Short Communication

Brefeldin A, a Specific Inhibitor of
Intracellular Translocation of
Vesicular Stomatitis Virus
G Protein: Intracellular
Accumulation of High-mannose
Type G Protein and Inhibition
of Its Cell Surface
Expression

Akira TAKATSUKI and Gakuzo TAMURA

Laboratory of Microbiology,
Department of Agricultural Chemistry,
The University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan

Received October 8, 1984

Synthesis and intracellular translocation of glycoproteins destined to be exported extracellularly or sequestered on cell surfaces have attracted much interest recently. Virus envelope glycoproteins have been the subject of intense studies because, in many cases, cellular macromolecular synthesis is shut off after virus infection and thus the subsequent synthesis and fate of virus components are facilitated.¹⁾ The polypeptide moiety of vesicular stomatitis virus (VSV) G protein is synthesized on membrane-bound polysomes and the oligosaccharide moiety is transferred *en bloc* from dolichol-linked lipid intermediates to the nascent polypeptide before completion of its synthesis.²⁾ The oligosaccharide moiety is processed during its translocation from the rough endoplasmic reticulum and matures in the Golgi apparatus.³⁾ Inhibitors such as tunicamycin have contributed to such studies.^{4,5)} The mechanism of intracellular translocation of glycoproteins remains to be solved. Specific inhibitors should be valuable probes in such studies as they have been in other studies. Brefeldin A (BFA) is a macrolide antibiotic having a potent inhibitory activity against virus multiplication.⁶⁾ Intracellular accumu-

lation of high-mannose type G protein and inhibition of its expression on cell surfaces in VSV-infected BHK cells in the presence of BFA will be described in this communication.

BFA was isolated from the culture broth of a soil fungus by silica gel column chromatography and crystallization in acetone, and identified by elementary and spectrometric analyses.⁶⁾ The same lot of BFA was used throughout the present experiments.

Confluent monolayer cultures of BHK-21 cells were infected with the New Jersey strain of VSV. At 4 hr infection, the medium was replaced with conditioned medium containing a designated amount of BFA. The cultures were supplemented with [³H]mannose or [³⁵S]-methionine after 1 hr pretreatment and incubated for a further 4 hr. The cultures were separated into medium and cell fractions, and acid-insoluble radioactivity was determined as described in the legend to Table I. [³H]-Mannose incorporation into the extracellular medium fraction was severely depressed in the presence of BFA, but, on the contrary, significant accumulation of [³H]mannose-labeled products was detected in the cell fraction (Table I). The incorporation of [³⁵S]-methionine into cellular acid-insoluble products was nearly the same among the samples, and no remarkable intracellular accumulation was observed on BFA-treatment. On the contrary, the incorporation of [³H]mannose was inhibited to a greater extent than that of [³⁵S]methionine with respect to the products in the medium. All virus-specific proteins, *i.e.*, L, G, N, NS and M proteins, were synthesized in nearly the same amounts as in the control, as revealed by autoradiography after sodium dodecylsulfate-polyacrylamide slab gel electrophoresis, except that G protein synthesized in the presence of BFA electrophoresed a little faster than the control G protein (data not shown). But BFA greatly suppressed virus particle formation, and the particles formed in the presence of BFA had about 1/10 the amount of G protein of the control. The discrepancy between the incorporation of [³H]mannose and [³⁵S]methionine

TABLE I. INCORPORATION OF RADIOLABELED PRECURSORS INTO ACID-INSOLUBLE PRODUCTS OF VSV-INFECTED BHK CELLS

Confluent monolayer cultures of BHK-21 cells in 24-well plastic plates (Nunc, Denmark) were infected with VSV at an input multiplicity of about 10 plaque-forming units per cell. The medium was replaced with a conditioned one containing a designated concentration of BFA at 4 hr infection. [^3H]Mannose (Amersham, England; 28.1 Ci/mmol) or [^{35}S]methionine (New England Nuclear, U.S.A.; 1236.8 Ci/mmol) was added to 5 $\mu\text{Ci}/\text{ml}$ or 3 $\mu\text{Ci}/\text{ml}$, respectively, after 1 hr pretreatment with BFA. After 4 hr of the labeling, the medium was put into test tubes. The cell layers were rinsed 3 times with cold PBS and disrupted by adding PBS containing 1% sodiumdodecyl sulfate. Aliquots of the medium and cell lysates were mixed with an equal volume of mixture of 10% trichloroacetic acid and 4% phosphotungstic acid, and incubated in melting ice for 2 hr to facilitate precipitate formation. The precipitates were collected on glass fiber filters (Whatman, England; GF/C), washed thoroughly with 95% ethanol and dried under an infrared lamp, and then the radioactivity retained on the filters was counted with a liquid scintillation counter. The figures are the means of three determinations, and those in parentheses are % incorporation.

Fraction	Precursor	DPM $\times 10^{-3}/\text{sample}$				
		Control	0.01	BFA ($\mu\text{g}/\text{ml}$) 0.05	0.1	0.5
Cell	[^{35}S]Methionine	398.1 (100)	408.6 (103)	414.8 (104)	411.7 (103)	394.9 (99)
	[^3H]Mannose	90.5 (100)	111.0 (123)	117.5 (123)	130.3 (144)	121.1 (134)
Medium	[^{35}S]Methionine	95.4 (100)	89.6 (94)	63.9 (67)	34.6 (36)	25.8 (27)
	[^3H]Mannose	170.0 (100)	166.4 (98)	97.5 (57)	15.1 (8.9)	3.9 (2.3)

into the medium fraction may be explained by the fact that VSV particles containing little G protein are formed in the presence of BFA, clearly indicating that BFA specifically inhibits egress of newly synthesized G protein.

To ascertain inhibition of surface expression of G protein in the presence of BFA, cells processed as described above were suspended in phosphate-buffered saline (PBS) containing a large amount of BFA, and cell-associated radioactivity was determined after designated incubation periods in the presence or absence of proteinase K. No substantial loss of radioactivity was observed with the cells labeled in the presence of BFA (Fig. 1B). As for the cells labeled in the absence of BFA relatively rapid loss of radioactivity was detected even in the presence of BFA, and this loss of cell-associated radioactivity was accelerated on proteinase K treatment (Fig. 1A). These results indicate inhibition of cell surface expression of G

protein synthesized in the presence of BFA, and also suggest that BFA does not interfere in the late stage(s) of intracellular translocation and surface expression of G protein.

The enzymes involved in the processing of the oligosaccharide moiety of glycoproteins are known to be compartmentalized in particular cell structures. To gain insight into the site of action of BFA, the structures of the carbohydrate chains of G protein were analysed. The carbohydrate moiety was metabolically labeled with [^3H]mannose, and cell and medium fractions were prepared as described above. Both fractions were digested exhaustively with pronase E and passed through a Sephadex PD-10 column. The glycopeptide fractions were collected and divided into two parts, one was treated with endo-*N*-acetylglucosaminidase H (Endo H) and the other was false-treated. The glycopeptides in the control extracellular medium fraction were no more sensitive to the

FIG. 1. Release of [^3H]Mannose-labeled G Protein Oligosaccharides from VSV-Infected BHK-21 Cells by Proteinase Treatment.

Confluent monolayer cultures of BHK-21 in 175-cm² flasks (Falcon Labware, U.S.A.) were infected with the New Jersey strain of VSV at an input multiplicity of about 10 plaque-forming units per cell. The medium was replaced with a conditioned one with or without BFA (0.5 $\mu\text{g}/\text{ml}$) at 4 hr infection. After 1 hr pretreatment with BFA, [^3H]mannose (Amersham, England; 28.1 Ci/mmol) was added to 10 $\mu\text{Ci}/\text{ml}$, and the mixture incubated for a further 4 hr. The cell layers were rinsed 5 times with cold PBS and supplemented with PBS containing 50 mM ethylenediaminetetraacetic acid, and then the cells were collected with the aid of a rubber policeman, washed twice with cold PBS by repeated centrifugation at 1,500 rpm in a clinical centrifuge and suspended in PBS containing 10 $\mu\text{g}/\text{ml}$ of BFA. The cell suspensions were divided into two equal portions, and proteinase K (Boehringer Mannheim Yamanouchi, Japan) was added to one to 50 $\mu\text{g}/\text{ml}$. The mixtures were incubated at 20°C, and 1 ml portions were withdrawn at designated times, centrifuged at 1,500 rpm for 5 min in a clinical centrifuge and washed with PBS. The remaining cell-associated radioactivity after incubation in the presence (●) or absence (○) of proteinase K was determined with a scintillation counter (Aloka). The results are presented as percentages of the initial cell-associated radioactivity. (A), control; (B), BFA-treated.

action of Endo H, and no change in the elution pattern on a Bio-Gel P-4 column was observed on Endo H treatment (Fig. 2A). The radioactivity in the medium fraction of the BFA-treated sample was too small to analyze by Bio-Gel P-4 column chromatography. As for the glycopeptides in the cellular fraction, two major radioactive peaks were observed (Fig. 2B and C). The late-eluting peak fraction was sensitive to Endo H on the whole and no

radioactive peak was detected on Endo H treatment at this position, with the appearance of a new radioactive peak eluting later than that in the Endo H false-treated samples. The fast-eluting peak was partially sensitive to Endo H digestion, and the radioactivity in this peak fraction decreased on the enzyme treatment, but the sensitivity was greater with the BFA-treated sample than the control. These results indicate that the majority of the oligosaccharides of cell-associated G protein are of the high-mannose type, in good agreement with the observation that the rate-limiting step in intracellular translocation is transport from *cis* to *trans* cisternae of the Golgi apparatus. On treatment with BFA, intracellular [^3H]-G protein accumulated (Table I), and it was Endo H sensitive (Fig. 2C) and contained no capping sugars such as *N*-acetylglucosamine, galactose, *N*-acetylneuraminic acid or fucose (data not shown). These observations suggest that translocation of G protein is stopped by BFA before or at, at the most, the *cis* cisternae of the Golgi apparatus. In connection with this, monensin has been reported to interfere with intracellular translocation of VSV G protein by perturbing the Golgi apparatus (for a review, see ref. 7), and the actions of BFA and monensin are similar to each other.

The abovementioned observations are in good agreement with the assumption that G protein containing high-mannose type oligosaccharides is accumulated intracellularly on treatment with BFA. Cell lysates prepared from BFA-treated cells showed an activity of removing mannose residues from VSV glycopeptides and also BFA did not interfere with glycosyltransferase activities *in vitro*. Swainsonine, an inhibitor of the processing enzyme, α -mannosidase II,⁸⁾ showed no inhibitory activity toward either surface expression of G protein or virus particle formation in our virus-cell system (unpublished observations), *i.e.*, processing of the oligosaccharide moiety is not a prerequisite for the intracellular translocation of G protein. These observations may exclude the possibility that the intracellular accumulation of high-mannose type G protein

FIG. 2. Bio-Gel P-4 Column Chromatograms of Pronase-Digested Glycopeptides before and after Endo H-Treatment.

VSV-infected cells were labeled with [^3H]mannose for 4 hr, starting at 5 hr infection, and collected as described in the legend to Fig. 1. The medium and washed cells were heated in boiling water to inactivate cellular enzymes and then exhaustively digested with pronase E (Kaken Kayaku, Japan) at 40°C for 4 days with daily addition of the enzyme. The digests were heated in a boiling-water bath to inactivate the enzyme and also to denature proteinaceous materials and, after removal of precipitates by centrifugation at $12,000 \times g$ for 10 min in a microfuge, they were passed through a column of PD-10 (Pharmacia, Sweden). Glycopeptide portions of the eluates were divided into two portions, and treated (---●---) or false-treated (—▲—) with 0.1 units/ml of Endo H (Seikagaku Kogyo, Japan) in 50 mM Na-citrate buffer (pH 5.5) for 20 hrs at 25°C. The digests were loaded on a Bio-Gel P-4 column (1 $\times$ 120 cm) and eluted with 4% acetic acid at a flow rate of 7 ml/hr. Fractions of 20 drops were collected and radioactivity in each fraction was determined with a liquid scintillation counter. The same column was used throughout the present experiments, and the elution profiles were superimposed. The elution positions of blue dextran (BD) and mannose (M) are indicated by arrow heads. (A), control medium; (B), control cellular fraction; (C), BFA-treated cellular fraction.

and inhibition of its surface expression in the presence of BFA are caused by direct inhibition by BFA of processing of the oligosaccharide moiety of G protein, suggesting that BFA may interfere with intracellular translocation of G protein to cell surfaces, perhaps before or at the Golgi apparatus as discussed above.

The details of the present study and other features of the action of BFA will be presented elsewhere. Inhibition of intracellular translocation of glycoproteins by BFA is not restricted to VSV G protein and such inhibition was observed with others. Specific inhibitors of intracellular translocation are eagerly sought as probes for studies on the mechanism of intracellular translocation and secretion or surface expression of glycoproteins. The mechanism of the BFA action has not been clarified as yet, but this antibiotic should be expected to contribute to such studies.

Acknowledgments. The present study was financially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. BHK-21 cells and VSV were gifts from Dr. K. Onodera of the University of Tokyo.

REFERENCES

- 1) L. Kääriäinen and O. Renkonen, "Cell Surface Reviews," Vol. 4, ed. by G. Poste and G. L. Nicolson, Elsevier/North-Holland Biochemical Press, Amsterdam, The Netherlands, 1977, p. 741.
- 2) J. E. Rothman and H. F. Lodish, *Nature* (London), **269**, 775 (1977).
- 3) S. Kornfeld: "The Glycoconjugates," Vol. 3, Part A, ed. by M. I. Horowitz, Academic Press, New York, U.S.A., 1982, p. 3.
- 4) G. Tamura, Ed., "Tunicamycin," Japan Scientific Societies Press, Tokyo, Japan, 1982.
- 5) K. Olden, J. B. Parent and S. L. White, *Biochim. Biophys. Acta*, **650**, 209 (1982).
- 6) G. Tamura, K. Ando, S. Suzuki, A. Takatsuki and K. Arima, *J. Antibiot.*, **21**, 160 (1968).
- 7) A. M. Tartakoff, *Cell*, **32**, 1026 (1983).
- 8) D. R. P. Tulsiani, T. M. Harris and O. Touster, *J. Biol. Chem.*, **257**, 7936 (1982).