Brefeldin A Inhibits Oligosaccharide Processing of Glycoproteins in Mouse Hypothyroid Pituitary Tissue at Several Subcellular Sites (42862)

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Abstract. **We have studied the effects of brefeldin A (BFA) and monensin on the processing of the oligosaccharides of thyrotropin (TSH), free α-subunits, and cellular glycoproteins of mouse pituitary tissue to clarify the subcellular sites of action of BFA. BFA was previously shown to inhibit the translocation of glycoproteins from the rough endoplasmic reticulum to the Golgi apparatus but action at other sites was possible. Pituitaries from hypothroid mice were incubated with [^{35}S]methionine, [^{3}H]mannose, [^{3}H] galactose, [^{3}H]fucose, *N*-[^{3}H]acetylmannosamine, or [^{35}S]sulfate for 2 hr in the absence or presence of 5 μg of BFA/ml or 2 μM monensin. TSH and free α-subunits were immunoprecipitated from tissue lysates and analyzed by sodium dodecyl sulfate-gel electrophoresis. The tryptic glycopeptides of TSH were separated using high-performance liquid chromatography. Total glycoproteins in cell lysates were precipitated using trichloroacetic acid. Labeled oligosaccharides were released from the tryptic glycopeptides of TSH and cellular glycoproteins by endoglycosidase H and they were analyzed by paper chromatography. Compared with control incubations, BFA caused the intracellular accumulation of glycoproteins having less than expected amounts of $Man_9GlcNAc_2$ units, but with excess $Man_8GlcNAc_2$, $Man_7GlcNAc_2$, $Man_6GlcNAc_2$, and $Man_5GlcNAc_2$ units. There was a lesser accumulation of glucose-containing oligosaccharides, especially $Glc_1Man_9GlcNAc_2$. Monensin also caused the accumulation of certain high mannose species, but the pattern differed from that seen for BFA, since $Man_9GlcNAc_2$ units were preserved and there was less excess of $Man_8GlcNAc_2$, $Man_7GlcNAc_2$, $Man_6GlcNAc_2$, and $Man_5GlcNAc_2$ units. BFA did not block the initial attachment of oligosaccharides at any of the three Asn-glycosylation sites of TSH, but caused the accumulation of $Man_{5-8}GlcNAc_2$ units at each site. Both monensin and BFA inhibited fucosylation, sulfation, and sialylation more markedly than mannose incorporation. Thus, in addition to its previously described action of inhibiting rough endoplasmic reticulum to Golgi transport, BFA appears to partially inhibit the glucose-trimming enzymes as well as some Golgi enzymes.** [P.S.E.B.M. 1989, Vol 190]

Brefeldin A (BFA) is a fatty acid derivative biosynthesized from palmitate in the fungus *Penicillium cyaneum* (1) with a structure similar to that of the prostaglandins (Fig. 1). Although this drug has been employed in few studies, it appears that one important action is to inhibit the translocation of glycoproteins from the rough endoplasmic reticulum (RER) to the Golgi (2–4). The purpose of this study is to use hypothyroid mouse pituitary tissue to learn whether or not BFA may act at a variety of subcellular sites. In some instances we compare the actions of BFA to those of monensin, a carboxylic acid ionophore that binds and transports monovalent cations across cellular membranes. The main site of action of monensin is though to be within the Golgi apparatus (5,6).

The model system chosen is well suited for this evaluation of BFA action. Thyroid-stimulating hormone (TSH) is a glycoprotein composed of noncovalently linked α- and β-subunits that are synthesized from separate messenger RNA (7–10). Thyrotropic cells also synthesize excess free α-subunits. The α-subunits have two asparagine-linked oligosaccharide units whereas β-subunits have only one. These species are cotranslationally glycosylated with $Glc_3Man_9GlcNAc_2$

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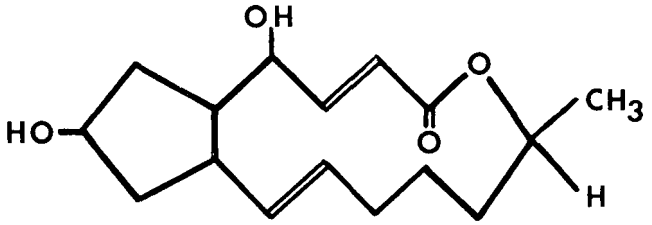

Figure 1. Structure of brefeldin A (1).

units. Following trimming of $Glc_3Man_9GlcNAc_2$ to $Man_9GlcNAc_2$ within the RER, further trimming to $Man_8GlcNAc_2$,$Man_7GlcNAc_2$, $Man_6GlcNAc_2$, and-$Man_5GlcNAc_2$ may occur within the RER by one or more endoplasmic reticulum (ER) (α1,2)-mannosidases or within the Golgi apparatus by the Golgi (α1,2)-mannosidases (11–19). The formation of complex oligosaccharides that may contain fucose, galactose, sulfate, and sialic acid is generally believed to occur in more distal compartments (11, 17–20).

In recent studies of TSH biosynthesis, we employed inhibitors of the intracellular translocation of proteins (monensin (17, 19), carboxyl cyanide *m*-chlorophenylhydrazone (17, 19), and BFA (12, 21)), as well as subcellular fractionation techniques, to study the sites of incorporation of various radioactive precursors and the sites of distribution of various processing enzymes. BFA caused the accumulation of $Man_{5-8}GlcNAc_2$ units within several subcellular compartments, beginning as early as the RER (12). Inhibition of translocation from RER to Golgi could partially explain this, but failed to explain an observed relative decrease in $Man_9GlcNAc_2$ units. It also failed to explain the distal accumulation of altered glycoproteins within the Golgi. Moreover, during chase incubations in the presence of BFA, only a portion of [^{35}S]sulfate- or [^{3}H]fucose-labeled TSH and free α-subunits appeared in media (21), suggesting that BFA might have some direct effect on the Golgi apparatus. In the present study we extend these observations by incubating hypothroid pituitary tissue with several radioactive precursors in the presence of BFA to gain insight into which subcellular compartments are disturbed by the drug.

Methods

Preparation of Tissues and Media. Hypothyroid LAF female mice that had been radiothyroidectomized 10–16 weeks before the experiment were decapitated and pituitary tissue was collected as described previously (17). Up to 60 mice were killed in a given experiment. One thyrotropic tumor weighing 1.5 g was used for studies on the tryptic glycopeptides of TSH. Tissues were minced to 1-mm^3 pieces with steel scalpel blades and incubated in a shaking water bath for 45 to 60 min at 37°C in moist 5% CO_2-95% O_2 in sterile tubes containing 600 μl of Dulbecco's modified Eagle's medium supplemented with 10 m*M* Hepes and 1 m*M* glutamine (Gibco) and devoid of glucose, $MgSO_4$, and methionine. Glucose (200 μg/ml) was added to incubations that were later to receive [^{3}H]mannose, [^{3}H]fucose, or *N*-[^{3}H]acetylmannosmaine, and glucose (100 μg/ml) was added to incubations that were later to receive [^{3}H]galactose. Glucose (1000 μg/ml) was added to the incubations that were later to receive [^{35}S]methionine or [^{35}S]sulfate. $MgSO_4$ (200 μg/ml) was added to all incubations except for the [^{35}S]sulfate incubations, where $MgCl_2$ (200 μg/ml) was substituted. Methionine (30 μg/ml) was added to all incubations except for the [^{35}S]methionine incubations.

Incubation of Tissues. Stock solutions of 2 mg of BFA/ml in methanol (21) and 1 m*M* monensin (Sigma, St. Louis, MO.) in ethanol (17) were prepared as described previously. BFA was generously provided by Dr. Akira Takatsuki, University of Tokyo, Tokyo, Japan. BFA, at a final concentration of 5 μg/ml, was added to the appropriate media 40 min before, whereas 2 μ*M* monensin was added 10 min before the addition of the respective radioisotopes. The final concentrations of the drugs tested were selected in view of our past experience (12, 17, 19, 21). The isotopes purchased from New England Nuclear (Boston, MA) were [^{3}H]mannose (1.5 mCi/ml; 13 Ci/mmol), [^{3}H]fucose (1.0 mCi/ml; 20 Ci/mmol), *N*-[^{3}H]acetylmannosamine (1.0 mCi/ml; 26 Ci/mmol), [^{3}H]galactose (1.0 mCi/ml; 337 mCi/mmol) and from Amersham/Searle (Arlington Heights, IL) were [^{35}S]sulfate (500 μCi/ml; 1019 Ci/mmol) and [^{35}S]methionine (1.0 mCi/ml; 900 Ci/mmol). These were added and incubations were continued for 2 hr. To analyze the tryptic glycopeptides of TSH, mouse thyrotropic tumor tissue, a richer source of TSH than pituitary tissue, was incubated with [^{3}H]mannose for 2 hr in the presence or absence of BFA. To terminate incubations, tissue minces were centrifuged at 500 *g* for 2 min at 24°C and washed once with ice-cold phosphate-buffered saline (pH 7.4) before freezing.

Immunoprecipitation of TSH or Free α-Subunits. Pituitary or tumor tissue was homogenized in 1000 μl of buffer containing 0.15 *M* sodium chloride, 0.02 *M* Tris HCl, 0.01 *M* EDTA, 1% (w/v) Triton X-100, and 100 units/ml aprotinin (pH 7.6). The mixtures were vortexed vigorously, incubated at 24°C for 1 hr with intermittent vortexing, and then centrifuged at 10,000 *g* for 30 min at 4°C. Solubilized radioactivity was immunoprecipitated by a sequential scheme employing specific antisera and *Staphylococcus A* (Pansorbin, Calbiochem, La Jolla, CA) as described previously (14).

Sodium Dodecyl Sulfate (SDS)-Gel Electrophoresis. Subunits were analyzed by electrophoresis in SDS-polyacrylamide slab gels using a 5% polyacrylamide stacking gel (1–2 cm) and a 12–20% linear resolving gradient gel (12 cm) as described previously (22). The following standards (2–5 μg each; obtained from Calbiochem or Sigma (St. Louis, MO)) were

mixed with each sample immediately before gel electrophoresis to serve as internal molecular weight markers: carbonic anhydrase (29,000), soybean trypsin inhibitor (20,000), myoglobin (17,000), and cytochrome *c* (12,000). Coomassie brilliant blue-stained gels were sliced into 1- or 2-mm sections, solubilized with hydrogen peroxide at 60°C for 16 hr, and counted in 10 ml of Opti-Fluor (Packard, Downers Grove, IL).

High Performance Liquid Chromatography (HPLC) Analysis of Tryptic Peptides. TSH subunits from mouse thyrotropic tumor tissue, incubated for 2 hr in the presence or absence of BFA, and eluted from *Staphylococcus A* pellets were reduced and alkylated using dithiothreitol and iodoacetic acid, followed by incubation in the presence of 10 μl of L-1-tosylamide-2-phenylethylchloromethyl ketone-trypsin (stock 20 mg/ml in 0.06 *M* ammonium bicarbonate, pH 8.0; Worthington, Freehold, NJ) as described previously (23). Tryptic peptides were lyophilized and resuspended in 10 μl of 0.05% trifluoroacetic acid (TFA; Baker Chemical, Phillipsburg, NJ) titrated to pH 3.3 with triethylamine (Sigma). Insoluble material was removed by centrifugation. The samples were loaded on a C18 reverse phase column (5-μm packing, 4.6 × 250 mm; ISCO, Lincoln, NE) equilibrated in 0.05% TFA titrated to pH 3.3 with triethylamine using model 2350 pumps and a Chemresearch control program (ISCO). Peptides were eluted with gradients of acetonitrile increasing from 0 to 8% for 24 min, then 8 to 40% for 40 min at a flow rate of 1.0 ml/min. The retention times for mouse TSH tryptic peptides have been determined previously (23).

Trichloroacetic Acid (TCA) Precipitation of Total Glycoproteins, Endoglycosidase H Treatment, and Paper Chromatography. After removal of TSH and free α-subunits from [^{3}H]mannose and [^{3}H]galactose-labeled specimens using specific antisera, 500-μl aliquots of the supernatants were incubated with an equal volume of 20% TCA for 1 hr at 4°C. The pellets were washed with 10% TCA followed by two washes with 0.06 *M* ammonium bicarbonate (pH 8.5) and were then incubated in 75 μl of the latter buffer containing 75 μg of trypsin for 4 hr at 37°C, stirring frequently. Specimens were centrifuged at 10,000*g* at room temperature for 4 min and the supernatants were removed. These specimens, as well as the tryptic glycopeptides of TSH separated using HPLC, were separately lyophilized and resuspended in 0.01 *M* sodium citrate buffer (pH 5.5) with 0.1% SDS and 1% 2-mercaptoethanol. After boiling for 5 min, each 30- to 50-μl specimen was incubated with 5 mIU of endoglycosidase H (Miles Scientific, Naperville IL) at 37°C for 16 hr. [^{14}C]Mannose-labeled oligosaccharides were then added to selected ^{3}H-labeled specimens as internal standards (15). Each specimen was spotted on Whatman No. 1 paper (Whatman, London, England) and subjected to descending chromatography using 1-propanol:nitromethane:water (5:2:4). After 39–44 hr the papers were dried at room temperature, lanes were cut at 0.5- or 1.0-cm intervals, and radioactivity was eluted with 0.5 ml of water in scintillation vials for 1 hr at room temperature. Radioactivity was then counted in 10 ml of Opti-Fluor using a program for double isotope analysis.

TCA Precipitation of Total Glycoproteins for Quantitation of Labeled Oligosaccharides in the Presence and Absence of Inhibitors. Aliquots (50 μl) of supernatants, obtained after removal of TSH and free α-subunits from specimens by using specific antisera, were incubated in triplicate in 100 μl of 20% TCA for 1 hr at 4°C. The pellets were washed with 200 μl of 10% TCA followed by a wash with 200 μl of 2% TCA, and were dissolved in 400 μl of 0.2 *N* sodium hydroxide at 4°C for 16 hr and counted in 10 ml of Opti-Fluor.

Results

Methionine. As an indicator of the toxicity of the drugs used in these experiments, we evaluated the effects of BFA or monensin on the incorporation of [^{35}S] methionine into total proteins in cells during 2–hr incubations in the presence or absence of these inhibitors. TCA precipitable radioactivity in the presence of 5 μg of BFA/ml or 2 μ*M* monensin was 140% and 108%, respectively, compared with that of control, indicating that no inhibition of protein synthesis occurred.

Mannose. Mouse pituitary tissue was incubated with [^{3}H]mannose for 2 hr in the absence or presence of BFA or monensin. TSH subunits (Fig. 2) and free α-subunits (Fig. 3) were immunoprecipitated from tissue lysates and analyzed by SDS-gel electrophoresis; cellular glycoproteins were precipitated in triplicate using TCA. As compared with the control incubations (Figs. 2a and 3a), 5 μg of BFA/ml did not affect the incorporation of [^{3}H]mannose into TSH substantially (Fig. 2, b vs a) and the incorporation of [^{3}H]mannose into free α-subunits was moderately decreased (Fig. 3, b vs a). BFA decreased the incorporation of [^{3}H]mannose into cellular glycoproteins to 60% of the control. In the presence of 2 μ*M* monensin, the incorporation of [^{3}H]mannose into either TSH (Fig. 2, c vs a) or free α-subunits (Fig. 3, c vs a) was not significantly affected and the incorporation into cellular glycoproteins was increased to 150% of control. Thus, the doses of the drugs, selected in the light of our past experience (12, 17, 19, 21), did not markedly inhibit biosynthesis as judged by [^{3}H] mannose labeling. As compared with control, TSH subunits (Fig. 2, a vs b and c) and free α-subunits (Fig. 3, a, vs b and c) appeared to be of slightly lower molecular size in the presence of BFA or monensin.

Aliquots of the [^{3}H]mannose-labeled glycoproteins in pituitary homogenates were precipitated with TCA and the high mannose oligosaccharides were released by endoglycosidase H and analyzed by paper chroma-

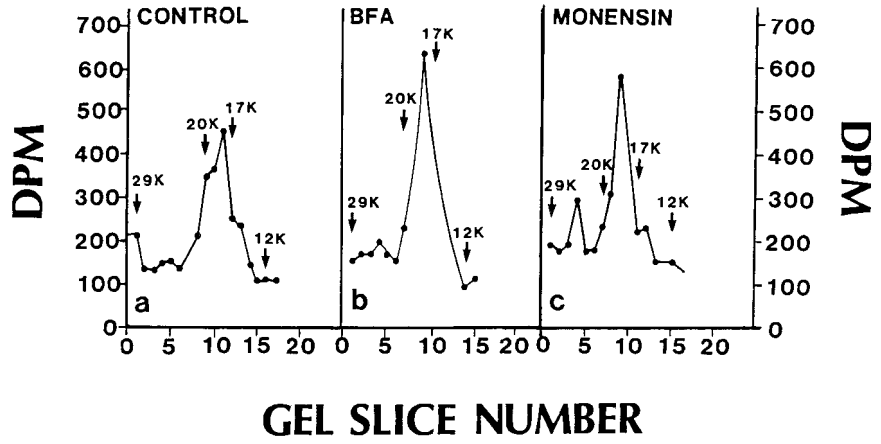

Figure 2. Electrophoretic profiles of [^{3}H]mannose-labeled TSH subunits. Pituitaries from hypothyroid mice were incubated with [^{3}H]mannose for 2 hr in the absence of inhibitors (a), or in the presence of 5 μg of BFA/ml (b) or 2 μM monensin (c). TSH was immunoprecipitated from tissue lysates with anti-TSHβ serum and *Staphylococcus A*. Internal M_r markers (arrows) were included in each lane and identified by Coomassie brilliant blue stain.

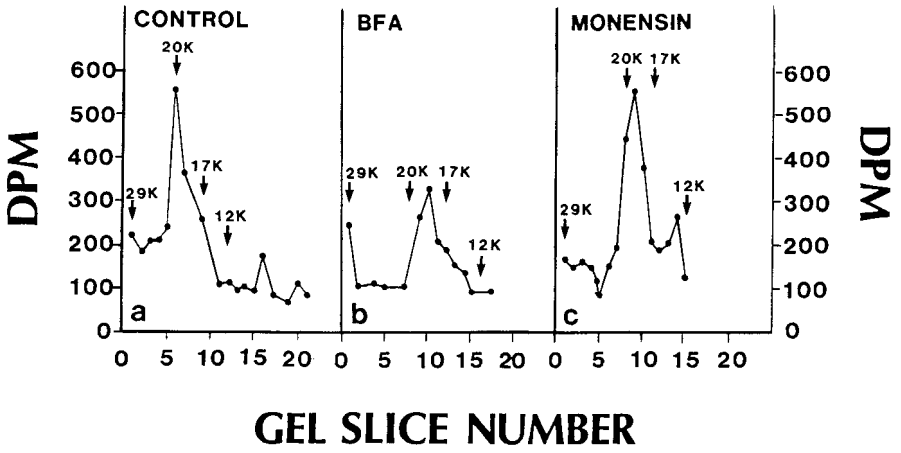

Figure 3. Electrophoretic profiles of [^{3}H]mannose-labeled free α-subunits. Pituitaries from hypothyroid mice were incubated with [^{3}H]mannose for 2 hr in the absence of inhibitors (a), or in the presence of 5 μg of BFA/ml (b) or 2 μM monensin (c). First, TSH was immunoprecipitated with anti-TSHβ serum and *Staphylococcus A* (see Fig. 2). Then anti-LHα serum was used to obtain free α-subunits which were analyzed by SDS-gel electrophoresis. Internal M_r markers (arrows) were included in each lane and identified by Coomassie brilliant blue stain.

tography. In the absence of inhibitors (Fig. 4a) the predominant oligosaccharide present was $Man_9GlcNAc$. There were also large amounts of $Man_8GlcNAc$ whereas $Man_7GlcNAc$ comprised a small amount of the high mannose oligosaccharides. $Glc_3Man_9GlcNAc$ was seen as a small peak of radioactivity preceding $Man_9GlcNAc$ and trace amounts of $Man_6GlcNAc$ and $Man_5GlcNAc$ were seen as a shoulder of radioactivity following $Man_7GlcNAc$. Incubation with 5 μg of BFA/ml (Fig. 4b) caused marked changes. There were approximately equal amounts of $Man_9GlcNAc$ (or $Glc_1Man_9GlcNAc$, which is not well resolved from $Man_9GlcNAc$) and $Man_8GlcNAc$, whereas $Man_7GlcNAc$, $Man_6GlcNAc$, and $Man_5GlcNAc$ units increased to comprise about half of the high mannose oligosaccharides seen. A trace amount of the units were $Glc_3Man_9GlcNAc$. In the presence of 2 μM monensin, $Man_9GlcNAc$ comprised the majority of the oligosaccharides present (Fig. 4c) and there was less increase of $Man_8GlcNAc$, $Man_7GlcNAc$, $Man_6GlcNAc$, and $Man_5GlcNAc$ units as compared with the incubation with BFA.

Processing of the [^{3}H]Mannose-Labeled Oligosaccharides of TSH at Individual Asn-Glycosylation Sites. Mouse thyrotropic tumor tissue was incubated with [^{3}H]mannose for 2 hr in the absence or presence of 5 μg of BFA/ml. TSH was immunoprecipitated from tissue lysates, reduced, alkylated, trypsinized, lyophilized, and resuspended in 0.05% TFA, titrated to pH 3.3 using triethylamine, prior to separation on HPLC. [^{3}H]Mannose-labeled glycopeptides containing Asn 56 and Asn 82 of the α-subunit and Asn 23 of the β-subunit were eluted in Fractions 66, 70, and 74, respectively (23, 24). The high mannose oligosaccharides from the three tryptic glycopeptides were released by endoglycosidase H and analyzed by paper chromatography.

In the absence of BFA, we noted almost equal amounts of $Man_9GlcNAc$ and $Man_8GlcNAc$ at both Asn 56 and Asn 82 of the α-subunit (Fig. 5, a and b, respectively); Asn 23 of the β-subunit contained predominantly $Man_9GlcNAc$ (Fig. 5c). In the presence of BFA there was an increase in $Man_{5-8}GlcNAc$ and a relative decrease in $Man_9GlcNAc$ at each of the three glycosylation sites (Fig. 5, d–f).

Glucose. As noted in a previous study (12) and confirmed in this study, the amount of $Man_9GlcNAc$ present on oligosaccharides in the presence of BFA decreased in relation to $Man_{5-8}GlcNAc$ units. In an attempt to clarify whether this drug affected very early processing events, we studied the processing of glucose-bearing high mannose oligosaccharides in the presence or absence of BFA. [^{3}H]Galactose was chosen for these

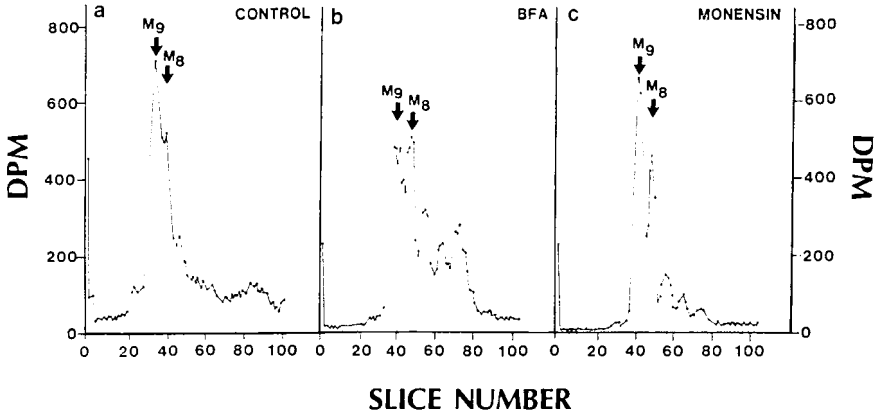

Figure 4. Paper chromatographic profiles of the ^{3}H-labeled high mannose oligosaccharides of cellular glycoproteins. Pituitaries from hypothyroid mice were incubated with [^{3}H]mannose for 2 hr in the absence of inhibitors (a) or in the presence of 5 μg of BFA/ml (b) or 2 μM monensin (c). After immunoprecipitation of TSH and free β-subunits (Fig. 2) as well as free α-subunits (Fig. 3) from tissue homogenates, the total cell glycoproteins remaining were precipitated with TCA. The oligosaccharides present were released by endoglycosidase H and analyzed by paper chromatography. The arrows mark the positions of migration of [^{14}C]mannose-labeled standards $Man_9GlcNAc$ and $Man_8GlcNAc$.

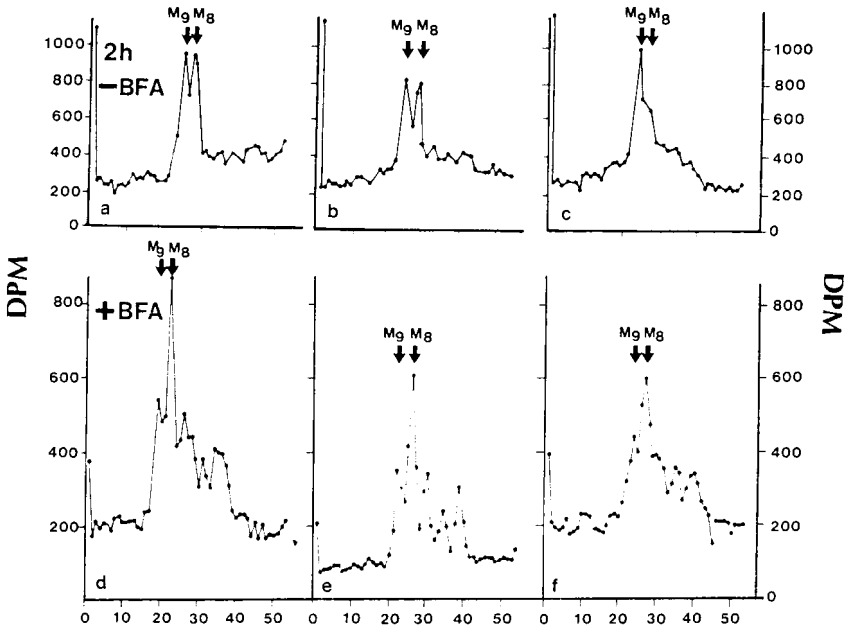

Figure 5. Paper chromatographic profiles of the ^{3}H-labeled high mannose oligosaccharides at the three Asn glycosylation sites of TSH. Mouse thyrotropic tumor tissue was incubated with [^{3}H]mannose for 2 hr in the absence of (a–c) or presence of 5 μg of BFA/ml (d–f). TSH was immunoprecipitated from tissue lysates, reduced, alkylated, and trypsinized. Tryptic glycopeptides were separated on reverse phase HPLC; glycopeptides containing Asn 56 (a and d) and Asn 82 (b and e) from α-subunits and the glycopeptide containing Asn 23 (c and f) from β-subunits eluted in Fractions 66, 70, and 74, respectively. The fractions were lyophilized, the oligosaccharides were released by endoglycosidase H and analyzed by paper chromatography. The arrows mark the positions of migration of [^{14}C]mannose-labeled standards $Man_9GlcNAc$ and $Man_8GlcNAc$.

experiments rather than [^{3}H]glucose because [^{3}H]galactose has been shown to label glucose and not mannose during short incubations whereas [^{3}H]glucose labels both glucose and mannose (16).

Pituitary tissue was incubated with [^{3}H]galactose for 2 hr in the absence or presence of 5 μg of BFA/ml. [^{3}H]Galactose-labeled TSH or free α-subunits were not detectable. The [^{3}H]galactose-labeled glycoproteins in pituitary homogenates were precipitated with TCA and the labeled oligosaccharides were released by endoglycosidase H and analyzed by paper chromatography (Fig. 6). In the absence of BFA (Fig. 6a), most of the [^{3}H]galactose oligosaccharides remained at the origin, indicating that little [^{3}H]galactose was incorporated into endoglycosidase H-sensitive [^{3}H]glucose-labeled species under these conditions. Peaks representing $Glc_3Man_9GlcNAc$, $Glc_1Man_9GlcNAc$, and another very rapidly migrating species were noted. This latter species migrated at the same rate as free [^{3}H]galactose. In the presence of 5 μg of BFA/ml (Fig. 6b), more radioactivity entered the chromatogram; species representing $Glc_{3-1}Man_9GlcNAc$ and $Glc_1Man_8GlcNAc$ (or $Glc_2Man_7GlcNAc$) were noted and free [^{3}H]galactose was detectable near the bottom of the chromatogram.

Fucose, Sulfate, and Sialic Acid. Mouse pituitaries were incubated with [^{3}H]fucose, [^{35}S]sulfate, or *N*-[^{3}H]acetylmannosamine (a precursor of sialic acid) for 2 hr in the absence or presence of BFA or monensin. TSH and free α-subunits were immunoprecipitated from tissue lysates and analyzed by SDS-gel electrophoresis; cellular glycoproteins were precipitated in triplicate using TCA. In the presence of 5 μg of BFA/ml, the incorporation of [^{3}H]fucose into TSH, free α-subunits, and cellular glycoproteins was 0, 40, and 40% of control, respectively. In the presence of 2 μM monensin, the incorporation was also 0, 40, and 40% of control, respectively. Thus, in contrast to their more limited effects on [^{3}H]mannose incorporation, both inhibitors markedly reduced [^{3}H]fucose incorporation into TSH, free α-subunits, and cellular glycoproteins.

In the presence of 5 μg of BFA/ml, the incorporation of [^{35}S]sulfate into free α-subunits and total glycoproteins was 0 and 7% as compared with that of control. In the presence of 2 μM monensin, the incorporation was 20% and 15% of control, respectively. Thus, both inhibitors markedly reduced [^{35}S]sulfate incorporation into free α-subunits and cellular glycoproteins. In the presence of 5 μg of BFA/ml or 2 μM monensin, the incorporation of *N*-[^{3}H]acetylmannosamine into cellular glycoproteins was 42 and 48%, respectively, compared with that of control.

Discussion

Brefeldin A is thought to be a relatively specific inhibitor of the intracellular translocation of newly synthesized proteins (2–4). When we tested this drug previously using chase incubations, we found that it markedly inhibited the secretion of [^{35}S]methionine-

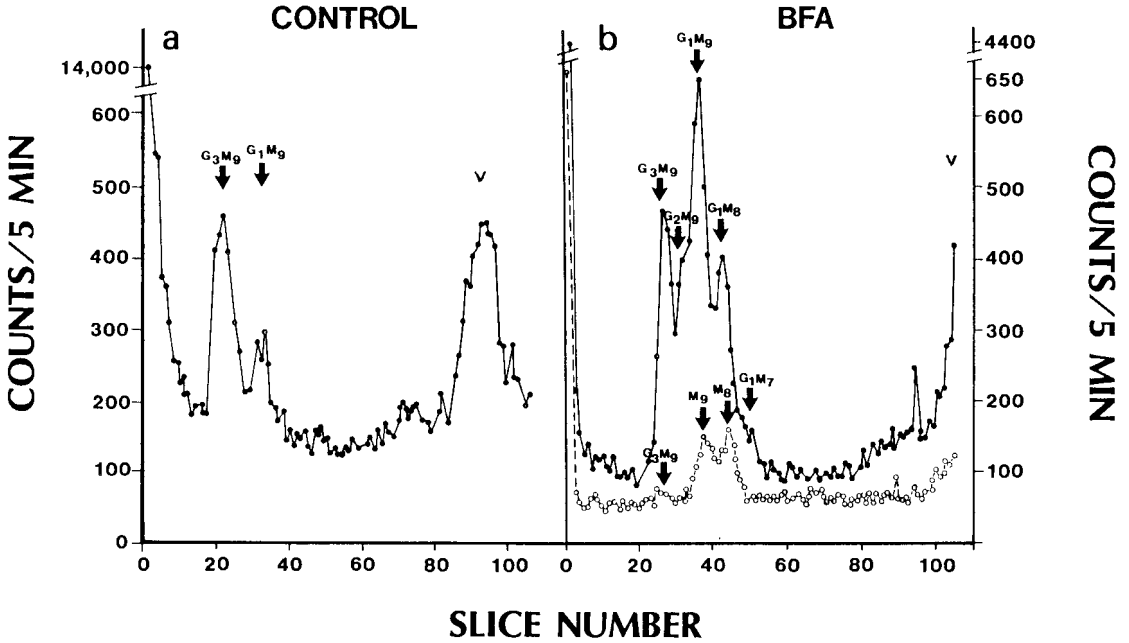

Figure 6. Paper chromatographic profiles of the [^{3}H]glucose-labeled high mannose oligosaccharides of cellular glycoproteins. Pituitaries from hypothyroid mice were incubated with [^{3}H]galactose for 2 hr in the absence (a) or presence (b) of 5 μg of BFA/ml. After immunoprecipitation of TSH as well as free α-subunits from tissue homogenates (both were undetectable), the total cell glycoproteins remaining were precipitated with TCA. The oligosaccharides present were released by endoglycosidase H and analyzed by paper chromatography (closed circles). The arrows mark the positions of migration of [^{14}C]mannose-labeled standards $Glc_3Man_9GlcNAc$, $Man_9GlcNAc$ and $Man_8GlcNAc$ (open circles, b). Other arrows pointing to peaks of radioactivity in closed circles show our identification of these species, relative to the positions of migration of the known [^{14}C]mannose-labeled standards. The V's mark the positions of migration of free [^{3}H]galactose.

labeled TSH and free α-subunits and partially inhibited the secretion of [^{35}S]sulfate- and [^{3}H]fucose-labeled TSH and free α-subunits. In some cases, species accumulated within cells with slightly lower than normal molecular size (21). We subsequently demonstrated that these smaller species had altered posttranslational processing of their high mannose units beginning as early as the RER (12). Cohorts of [^{3}H]mannose-labeled molecules, chase incubated in the presence of 5 μg of BFA/ml, demonstrated a relative increase in $Man_{5-8}GlcNAc_2$ units and an unexplained relative decrease in $Man_9GlcNAc_2$ units (12). An accumulation of $Man_{5-8}GlcNAc_2$ units within Golgi-enriched subcellular fractions was also noted and raised the possibility that BFA might also have a direct effect on Golgi. The goal of this study was to infer whether or not BFA was acting at multiple subcellular sites, even though its chief action was to inhibit RER to Golgi transport.

According to a general model of posttranslational processing, asparagine-linked oligosaccharide assembly begins in the RER with the en bloc transfer of a lipid-linked oligosaccharide, $Glc_3Man_9GlcNAc_2$, to an asparagine in a nascent polypeptide (11). Processing is then initiated, either cotranslationally or posttranslationally, via the enzymatic removal of the three glucose molecules; the first glucose by the enzyme glucosidase I and the next two by the enzyme glucosidase II, itself a glycoprotein with a half-life of 50 min (11). At least one mannose residue is trimmed from high mannose oligosaccharides within the RER by an ER (α1,2)-mannosidase (11,15). After additional residues are trimmed, other residues such as fucose, sialic acid, or sulfate are added to form complex oligosaccharides.

In this study, we assumed that [^{35}S]methionine and [^{3}H]mannose were incorporated into molecules in the RER and that most of [^{3}H]fucose, [^{3}H]sialic acid (*N*-[^{3}H]acetylmannosamine is a sialic acid precursor), and [^{35}S]sulfate were incorporated in the Golgi. [^{3}H]Galactose may be incorporated either as [^{3}H]galactose or [^{3}H]glucose and has been shown previously to be a good label of glucosylated early oligosaccharides which exist initially in the RER (16). Thus, as the incorporation of each radioactive precursor was assumed to be associated with a subcellular compartment, we hoped that incubating tissue with each precursor in the presence or absence of BFA would lend insight into whether or not BFA was disturbing events in that subcellular compartment. The effects of monensin, a Golgi-active drug, were examined for comparison.

First, we documented that neither 5 μg of BFA/ml nor 2 μM monensin substantially inhibited incorporation of [^{35}S]methionine or [^{3}H]mannose into glycoproteins, suggesting that protein synthesis and Asn glycosylation remained grossly intact in the presence of these dosages of the drugs. Second, we analyzed the structures of the high mannose oligosaccharides of glycoproteins and found that these structures differed in the presence of BFA or monensin as compared with the control. Moreover, each drug caused the accumulation of a somewhat different array of high mannose structures. BFA, to a greater extent than monensin, caused species with $Man_{5-8}GlcNAc_2$ to become more predominant and there were fewer $Man_9GlcNAc_2$ units in the presence of BFA. Additionally, by using [^{3}H]galactose labeling, it was clear that a secondary effect of BFA was to interfere with glucose trimming from early high mannose oligosaccharides. We interpret these [^{3}H]galactose and [^{3}H]mannose data as follows: (i)BFA causes a morphologic distortion of the RER (21) and thus interferes with the action of the glucosidases. Thus, $Glc_{3-1}Man_9GlcNAc_2$ units become more predominant on species due to less efficient glucose trimming. The action of glucosidase II may be affected because the enzyme itself is a short-lived glycoprotein (11) that may be aberrantly synthesized in the presence of BFA. (ii) Ongoing mannose trimming by ER (α1,2)-mannosidases, which may remove mannose residues prior to the removal of the last glucose (11), may explain the lesser predominance of $Man_9GlcNAc_2$ in the presence of BFA. (iii) $Man_{5-8}GlcNAc_2$ units accumulate on species as molecules are retained in the RER by BFA; we speculate that ER (α1,2)-mannosidases may trim units as far as $Man_5GlcNAc_2$ under these conditions (11). (iv) The impressive accumulation of $Man_5GlcNAc_2$ seen with BFA may also indicate a direct or indirect effect on the action of the Golgi enzyme GlcNAc transferase I, as is believed to occur in the presence of monensin (25). Such a Golgi-localized action of BFA is reasonable in view of the other incorporation data to be discussed below.

We found that although BFA and monensin did not markedly impair incorporation of [^{35}S]methionine or [^{3}H]mannose, both BFA and monensin did impair incorporation of [^{3}H]fucose, *N*-[^{3}H]acetylmannosamine, and [^{35}S]sulfate. We interpret these data as indicative of direct Golgi actions of these inhibitors. Alternatively, Golgi-localized processing could decline in the presence of BFA because fewer substrates were arriving in the Golgi from the RER, but we presently discount this possibility because our prior subcellular fractionation studies (12) appeared to show a substantial leak of substrate from RER to Golgi at this dose of BFA, as well as altered oligosaccharide structures of glycoproteins within Golgi-enriched fractions. It is unclear at present whether the different degrees of inhibition of the incorporation of these precursors by TSH, free α-subunits, and total glycoproteins are physiologically meaningful, although Stannard *et al.* (26) have postulated that TSH and free α-subunits may follow somewhat different intracellular secretory pathways.

We have previously noted differential rates of processing at the three glycosylation sites of TSH (23) and an increased susceptibility to *N*-glycanase at Asn 82 (24). We studied the tryptic glycopeptides of TSH to

exclude the possibility that BFA might inhibit glycosylation at one of the Asn sites and thereby result in the smaller weight TSH subunits that have been observed (21). After tissue incubation with BFA, however, we noted that TSH was glycosylated at each of the three Asn sites.

The impaired secretion of labeled glycoproteins from cells chased in the presence of BFA may be due to another inhibitory effect of BFA at the cell surface, but little data are currently available on this point. In this report, in addition to its known major inhibitory action on RER to Golgi transport, we have demonstrated additional effects of BFA on the early processing of glucose-containing high mannose oligosaccharides and on intra-Golgi processing. Although BFA will remain a useful drug to inhibit RER to Golgi intracellular transport, our recent studies indicate that BFA may have actions at multiple sites within cells.

Note Added in Proof. In a recent paper (27) evidence is presented that BFA directly affects the Golgi apparatus in rat hepatocytes, in agreement with our data concerning BFA perturbation of Golgi function.

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