HisTRXG1L expressed in *E. coli*. Expression was induced, cells were lysed, the soluble fraction was loaded onto a gel, electrophoresis was carried out, and proteins were transferred to a nitrocellulose membrane. HisTrxG1 was probed with anti-His (GE Healthcare) and was observed as a single band corresponding to the full-length protein. (**b**) *In vitro* translation of G1. The mRNA G1 (10 ng/µl) was used to program RRLs; after 30 min, protein translation was terminated and samples were then incubated for an additional 1 hour at the indicate temperatures (lanes 3-5). The negative control (lane 1) was incubated for 30 min without RNA. Protein synthesis was monitored by electrophoresis in a 17.5% acrylamide gel; the position of the uncleaved G1 is indicated by a black arrow. (**c**) *In vitro* translation of G1 in the presence of zinc. The mRNA G1 (10 ng/µl) was used to program RRLs in which zinc chloride was added to the translation mixture on ice prior to incubation at the indicated concentrations (lanes 2-3). Protein analysis was performed as described in the legend to panel *b*. Uncleaved G1 is indicated by a black arrow. (**d**) Immunoprecipitation of HeLa cells transfected with either empty pCDNA3.1 (lanes 1 and 2) or pCDNA3.1G1HA (lanes 3 and 4). After 48 hours, cells were lysed with RIPA buffer and G1HA present in the soluble fraction was immunoprecipitated using Anti-HA beads. The precipitate material was separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After lysis with RIPA buffer, the insoluble fraction was also examined on the gel (lanes 2 and 4). G1 was probed with anti-HA (3F10) from Roche (black arrow). Heavy and light chains of secondary antibodies are indicated by an asterisk

Fig. 2C). Moreover, G1 cleavage was also inhibited when the specific proteasome inhibitors lactacystin and MG132 were used (Table 1 and Fig. 2C and D). Although acting on different targets, these three inhibitors share the ability to impair vaccinia virus late gene expression [23, 29, 30]. Therefore, one cannot rule out the requirement of a late viral cofactor for G1 proteolytic activity. None of the other inhibitors tested had the ability to inhibit G1 cleavage (Table 1).

G1L itself is a late gene [30], and both AraC and MG132 affected its expression, as can be seen in Fig. 2C. The vG1Li virus used in this study has its T7 polymerase under the control of a vaccinia virus late promoter and G1 under the control of a T7 promoter [3]. Therefore, G1 has

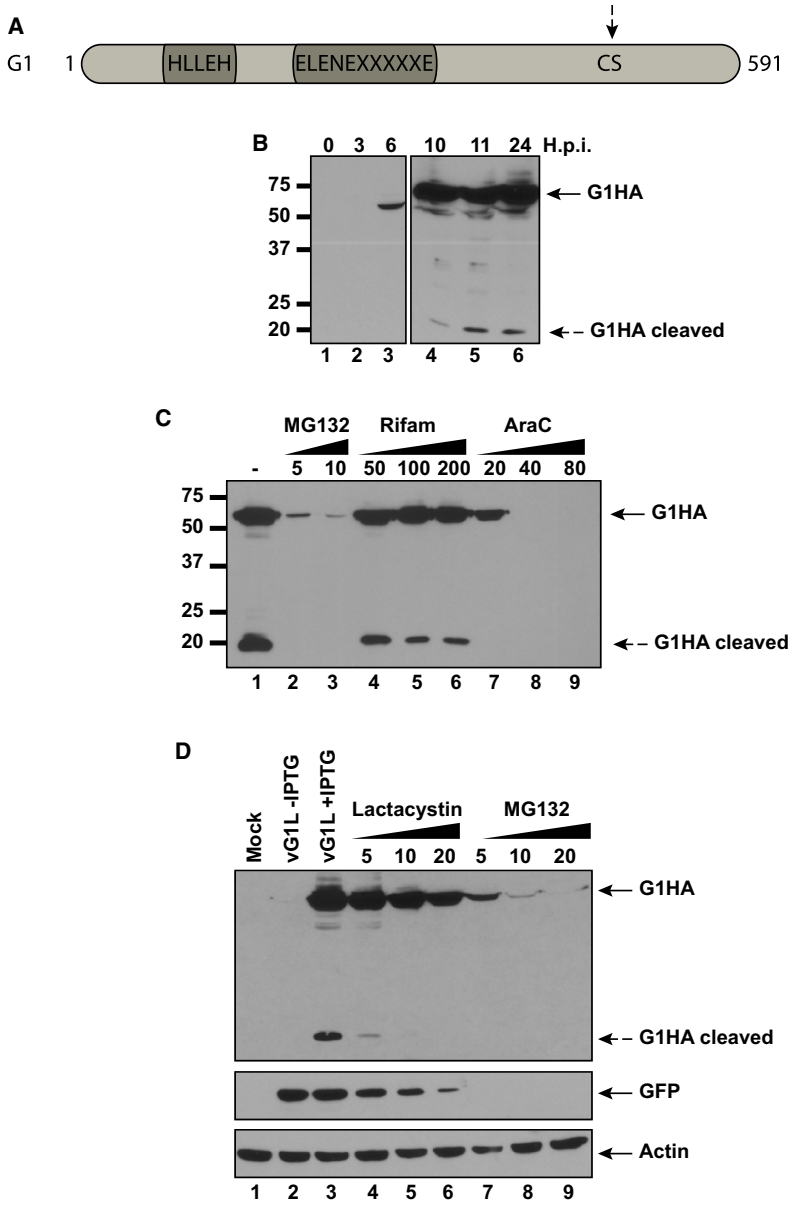

Fig. 2 G1 cleavage relies on the activity of other proteins. (**a**) Schematic diagram of the G1 protein. The zinc-binding motif (HLLEH) and the zinc ion coordination motif (ELENEXXXXXE) are indicated by dark boxes. The approximate location of the cleavage site (CS) is indicated by a dashed arrow. (**b**) Western blot of BSC40 cells infected with vG1Li at an MOI of 1, in the presence of 50 μM IPTG. At the indicated times, cells were lysed with CelLytic M buffer plus protease inhibitor cocktail (Sigma P8340), and 20 μg of whole protein extract was separated by electrophoresis and transferred to a nitrocellulose membrane. The position of uncleaved G1HA (black arrow) and cleavage products (dashed arrow) are shown on the right. (**c**) Western blot of BSC40 cells infected with vG1Li at an MOI of 1 in the presence of 50 μM IPTG and treated or not (lane 1) with increasing concentrations (μM) of MG132 (lanes 2-3), rifampicin (lanes 4-6) and AraC (lanes 7-9). After 30 hours, cells were lysed with CelLytic M buffer plus protease inhibitor cocktail (Sigma P8340), and 20 μg of whole protein extract was separated by electrophoresis and transferred to a nitrocellulose membrane. The position of uncleaved G1HA (black arrow) and cleavage products (dashed arrow) are shown on the right. (**d**) Western blot of BSC40 cells that were mock infected (lane 1) or infected with vG1Li at an MOI of 1 in the presence of 50 μM IPTG (lanes 3-9) or without IPTG (lane 2). Cells were treated with increasing concentrations (μM) of lactacystin (lanes 4-6) or MG132 (lanes 7-9). After 30 hours, cells were lysed with CelLytic M buffer plus protease inhibitor cocktail (Sigma P8340), and 20 μg of whole protein extract was separated by electrophoresis and transferred to a nitrocellulose membrane. The position of uncleaved G1HA (black arrow) and cleavage products (dashed arrow) are shown on the right. GFP is used as a marker for late viral expression (middle panel)

Table 1 G1 cleavage in vaccinia virus infected HeLa cells in the presence of the listed substances

Inhibitor	Site of inhibition	Concentrations tested (μM)	Cleavage of G1
Batimastat	Metalloproteases	5, 10, 20	+
E64d	Papain proteases	5, 10, 20	+
Lactacystin	**Proteasome**	**5, 10, 20**	**-**
MG132	**Proteasome**	**5, 10, 20**	**-**
zVAD.fmk	Caspases	5, 10, 20, 40	+
AraC	**VV DNA replication**	**20, 40, 80**	**-**
Cidofovir	VV DNA synthesis	32,5; 65, 130	+
Leptomycin B	Nuclear transport	0,0025; 0,005; 0,01	+
LY294002	PI3K pathway	10, 20, 40	+
SP600125	JNK pathway	20, 40, 80	+
PD98059	MEK pathway	12; 25, 50, 100	+
Rifampicin	Virus assembly	50, 100, 200	+

Substances in bold type inhibited cleavage of G1

its expression temporally controlled as a late gene, even in the presence of IPTG. Another important feature of this virus is that it contains a GFP gene regulated by the late P11 promoter replacing the original G1L gene. Thus, we can assay viral late gene expression through GFP expression. Indeed, in our hands, treatment with MG132 impairs viral late gene expression, as is shown by the lack of GFP (Fig. 2D) as reported previously [23, 29]. However, we observed a less potent effect on late gene expression when cells were treated with lactacystin, a highly specific, irreversible proteasome inhibitor (Fig. 2D). This raises the possibility that proteasome activity is necessary for G1 processing, as has been described for the Gag polyprotein of HIV [24].

Together, these observations indicate that G1 cleavage is regulated by the expression of other virus late genes and, either directly or indirectly, via the activity of a cellular protein. More-detailed investigations involving the co-expression of G1 and each of the 80 vaccinia virus late proteins or the investigation of the proteasome as a G1 regulator will be necessary to clarify this issue. Further questions to be investigated include the role of the conserved HXXEH sequence and whether G1 coordinates a zinc ion or not. The data presented here provide a platform for a better understanding of the late events that are necessary for the complex poxvirus life cycle.

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Compliance with ethical standards

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Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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