Short title: BFA blocks translation

DIFFERENTIAL INHIBITION OF CELLULAR AND SINDBIS VIRUS TRANSLATION BY BREFELDIN A

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Abbreviations used: ARF1, ADP-ribosilation factor 1; BFA, Brefeldin A; BHK, Baby Hamster Kidney; DMEM, Dulbecco's Modified Eagle's medium; GEFs, GTP-exchange factors; eIF, eukaryotic initiation factor; luc, Luciferase; nsp, non-structural proteins; SV, Sindbis virus; SV-luc, Sindbis virus expressing luciferase.

SUMMARY

Brefeldin A is a macrolide compound that interferes with the secretory pathway and also affects protein synthesis in mammalian cells. As a result, this antibiotic impedes the maturation of viral glycoproteins of enveloped viruses and viral genome replication in several virus species. In the present work, we show that translation of subgenomic mRNA from Sindbis virus, which in contrast to cellular translation, is resistant to brefeldin A after prolonged treatment. The phosphorylation of eIF2α as a result of brefeldin A treatment correlates with the inhibition of cellular translation, while late viral protein synthesis is resistant to this phosphorylation. The effect of brefeldin A on Sindbis virus replication was also examined using a Sindbis virus replicon. Although brefeldin A delayed viral RNA synthesis, translation by non-replicative viral RNAs was not affected, reinforcing the idea that brefeldin A delays viral RNA replication, but does not directly affect Sindbis virus protein synthesis.

Key words: Brefeldin A; Sindbis virus; cellular translation; viral protein synthesis; Sindbis virus replication

INTRODUCTION

Sindbis virus (SV) is the prototype member of the *Alphavirus* genus of the *Togaviridae* family. The genome, a positive-strand non-segmented RNA of almost 12 kb, consists of two open reading frames: the first two-thirds encode non-structural proteins (nsPs), while the remaining third encodes structural proteins [1, 2]. The genomic 49S RNA also serves as a template for the synthesis of negative RNA which is used to generate more genome copies and to transcribe the subgenomic 26S mRNA from an internal promoter [2]. The lytic cycle of SV infection has two well-defined stages. In the first one, SV genomic RNA is translated to render the non-structural proteins, nsp1, 2, 3 and 4. These proteins are required for viral replication and transcription [3]. At about 2-4 hours post infection (h p. i.), the pattern of protein synthesis drastically changes, and subgenomic RNA translation increases notably while genomic RNA is encapsidated in new virus particles. At the time of infection, a rapid inhibition of host protein synthesis occurs [2, 4, 5] (38). The ability of a virus to inhibit the translation of cellular mRNAs under conditions in which viral mRNAs are translated has been observed for a variety of positive- and negative-strand RNA viruses, including poliovirus and influenza virus, and for many species of DNA viruses such as adenoviruses, herpesviruses and poxviruses [6-8]. How this blockade is accomplished remains still poorly understood in many cases, and different viruses may use different mechanisms to achieve this differential inhibition of translation [6-8].

Brefeldin A (BFA) is a macrolide compound capable of disrupting the vesicular system and blocking glycoprotein secretion in eukaryotic cells [9-13]. The molecular target of BFA is a subset of Sec7-type GTP-exchange factors (GEFs), which activate a GTP-binding protein known as ADP-ribosylation factor 1 (ARF1) [14]. ARF1 recruits the COPI coat and AP-1/clathrin coat protein complexes involved in the formation of

transport vesicles [15]. BFA inhibits the GDP-GTP exchange reaction between GEF and ARF1, leading to the inhibition of ARF activation [14, 16]. As a consequence, coat proteins are released from Golgi membranes, provoking the loss of control of fusion and budding membranes [9, 12, 15, 17-21]. BFA possesses antiviral activity against enveloped viruses, since it impedes the maturation of viral glycoproteins and the production of infectious particles which mature on the plasma membrane or within the cell [22-26]. The replication of viruses such as poliovirus or Vesicular Stomatitis Virus is inhibited by BFA, since RNA replication of these viruses requires continuous synthesis of lipids to provide new membranes to which viral replication complexes attach. Therefore, this compound acts against some non-enveloped viruses, interfering with genome replication, which requires an intact vesicular system [22, 27-30]. BFA also decreases protein synthesis in culture cells. Thus, BFA treatment of rat GH3 pituitary cells leads to an inhibition of protein synthesis at the initiation level [31, 32]. Presumably, this effect is due to the stress situation of the endoplasmic reticulum caused by the disorganization of the membrane system [32-34]. We have now analyzed the action of BFA on the translation of BHK-21 cells and SV infected cells and also tested the effect of BFA on SV mRNA synthesis. Our results indicate that BFA delays viral translation by retarding viral RNA synthesis.

MATERIALS AND METHODS

Cell culture and viruses

Baby hamster kidney (BHK-21) cells were grown at 37ºC in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) and non-essential amino acids. Wild-type (wt) SV and SV-Luc stocks were obtained from the cDNA clones pT7SVwt [35, 36] and pToto1101/Luc, respectively (generously provided by Charles M. Rice, Rockefeller University, NY) [37]. They were propagated and titered in BHK-21 cultures.

Plasmids

The replicon *repL26S C-luc* was obtained by *in vitro* transcription from the plasmid pT7 *repL26S C-luc*. This plasmid is derived from pT7SVwt and contains the luciferase gene between the SV C protein sequence and the SV 3'noncoding region with deletion of the sequence encoding for E3, E2, 6K and E1 proteins. The first three codons encoding for E3 are maintained to facilitate the autoproteolytic cleavage of C protein.

The non-replicative RNA *49S-luc* was obtained by *in vitro* transcription from *pToto1101/Luc* digested with BssH II, which eliminates the 3' non-coding region. The resulting RNA was polyadenylated post-transcriptionally with PolyA polymerase (Invitrogen).

The non-replicative RNA *L26S C-luc* was obtained by *in vitro* transcription from pT7 C+ Luc. This plasmid contains the subgenomic sequence from *repL26S C-luc* after the T7 promoter sequence, which permits the *in vitro* production of this mRNA.

Viral infections

BHK-21 cells were infected with wild type SV or SV-Luc at a multiplicity of infection of 10 PFU/cell. After 30 minutes of adsorption, the medium was removed and culture plates were incubated with fresh DMEM medium supplemented with 5% FCS.

Effects of different compounds on SV and cellular translation

BFA (5 μg/ml) (Sigma) was added to the wt SV infected cultures at different h p. i. At the indicated times, proteins were labelled for 30 min with 200 μl methionine/cysteine-free DMEM supplemented with 1 μl Trans label [^{35}S] Met-Cys (15 mCi/ml, Amersham Biosciences) per well in the presence or absence of the inhibitor. Cells were collected in sample buffer, boiled for 4 min and analyzed by SDS-PAGE and fluorography.

Western blot analysis

The phosphorylation state of the translation initiation factor eIF2 was determined by Western blotting [38]. BHK 21 cells were infected, treated with BFA, or infected and treated with BFA after virus adsorption. At different h p. i., cells were collected in sample buffer and proteins were fractionated by SDS-PAGE in 15% polyacrylamide gels and transferred to nitrocellulose membranes by wet transfer. Membranes were blocked with PBS containing 5% low-fat dry milk. Anti-eIF2 (Santa Cruz) or anti-phosphorylated-eIF2 (Biosource) antibodies were then added, and the membranes were washed with PBS containing 0.2% Tween 20. Goat anti-rabbit horseradish peroxidase-conjugated antibodies (Pierce) and the ECL kit (Amersham Biosciences) were used to detect bound antibodies. Chemiluminescence was detected by exposure to Agfa X-ray film.

Measurement of luciferase activity

BHK-21 cells were infected with SV-Luc or electroporated with 20 μg of RNA. At different hours post-electroporation (h p. e.), cells were lysed in a buffer containing 0.5% Triton X-100, 25 mM glycylglycine (pH 7.8) and 1 mM dithiothreitol. Luciferase activity was determined using a Monolight 2010 apparatus (Analytical Luminescence Laboratory), as described previously [39].

Analysis of mRNA by real-time PCR

SV RNA levels in infected cells were determined by real-time quantitative reverse transcription (RT)-PCR as previously described [40, 41]. Briefly, total RNA was extracted from $2x10^5$ cells at the times indicated in each figure, using the RNeasy commercial kit (Qiagen) following the manufacturer's recommendations. The isolated RNA was resuspended in 30 μl of nuclease-free water, and 3 μl were subjected to analysis. Real-time quantitative RT-PCR was performed with the LightCycler thermal cycler system (Roche Diagnostics) using the RNA Master SYBR Green I kit (Roche Diagnostics) as described [40, 41]. Data analysis was done using the Roche Molecular Biochemicals LightCycler software, version 3.3. The specificity of the amplification reactions was confirmed by analyzing the corresponding melting curves.

RESULTS

Effect of BFA on protein synthesis in BHK-21 and SV infected cells

To test the effect of BFA on protein synthesis, SV-infected or uninfected BHK-21 cells were treated with different concentrations of BFA after viral adsorption. Concentrations of BFA ranged from 1 to 20 μg/ml. Cellular (Figure 1A) and late viral protein synthesis (Figure 1B) were analyzed after 6 hours of BFA treatment by metabolic labeling with ^{35}S-Met/Cys. At all the concentrations analyzed, BFA induced an inhibition of cellular protein synthesis of around 80%, whereas very little effect was observed on viral translation. Notably, SV protein synthesis was not inhibited at the highest concentration of BFA whereas cellular translation was strongly blocked by only 1 μg/ml of this antibiotic. The comparative activity of BFA on cellular and viral protein synthesis is represented in Figure 1C. There is a clear differential inhibition caused by BFA on cellular as compared to subgenomic SV mRNA translation.

Our next aim was to examine the action of BFA on the early and late phases of the viral cycle. This was achieved by adding BFA after virus adsorption or at 2 h p. i. and analyzing the synthesis of structural viral proteins at 2, 4 and 6 h p. i. by protein radiolabeling (Figure 2B). As a control, translation in untreated cells infected by SV was estimated (Figure 2A). Addition of BFA after viral adsorption caused 91% inhibition of viral translation at 2 h p. i. This inhibition decreased throughout the course of infection, and was only 10% at 6 h p. i. However, when BFA was added at 2 h p. i., the reduction in viral translation was about 39% at the beginning of infection, and no differences in protein synthesis with respect to the untreated cells were observed at 6 h p. i. Therefore, BFA only delays but does not inhibit synthesis of SV structural proteins.

In culture cells, such as rat GH3 pituitary cells, BFA induces eIF2α phosphorylation, which could be responsible for the inhibition of translation [32].

Therefore, phosphorylation of eIF2α during BFA treatment and after SV infection of BFA-treated or non-treated cells was analyzed (Figure 3). To avoid the potential inhibition of virus entry by BFA, this compound was added after virus adsorption. Cells were collected at the times indicated in the figure, and phosphorylated or total eIF2 were detected by Western blotting with specific antibodies. As a control, eIF2α phosphorylation was estimated in mock-infected non-BFA-treated cells. In BFA-treated cells, the phosphorylated form is detectable from 2 hours of treatment, both in mock infected and SV infected cells, while phosphorylation of eIF2α in SV-infected non-treated cells was detected from 4 h p. i., as described previously [38]. In addition, eIF2 remains phosphorylated at 4 and 6 h p. i. in SV-infected BFA-treated cells when SV translation reaches the levels of non-treated controls. This result suggests that, although in BFA-treated cells the eIF2α phosphorylation was initially induced by BFA treatment, it did not affect SV RNA translation, which is consistent with previously published data (38). On the other hand, inhibition of cellular protein synthesis can be observed after 2 hours of treatment with BFA, which coincides with an increase in the phosphorylated form of eIF2α.

To analyze the effect of BFA on translation directed by SV genomic RNA, a recombinant virus SV-Luc with the luciferase gene inserted into the nsp3 sequence was employed [37]. BHK cells were treated with 5 μg/ml of BFA at 2 hours before infection with SV-Luc, after virus adsorption, or at 2 hours post infection. Although in SV infected cells, the synthesis of non-structural proteins ceases at 4 h p. i., SV-luc has a delayed early phase. For this reason, luciferase activity was measured at 2, 4 and 6 h p. i., the time when genomic RNA is being translated (38). The greatest inhibition of luciferase synthesis occurred when cells were treated with BFA before infection (Figure 4A). When BFA was present from the beginning of infection or from 2 h p. i., luciferase

synthesis was about 70% as compared to the non-treated control at 4 h p. i., and no effect was observed at 6 h p. i. These results indicate that the expression of genomic and subgenomic mRNAs of SV is delayed by BFA treatment. Interestingly, these data also suggest that the inhibitory effect of BFA would be independent on eIF2α phosphorylation, given that it has been described to inhibit translation of SV genomic RNA (38).

Effects of BFA on SV RNA synthesis

Since the amount of viral proteins synthesized is dependent on the number of copies of RNA, we next determined the effect of BFA on SV RNA synthesis. In this experiment, SV-infected BHK cells were treated with BFA immediately after infection, and at 2, 4 and 6 h p. i. RNA was extracted and quantitated by real-time PCR, using specific oligonucleotides for measuring total RNA (Figure 4B) or genomic RNA (Figure 4C). The amount of total RNA in BFA-treated cells was lower than in non-treated cells during the initial hours of infection. However, at later times a similar amount of total RNA was detected in BFA-treated and non-treated cells. These findings suggest that SV replication is delayed by BFA, thus accounting for the decreased synthesis of both structural and non-structural proteins. Analysis of genomic RNA levels revealed that they were lower in BFA-treated cells during the entire treatment period, which suggests that BFA interferes with SV RNA synthesis rather than protein synthesis.

Effect of BFA on viral replication

Since BFA blocks genome replication of some viruses [22, 27-29], our next aim was to determine whether BFA inhibited SV replication using the SV replicon *repL26S C-luc* (Figure 5A). BHK cells were treated for 4 hours with 5 μg/ml BFA and then electroporated with *repL26S C-luc*. BFA was maintained after electroporation, and cells

were collected at 2, 4, and 6 h p. e. Luciferase activity, which reflects the level of proteins translated from subgenomic mRNA, is shown in Figure 5B. An acute effect of BFA can be observed within the first hours of electroporation, while at 6 h p. e. the luciferase activity is similar to that found in untreated controls. In addition, the effect of BFA on translation of the non-replicative RNAs *49S-luc* and *L26SC-luc* was assayed. A schematic representation of these RNAs is shown in Figure 5A. Both RNAs were electroporated in BHK cells previously treated with BFA for 4 hours. BFA was maintained during and after electroporation, and cells were collected at 2, 4 and 6 h p.e to measure luciferase activity (Figures 5C and 5D). In both cases, luciferase activity in BFA-treated cells was higher than in untreated control cells, indicating that BFA had no inhibitory effect on the translation of non-replicative RNAs. The fact that neither the SV 49S-derived RNA was affected by BFA is consistent with the results obtained with the SV-Luc virus (Figure 4A).

These findings suggest that BFA interferes with the SV replicative RNA but does not inhibit the non-replicative forms, supporting the idea that the action of BFA on SV is due to the delay in RNA synthesis.

DISCUSSION

The fungal metabolite BFA is a well characterized inhibitor of the secretory pathway in mammalian cells [12, 19, 20]. BFA also causes a potent inhibition of cellular protein synthesis, presumably due to the unfolded protein response (UPR) triggered in the endoplasmic reticulum as a consequence of disorganization of the membrane system [31-34]. Notably, SV protein synthesis is maintained after a prolonged treatment with this compound, thus reflecting a differential behaviour between cellular and viral mRNAs. Indeed, BFA strongly inhibits cellular protein

synthesis, whereas SV translation is only slightly delayed. Notably, the amount of phosphorylated eIF2-α increases after two hours treatment of BHK cells with BFA, in agreement with previous results reported for GH3 pituitary cells [32]. This finding can be interpreted as a consequence of UPR activation [10, 42, 43]. However, this phosphorylation might not be the sole cause of translation inhibition induced by BFA, since cellular protein synthesis is not completely abrogated, and neither the synthesis of luciferase from SV-Luc and the SV-derived genomic RNA in BFA-treated cells is inhibited. Moreover, the translatability of exogenous mRNAs transfected in BFA-treated cells was not affected (data not shown).

On the other hand, SV protein synthesis is reduced in the presence of BFA only at early times after infection. Viral translation recovers throughout the time of treatment, so that the levels of SV protein synthesis reach those observed in untreated infected cells. The inhibitory effect of BFA is lower when added later during infection, suggesting that BFA has an indirect effect on SV protein synthesis. Interestingly, the amount of genomic RNA is lower in the presence of BFA as compared to untreated cells, indicating that BFA interferes with viral RNA replication. This possibility is reinforced by the observation that BFA only reduced the expression of the SV derived replicon, whereas non-replicative mRNAs were translated under these conditions. The finding that the 49S non-replicative RNA is not inhibited by BFA treatment agrees with the idea that the action of BFA on SV is exerted on replication. The resistance of 26S mRNA to BFA could be due to its hairpin structures, which may provide translational resistance to the conditions induced by this compound.

The action of BFA on the vesicular system impairs the maturation of viral glycoproteins, suppressing the formation of viral particles of enveloped viruses [22-26]. In addition, BFA also has an inhibitory effect on replication of many non-enveloped

viruses without glycoproteins, such as enteroviruses and rhinoviruses. In these cases, BFA abrogates viral replication, since this process is associated with the formation of vesicular structures [27-30]. BFA is known to affect the control of fusion and budding of membranes, and so also influences the process of vacuole formation [9, 12, 15, 17-21]. Replication of SV alters intracellular membranes, creating new vesicles which are linked to viral RNA synthesis [44-46]. Most probably, BFA interferes with the formation of the new vesicles by decreasing the replication efficiency, and hence affecting viral protein synthesis [27, 28, 30]. This hypothesis also explains the differential effect observed when BFA is added at different times after infection, with the greatest effect when BFA was present before infection. When SV infection takes place in cells previously treated with BFA, the vesicular system is disorganized before the replicative complexes are formed. In this case, replication may be more affected than when BFA is added after virus adsorption or at 2 h p. i. The formation of the vesicles needed for viral replication before BFA addition may result in a lower inhibition of replication. However, this inhibition is partial even when BFA is added before infection. Thus, viral replication is not completely abolished by the presence of BFA, since RNA and protein synthesis increase throughout infection. Therefore, BFA may disturb and delay, but not completely block, the formation of viral replication complexes. In such conditions, more time is needed to reach adequate levels of SV non-structural proteins in BFA-treated cells. Thus, the amount of viral RNA and protein synthesis finally recovered in the presence of BFA and at 6 h p. i. is comparable to non-treated controls.

Taken together, these results provide further insight into effect of the macrolide compound BFA on alphavirus infection.

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FIGURE LEGENDS

Figure 1. Effect of BFA on cellular and SV late protein synthesis. Mock-infected (A) or SV infected BHK cells (multiplicity of infection, 20 PFU/cell) (B) were treated for 6 hours and then labeled with [^{35}S]Met-Cys during 30 minutes in the presence of the same concentrations of BFA. Samples were processed by SDS-PAGE, fluorography and autoradiography as indicated in Materials and Methods. (C) SV C protein and a cellular protein, both indicated with arrows, were subjected to densitometric analysis to estimate the percentage of protein synthesis compared to untreated controls.

Figure 2. Kinetics of cellular and SV late protein synthesis: Effect of BFA. (A) Mock-infected cells were treated with BFA and radiolabeled after 2, 4 or 6 hours of treatment. (B) SV-infected cells were treated at 0 or 2 h p. i. and radiolabeled at 2, 4 and 6 h p. i. during 30 minutes. In both cases, BFA was present during labeling. Samples

were processed by SDS-PAGE, followed by fluorography and autoradiography. The numbers below each lane represent the percentage of protein synthesis with respect to non-treated controls calculated by densitometry of the proteins indicated by the arrows.

Figure 3. Phosphorylation of eIF2α. SV-infected or mock-infected cells were treated with BFA, added after virus adsorption. After 2, 4 or 6 hours, cells were collected in sample buffer and subjected to SDS-PAGE and Western blotting, as described in Materials and Methods, using specific antibodies for the phosphorylated (eIF2-P) and the non-phosphorylated form of eIF2. As a control, non-treated mock-infected BHK cells were used.

Figure 4. Effect of BFA on SV non-structural proteins and RNA synthesis. (A) SV-luc infected cells were treated with 5μg/ml of BFA two hours before infection and at and 2 h p. i. Cells were collected at 2, 4 and 6 h p. i. to measure luciferase activity as described in Materials and Methods. (B) Quantitation of total RNA and genomic RNA. (C) Genomic RNA molecules in SV-infected cells in the presence or absence of 5 μg/ml of BFA. BFA was added at 0 h p. i. and cells were collected at 2, 4 and 6 h p. i. RNA was extracted and quantified by real time PCR as described in Materials and Methods. Data are presented as percentages of number of molecules of RNA in BFA-treated with respect to non-treated cells.

Figure 5. Effect of BFA on SV replication. (A) Schematic representation of the different viral RNAs *Rep L26S C+luc* and *L26SC-luc*. (B) Luciferase activity at 2, 4 and 6 h p. e. of BHK cells with the replicative RNA *Rep L26S C+luc*, in the presence or absence of BFA. (C) and (D) show the measurement of luciferase activity at 2, 4 and 6 h

p. e. in BHK cells electroporated with the non-replicative RNAs *L26SC-luc* and *49S-luc* respectively, in the presence or absence of BFA.