Action of brefeldin A on translation in Semliki Forest virus-infected HeLa cells and cells doubly infected with poliovirus

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Brefeldin A (BFA) is a macrolide antibiotic that blocks membrane traffic through the vesicular system and affects the glycosylation of viral glycoproteins. Treatment of HeLa cells infected with Semliki Forest virus (SFV) with BFA enhances the synthesis of late viral proteins. Proteolytic cleavage of p107 is partially blocked and viral glycoproteins accumulate in BFA-treated cells. This enhanced synthesis of late SFV proteins is due, at least in part, to an increase in the formation of the subgenomic SFV 26S mRNA. Since BFA blocks the replication of poliovirus genomes without affecting the cleavage of the translation initiation factor p220, protein synthesis was analysed in doubly infected cells. HeLa cells infected with SFV and poliovirus at the same multiplicity predominantly synthesize poliovirus proteins. But if these cells are treated with BFA they synthesize significant amounts of SFV capsid protein C for several hours, despite the fact that p220 has been degraded.

Introduction

The replication of several animal viruses is blocked by brefeldin A (BFA), an inhibitor of vesicular traffic (Hunziker et al., 1992). Since transport of proteins into post-Golgi compartments in the cell is potently blocked by BFA (Klausner et al., 1992) so too is the delivery of viral glycoproteins to the plasma membrane (Misumi et al., 1986; Oda et al., 1990; Ulmer & Palade, 1990; Chen et al., 1991; Whealy et al., 1991; Cheung et al., 1991). In this manner, enveloped viruses that mature from the plasma membrane or mature intracellularly are inhibited by this macrolide antibiotic (Ulmer & Palade, 1990; Chen et al., 1991; Whealy et al., 1991; Cheung et al., 1991). The effects of BFA on late protein synthesis by animal viruses vary according to the virus species examined (Irurzun et al., 1992). Amongst the picornaviruses, rhinoviruses and poliovirus are sensitive to the antibiotic, whereas encephalomyocarditis virus (EMCV) is resistant. A negative-stranded RNA virus, such as vesicular stomatitis virus, is blocked by BFA whereas vaccinia virus, a cytoplasmic DNA virus, is resistant (Irurzun et al., 1992). These findings suggest that even viruses that do not have a lipid envelope and do not synthesize glycoproteins may require newly made membranes to allow the synthesis of new genomes (Irurzun et al., 1992; Maynell et al., 1992). Indeed, when this macrolide antibiotic is added late during infection it has no effect on poliovirus protein synthesis, whereas the replication of virus genomes is strongly blocked (Irurzun et al., 1992). BFA, like other inhibitors of poliovirus genome replication (Bonneau & Sonenberg, 1987; Pérez & Carrasco, 1992), has no effect on the extent or the kinetics of p220 cleavage, though the inhibition of host translation by poliovirus is not so drastic when BFA is present (Irurzun et al., 1992). It has been suggested that cleavage of the cellular polypeptide p220, a component of the translation factor eIF-4F, is responsible for the inhibition of cellular protein synthesis that takes place after poliovirus infection (Etchison et al., 1982; Carrasco & Castrillo, 1987; Sonenberg, 1990). The evidence that degradation of the p220 component of the eIF-4F is involved in the shut-off of host translation by poliovirus has been summarized (Sonenberg, 1990). However, several lines of evidence do not support this model and suggest that additional factors are involved in the inhibition of host translation by poliovirus. Thus, the kinetics of p220 cleavage and the shut-off of host translation differ in poliovirus-infected cells (Etchison et al., 1982; Bonneau & Sonenberg, 1987; Perez & Carrasco, 1992; Lloyd & Bovee, 1993). Moreover, transient expression of poliovirus protein 2A, the protease that induces p220 cleavage, has a major effect on transcription of a reporter gene, as compared to its translation (Davies et al., 1991).

In general, poliovirus efficiently interferes with viral protein synthesis in doubly infected cells (Carrasco & Castrillo, 1987; Carrasco et al., 1989). Even the translation of another picornavirus RNA, such as EMCV RNA, is efficiently blocked by poliovirus when similar multiplicities of infection are used (Alonso & Carrasco, 1982b), indicating that the poliovirus-induced inhibition
of EMCV mRNA translation is possibly not mediated by the blockage of cap recognition factors. Under some conditions, particularly when low poliovirus m.o.i.s are used, translation of other viral mRNAs, including capped mRNAs, occurs in poliovirus-infected cells (Schrader & Westaway, 1990; Muñoz et al., 1984, 1985a; Alonso & Carrasco, 1982a). Over a decade ago, it was shown that cells doubly infected with two different viruses, such as poliovirus and Semliki Forest virus (SFV), translate the SFV 26S mRNA, which has a 5' cap structure (Alonso & Carrasco, 1982a). More recently, the translation of several capped mRNAs was achieved in poliovirus-infected cells, including SFV late 26S mRNA (Schrader & Westaway, 1990). However, the integrity of p220 could not be analysed in these early studies.

**Methods**

**Viruses and cell culture.** HeLa cells were grown on 100 mm plates in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum (Gibco). Infection with SFV or poliovirus type 1 (Mahoney strain) was carried out in DMEM supplemented with 2% calf serum at 37 °C in a CO₂ incubator.

**Antibiotics.** Brefeldin A (Sigma) was stored as a 1 mg/ml stock solution in DMSO (Carlo Erba).

**Electrophoretic analysis of protein synthesis.** Cells grown in 24-well plates were infected at an m.o.i. of 50 p.f.u./cell. After virus adsorption (time 0 of infection) the cells were incubated in DMEM plus 2% calf serum. Protein labelling was performed with 20 μCi/ml [³⁵S]methionine (1.45 Ci/mmol; Amersham) added to methionine-free medium. The radiolabelled cell monolayers were dissolved in sample buffer (62.5 mM-Tris–HCl pH 6.8, 2% SDS, 0.1 mM-DTT, 17% glycerol and 0.024% bromophenol blue as indicator). Samples were heated at 90 °C for 5 min, applied to a 15% polyacrylamide gel and run overnight at 80 V.
Fluorography was carried out in 1 M-sodium salicylate. The gels were finally dried and exposed to X-ray films (Castrillo et al., 1986).

Immunoblot analysis. Immunoblot analysis of p220 antigens was performed using a mouse monoclonal antibody (MAb) directed against p220 (Etchison & Etchison, 1987). Cell monolayers were harvested in a 1 : 1 mixture of 1% SDS, 0.1 M-NaOH and sample buffer (described above), containing 1 mM-PMSF (Boehringer) to avoid non-specific degradation of p220. After sonication and boiling, samples equivalent to 10^6 cells were applied and run on a 7.5% SDS–polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane (Trans-Blot transfer medium, Bio-Rad), overnight at 200 mA in transfer buffer (50 mM-Tris, 380 mM-glycine and 20% methanol). The nitrocellulose sheet was stained with Ponceau S (Sigma). A 5% (w/v) solution of non-fat dried milk in 20 mM-Tri~HCl pH 7.4, 500 mM-NaCl was used to block the membrane, by incubation for 2 h at room temperature. After blocking, the anti-p220 MAb diluted in 1% dried milk plus 0.01% sodium azide solution was added. The blot was incubated with the antibody for 18 h and then washed with four changes of the saline buffer described above. A second incubation with a rabbit-anti-mouse antibody (Nordic Immunology) was carried out for 1 h before washing four times and detection with 0.1 μCi/ml 125I-labelled Protein A (30 mCi/mg; Amersham). Finally, the blot was washed, dried and exposed to an X-ray film at -70°C.

Measurements of SFV-specific RNA
(i) Northern blot analysis of RNA. Viral RNA synthesis was studied in HeLa cell monolayers grown in 60 mm dishes and infected with SFV and/or poliovirus at an m.o.i. of 50. BFA was added after adsorption of the virus. At the times indicated the cells were lysed in a buffer containing 10 mM-Tris–HCl pH 7.4, 1 mM-EDTA, 150 mM-NaCl and 0.65% NP40. After removal of the 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 the RNA was precipitated with 2 vol. of ethanol. Of the denatured RNA 5 μg was 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 strand-specific SFV RNA probe generated by in vitro transcription of a cDNA encoding the SFV 26S gene (pGEMI-SFV), kindly provided by Dr P. Liljeström (Karolinska Institute, Stockholm, Sweden).

(ii) Dot blot analysis. RNA samples (3 μg) were denatured with 10 x SSC:7% formaldehyde, incubated at 55°C for 1 h and transferred to a nitrocellulose membrane under vacuum. The spots obtained in the films were quantified in a computing densitometer (model 300A, Molecular Dynamics).

Results
BFA enhances the synthesis of SFV structural proteins
The translation of the SFV subgenomic RNA yields a polyprotein which is proteolytically processed (Melancon & Garoff, 1987). The maturation of the SFV structural proteins, and particularly their glycosylation, is coupled to membrane traffic through the vesicular system (de Curtis & Simons, 1988). The antibiotic BFA blocks this pathway between the endoplasmic reticulum and the Golgi apparatus (Alcalde et al., 1992; Chege & Pfeffer, 1990; Griffiths & Simons, 1986; Helms & Rothman, 1992; Hunziker et al., 1992; Klausner et al., 1992; Ladinsky & Howell, 1992; Rothman & Orci, 1992; Lippincott-Schwartz, 1993). To investigate the effect of BFA on the synthesis of SFV late proteins, SFV was allowed to enter HeLa cells for 1 h before treatment of the cells with 5 μg/ml BFA. Protein synthesis in the infected cells was analysed by labelling with [35S]methionine followed by SDS–PAGE (Fig. 1). BFA stimulated the synthesis of SFV late proteins (especially the 107K precursor) and changed the mobility of the viral glycosylated proteins (p62, E1 and E2). The stimulation of SFV protein synthesis did not depend on the time of BFA addition to HeLa cells, since the same effect was observed when it was added later during infection [3 h post-infection (p.i.)]. BFA interferes with the trimming of glycoproteins since the exocytic pathway is impaired (Lippincott-Schwartz, 1993). Thus, not only is the glycosylation of SFV glycoproteins modified by BFA, but these glycoproteins also accumulate in the cell. BFA also enhances the synthesis of the coat protein C which does not follow the membrane pathway, suggesting that it affects SFV protein synthesis in multiple ways. When the effect of BFA was analysed in other cell lines, such as BHK cells, no stimulation of protein synthesis was observed (data not shown), although changes in the mobility of the glycoproteins E1 and E2 were evident, indicating that BFA was functioning in this cell line.
SFV 26 S mRNA synthesis is stimulated by BFA

An inhibitory effect of BFA on poliovirus RNA synthesis has been described recently (Irurzun et al., 1992; Maynell et al., 1992). To test the effect of this compound on SFV RNA synthesis, the cytoplasmic RNA of SFV-infected cells was extracted and separated on partially denaturing agarose gels, transferred to a nitrocellulose filter and hybridized with a [32P]CTP-labelled riboprobe specific for the SFV 26S mRNA (Fig. 2). In untreated infected cells there are two main RNA species of Mr greater than the 26S marker. These are partial hybrids between the SFV 49S negative-strand RNA and the 49S (genomic) or 26S (subgenomic) plus-strand RNA. The presence of BFA from 0, 2 or 4 h p.i. increases the level of both RNA species, suggesting that the stimulation of protein synthesis by BFA results from an increased amount of the viral subgenomic plus-strand RNA in SFV-infected HeLa cells.

Mixed poliovirus and SFV infections

(i) Effects of BFA on poliovirus and SFV mRNA translation

Both SFV and poliovirus induce a drastic inhibition or shut-off of host protein synthesis during infection (Carrasco & Castrillo, 1987; Carrasco et al., 1989). As BFA has the opposite effect on the synthesis of viral macromolecules in poliovirus- or SFV-infected cells we thought that BFA would be an interesting tool to analyse the inhibition of protein synthesis on capped mRNAs by poliovirus. The mixed infection system consisting of cells infected with SFV plus poliovirus in the presence of BFA constituted a good model to analyse the translation of a capped viral RNA in cells where poliovirus genome replication is restricted and p220 is degraded (Irurzun et al., 1992). Fig. 3 shows the electrophoretic analysis of protein synthesis at different times in HeLa cells doubly infected with poliovirus and SFV. In cells infected with poliovirus and SFV at the same m.o.i., the synthesis of both cellular and SFV proteins is strongly inhibited. However, if the doubly infected cells are incubated with 5 μg/ml BFA (SFV/PV + BFA panel) some SFV structural proteins can be observed (mostly the coat protein C).

Cleavage of a 220K cellular factor, known as p220, that is thought to be involved in the initiation of translation of capped mRNAs, has been invoked as the explanation for the poliovirus-induced shut-off of translation (Etchison et al., 1982; Carrasco & Castrillo, 1987; Sonenberg, 1990). By using BFA we have been able to detect significant synthesis of SFV C protein for 4 to 5 h in poliovirus-infected cells. We therefore tested p220 integrity in this situation by immunoblot analysis using a MAb against human p220 (Fig. 4a). The p220 remains
Translation in doubly infected cells

Fig. 4. Immunoblot analysis of p220 and dot blot analysis of SFV-specific RNA in SFV or SFV plus poliovirus infected cells: effects of BFA. (a) An anti-p220 MAb was used for the Western blot analysis of the following samples: lane 1, mock-infected cells; lane 2, PV-infected cells at 2 h p.i.; lane 3, PV + SFV, 2 h p.i.; lane 4, SFV, 7 h p.i.; lanes 5, 6 and 7, PV + SFV at 2, 4 and 6 h p.i., respectively in the presence of 5 μg/ml BFA. The position of intact p220 or the cleavage products is indicated. (b) Samples were collected at 5 h p.i. with both viruses, in parallel with the experiment shown in (a). The RNA was extracted and analysed by dot blot. SFV input: RNA extracted 1 h p.i. with SFV. HeLa: RNA extracted from uninfected HeLa cells. (c) Quantification by densitometry of the penultimate (third) row of samples in the dot blot (b).

(ii) Effect of BFA on SFV RNA synthesis in mixed poliovirus/SFV infections

Because BFA has opposite effects on the synthesis of viral RNA in poliovirus- and SFV-infected cells it was of interest to test the synthesis of SFV RNA in the doubly infected cells. As shown above, the amount of SFV plus-strand-specific RNA increases in cells treated with BFA (Fig. 4b and c). In the cells infected with SFV and poliovirus there is a marked reduction in the level of SFV RNA. BFA, when present from 0 h p.i., prevents this reduction. Moreover, the enhancement of SFV RNA synthesis by BFA is seen in the doubly infected cells. Thus, BFA induces alterations in membrane traffic that enhance SFV 26S mRNA and late protein synthesis. This increase in SFV 26S mRNA induced by BFA is maintained in mixed infections with poliovirus.

Discussion

Membrane traffic through the vesicular system is required for the proper maturation of some cellular and viral proteins (Rothman & Orci, 1992; Griffiths & Simons, 1986). Interference with membrane traffic by BFA inhibits the trimming of viral glycoproteins in a number of enveloped viruses (Chen et al., 1991; Cheung et al., 1991; Misumi et al., 1986; Oda et al., 1990; Ulmer & Palade, 1990; Whealy et al., 1991; Collins & Mottet, 1992). In addition, the proper functioning of the vesicular system plays a part in the replication of viral genomes in some RNA-containing viruses that replicate in the cytoplasm. Inhibition of new membrane formation by the antibiotic cerulenin blocks the replication of vesicular stomatitis virus, SFV, EMCV and poliovirus genomes (Guinea & Carrasco, 1990; Pérez and Carrasco, 1991; Pérez et al., 1991), whereas the disruption of membrane traffic by BFA blocks poliovirus RNA replication (Irurzun et al., 1992; Maynell et al., 1992), but not that of EMCV (Irurzun et al., 1992), suggesting that EMCV RNA synthesis needs continuous membrane formation,
but that replication complexes are functional even in the presence of BFA. SFV constitutes an interesting example of a cytoplasmic virus that also requires continuous membrane formation for RNA synthesis, but does not require an intact vesicular system to make the 26S mRNA. Our present results indicate that the subgenomic 26S mRNA is synthesized in BFA-treated cells and is functional. The replication complexes in SFV-infected cells are closely associated with membrane structures, termed cytopathic vacuoles (Acheson & Tamm, 1967; Froshauer & Kartenbeck, 1988; Griffiths et al., 1989). It is not known whether the same replication complexes synthesize both 42S genomic RNA and subgenomic 26S RNA, but the present findings show that interference with the vesicular system in HeLa cells by BFA enhances the formation of 26S mRNA.

Both SFV and poliovirus induce a profound inhibition of host translation soon after infection of susceptible cells. The mechanism of this inhibition in the case of SFV is connected with the modification of membrane permeability (Muñoz et al., 1985b; Carrasco, 1978; Garry et al., 1979; Ulug et al., 1984). Thus, alterations in the levels of monovalent cations closely parallels the blockade of host protein synthesis (Garry et al., 1979). In addition, translation of the late SFV 26S mRNA in cell-free systems is optimal at concentrations of monovalent ions higher than those required for the translation of host or early viral 42S mRNAs (Carrasco et al., 1979). The possibility that coat protein C blocks translation (van Steeg et al., 1984) is not supported by recent results indicating that shut-off is impaired in a Sindbis virus mutant in protein 6K (Schlesinger et al., 1993), a hydrophobic membrane-associated protein that shares homology with ion channel proteins (Carrasco et al., 1993). Mixed infection experiments with SFV and poliovirus indicated that SFV 26S mRNA can be translated in the infected cells and this translation was regulated by the concentration of monovalent cations in the culture medium (Alonso & Carrasco, 1982a). Despite the fact that SFV 26S mRNA has a cap structure at its 5' end it is translated in poliovirus-infected cells. Presumably the p220 polypeptide, which forms part of the translation initiation factor eIF-4F, was cleaved by poliovirus in those experiments, but direct evidence of this cleavage was not provided. Our present results provide evidence that p220 cleavage does take place in cells doubly infected with SFV and poliovirus even when poliovirus replication is restricted. In addition, the blockage of SFV translation by poliovirus can be regulated by the use of inhibitors of poliovirus RNA synthesis.

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Translation in doubly infected cells

2203


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