Volume 336, number 3, 496–500 FEBS 13467 December 1993

Brefeldin A blocks protein glycosylation and RNA replication of vesicular stomatitis virus

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Received 27 October 1993

Brefeldin A is a macrolide antibiotic that interferes with membrane traffic and blocks the growth of several animal viruses including vesicular stomatitis virus (VSV). The inhibition of VSV by brefeldin A takes place at least at two different steps during the growth cycle: the glycosylation of VSV G protein and the replication of viral genomes. Our results indicate that interference with membrane traffic leads not only to inhibition of viral protein glycosylation, but also to the blockade of virus genome replication in several cytoplasmic RNA-containing viruses.

Brefeldin A; Vesicular stomatitis virus; RNA replication; Protein glycosylation

1. INTRODUCTION

Vesicular stomatitis virus (VSV) is a member of the *Rhabdoviridae* family which has been well characterized at the molecular level [1]. The bullet-shaped virus particle is enveloped by a lipid bilayer [2]. After virus entry and partial decapsidation, the minus-strand RNA is first transcribed by the virion polymerases that form part of the nucleocapsid to produce five distinct mRNA molecules, each encoding a single viral polypeptide [3,4]. Translation of the VSV mRNAs produces five proteins known as N, NS, M, G and L, encoded in this order in the RNA genome (3′ to 5′) [3,4]. The progeny genomes are produced in two stages; first the genome is copied to produce a full-length positive strand RNA molecule, which represents an exact copy of the genome (−) RNA [3,4]. These (+) RNAs then act as templates for the synthesis of more genomic copies of (−) RNA [3,4]. Both types of RNA molecules are encapsidated in nucleocapsids but only the (−) RNA are assembled, after budding, in whole virions [1]. Although detailed studies have been carried out on the requirements of VSV transcription in cell-free systems, much less is known about the cellular processes that govern VSV RNA synthesis in intact cells [3,4].

We have reported that the replication of poliovirus, Semliki Forest virus and vesicular stomatitis virus RNA requires continuous synthesis of lipids to provide new membranes to which viral replication complexes attach [5–7]. Thus, cerulenin an inhibitor of lipid synthesis diminishes viral genome replication [5–7]. In addition, the inhibition of vesicular traffic by the antibiotic brefeldin A (BFA) arrests poliovirus genome replication [8,9], leading to the idea that proper assembly of functional replication complexes requires vesicular transport through the Golgi complex.

BFA is a fungal antibiotic that blocks protein secretion and vesicular traffic. It induces a rapid disaggregation of the Golgi complex that redistributes into the ER [10,11]. Therefore, transport of proteins into post-Golgi compartments in the cell is potently blocked by BFA [12]. Consequently, delivery of vesicular stomatitis virus (VSV) G glycoprotein to the plasma membrane is blocked by BFA [12–15]. This compound prevents the proper glycosylation of the VSV G protein, in accord with the idea that transport from the ER to the Golgi complex is hampered. In addition to VSV, an increasing number of animal viruses are reported to be affected by this inhibitor [16–19]. BFA inhibits the processing and transport of glycoproteins which are present in all these viruses. Thus, production of infectious particles of enveloped viruses that mature from the plasma membrane or intracellularly is inhibited by this macrolide antibiotic [16–19]. Since BFA blocks poliovirus genome replication [8,9], we wished to determine whether replication of genomes of other RNA-containing viruses is also affected by the inhibition of membrane traffic. We now report a novel mode of action of BFA against vesicular stomatitis virus, i.e. the blockade of genome replication, suggesting that proper assembly of functional replication complexes requires vesicular transport through the Golgi complex.

2. MATERIALS AND METHODS

2.1. *Cell cultures and virus*

Dulbecco-modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (E4D2) was used for growth and maintenance of HeLa cell cultures.

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 Published by Elsevier Science Publishers B.V.

Vesicular Stomatitis Virus (VSV), Indiana strain, was propagated and titered in monolayers of BHK-21 cells. The VSV infections were performed at a multiplicity of infection (m.o.i.) of 50 pfu/cell in DMEM with 2% calf serum (E4D2).

2.2. *Antibiotics*

Brefeldin-A was purchased from Sigma. Actinomycin D was supplied by Boehringer-Mannheim.

2.3. *Radioactive compounds*

[^{35}S]Methionine (1220 Ci/mmol), D-[6-^{3}H]glucosamine (20 Ci/mmol) and cytidine 5′-[α^{32}P]triphosphate (400 mCi/mmol) were purchased from Amersham.

2.4. *Analysis of proteins by polyacrylamide gel electrophoresis and autoradiography*

HeLa cell monolayers were infected as indicated in E4D2. Adsorption was allowed to continue for 60 min at 37°C. At the times indicated, proteins were labelled by the addition of 20 μCi/ml [^{35}S]methionine contained in methionine-free DMEM. To label glycoproteins with [^{3}H]glucosamine, glucose-free DMEM supplemented with pyruvic acid was used. After 1.5 h of incubation with the radioactive medium, the label was removed and the cells were harvested in 0.1 ml of buffer containing 62.5 mM Tris-HCl pH 6.8, 2% SDS, 0.1 M dithiothreitol, 17% glycerol and 0.024% Bromophenol blue. Samples were sonicated at 12 Hz and boiled for 5 min. The samples were electrophoresed overnight at 80 v in PAGE-SDS.

2.5. *Incorporation of [^{3}H]uridine into RNA*

Monolayers of 5×10^5 HeLa cells were infected with VSV at a m.o.i. of 50 in the presence of 5 μg/ml actinomycin D. 10 μCi/ml of [5′-^{3}H]uridine were added at the indicated times. After a 60 min labelling period the medium containing the label was discarded, and the cells were treated with 0.5 ml 5% trichloroacetic acid, washed twice with ethanol, dried under an infrared lamp and dissolved in 200 μl 0.1 N NaOH/1% SDS. 150 μl samples were counted in a liquid scintillation spectrometer.

2.6. *Northern blot analysis of RNA*

Viral RNA synthesis was studied in HeLa cell monolayers grown in 60 mm dishes and infected with VSV at a m.o.i. of 50 PFU/cell. BFA was added at the times indicated. The cells were lysed in a buffer containing 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 150 mM NaCl and 0.65% Nonidet P-40. After removal of nuclei by low speed centrifugation, supernatants were mixed with an equal volume of a buffer containing 20 mM Tris-HCl (pH 7.8), 350 mM NaCl, 20 mM EDTA and 1% SDS. Samples were extracted with a mixture of phenol/chloroform/isoamyl alcohol (24:24:1, v/v/v) and RNA was precipitated with 2 vol. of ethanol. 5 μg of denatured RNA were subjected to electrophoresis in a 1.2% agarose/formaldehyde gel and transferred to nitrocellulose filters. RNA samples immobilized on nitrocellulose were hybridized under standard conditions to plus or minus strand-specific M gene RNA probes generated by in vitro transcription of a cDNA of the M gene of VSV flanked by the T7 and T3 promotors (kindly provided by Dr. R.R. Wagner, University of Virginia).

3. RESULTS

3.1. *Effects of brefeldin A on VSV translation*

To analyze the effect of brefeldin A on vesicular stomatitis virus protein synthesis, different concentrations of this antibiotic were added at different times post-infection and proteins were labelled from 6 to 7 h.p.i. When brefeldin A is added after virus entry (0 h.p.i.) or

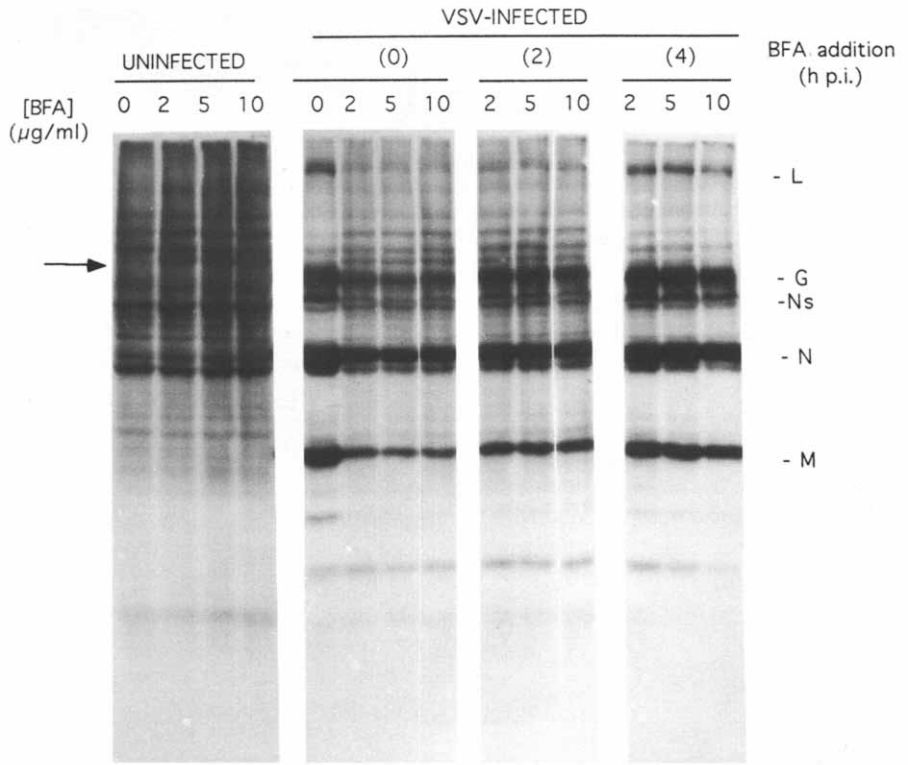

Fig. 1. Effects of brefeldin A (BFA) on VSV protein synthesis. At the times indicated, different concentrations of BFA were added to VSV-infected HeLa cells (m.o.i. 50 PFU/cell). Where indicated, BFA was also added to uninfected cells after mock infection. Proteins were labelled for one hour with radioactive methionine at 6 h.p.i. and analyzed as described above. The arrow shows the cellular protein induced by BFA. The migration of VSV proteins are also indicated.

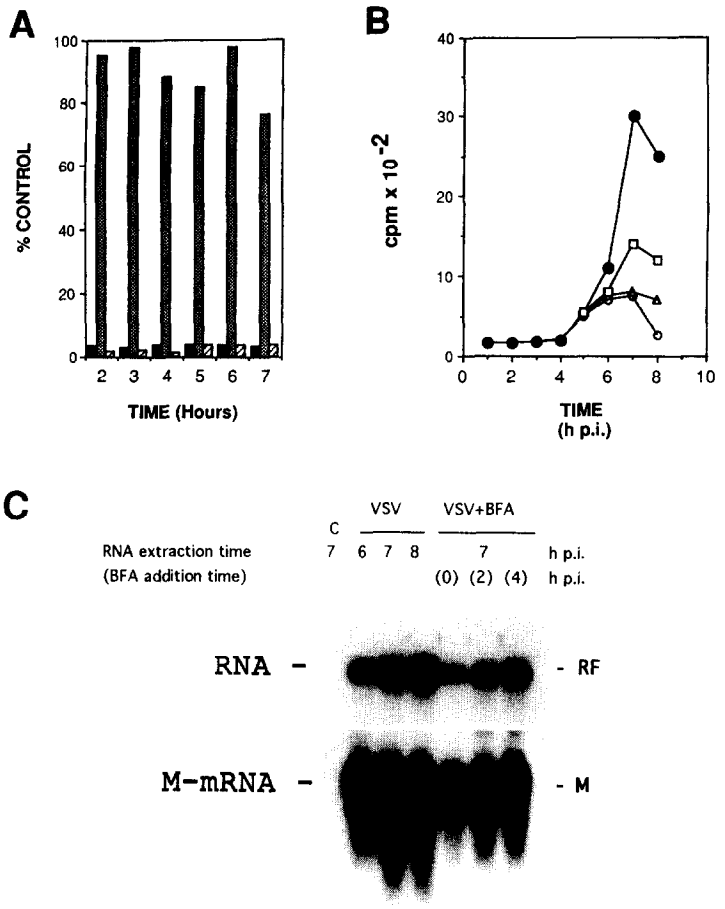

Fig. 2. Effect of BFA on RNA synthesis in both control and VSV-infected cells. (A) Time-course of [^{3}H]uridine incorporation in uninfected HeLa cells. Uninfected HeLa cells treated with 5 μg/ml BFA from time '0' (stippled bars), or treated with 5 μg/ml actinomycin D from time '0' (black bars), or with both antibiotics (hatched bars) were labelled at the times indicated with 10 μCi/ml [^{3}H]uridine for 60 min. Data are presented as percentage of control cells (untreated and uninfected). (B) Time-course of [^{3}H]uridine incorporation in VSV-infected HeLa cells. HeLa cells infected with VSV (m.o.i. 50 pfu/cell) were incubated in the presence of 5 μg/ml actinomycin D (black circles). 5 μg/ml BFA were added at 0 (open circles), 2 (open triangles) or 4 h.p.i. (open squares). Cultures were labelled with 10 μCi/ml [^{3}H]uridine for 60 min and the radioactivity in viral RNA was estimated as described in section 2. (C) Northern-blot analysis of RNA from VSV-infected cells. RNA was extracted from VSV-infected HeLa cells and the level of M gene mRNA and RF RNA was estimated by using two complementary riboprobes hybridizing with the plus or minus strand of the M gene, respectively. The figure shows the kinetics of VSV RNA accumulation in virus-infected cells at hours 6, 7 and 8 p.i. 5 μg/ml BFA was added when indicated at 0, 2 or 4 h.p.i. and RNA was extracted at 7 h.p.i. RF, replicative form; M-mRNA, messenger RNA for M protein.

even at 2 h.p.i. there is a substantial reduction of viral protein synthesis (Fig. 1). This inhibition of VSV translation is lower when BFA is added later (4 h.p.i.), suggesting that this antibiotic does not have a direct effect on viral protein synthesis. As a control, the action of BFA on cellular translation was tested. Fig. 1 shows that incubation of HeLa cells with the macrolide antibiotic for six hours does not interfere with cellular translation, although there is a clear induction of a 70 kDa protein (Fig. 1).

3.2. *Brefeldin A blocks VSV RNA synthesis*

After entry of vesicular stomatitis virus into cells and uncoating, the viral nucleocapsid transcribes the negative strand RNA genome to generate the viral mRNAs that participate in early gene expression. The proteins thus made are involved in the replication of the genome, that in turn, will be used as template to generate more mRNAs. To assay the effect of BFA on VSV transcription and the replication of genomes we first measured the incorporation of [^{3}H]uridine in VSV-infected HeLa cells in the presence of actinomycin D to inhibit cellular but not viral RNA synthesis. An increase in viral RNA synthesis is observed from the fifth h.p.i. peaking at the seventh h.p.i. Addition of BFA at 0, 2 or 4 h.p.i. leads to a reduction in VSV RNA synthesis (Fig. 2B). Some [^{3}H]uridine incorporation is observed even when BFA was present from 0 h.p.i. perhaps indicating that transcription of viral RNA from the input VSV nucleocapsid occurs. On the other hand, BFA significantly reduced viral RNA synthesis even when added 4 h.p.i., suggesting that this is one of the targets of BFA action on VSV growth. The inhibition of [^{3}H]uridine incorporation by BFA was selective for viral RNA synthesis and almost no effect on cellular RNA synthesis was seen even after seven hours of incubation with the antibiotic

Fig. 3. Effect of BFA on glycosylation of VSV G protein. HeLa cells were infected with VSV (m.o.i. 50 PFU/cell). Different concentrations of BFA were added at 5 h.p.i. Thirty minutes later, proteins were labelled for 1.5 h with radioactive methionine (20 μCi/ml) or glucosamine (20 μCi/ml). The proteins were analyzed by SDS-PAGE as described in section 2. The migration of VSV G protein is indicated.

(Fig. 2A). To analyze the effect of BFA on VSV RNA synthesis by Northern blot hybridization the RNA was extracted from untreated cells at different times post-infection, or at 7 h.p.i. from cells treated with BFA from 0, 2 or 4 h.p.i. Northern blots were hybridized with plus- and minus-strand specific probes covering the M gene region of the VSV genome to detect the RF RNA and mRNA respectively. Fig. 2C shows that BFA reduces the amount of RF RNA or viral mRNA, particularly if the compound was added at 0 h.p.i. Notably, some synthesis of M mRNA is detected when BFA is present from the beginning of infection suggesting that the early transcription of viral mRNA may not be inhibited by the antibiotic. The presence of this amount of mRNA could account for the partial synthesis of viral proteins when BFA is added at 0 h.p.i. (Fig. 1).

3.3. *Effect of BFA on VSV G protein glycosylation*

VSV possesses a single glycoprotein (G) which is inserted into the lipid bilayer that surrounds the virion particles. Glycosylation of the G protein was analyzed by labelling with radioactive glycosamine from 5.5 to 7 h.p.i. in the presence of two different concentrations of BFA (Fig. 3). In agreement with the results shown in Fig. 1, BFA had no significant effects on viral protein synthesis when added late during infection, whereas the antibiotic strongly decreased the incorporation of glycosamine into VSV G protein.

These results are in agreement with previous findings and indicate that glycosylation is another target of BFA activity on VSV.

4. DISCUSSION

The maturation of membrane proteins in eukaryotic cells involves passage through the ER, and sequential migration through the *cis*- medial- and *trans*-compartments of the Golgi complex to an organelle known as the *trans*-Golgi network (TGN) [20–23]. Several routes can be followed by the proteins from the Golgi apparatus, including incorporation into the plasma membrane, transport to endosomes, lysosomes, etc. [20–23]. Recent results with poliovirus indicate that an intact vesicular system is required for the formation of functional RNA replication complexes, implying that the replication of viral genomes in the cytoplasm of eucaryotic cells is connected with the formation of new membranous structures [5–9]. Our present findings show that, in addition to poliovirus, VSV RNA synthesis is also inhibited by BFA, lending support to the concept that cytoplasmic genome replication of RNA-containing animal viruses is functionally coupled to vesicular transport. In addition to this effect, it has been well documented that brefeldin A also interferes with the glycosylation of proteins [12,13]. Cellular and viral glycoproteins must travel through the vesicular system to be properly glycosylated, a process blocked by BFA.

Thus, at least two different targets of action of BFA exist in the case of VSV, genome replication and the glycosylation of protein G. Both events may have consequences for virus budding, because the formation of nucleocapsids and the proper location of G protein in the plasma membrane are necessary for the formation of new virions to occur.

Our present findings show that not only positive-stranded viruses, such as poliovirus, but also a negative-stranded virus such as VSV require an intact vesicular system for virus genome replication. The exact understanding of the molecular mechanisms that govern the interaction of replication complexes with membranes will shed light on the synthesis of viral genomes and provide a rationale for development of antiviral agents against these viruses. The specificity of such an antiviral approach could rely upon the selective inhibition of viral proteins that connect the RNA replication complexes with cellular membranes.

Acknowledgements: The expert technical assistance of Mr. M.A. Sanz is acknowledged. L.P. is the holder of a Comunidad Autónoma de Madrid fellowship. Plan Nacional project number /BIO 92-07151, DGICYT project number PB90-0177 and Fundación Ramón Areces are acknowledged for financial support.

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