THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 267, No. 7, Issue of March 5, pp. 4440-4455, 1992
Printed in U.S.A.

The Spectrum of Incomplete *N*-Linked Oligosaccharides Synthesized by Endothelial Cells in the Presence of Brefeldin A*

(Received for publication, September 18, 1991)

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Previous studies in many cell lines have shown that Brefeldin A (BFA) inhibits the forward movement of newly synthesized glycoconjugates by fusing the *cis*-, *medial*-, and *trans*-Golgi compartments with the rough endoplasmic reticulum. Studies on the oligosaccharide processing of individual glycoproteins have yielded confusing and incomplete results regarding the location of the block. Assuming that all glycoproteins with *N*-linked oligosaccharides follow the same endoplasmic reticulum to the Golgi pathway, a more complete picture on the location and nature of the block can be determined by analyzing *N*-linked oligosaccharides synthesized in the presence of BFA. In bovine pulmonary artery endothelial cells, BFA (0.1 μg/ml) reversibly inhibits the secretion of >95% of Tran^{35}S and [^{3}H]Man-labeled glycoproteins without affecting protein synthesis or *N*-linked glycosylation. In addition, BFA inhibits the synthesis and secretion of $^{35}SO_4$-labeled oligosaccharides. Initial oligosaccharide trimming is uninhibited, but further processing is affected since the majority (65%) of the chains terminate only in β-GlcNAc residues. Concomitantly, the proportion of [^{3}H]Man-labeled *N*-linked anionic oligosaccharides is reduced from 60 to 20%, and the great majority of the charge is due to one sialic acid. The rate-limiting step for sialylation appears to be the branch selective addition of β-Gal residues. The remaining charge is due to sulfate esters (0.6%) which normally account for >10% of the anionic substituents. BFA also reduces the amount of phosphorylated chains by 80% and greatly diminishes further phosphodiester processing since the majority of these oligosaccharides (60%) contain a Man-6-PO_4 residue in an acid-sensitive diester linkage. The addition of all polylactosamine chains, outer-branch fucose and terminal α-Gal residues are completely inhibited by BFA. Secretion, fucosylation, and sialylation are completely restored when BFA is removed, but the other modification steps are only partially restored. Our results indicate that addition of sulfate esters, terminal α-Gal residues, polylactosamine chains, outer-branch fucose residues, some initial phosphorylation, and most phosphodiester processing may occur beyond a compartment where some β-Gal and sialic acid residues can be added. Essentially, all of the effects on oligosaccharide processing are partially or completely reversible.

N-Linked oligosaccharides are initially added to proteins in the lumen of the endoplasmic reticulum, but much of the subsequent maturation of the hundreds of known structures occurs within the Golgi apparatus (1). Here, an interdependent series of sequential and parallel trimming and synthetic steps are carried out by specific glycosidases and glycosyltransferases, using sugar nucleotides that are concentrated by specific transporters (1, 2). From there, proteins containing the mature oligosaccharides are dispatched to the secretory pathway, to membranes, or into specific subcellular compartments (3–7).

A large body of cytological, immunochemical, and biochemical data suggest that the enzymes involved in oligosaccharide processing reside in ordered subcompartments of the Golgi (6–9). Initial steps are believed to occur in the *cis*-Golgi, intermediate steps in the *medial*-Golgi, and later steps in the *trans*-Golgi (6). Depending upon the cell type, the *trans*-Golgi or a group of tubules/vesicles called by various names including the *trans*-Golgi network (TGN)[1] or *trans*-Golgi reticulum, may add the finishing touches to the oligosaccharide (3, 10, 11). Although the apparent stacking order of the synthetic enzymes fits neatly into the known order of sugar chain processing, a morphologically defined Golgi apparatus is not required for normal processing to occur (12, 13). However, for a particular glycosylation reaction to take place, the simultaneous presence of the glycosyltransferase, an appropriate acceptor, and the corresponding sugar nucleotide transporter in the same Golgi stack is required (2, 14).

Brefeldin A (BFA) inhibits the secretion of all proteins using the ER-Golgi pathway (15–21). At the structural level, BFA appears to fuse the endoplasmic reticulum and the *cis*-, *medial*-, and *trans*-Golgi compartments, but not the *trans*-Golgi network (21–26). BFA action appears to involve interference with reversible binding of a soluble protein called βCOP110 to the cytoplasmic side of the Golgi. This halts the forward movement of itinerant proteins by vesicular transport (27–29). Many studies have used BFA to study the secretion and routing of [^{35}S]Met-labeled polypeptide chains (16, 18, 19, 21, 24, 30–38). Indirect approaches such as lectin binding, changes in isoelectric point, size, and endo-β-*N*-acetylglucosaminidase H sensitivity of the peptide were used to infer effects on oligosaccharide processing and to correlate this with the Golgi subcompartments affected by BFA. This is a useful approach, but it has yielded some confusing results about sugar chain processing. For example, two studies con-

* This research was supported by United States Public Health Service Grant RO1-CA38701. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TGN, *trans*-Golgi network; BFA, Brefeldin A; CPAE, bovine pulmonary artery endothelial cells; SLIM, sulfate label incorporation medium; ConA, concanavalin A; α-MeMan, α-methylmannoside; α-MeGlc, α-methylglucoside; SDS, sodium dodecyl sulfate; Fuc, fucose; ER, endoplasmic reticulum; HPLC, high performance liquid chromagraphy; PNGaseF, peptide *N*-glycosidase F.

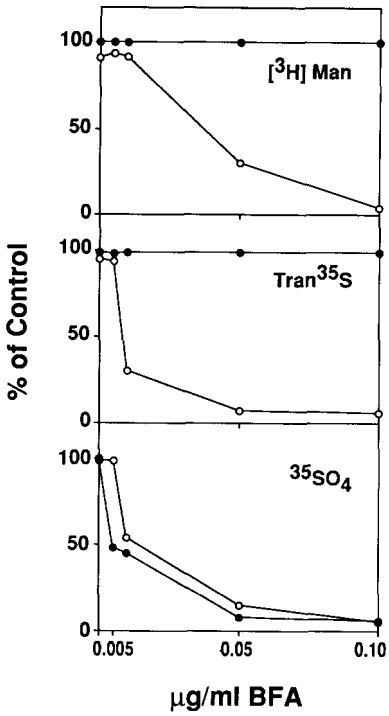


FIG. 1. **Effects of BFA on the synthesis and secretion of macromolecules.** CPAE cells were preincubated for 30 min in the presence of 0.005–0.1 µg/ml of BFA. [2-^{3}H]Man (*upper panel*), Tran^{35}S-label (*center panel*), or $^{35}SO_4$ (*lower panel*) was added for 30 min and the label chased for 3 h as described under "Experimental Procedures." The relative amount of label incorporated into cell-associated macromolecules or those secreted into the medium was determined by precipitation with trichloroacetic acid/phosphotungstic acid. All values were normalized to protein content and expressed as a percentage of control with no added BFA. Cellular control values were: 57,130 cpm [^{3}H]Man, 32,571 cpm Tran^{35}S-label, and 5,170 cpm $^{35}SO_4$. The media contained 55–65% of each label. ○, secreted; ●, cell-associated.

cluded that there was complete inhibition of sialylation (21, 25), while others showed variable decreases (31, 33, 36). A more direct way to study oligosaccharide processing in BFA is to metabolically label the oligosaccharide chain itself. This has been done only for a few specific proteins (30, 32), but analysis was limited by the amounts of labeled material obtained from single polypeptides. Since *all* proteins with *N*-linked oligosaccharides must pass through the Golgi, we reasoned that analysis of the total cellular *N*-linked oligosaccharides offers an opportunity to simultaneously assess the effects on many different processing steps for many proteins. By using [2-^{3}H]Man to metabolically label the core of *N*-linked oligosaccharides, it is possible to generate a synchronous wave of newly synthesized glycoproteins with most of the label located in the core of the *N*-linked oligosaccharides, proximal to the block imposed by BFA.

In previous work, we characterized an array of sulfated/sialylated oligosaccharides from an endothelial cell line (CPAE) (39). Since this cell line also synthesizes phosphorylated high mannose chains and secretes a large portion of its glycoproteins, it provides an ideal system to examine the effects of BFA on the processing of many different structures. Here, we report a distinct hierarchy of BFA effects on *N*-linked sugar chain processing; some early steps are unaffected, but others are completely blocked, while still others appear to "straddle" the block. Surprisingly, we found that rate-limiting β-Gal addition is tightly coupled to sialylation and is only partially affected by BFA, whereas phosphorylation and sub-

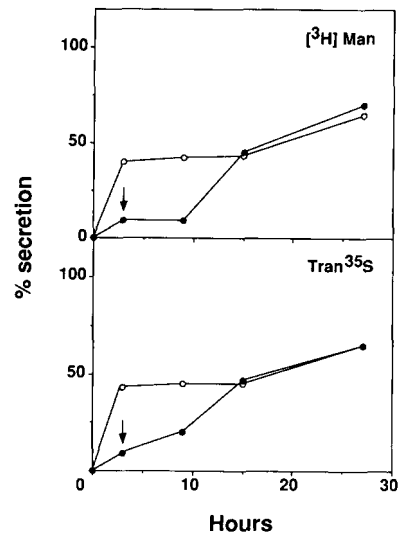


FIG. 2. **The effects of BFA on protein secretion are reversible.** Cells were incubated in the presence or absence of 1 µg/ml of BFA for 30 min as in Fig. 1 and then labeled for 30 min with [^{3}H] Man (*upper panel*) or Tran^{35}S-label (*lower panel*) in the continuous presence of BFA. The cells were chased in the presence or absence of BFA for an additional 3 h, at which time the BFA was removed (*arrow*). Samples were taken at the indicated times, and the amount of label in the cells or secreted into the media was determined by trichloroacetic acid/phosphotungstic acid precipitation. All values were normalized and expressed as indicated in the legend to Fig. 1. ○, control; ●, BFA.

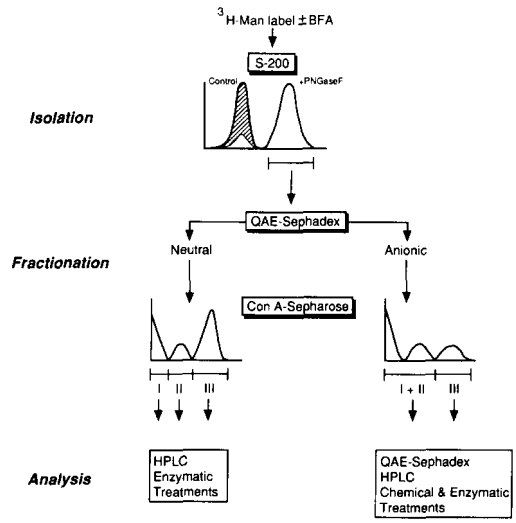


FIG. 3. **Flow diagram for the fractionation and analysis of [^{3}H]Man-labeled *N*-linked oligosaccharides.** CPAE cells were labeled in the presence or absence of BFA, the cell lysates denatured in boiling SDS, and the ^{3}H-labeled macromolecules were isolated from the void volume region of a Sephacryl S-200 column. The pool was acetone-precipitated, redissolved, and digested with PNGaseF as described under "Experimental Procedures." The released oligosaccharides (92% of total cpm in each case) were pooled, desalted, and separated into neutral and anionic fractions on QAE-Sephadex. These were desalted and fractionated by lectin affinity chromatography on concanavalin A-Sepharose into three fractions: *I*, buffer alone; *II*, buffer with 10 mM α-MeGlc; and *III*, buffer with 100 mM α-MeMan). Each neutral ConA fraction and the anionic ConA III fraction was analyzed separately whereas anionic ConA I and ConA II were combined prior to analysis. Each of the fractions was subjected to a battery of chemical treatments and enzymatic digestions followed by analysis by QAE-Sephadex and HPLC to determine the residual charge, size, and sequence.

TABLE I

Distribution of peptide:N-glycosidase F-released oligosaccharides synthesized in the presence and absence of BFA and following recovery from BFA

Samples labeled with [^{3}H]Man were digested with PNGaseF, and the released oligosaccharides fractionated as described under "Experimental Procedures," and as shown in Fig. 3. Samples in the initial BFA experiments are only from cells, while the recovery experiment analyzed both cells and media.

Fraction	Initial BFA experiment			Recovery experiment	
	Control	0.1 μg/ml BFA	1.0 μg/ml	Control	1.0 μg/ml
	% of peptide:N-glycosidase F-released [^{3}H]				
Neutral	40.0	80.0	86.0	51.0	49.0
ConA I	17.0	30.0	32.0	20.0	19.0
ConA II	4.0	18.0	20.0	5.0	5.0
ConA III	19.0	32.0	34.0	26.0	25.0
Anionic	60.0	20.0	14.0	49.0	51.0
ConA I	48.0	14.0	10.0	39.0	40.8
ConA II	6.0	2.0	1.5	5.0	5.0
ConA III	6.0	4.0	2.5	5.0	5.0
ConA I+II negative charge					
1	22.0	12.0	9.0	20.0	29.0
2	23.0	3.0	1.5	16.0	12.0
3	5.0	1.0	1.0	5.0	3.0
4	2.0	0.0	0.0	2.0	1.0
>4	2.0	0.0	0.0	1.0	1.0

sequent processing of oligosaccharides on lysosomal enzymes is greatly reduced.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Effects of BFA on Incorporation and Secretion of Labeled Precursors—CPAE cells were pulse-labeled with Tran^{35}S or [^{3}H]Man for 30 min in the presence of various concentrations of BFA and then chased for 3 h under the same conditions in the absence of label. Cell-associated macromolecules or those secreted into the chase medium were precipitated and the results are shown in Fig. 1. Incorporation of both labels was unaffected by BFA, but secretion of both was reduced by >95% with 0.1 μg/ml of BFA. In contrast, in cells labeled with $^{35}SO_4$ for 30 min and chased for 3 h, the overall incorporation of this label was reduced by >95% and secretion was < 1% of control. The effects were the same when the BFA concentration was raised to 1 μg/ml (not shown). This result is consistent with the notion that sulfation is a late Golgi processing event and that BFA prevents the movement of appropriate sulfate acceptors into such a compartment. Normal secretion of previously labeled molecules (Tran^{35}S-label or [^{3}H]Man) resumes within 12 h when BFA is removed (Fig. 2). The time required to complete secretion of these arrested molecules is longer than the time usually required to restore a morphologically normal Golgi after BFA removal (21, 23).

Labeling, Release, and Fractionation of N-Linked Oligosaccharides—[2-^{3}H]Man preferentially labels the core of *N*-linked oligosaccharides because metabolic conversion to molecules other than [^{3}H]Fuc yields [^{3}H]$_2$O (14, 40, 41). We therefore pulse-labeled cells with [2-^{3}H]Man to maximize incorporation at a single point that was unaffected by BFA. Further processing of these core-labeled molecules during the chase should involve steps that occur proximal to the BFA block in the "Brefeldin compartment" created by the ER-Golgi fusion. The flow chart in Fig. 3 shows how the labeled material was fractionated and analyzed. Following the chase, the *N*-linked oligosaccharides were released by PNGaseF digestion and separated from the unreleased material by gel filtration. The digestion released >90% of the total label from both control cells and those labeled in the presence of 0.1 or 1.0 μg of BFA (Fig. 4). Control incubations without PNGaseF showed only 0.8% of the radioactivity in the region pooled for analysis. The majority of released radioactivity was incorporated into [^{3}H]Man (70% for both samples) and the remainder into [^{3}H]Fuc as determined by thin layer chromatography analysis of strong acid hydrolysates (data not shown). After desalting, neutral and anionic chains were batch-separated on QAE-Sephadex. As shown in Table I, the proportion of anionic oligosaccharides was decreased from 60% in control samples to 20% in BFA samples. This decrease was not surprising since sulfation and sialylation are considered to be late Golgi events, and previous studies predicted a decrease in sialylation (25, 31, 33, 36) and sulfation (30) of *N*-linked oligosaccharides with BFA.

The neutral and anionic molecules were desalted again and each was fractionated on concanavalin A-Sepharose into three separate pools (42, 43). Fraction I did not bind to the column and is expected to contain tri- and tetraantennary chains and possibly bisected chains. Fraction II is eluted by 10 mM α-methylglucoside and should consist of biantennary chains and certain partially processed high mannose chains or hybrid molecules. Fraction III is eluted by 100 mM α-methyl mannoside and should consist of the remaining partially processed high mannose-type chains or hybrid molecules. BFA treatment caused some alteration in the relative distribution into the three ConA fractions (Table I), indicating that there were some changes in the degree of processing or branching. For the remainder of the study, anionic Fractions I and II were combined and analyzed together. Anionic and neutral chains from Fraction III were used to study phosphorylated oligosaccharides and neutral high mannose-type oligosaccharides, respectively.

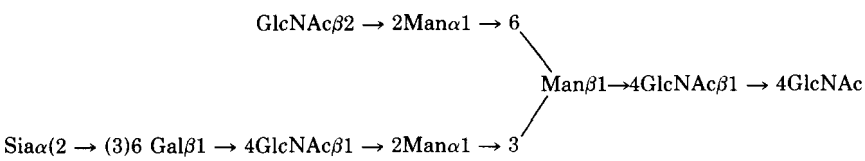
Analysis of the Spectrum of Oligosaccharides Synthesized in the Presence of BFA—We used a combination of chemical and enzymatic treatments to analyze each of the individual fractions shown in Fig. 3. As a guide to the use of these procedures, the reader is referred to Fig. 5, which depicts two generic composite *N*-linked oligosaccharides that have all of the potential structures discussed in this study. With a few exceptions, we were able to characterize most of the structures in all of the fractions. To simplify the presentation of results, relevant data from the various fractions will be grouped and summarized here for each set of processing reactions. Much of the actual supporting data is presented individually, by fraction, in the Miniprint, and Table IV gives the relative amounts of each type of structure found.

α-Glucose Processing Is Unaffected and α-Man Processing Is Increased—Final removal of the 3 α-glucose residues from the starting $Glc_3Man_9GlcNAc_2$ oligosaccharide must proceed normally because no residual glucose units were found. The proportion of high mannose-type chains is unchanged by BFA (Table I). However, their average size, (Man 6.6) is smaller than in untreated cells (average size Man 7.6) (Fig. 14, *panels A* and *E*, and Table IV). This is probably due to the prolonged residence time in the Brefeldin compartment where they are continually exposed to Golgi α-mannosidase I.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," and Figs. 4, 6, 9, 11–16) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

Initiation of Multiantennary Chains Is Normal, but the Extent of Branching Is Decreased—Multiantennary chains arise by the action of Golgi mannosidase I and II and a series of GlcNAc transferases (1, 44). Depending upon the individual glycosylation site, hybrid (monoantennary), bisected, bi-, tri-, and tetraantennary chains can be found. The overall proportion of complex-type chains is unaffected by BFA (Table I), showing that prolonged exposure to the Golgi processing mannosidases does not change the extent of Man processing at a given glycosylation site. However, the proportion of complex biantennary chains is increased from 2 to 20% with BFA, and a corresponding loss of tri- and tetraantennary chains occurred. This indicates that access to the later acting GlcNAc transferases (III, IV, or V) is partly affected by BFA.

Addition of β-Galactose and Sialic Acid Is Coupled but Decreased—In the absence of BFA, oligosaccharides are converted to a broad spectrum of different structures as described below. However, in the presence of BFA, there is a dramatic decrease in the number of oligosaccharides with β-Gal residues (35% of control). This results in oligosaccharides with many terminal GlcNAc-β-Man sequences which are hardly ever found in normal mature mammalian oligosaccharides. Since β-Gal residues are required for any further chain extension, the addition of these residues appears to be rate limiting in the presence of BFA and processing is halted at this point. Based on the identification of the structure shown below as a major sialylated oligosaccharide, the β1→4-galactosyltransferase appears to preferentially add β-Gal residues to the α1→3 Man branch of biantennary chains.



This conclusion is partly based on the fact that the above structure elutes exclusively in anionic ConA Fraction III (Table I and Fig. 7) and on the HPLC run shown in Fig. 8. If the single β-gal residue is instead added to the α1→6 Man branch, that molecule should elute in ConA Fraction II(42). This preference is also in keeping with prior knowledge concerning the branch specificity of this enzyme (45). The second β-Gal residue can then be added to the other branch on the α1→6 arm, but a third β-Gal residue is rarely added to the more highly branched chains (Figs. 11 and 13). However, terminal β-Gal residues are very uncommon in the neutral fractions and are almost invariably (>90%), capped by α2→6- or α2→3-linked sialic acids (Fig. 10) (see Miniprint for details). We interpret this to mean as soon as β-Gal is added, it is immediately sialylated. This suggests that the two transferases are in close functional association and that the addition of β-Gal is rate-limiting. When BFA is removed and the cells are allowed to recover for 12 h, the previously arrested chains are fully sialylated (see Table I).

Addition of Terminal α-Gal Residues Is Greatly Diminished—Terminal β-Gal residues are rare for oligosaccharides made in control cells. Almost all the anionic chains contain sialic acid residues, and nearly all the complex-type neutral and anionic chains are terminated by one or more α-Gal residues. Some of the neutral chains normally have as many as 4 α-Gal residues (Fig. 11*C*) based on their size and resistance to β-galactosidase digestion (Fig. 11*D*). Although it is thought that sialylation and α-galactosylation are competing reactions, we found a complete loss of α-Gal residues in the presence of BFA. After recovery from BFA, only about 50% of the normal α-Gal residues are added to the arrested chains, leaving a considerable portion remaining with terminal β-Gal residues (see Fig. 15). Interestingly, the proportion of sialylated residues is not increased after recovery, suggesting that α-galactosylation and sialylation reactions are still not competitive after removal of BFA.

Formation of Polylactosamine Chains Is Also Markedly Diminished—About 10% of the oligosaccharides made in the absence of BFA contained polylactosamine chains that were terminated by either α-Gal (~3%) or sialic acid residues (~7%). We did not determine the precise number of repeats nor which antennae carried them. However, none of these chains were found on oligosaccharides made in the presence of BFA, and a portion was subsequently restored when the BFA was removed (5%). However, only sialylated oligosaccharides regained polylactosamines. Since the key event in the formation of these chains is the action of Galβ1→4GlcNAc:GlcNAcβ1→3GlcNAc transferase (44, 46–48), the results suggest that this enzyme is also located beyond the BFA block. However, these findings could also be due to a low level of β-galactosyltransferase activity, which is also required for polylactosamine chain elongation.

Sulfation of Oligosaccharides—CPAE cells normally synthesize a considerable amount of sulfated/sialylated oligosaccharides (39). The chains represent 10–15% of the total label incorporated into CPAE cells from inorganic $^{35}SO_4$, and the rest of the label is mostly in the glycosaminoglycan chains of typical proteoglycans (49). BFA reduces the synthesis of all $^{35}SO_4$-labeled molecules by >95% (Fig. 1). Incorporation of the residual label may occur because a small amount of acceptors for sulfation are still in transit from the cells but are beyond the BFA block. The label incorporated was studied by PNGaseF release and QAE-Sephadex analysis. BFA inhibition was not selective for any class of these sulfated molecules (data not shown). A corresponding marked decrease is seen in the amount of [^{3}H]Man-labeled sulfated *N*-linked oligosaccharides (decreased from 10 to 0.6%). About half of this sulfate is normally found as GlcNAc-6-SO_4 located on an outer branch of the oligosaccharide (39). This was shown by its sensitivity to human β-hexosaminidase A, which will cleave this residue when it is terminal, and the digestion is done at pH 3.5 (39, 50). When BFA is removed, about half (44%) of the expected amount of sulfated/sialylated chains return. They have a normal distribution of 1, 2, and >2 negative charges. The proportion of outer branch GlcNAc-6-SO_4 (~50%) is the same as in controls that were never blocked by BFA. The precise location of the remainder of the sulfate esters is not known in the control or recovered samples.

Phosphorylated Oligosaccharides—Most lysosomal enzymes contain Man-6-P and are targeted to the lysosome by binding to phosphomannosyl receptors (5, 51). While most of the receptor is found in the endosomes and in the *trans*-Golgi network (5, 7, 51–53), some phosphorylation of the oligosaccharides occurs as an early Golgi or pre-Golgi event (4, 54, 55). Structural studies indicated the first GlcNAc-1-P is trans-

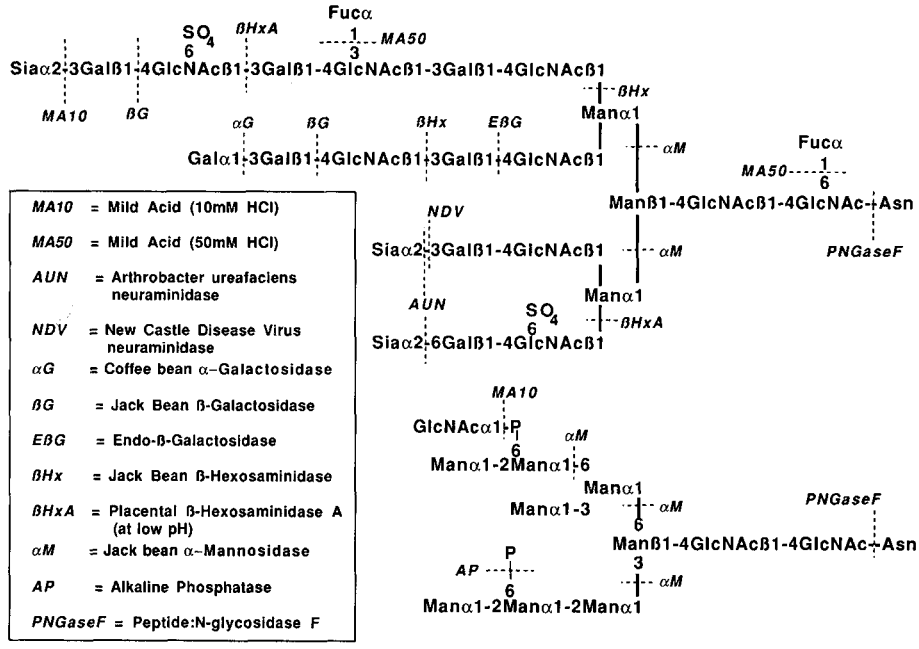


FIG. 5. **Composite structures of *N*-linked oligosaccharides from CPAE cells showing the typical sites of action of various chemical and enzymatic treatments.** The abbreviations are used throughout the figures. Note that all possible sites of action of each treatment are not shown, and that the branch specificity of the various types of antennae is not specified for the complex-type oligosaccharide.

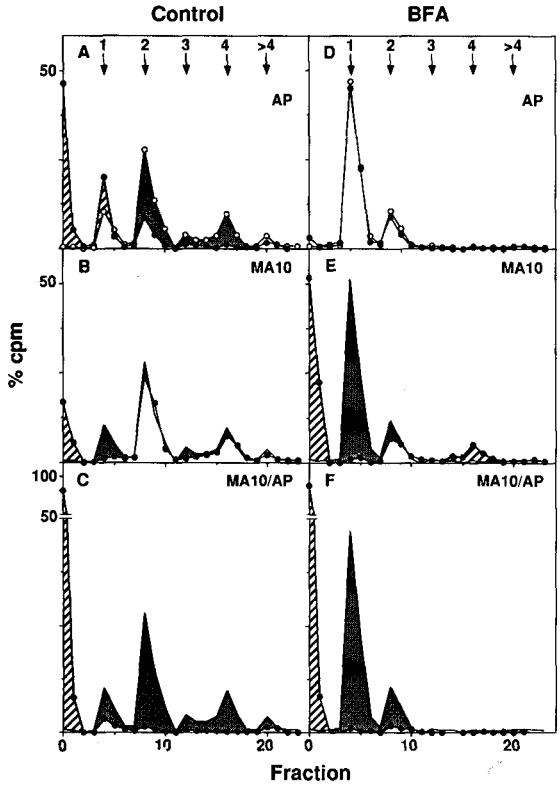


FIG. 7. **QAE-Sephadex analysis of ConA III anionic oligosaccharides.** Anionic oligosaccharides isolated from the ConA III fractions were analyzed on QAE-Sephadex without any treatment or with the treatments indicated for each panel. In each case, a decrease in peak area compared to the control is *shaded*, and an increase in area is shown in *stripes*. All figures are presented as percent of the recovered radioactivity. Controls are shown on the *left* (*panels A–C*) and BFA samples on the *right* (*panels D–F*). —— or O——O, no treatment; ●——●, treated.

ferred exclusively to the 6-position of one of the Man residues found on the arm of a high mannose oligosaccharide linked α1→6 to the β-linked Man (56, 57). This reaction is presumed to be a late ER or early *cis*-Golgi event (4, 54, 55). A second GlcNAc-1-P is then added preferentially to the α1→3-linked branch in a later compartment (54, 55). Subsequently, a specific phosphodiester glycosidase excises the GlcNAc residues creating the monoester forms, which have a high affinity for the receptor (5).

In control cells, we found that phosphorylated high mannose oligosaccharides (presumably from lysosomal enzymes) carried mainly one or two phosphomonoesters/chain. In the presence of BFA, the overall amount of phosphorylation decreases by almost 5-fold, and most of the chains have only one phosphate in a diester-linkage (Table II and Fig. 7). The addition of the second phosphate and conversion of the diesters to the monoesters are even further inhibited by BFA. These results suggest that some phosphorylation and nearly all conversion to the monoester are abolished, indicating that the phosphodiester glycosidase may not be a *cis*-Golgi enzyme as previously thought. Individually, each of these effects is less dramatic than those seen for the addition of sulfate and α-Gal residues and polylactosamine chains but are greater than the effects on sialylation. When BFA is removed, about half of the phosphorylation in the cellular oligosaccharides is restored on the previously labeled chains. Moreover, the ratio of chains with one and two phosphates returns to normal, and a substantial fraction occurs as phosphomonoesters. The failure to recover all phosphorylation is not due to enhanced secretion of phosphorylated lysosomal enzymes during the block (data not shown).

Core Fucosylation Is Increased, but Addition of Outer Fucose Residues Linked to GlcNAc Is Decreased—In the absence of BFA, ^{3}H-labeled or non-labeled Fuc may be added to GlcNAc residues both in the chitobiose core and on the outer lactosamine branches. BFA increased the percentage of label that bound to *Pisum sativum*-agarose (pea lectin) from 10 to 33%, indicating an increase in molecules with core Fuc residues (data not shown). On the other hand, it reduced the amount of [^{3}H]Fuc in oligosaccharides that did not bind to pea lectin from 25 to 2% (analyzed by thin layer chromatography, data not shown). This label should be in outer branch Fuc residues. This effect on outer Fuc residues is corroborated by sequential exoglycosidase digestion studies. Thus, core fucosylation appears to be increased, while outer branch fucosylation is greatly reduced in the presence of BFA. When BFA is re-

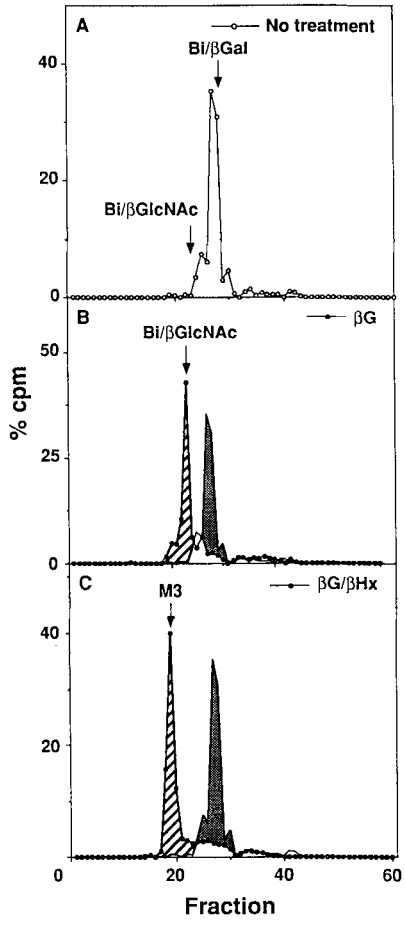


FIG. 8. **HPLC analysis of ConA III anionic molecules from BFA samples following desialylation by mild-acid hydrolysis.** The oligosaccharides neutralized by mild-acid treatment of BFA anionic ConA fraction III (Fig. 7, *panel E, striped area* at extreme left) were reduced and analyzed by HPLC as described under "Experimental Procedures." *Panel A* shows the size without any treatment. *Panels B* and *C* show the control again together with the indicated digestion. *Shaded areas* show the loss of area from the control and *striped areas* show the increase over control regions for each treatment. Abbreviations for the indicated digestions are presented in Fig. 5. The elution position of the standards is indicated (see Table III for full names). The results are shown as percentage of the total radioactivity.

moved, outer branch fucosylation of the arrested chains is fully restored, surprisingly as [^{3}H]Fuc that does not bind to pea lectin. Also, during recovery the percent of pea lectin binding is lowered to 10% as in the control, indicating that the increase in core fucosylation with BFA may be reversible (data not shown).

Effects of Reversal of BFA—The effects of removal of BFA have been mentioned above in each section. In essence, all of the effects on oligosaccharide processing, except for excessive Man trimming, are substantially reversed after BFA is removed (ranging from 43 to 100%, for different steps, see Tables I and II and Fig. 17). This shows that processing is only temporarily arrested and that the majority of the incomplete chains are stable in the BFA compartment. These analyses were done on oligosaccharides isolated from total cells and medium 12 h after removal of BFA. This period was selected because it is the earliest time when full secretion of proteins and glycoproteins returns when cells are incubated with 0.1 or 1.0 μg/ml BFA. We assume that intracellular processing of oligosaccharides is probably also maximally restored by then.

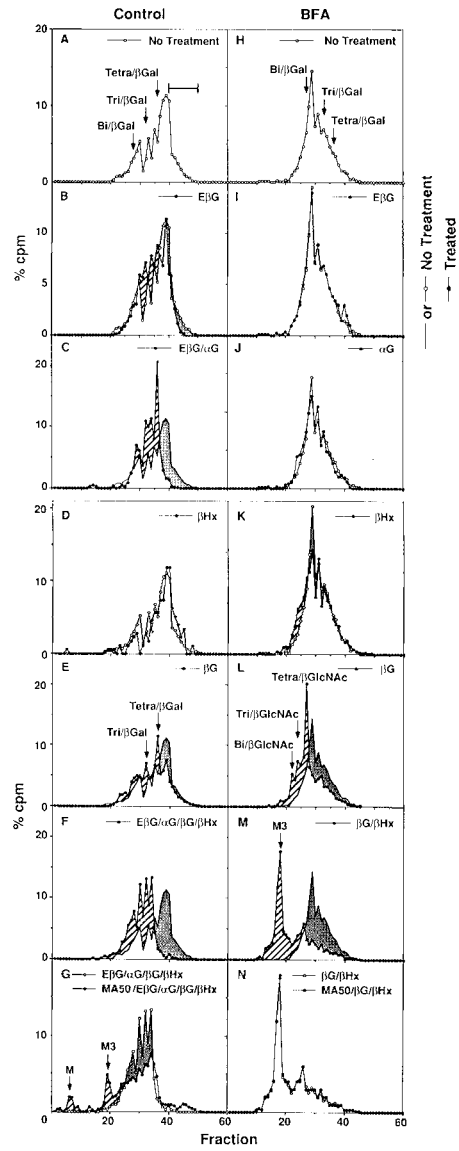


FIG. 10. **HPLC analysis of desialylated oligosaccharides from ConA I+II.** Oligosaccharides were desalted, reduced, and analyzed by HPLC as described with the indicated digestions. Controls are on the *left* (*panels A-G*) and BFA samples are shown on the *right* (*panels H-N*). The elution position of the standards is indicated (see Table III for full names). The *bracket* (|——|) on the right side of *panel A* indicates the positions of standard glycopeptides from the hLAMP protein containing a variable number of polylactosamine repeating units. In the case of *panels G* and *N*, an additional acid treatment (0.05 N HCl for 30 min at 100 °C) was used to remove potential Fuc residues prior to the digestions. The results presented in these panels are shaded to show the differences caused by the acid treatment. Similar acid treatment of BFA material (*panel N*) shows no effect. —— or O——O, no treatment; ●——●, treated.

DISCUSSION

A proposed mechanism of BFA in blocking protein secretion has been recently described. BFA prevents the reversible association of a cytoplasmic protein, βCOP110, with elements of the Golgi, and this causes a fusion of several normally distinct Golgi stacks via tubular connections (23, 26, 28, 29). Morphologically, some Golgi elements, such as the *trans*-Golgi network, appear to be excluded from this rearrangement. Previous studies using BFA have examined its effects on individual proteins, mostly using indirect indicators of oligosaccharide processing such as acquisition of endo-β-*N*-acetylglucosaminidase H resistance, change in pI, and alterations

in lectin binding (16, 18, 19, 21, 25, 31, 33–38). These results were used to infer which processing steps were affected by BFA. This type of approach has some limitations in that the structural interpretations are indirect and because single proteins may not be representative. On the other hand, when labeled oligosaccharides from individual proteins were directly studied detailed structural studies were not possible because of limited amounts of material. When considered together, these studies lead to different conclusions about the distribution of key synthetic enzymes such as sialyltransferases and suggest that enzyme distribution may be less restricted than previously predicted. In this study, we approached the question from a broader-based viewpoint and asked how BFA quantitatively affects total *N*-linked oligosaccharide processing and what this might reveal about the distribution of the relevant enzymes. Assuming that all proteins with *N*-linked oligosaccharides must traverse the Golgi, the entire spectrum of structures can be studied and the results should be applicable to the vast majority of glycoproteins. In interpreting the results of this study, we made several assumptions that we believe are reasonable. First, that the sequence and the structural requirements of the acceptors for each step of *N*-linked oligosaccharide processing are the same as described in many other systems (1). Second, that BFA does not directly inhibit any of the known enzymes in the processing pathway. Third, that the addition of sugar units in a particular compartment requires the simultaneous presence of a transferase, an acceptor, and a transporter to provide the sugar nucleotide (2, 14).

To determine the effects of BFA on oligosaccharide maturation, the biosynthetic label must be incorporated at a single step that is unaffected by BFA. [2-^{3}H]Man closely approximates these requirements. It is not converted into other sugars except Fuc, the majority is incorporated only at the time of protein synthesis, and incorporation is not affected by BFA (Fig. 1). The oligosaccharides labeled with [2-^{3}H]Man in the presence of BFA were chased for 3 h to allow the action of enzymes that are in the Brefeldin compartment. As summarized in Table IV and Fig. 17, BFA has little effect on the early steps of oligosaccharide processing, including trimming of Glc and Man residues and the addition of the first 2 GlcNAc residues. The extent of further branching of *N*-linked oligosaccharides was slightly decreased (35%), suggesting that the GlcNAc transferases III, IV, or V may functionally straddle the block. The addition of β-Gal is even further decreased by 65%. Since subsequent elongation of complex chains requires β-Gal residues, this appears to be the rate-limiting step in further processing. Prior *in vitro* studies using purified β-galactosyltransferase and defined acceptors showed a strong preference for the GlcNAc residues found on the α1→3-linked branch of biantennary chains (45). In fact, this structure was identified as the major product in the anionic ConA III fraction from BFA samples and accounts for 4% of the total chains. The failure to add more β-Gal residues may simply be due to branch specificity and preference under conditions of limited amounts of β-galactosyltransferase or UDP-Gal. A less likely possibility is that these residues are added only by newly synthesized β-galactosyltransferase that is trapped in the BFA compartment during the 3-h chase period.

Virtually all of the β-Gal residues added in the presence of BFA are sialylated, suggesting that β-galactosyltransferase and sialyltransferases must be closely coupled. The decrease

TABLE II

Analysis of ConA III anionic species

This fraction was isolated as described under "Experimental Procedures" and as shown in Fig. 3.

	Initial BFA experiment		Recovery experiment	
	Control	BFA	Control	BFA recovered
% of total oligosaccharides	6.00	4.0	5.0	5.0
% of total oligosaccharides with				
Sialic acid[a]	1.38	3.0	1.2	3.5
Phosphate[b]	4.62	1.0	3.8	1.5
Diesters[c]				
I	0.39 (8)[d]	0.60 (60)	0.30 (8)	0.60 (45)
II	0.39 (8)	0.24 (24)	0.30 (8)	0.30 (15)
Monoesters[e]				
I	2.88 (63)	0.08 (8)	2.40 (66)	0.40 (30)
II	0.96 (21)	0.08 (8)	0.80 (22)	0.20 (15)

[a] Defined as material neutralized (not bound to QAE-Sephadex) by mild-acid treatment (MA10).

[b] Defined as the percentage which can be neutralized by alkaline phosphatase alone or a combination of MA10 and alkaline phosphatase.

[c] Resistant to alkaline phosphatase unless treated by MA10. Assumed to be GlcNAc-1-P-6-Man.

[d] Numbers in parentheses indicate that amount of each fraction as a percent of the total phosphorylated species.

[e] Neutralized by alkaline phosphatase digestion alone.

TABLE III

AX-5 HPLC standards

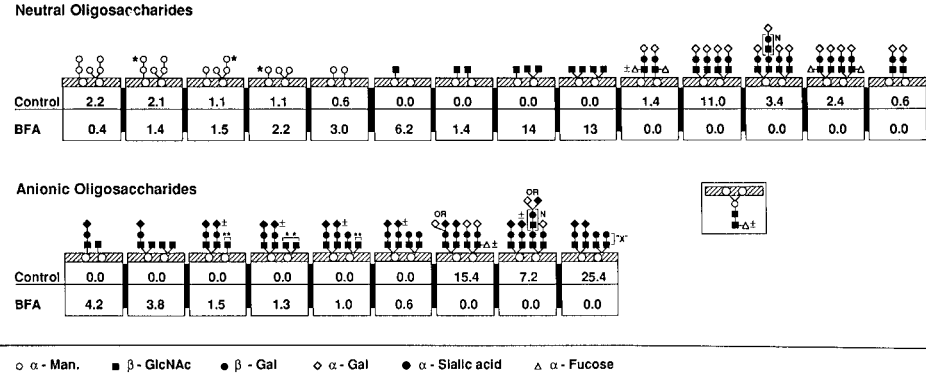
Standards for the HPLC analyses shown in the figures are indicated by the abbreviations shown above. The high mannose-type oligosaccharides (M5-M9) were derived by PNGase F release of [2-^{3}H]Man-labeled oligosaccharides from BW5147 lymphoma cells and reduced with sodium borotritide. All others were purchased from the Dionex Corporation and were reduced with sodium borohydride, desalted, and where needed, digested with the appropriate glycosidases.

Abbreviations	Oligosaccharides
M	Man
M1	$ManGlcNAc_2$
M3	$Man_3GlcNAc_2$
M5	$Man_5GlcNAc_2$
M6	$Man_6GlcNAc_2$
M7	$Man_7GlcNAc_2$
M8	$Man_8GlcNAc_2$
M9	$Man_9GlcNAc_2$
Bi/βGlcNAc	Biantennary chains terminated with 2 β-*N*-acetylglucosamine residues
Bi/βGal	Biantennary chains terminated with 2 β-galactose residues
Tri/βGlcNAc	Triantennary chains terminated with 3 β-*N*-acetylglucosamine residues
Tri/βGal	Triantennary chains terminated with 3 β-galactose residues
Tetra/βGlcNAc	Tetraantennary chains terminated with 4 β-*N*-acetylglucosamine residues
Tetra/βGal	Tetraantennary chains terminated with 4 β-galactose residues

TABLE IV

Spectrum of N-linked oligosaccharides synthesized in the presence and absence of BFA

A summary of all the types of structures found and their relative abundance as a % of total oligosaccharides in control and BFA samples are presented (excepting phosphorylated molecules). The $Man_3GlcNAc_2$ Core structure (±Fuc) common to all the molecules is shown in the inset. Calculations are based on the amount of radioactivity eluting in the fractions described in Fig. 3, and on relative amounts of label that clearly shifted using the various digestions (shown by the *shaded areas*) in the figures. In the case of high mannose-type structures, the amounts have been normalized for mannose content, and for all others the numbers are based on an assumed $Man_3GlcNAc_2$ core structure. In each case, no branch specificity is implied for any of the structures. Location of the outer branch Fuc residues is shown as linked to GlcNAc based on the size range of the structures and their resistance to β-hexosaminidase digestion. *Alternate Man residue that could have been removed during processing. **Position where a non-sialylated β-galactose residue may be added to a small portion of this structure. × Likely position of the unknown acid-resistant substituent.



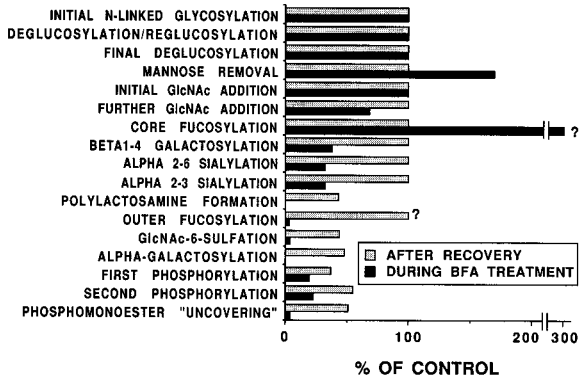


FIG. 17. **Summary of the effects of Brefeldin A on *N*-linked oligosaccharide biosynthesis in CPAE cells, and the extent of recovery following removal of the compound.** The various steps of *N*-linked oligosaccharide processing explored in this study are listed. The effects of BFA on each step are indicated as a percent of that seen in the control cells (*dark bars*). The extent of recovery after removal of BFA is indicated where appropriate (*open bars*). The *question marks* indicate uncertainty as to the magnitude of the effect.

in sialylation seen in the presence of BFA is thus a secondary consequence of reduction of β-Gal-containing acceptors. However, the other type of capping unit, the α-Gal residue is completely absent. This implies that α-galactosyltransferase, which should normally compete with sialyltransferase (58, 59), is not as accessible to the potential acceptors. The same is true for the addition of polylactosamine chains and sulfate esters. Based on this, we predict that BFA would have modest effects on proteins that carry high mannose-type or biantennary complex-type chains and major effects upon those with sulfate esters, polylactosamines, α-Gal units, or highly branched chains.

The effects of BFA on phosphorylated oligosaccharide synthesis and processing are complex, and somewhat surprising, particularly the substantial decrease in total amount of phosphorylated chains. This could be theoretically explained by hyperactivity of, or longer exposure to, Golgi α-mannosidase I which would cleave the critical α1→2-linked terminal Man residues needed for the phosphorylation reaction (5). However, the restoration of a major portion of the phosphorylation when BFA is removed rules out this as the sole explanation. The addition of the first GlcNAc-1-P residue on the α1→6 arm of the oligosaccharide is thought to occur in the ER salvage compartment or very early *cis*-Golgi, recently called the "*cis*-Golgi network" (60). This compartment is distinct from the one where the second GlcNAc-1-P residue is added to the (originally glucosylated) α1→3-linked arm. Therefore, the effects of BFA on the addition of the second GlcNAc-1-P can be considered as distinct from the addition of the first one. The existence of multiple GlcNAc phosphotransferases (or modulators of the transferase) is currently unexplored but is consistent with the existence of multiple complementation groups in the diseases of lysosomal enzyme phosphorylation (61, 62). The conversion of the phosphodiester to the phosphomonoester by the specific phosphodiester glycosidase "uncovering enzyme" is markedly reduced by BFA. This effect was previously reported for the processing of lysosomal cathepsin D in the presence of BFA (32). Exposure of cells to ammonium chloride also prevents the conversion of the diester to the monoester (63). Together, the results of these studies suggest that the uncovering enzyme may reside in a compartment (64) beyond the block imposed by BFA and not in the *cis*-Golgi, as suggested by others (65, 66).

BFA inhibits incorporation of sulfate into all macromolecules by >95%, suggesting that sulfation reactions are very late Golgi events. In corroboration with this, the [^{3}H]Man-labeled molecules do not encounter sulfotransferases. Also, recent studies in human melanoma cells show that chondroitin sulfate chain initiation is unaffected by BFA but that chain elongation and sulfation are reversibly inhibited (67). When BFA is removed, sulfation of *N*-linked oligosaccharides is partly restored, showing that the molecules are still competent as acceptors and that the sulfotransferases are still active. Similarly, the addition of terminal α-Gal and polylactosamine chains are also reversibly inhibited by BFA.

The effects of BFA on fucosylation are significant but

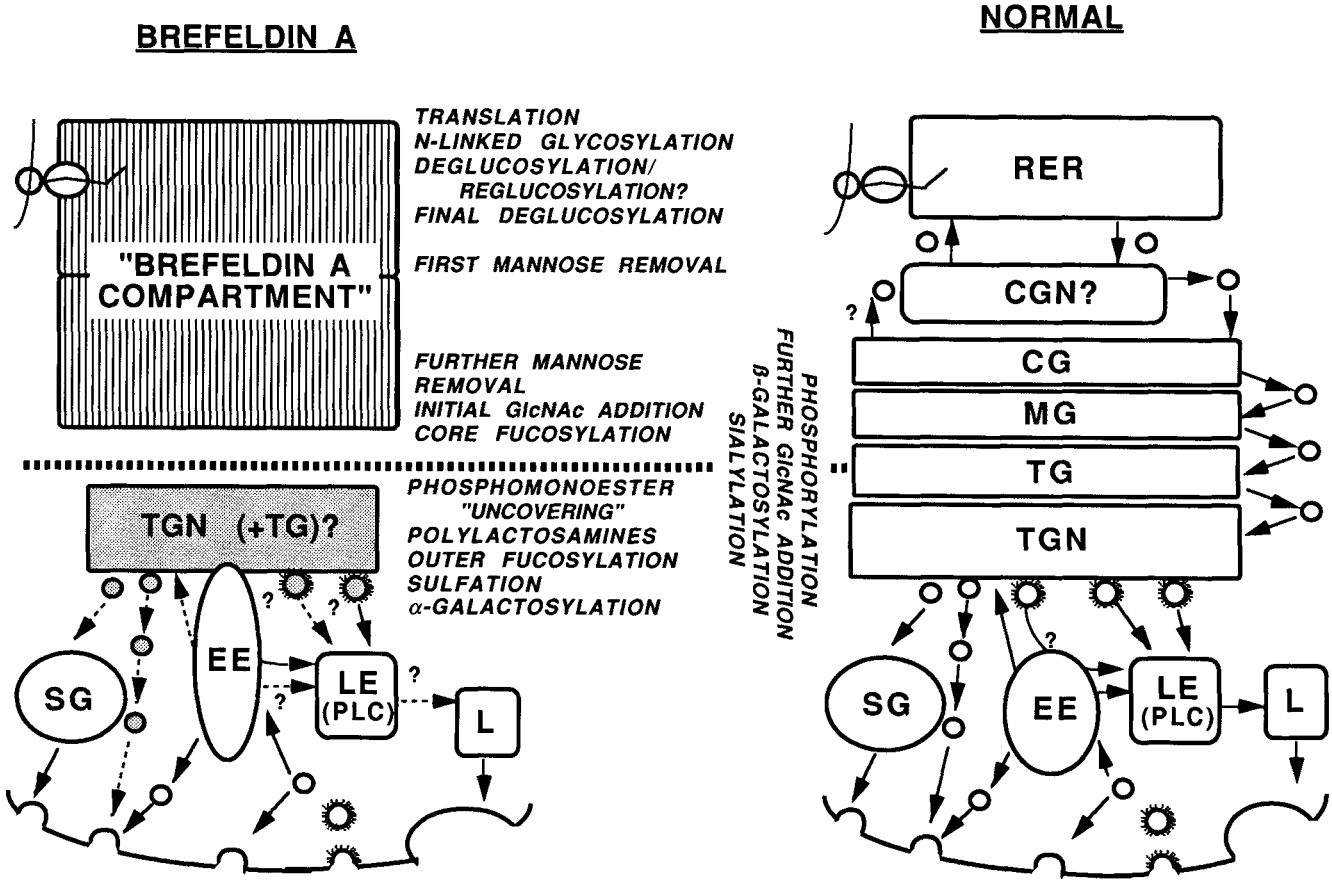


FIG. 18. **Proposed site of the transport block caused by Brefeldin A in CPAE cells, relative to the steps of *N*-linked oligosaccharide processing.** The *drawing* on the *right* indicates the normal pathways for vesicular transport via the ER-Golgi pathway (*RER*, rough endoplasmic reticulum; *CGn*, *cis*-Golgi network or "salvage compartment"; *CG*, *cis*-Golgi stack(s); *MG*, *medial*-Golgi stack(s); *TG*, *trans*-Golgi stack(s); *TGn*, *trans*-Golgi network; *SG*, secretory granule; *EE*, early endosome; *LE*, late endosome or "prelysosomal compartment;" and *L*, lysosomes). The *left side* of the figure shows the morphological effect of BFA schematically, with the proposed level of the block in transport indicated by the *heavy dotted line*. The *hatched area* indicates the abnormal compartment created by the fusion of parts of the Golgi apparatus with the RER, and *shaded areas* indicate the distal regions that would be rapidly depleted of acceptors by the block in transport. The central portion of the figure lists the various steps in *N*-linked oligosaccharide processing that were explored in this study and their proposed sites of action relative to the level of the block.

confusing. The 3-fold increase in the percent of label binding to pea lectin should indicate a significant increase in core fucosylation. However, during recovery from BFA, these previously labeled molecules show a decrease in pea lectin binding back to the level seen in control cells. This implies a novel process in which core fucose residues are added and then removed. Alternatively, the increase in pea lectin binding could be due to some as yet unknown type of processing intermediate. In contrast to core fucosylation, outer lactosamine chain fucosylation appears to be markedly reduced in the presence of BFA, as measured by both changes in the HPLC profiles, and by the direct measurement of the amount of [^{3}H]Fuc that does not bind to pea lectin. This type of fucosylation is restored during recovery from BFA. However, it is somewhat surprising that the restoration consists of *labeled* fucose residues, at exactly the same level as in the control. Overall, it is clear that further work is needed to accurately define the effects of BFA on fucosylation. This is beyond the scope of the present study.

The most likely explanation for all of these results is that some of the relevant Golgi enzymes reside in the *trans*-Golgi network beyond the BFA block. Other less likely explanations must be mentioned. BFA could affect the activity or localization of sugar nucleotide transporters (2) or the pyrophosphatases that influence sugar nucleotide concentration (22, 68, 69). Alternatively, BFA could inhibit a subset of the transferases directly. Another possibility is that calcium concentrations in the Brefeldin compartment are high because of fusion with the ER and that this selectively affects a subset of Golgi enzymes. These explanations appear much less likely because BFA would have to severely affect the function of some transferases and transporters, and yet not affect others at all. Furthermore, BFA is now known to have a highly specific mode of action on the structure of the Golgi itself.

Fig. 17 shows a summary of the relative effects of BFA on each processing step of *N*-linked oligosaccharide biosynthesis in these cells; it also gives an indication of how efficiently each type of structure is restored after the BFA is removed. The quantitative effects of BFA on the formation of each type of oligosaccharide are shown in Table IV. Fig. 18 shows a schematic diagram of *N*-linked oligosaccharide processing and protein secretion in the presence and absence of BFA derived from this study. The BFA compartment arises from fusion of the ER, the "salvage compartment" between the ER and the Golgi (also called the *cis*-Golgi network), and the functionally defined equivalents of the *cis*-, *medial*-, and part of the *trans*-

Golgi. Most oligosaccharide modifications that occur in these fused compartments are either totally unaffected or even exaggerated in the presence of BFA. On the other hand, secretion stops and late oligosaccharide processing events are totally inhibited, presumably because the TGN compartment is devoid of newly delivered acceptor glycoproteins. In between these two extremes are the processing reactions that straddle the BFA block. These modifications include the later branching addition of GlcNAc residues, β-galactosylation/sialylation of preferred branches on some chains, and the initial phosphorylation of a portion of the potential acceptors. In physical terms, this probably means that different sets of these transferases are segregated into BFA-sensitive and BFA-resistant compartments and that the itinerant proteins are preferentially trapped in the former. Alternatively, a group of selected proteins may have preferred access to these transferases. Yet another possibility is that the effects of BFA are partial and that processing events more distal to the partial block are more likely to be completely inhibited by BFA than those more proximal. Our results cannot completely distinguish between any of these possibilities.

Whatever the reason, these data do indicate several novel segregations and/or associations of either the acceptors or the transferases. For instance, many previous studies have used β-galactosyl and sialyltransferases as markers of the *trans*-Golgi and the TGN, respectively (3–7, 21, 25, 70). The separate localization of these enzymes is cell-type dependent, and when they are co-localized, it is thought to be in the TGN. Our results would argue that in CPAE cells, neither galactosyl nor sialyltransferases are accurate markers for the TGN. Rather, α-galactosyltransferase would be a better marker for the TGN, and its oligosaccharide products could be detected with *Griffonia* lectin. The dramatic effects of BFA on phosphorylation and phosphodiester processing suggests that most of the phosphorylation might occur in the TGN where the majority of the cation-independent phosphomannosyl receptor is located (5). Since this receptor has also been found in the *cis*-Golgi in some cell types (53), it is possible that the BFA-sensitive phosphorylation is different in different cell types. In addition, because the GlcNAc phosphotransferase appears to be quite different from all other Golgi transferases (being of very high apparent molecular weight and extremely labile to purification), it is possible that it is selectively inhibited by BFA-induced membrane redistribution.

This study is the first to report an analysis of the total spectrum of *N*-linked oligosaccharides synthesized by normal cultured endothelial cells. This was necessary to provide a basis for comparison to BFA samples. These data should also be useful as a guidepost for future studies on the *N*-linked oligosaccharides in endothelial cells, since we identified most of them in this cell type. However, a substantial number of tri/tetra-branched oligosaccharides were difficult to digest down to the core region with sequential exoglycosidases. This could be partly explained by a bisecting GlcNAc residue, which is often difficult to remove with enzymes. However, the complexity of the profile after all the digestions suggest that some of the chains have an unidentified (neutral) substituent on the outer GlcNAc residues that prevented complete digestion with β-hexosaminidase (71). Structural characterization of this phenomenon requires further work.

Acknowledgments—We acknowledge the contributions of Kaneyuki Kubushiro for preliminary experiments, Henny Bierhuizen for her extensive help in the preparation of the manuscript, and Bradley Hays for oligosaccharide standards.

Note Added in Proof—Since the acceptance of this manuscript, several papers have appeared indicating that pathways for endocytosis and transcytosis and the organization of the *trans*-Golgi network can also be affected by BFA (76–82). However, since the present study followed the fate of a [2-^{3}H]mannose pulse-label originating in the ER, most of the interpretations of our data remain unchanged. The exception might be the changes noted in outer fucose residues, which could be directly labeled by [2-^{3}H]fucose beyond the BFA-imposed block in the secretory pathway.

REFERENCES

1. Kornfeld, R., and Kornfeld, S. (1985) *Annu. Rev. Biochem.* **54,** 631–664
2. Hirschberg, C. B., and Snider, M. D. (1987) *Annu. Rev. Biochem.* **56,** 63–87
3. Griffiths, G., and Simons, K. (1986) *Science* **234,** 438–443
4. Pelham, H. R. B. (1989) *Annu. Rev. Cell Biol.* **5,** 1–23
5. Kornfeld, S., and Mellman, I. (1989) *Annu. Rev. Cell Biol.* **5,** 483–525
6. Rothman, J. E., and Orci, L. (1990) *FASEB J.* **4,** 1460–1468
7. Farquhar, M. G. (1991) in *Intracellular Trafficking of Proteins* (Steer, C. J., and Hanover, J., eds) Cambridge University Press, New York
8. Roth, J., Taatjes, D. J., Weinstein, J., Paulson, J. C., Greenwell, P., and Watkins, W. M. (1986) *J. Biol. Chem.* **261,** 14307–14312
9. Pavelka, M., and Ellinger, A. (1991) *J. Electron Microsc. Tech.* **17,** 35–50
10. Roth, J., Taatjes, D. J., Lucocq, J. M., Weinstein, J., and Paulson, J. C. (1985) *Cell* **43,** 287–295
11. Geuze, H. J., and Morré, D. J. (1991) *J. Electron Microsc. Tech.* **17,** 24–34
12. Rogalski, A. A., Bergmann, J. E., and Singer, S. J. (1984) *J. Cell Biol.* **99,** 1101–1109
13. Stults, N. L., Fechheimer, M., and Cummings, R. D. (1989) *J. Biol. Chem.* **264,** 19956–19966
14. Varki, A. (1991) *FASEB J.* **5,** 226–235
15. Misumi, Y., Misumi, Y., Miki, K., Takatsuki, A., Tamura, G., and Ikehara, Y. (1986) *J. Biol. Chem.* **261,** 11398–11403
16. Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A., and Ikehara, Y. (1988) *J. Biol. Chem.* **263,** 18545–18552
17. Yewdell, J. W., and Bennink, J. R. (1989) *Science* **244,** 1072–1075
18. Ulmer, J. B., and Palade, G. E. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 6992–6996
19. Lippincott-Schwartz, J., Yuan, L. C., Bonifacino, J. S., and Klausner, R. D. (1989) *Cell* **56,** 801–813
20. Nuchtern, J. G., Bonifacino, J. S., Biddison, W. E., and Klausner, R. D. (1989) *Nature* **339,** 223–226
21. Doms, R. W., Russ, G., and Yewdell, J. W. (1989) *J. Cell Biol.* **109,** 61–72
22. Fujiwara, T., Oda, K., and Ikehara, Y. (1989) *Cell Struct. Funct.* **14,** 605–616
23. Lippincott-Schwartz, J., Donaldson, J. G., Schweizer, A., Berger, E. G., Hauri, H.-P., Yuan, L. C., and Klausner, R. D. (1990) *Cell* **60,** 821–836
24. Yoshida, T., Chen, C., Zhang, M., and Wu, H. C. (1991) *Exp. Cell Res.* **192,** 389–395
25. Chege, N. W., and Pfeffer, S. R. (1990) *J. Cell Biol.* **111,** 893–899
26. Strous, G. J., Berger, E. G., Van Kerkhof, P., Bosshart, H., Berger, B., and Geuze, H. J. (1991) *Biol. Cell* **71,** 25–31
27. Orci, L., Tagaya, M., Amherdt, M., Perrelet, A., Donaldson, J. G., Lippincott-Schwartz, J., Klausner, R. D., and Rothman, J. E. (1991) *Cell* **64,** 1183–1195
28. Donaldson, J. G., Lippincott-Schwartz, J., Bloom, G. S., Kreis, T. E., and Klausner, R. D. (1990) *J. Cell Biol.* **111,** 2295–2306
29. Donaldson, J. G., Lippincott-Schwartz, J., and Klausner, R. D. (1991) *J. Cell Biol.* **112,** 579–588
30. Perkel, V. S., Miura, Y., and Magner, J. A. (1989) *Proc. Soc. Exp. Biol. Med.* **190,** 286–293
31. Takami, N., Oda, K., Fujiwara, T., and Ikehara, Y. (1990) *Eur. J. Biochem.* **194,** 805–810
32. Radons, J., Isidoro, C., and Hasilik, A. (1990) *Biol. Chem. Hoppe Seyler* **371,** 567–573
33. Shite, S., Seguchi, T., Shimada, T., Ono, M., and Kuwano, M. (1990) *Eur. J. Biochem.* **191,** 491–497
34. Shite, S., Seguchi, T., Mizoguchi, H., Ono, M., and Kuwano, M.

(1990) *J. Biol. Chem.* **265,** 17385–17388
35. Whealy, M. E., Card, J. P., Meade, R. P., Robbins, A. K., and Enquist, L. W. (1991) *J. Virol.* **65,** 1066–1081
36. Ulmer, J. B., and Palade, G. E. (1991) *J. Biol. Chem.* **266,** 9173–9179
37. Chen, S.-Y., Matsuoka, Y., and Compans, R. W. (1991) *J. Virol.* **65,** 1427–1439
38. Bosshart, H., Straehl, P., Berger, B., and Berger, E. G. (1991) *J. Cell. Physiol.* **147,** 149–156
39. Roux, L., Holojda, S., Sundblad, G., Freeze, H. H., and Varki, A. (1988) *J. Biol. Chem.* **263,** 8879–8889
40. Li, E., Tabas, I., and Kornfeld, S. (1978) *J. Biol. Chem.* **253,** 7762–7770
41. Huffaker, T. C., and Robbins, P. W. (1982) *J. Biol. Chem.* **257,** 3203–3210
42. Merkle, R. K., and Cummings, R. D. (1987) *Methods Enzymol.* **138,** 232–259
43. Cummings, R. D., Merkle, R. K., and Stults, N. L. (1989) *Methods Cell Biol.* **32,** 141–183
44. Schachter, H. (1986) *Adv. Exp. Med. Biol.* **205,** 53–85
45. Pâquet, M. R., Narasimhan, S., Schachter, H., and Moscarello, M. A. (1984) *J. Biol. Chem.* **259,** 4716–4721
46. van den Eijnden, D. H., Koenderman, A. H. L., and Schiphorst, W. E. C. M. (1988) *J. Biol. Chem.* **263,** 12461–12471
47. Fukuda, M., Dell, A., Oates, J. E., and Fukuda, M. N. (1984) *J. Biol. Chem.* **259,** 8260–8273
48. Fukuda, M. N., Dell, A., and Scartezzini, P. (1987) *J. Biol. Chem.* **262,** 7195–7206
49. Kjellén, L., and Lindahl, U. (1991) *Annu. Rev. Biochem.* **60,** 443–475
50. Kresse, H., Fuchs, W., Glossl, J., Holtfrerich, D., and Gilberg, W. (1981) *J. Biol. Chem.* **256,** 12926–12932
51. von Figura, K., and Hasilik, A. (1986) *Annu. Rev. Biochem.* **55,** 167–193
52. Griffiths, G., Hoflack, B., Simons, K., Mellman, I., and Kornfeld, S. (1988) *Cell* **52,** 329–341
53. Brown, W. J., and Farquhar, M. G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 9001–9005
54. Lazzarino, D. A., and Gabel, C. A. (1989) *J. Biol. Chem.* **264,** 5015–5023
55. Lazzarino, D. A., and Gabel, C. A. (1988) *J. Biol. Chem.* **263,** 10118–10126
56. Varki, A., and Kornfeld, S. (1983) *J. Biol. Chem.* **258,** 2808–2818
57. Freeze, H. H., Yeh, R., Miller, A. L., and Kornfeld, S. (1983) *J. Biol. Chem.* **258,** 14874–14879
58. Joziasse, D. H., Shaper, J. H., Van den Eijnden, D. H., Van Tunen, A. J., and Shaper, N. L. (1989) *J. Biol. Chem.* **264,** 14290–14297
59. Smith, D. F., Larsen, R. D., Mattox, S., Lowe, J. B., and Cummings, R. D. (1990) *J. Biol. Chem.* **265,** 6225–6234
60. Hsu, V. W., Yuan, L. C., Nuchtern, J. G., Lippincott-Schwartz, J., Hammerling, G. J., and Klausner, R. D. (1991) *Nature* **352,** 441–444
61. Little, L. E., Mueller, O. T., Honey, N. K., Shows, T. B., and Miller, A. L. (1986) *J. Biol. Chem.* **261,** 733–738
62. Mueller, O. T., Honey, N. K., Little, L. E., Miller, A. L., and Shows, T. B. (1983) *J. Clin. Invest.* **72,** 1016–1023
63. Isidoro, C., Radons, J., Baccino, F. M., and Hasilik, A. (1990) *Eur. J. Biochem.* **191,** 591–597
64. Deutscher, S. L., Creek, K. E., Merion, M., and Hirschberg, C. B. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 3938–3942
65. Goldberg, D. E., and Kornfeld, S. (1983) *J. Biol. Chem.* **258,** 3159–3165
66. Pohlmann, R., Waheed, A., Hasilik, A., and von Figura, K. (1982) *J. Biol. Chem.* **257,** 5323–5325
67. Spiro, R., Freeze, H., Sampath, D., and Garcia, J. (1991) *J. Cell. Biol.* **115,** 1463–1473
68. Kean, E. L., and Bighouse, K. J. (1974) *J. Biol. Chem.* **249,** 7813–7823
69. Byrd, J. C., Fearney, F. J., and Kim, Y. S. (1985) *J. Biol. Chem.* **260,** 7474–7480
70. Farquhar, M. G. (1985) *Annu. Rev. Cell Biol.* **1,** 447–488
71. Kobata, A. (1979) *Anal. Biochem.* **100,** 1–14
72. Marshall, R. D., and Neuberger, A. (1972) in *Glycoproteins, Their Composition, Structure and Function* (Gottschalk, A., ed) Elsevier, Amsterdam
73. Freeze, H. H., Willies, L., Hamilton, S., and Koza-Taylor, P. (1989) *J. Biol. Chem.* **264,** 5653–5659
74. Wang, W.-C., Clark, G. F., Smith, D. F., and Cummings, R. D. (1988) *Anal. Biochem.* **175,** 390–396
75. Varki, A., and Diaz, S. (1984) *Anal. Biochem.* **137,** 236–247
76. Hudson, T. H., and Grillo, F. G. (1991) *J. Biol. Chem.* **266,** 18586–18592
77. Pelham, H. R. B. (1991) *Cell* **67,** 449–451
78. Wood, S. A., Park, J. E., and Brown, W. J. (1991) *Cell* **67,** 591–600
79. Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L., and Klausner, R. D. (1991) *Cell* **67,** 601–616
80. Hunziker, W., Whitney, J. A., and Mellman, I. (1991) *Cell* **67,** 617–627
81. Sandvig, K., Prydz, K., Hansen, S. H., and van Deurs, B. (1991) J. Cell Biol. **115,** 971–981
82. Kakiuchi, T., Takatsuki, A., Watanabe, M., and Nariuchi, H. (1991) *J. Immunol.* **147,** 3289–3295

SUPPLEMENTAL MATERIAL TO THE SPECTRUM OF INCOMPLETE *N*-LINKED OLIGOSACCHARIDES SYNTHESIZED BY ENDOTHELIAL CELLS IN THE PRESENCE OF BREFELDIN A

By

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EXPERIMENTAL PROCEDURES

MATERIALS

Enzymes. Flavobacterium meningosepticum peptide:N-glycosidase F was purchased from Genzyme. Arthrobacter ureafaciens sialidase was from Calbiochem. E. coli Alkaline Phosphatase was obtained from Sigma. Chicken liver β-galactosidase and coffee bean α-galactosidase were from Oxford Glycosystems. Human placental β-hexosaminidase A was generously provided by Dr. Don Mahuran, Hospital for Sick Children, Toronto, and Dr. Mario Ratazzi, North Shore University Hospital, Manhasset, N.Y. Escherichia freundii endo-β-galactosidase was a kind gift from Dr. Michiko Fukuda, La Jolla Cancer Research Foundation. Jack bean α-mannosidase was generously donated by Dr. Robert Trimble, New York State Department of Health, Albany, N.Y.

Radioisotopes. Tran^{35}S (1100Ci/mmol) and $Na^{35}SO_4$ (43Ci/mg) were purchased from ICN. [2-^{3}H] D-Man (15Ci/mmol) was purchased from American Radiolabeled Chemicals.

Cell Lines. Bovine pulmonary artery endothelium cells (CPAE) were obtained from the American Type Culture Collection (ATCC CCL 209). Low passage (<20) cultures were maintained in monolayers in Alpha Modified Eagles Medium-alpha (α-MEM) supplemented with 10% fetal calf serum, 2mM L-Glutamine, and 100 units/ml penicillin/ 100 μg/ml streptomycin at 37°C in a 5% CO_2 atmosphere.

Other Materials. N-linked oligosaccharides with defined structures (from Dionex Corporation, confirmed by NMR) were labelled at the reducing terminus with tritiated borohydride. Brefeldin A was purchased from Epicentre Technologies, Madison, WI and stored as a stock solution of 1 mg/ml in 100% ethanol at -20°C. Modified Eagles Medium Alpha (α-MEM), Jokliks Spinner Salts, penicillin, streptomycin, and L-glutamine were purchased from Irvine Scientific. Fetal Calf Serum was obtained from Tissue Culture Biologicals, Tulare, CA. Sephadex G-25 , G-15, and QAE-Sephadex were purchased from Sigma. Sephacryl S-200 and Concanavalin A-Sepharose were purchased from Pharmacia. *Griffonia simplicifolia*-1 agarose and *Pisum sativum*-agarose were from E-Y Laboratories, Inc. Micropak AX-5 HPLC columns (30cm x 4mm) were purchased from Varian Instruments, Walnut Creek, CA. All other chemicals used were reagent grade.

METABOLIC LABELLING OF CELLS

BFA Dose Effect. CPAE cells grown in 60mm culture dishes to 75% confluency were pre-incubated in the presence and absence of BFA in complete α-MEM for 30 min and labeled with $^{35}SO_4$ (33μCi/ml), [2-^{3}H]Man (67μCi/ml), or Tran^{35}S (20μCi/ml) in the same concentrations of BFA. Cells were labeled in low glucose (0.1mg/ml) α-MEM for 30 min with [2-^{3}H]Man or Tran^{35}S, while those labeled with $Na^{35}SO_4$ were in medium containing 15μM inorganic sulfate (Sulfate Labelling Incorporation Medium) for 30 min(39). Cells were chased for 3 h in the continued presence of BFA in complete α-MEM, harvested with a rubber policeman, and lysed in 0.1% Triton X-100. The amount of incorporated radioactivity in samples from cells and media was determined by precipitation with 10% trichloroacetic acid/2%phosphotungstic acid (TCA/PTA) and normalized to protein content. Protein determination was done with the BioRad Microassay System.

Characterization of Oligosaccharides Synthesized in the Presence of BFA. Pre-incubation and chase conditions were similar to those mentioned above. 75% confluent cells were metabolically labeled in 10cm culture dishes with [2-^{3}H]Man (333via/ml) or Tran^{35}S (8.3μCi/ml) in the presence or absence of BFA in low glucose medium for 30 min and chased for 3 h in unlabeled medium with/without BFA. Cells labeled with $^{35}SO_4$ were labeled for 6 h in SLIM with 15uM inorganic sulfate(39). Cells were harvested and lysed for isolation of N-linked oligosaccharides as described (39).

Recovery from BFA. Cells labeled with [2-^{3}H]Man and Tran^{35}S were pre-incubated, pulsed, and chased under conditions described above. Following a 3 h chase in the presence of 1.0μg/ml BFA, the cells were incubated for 12 h in its absence, then were harvested, lysed as described (39), and the amount of incorporated radioactivity was determined by 10%TCA/2%PTA precipitation. The chase medium was dialyzed against 2mM Tris pH 7.5 for 24 h, concentrated on a Centricon 10 microconcentrator (10kDa cutoff), and lyophylized. The dried medium was combined with the pre-lysed cells and heated in 0.2% SDS, 0.05M Tris-HCl, pH 7.5 for isolation of N-linked oligosaccharides.

ISOLATION AND ANALYSIS OF N-LINKED OLIGOSACCHARIDES

Release and Purification of N-linked Oligosaccharides. Labeled N-Linked oligosaccharides were released and purified as previously described (39). Briefly, the void volume fractions from a 0.7x50 cm S-200 Sephadex column run in 0.2% SDS were collected and acetone-precipitated. The precipitate was resuspended in 200µl 0.5% SDS, 30mM βME, boiled for 5min, 2.5% NP-40 added, and incubated with 1.0 unit (1nmole/min at 37°C) of PNGaseF at 30°C overnight. The digest was passed over the same S-200 column, the included (released) fractions pooled, KCl precipitated to remove SDS, and desalted on a Sephadex G-25 column (see Figure 4 for column profiles, flow chart in Figure 3 for summary, and Ref. 39 for full details).

Enzymatic Treatments. The following digests were performed as previously described: Arthrobacter ureafaciens sialidase, Alkaline Phosphatase, and Newcastle Disease Virus sialidase (39). The following digests were carried out at 37°C overnight and terminated by boiling for 10 min, cooling and spinning the mixture at 10,000xg for 2 min in a microcentrifuge.

α-Mannosidase - Dried samples were resuspended in 20µl of 150mM citrate-phosphate buffer pH 5.0 and 1.0U of jack bean α-mannosidase.

β-Galactosidase - Dried samples were resuspended in 100µl of 150mM sodium citrate-phosphate pH 4.0 with 50mU of the enzyme.

α-Galactosidase - Dried samples were resuspended in 20µl of 5x digest buffer (500mM sodium citrate-phosphate pH 6.0), 1.0U of enzyme (25U/mg), and diluted to 100µl with H_2O.

Endo-β-Galactosidase - Dried samples were resuspended in 25µl of 200mM NaAcetate pH 5.5 and 25µl of enzyme (.31U/ml) were added to the mixture.

β-Hexosaminidase A - Dried samples were resuspended in 100µl of either 25mM NaFormate pH 4.5 or 50mM pH 3.5 (for cleavage of GlcNAc-6-SO_4) and 1.0U of enzyme was added.

Sequential Digestions - The sequential digestions were performed in a single tube to minimize sample loss. In each case, the first incubation was terminated by heating, followed by drying, and addition of the appropriate enzyme buffer to adjust the pH before the next digestion.

Chemical Treatments.

Mild Acid Hydrolysis for cleavage of phosphodiesters and removal of sialic acids was carried out in 10mM HCl at 100°C for 30 min (39). Stronger acid (50mM HCl at 100°C for 30 min) was used for removal of fucose residues(72). This approach was taken because commercially available fucosidases are generally contaminated with significant levels of other glycosidases.

Solvolysis for removal of sulfate esters was performed as described previously(39,73).

Chromatographic Analysis.

QAE-Sephadex - Analysis of the negative charges on N-linked oligosaccharides (1500-2000 cpm) was performed as described, by sequential batch elution with increasing concentrations of NaCl (39).

Concanavalin A-Sepharose - Analysis on Concanavalin A-Sepharose was performed in 2x0.5cm columns constructed in pasteur pipets. Samples were applied in 1.5ml of 150mM NaCl 10mM NaPi (PBS), pH 6.5 and washed with 3 x 1.5ml of the same buffer (Fraction I). Elutions were performed with 4 x 1.5ml washes of 10mM α-methyl-glucoside (Fraction II) and 100mM α-methyl-mannoside (Fraction III) at 55°C in PBS.

Griffonia simplicifolia-1 Agarose - 3 x 0.5cm columns were constructed in pasteur pipets and equilibrated in PBS, pH 6.5. Samples (1500-2000 cpm) were applied in 0.5ml of this buffer and washed with 10 x 0.5ml. Elutions were performed 10 x 0.5 ml of 100mM α-methyl-galactoside in PBS(74). Each run included an internal standard of [^{14}C]Acetyl-AsnGlcNAc$_2$Man$_6$ to mark the unretarded volume.

Pisum sativum Agarose Analysis was performed with a 20x0.5cm column equilibrated in Tris-buffered saline containing 0.01 M Tris, pH 8.0, 0.15M NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$, and .02% NaN_3 (TBS-NaN_3). Samples were applied in 1.0ml of TBS-NaN_3 and the column was washed with 20 ml of the same buffer. Elutions were performed with 20ml of TBS-NaN_3 containing 0.5M α-methyl D-mannoside. Each run included an internal marker of ^{14}C glucose to mark the unretarded volume. Fractions of 0.2ml were collected and counted.

HPLC Analysis - Neutral or neutralized oligosaccharides (2000-3000 cpm) were analyzed on a 30cm x 4mm Micropak AX-5 column (Varian Instruments) using a 70-40% gradient of acetonitrile in 25mM NaPi, pH 6.5 in 60 min, at a flow rate of 1ml/min. Fractions of 1ml were counted. Each run included an internal standard of [^{14}C]AcAsnMan$_6$GlcNAc$_2$ which was used to align the column fractions. Elution of the internal standard initially occurred at fraction 31 and with prolonged column use moved to fraction 28. However, related samples were always analyzed one after the other. The columns were also calibrated with defined N-linked oligosaccharides (from Dionex Corporation) that were labelled at the reducing terminus with tritiated borohydride. Elution of internal and external standards did not vary by more than one fraction within any given set of samples.

Scintillation counting - In all cases, the samples were counted at constant quench in aqueous-compatible scintillation fluid long enough to give 95% confidence level. Most of the results are expressed as a percentage of the total radioactivity for convenience.

RESULTS

Effects of BFA on the Addition of Anionic Substituents. The overall percent of anionic oligosaccharide chains was reduced from 60% in control cells to 20% in BFA samples cells. The number of negative charges per oligosaccharide chain in ConA fractions I+II was determined by step-wise elution from QAE-Sephadex. As shown in Figure 6 and in Table I, control cells contained a range of species with 1 to 4 negative charges, but in the presence of BFA most oligosaccharides carried primarily 1 negative charge, a few 2 charges and <1.0% contained 3 charges. When the oligosaccharides were treated with *A. ureafaciens* sialidase to remove sialic acids, the majority of control (74%) and BFA samples (82%) chains were converted to neutral molecules, indicating that although sialylation was reduced in the presence of BFA, it was clearly not eliminated (data not shown). To determine whether BFA selectively inhibits the addition of α2→3- or α2→6-linked sialic acids, the chains were digested with the α2→3-specific sialidase from Newcastle Disease Virus. This digestion neutralized 20% of the anionic material from both samples, showing that both α2→3 and α2→6 sialyltransferases were active in the same ratio in BFA treated cells (data not shown). Some sialic acids can be relatively insensitive to sialidase, but all are released by mild-acid hydrolysis (75). Acid treatment (10mM HCl 100°C, 30 min) (MA10) neutralized slightly more material (80% in control and 95% in BFA) than sialidase alone (74% in control and 82% in BFA) in both samples (data not shown), indicating that the majority of sialic acids are sensitive to sialidase treatment.

The oligosaccharides that remained bound to QAE-Sephadex following mild-acid treatment (20% of anionic chains in control cells and 5% in BFA-treated cells) must contain another negatively charged group. We have previously shown that about 15% of the *N*-linked oligosaccharides from this cell line are sulfated (39). Nearly one-half of this sulfate is found as GlcNAc-6-SO_4 on the outer branches of tri- and tetraantennary chains. This sulfated sugar can be released by human placental β-hexosaminidase A at low pH (39,50), after removal of sialic acid and β-galactoside residues. Such a digestion of [^{3}H]Man-labeled oligosaccharides from control cells converted ~40% of the desialylated molecules into neutral species (data not shown). Subsequent digestion with α-mannosidase released >40% of the label as free [^{3}H]Man showing that some of this material probably had an exposed tri-mannosyl core following the β-hexosaminidase digestion. The desialylated oligosaccharides from BFA samples that still bound to QAE-Sephadex were neutralized by the same sequential exoglycosidase digestion (20%), and the remainder by solvolysis. Since the total amount of anionic oligosaccharides is reduced to 20% by BFA, the latter actually represents only 0.6% of total oligosaccharides compared with the 10-15% seen in control cells. All of the neutralized material could be accounted for by loss of molecules with one negative charge and less than 10% [^{3}H]Man was released by subsequent α-mannosidase digestion (data not shown). The oligosaccharides that retained a negative charge after the sequential digestion (<1% of the total) were not characterized further in this study.

Effects of BFA on the Synthesis of $^{35}SO_4$-Labelled Oligosaccharides. As shown in Figure 1, BFA drastically reduces the amount of sulfate incorporated into TCA/PTA precipitable material. To examine this material in greater detail, cells were labeled with $^{35}SO_4$ with and without BFA and following a chase, the sulfated *N*-linked oligosaccharide chains were cleaved with PNGaseF. BFA affected the incorporation of $^{35}SO_4$ into PNGaseF sensitive (15% of total label in control cells) and insensitive chains equally(data not shown). Analysis of released *N*-linked chains from control and treated samples on QAE-Sephadex showed a broad range of sulfated/sialylated species (data not shown) (See Ref. 39). Mild acid treatment (MA10) or sialidase digestion reduced the charge on most of the sulfate-labeled species from control or BFA samples cells. Inorganic $^{35}SO_4$ is not released under these conditions (39). About one-half of the $^{35}SO_4$ label in desialylated N-linked oligosaccharides was released from both samples by the sequential treatment with β-galactosidase and human placental β-hexosaminidase A showing that the relative distribution of β-hexosaminidase A-sensitive and resistant sulfated residues was unaffected by BFA treatment (data not shown). Thus, BFA markedly reduces $^{35}SO_4$ incorporation into *N*-linked chains and other macromolecules (presumably *O*-linked glycosaminoglycan chains) equally, and maintains the same relative distribution of sulfated sugars on the *N*-linked chains as well. The low level of $^{35}SO_4$ incorporation in BFA is presumably in acceptors that were already in the late Golgi when BFA is added. Since no new acceptors are available in the presence of BFA, the incorporation stops quickly (see Figure 1).

Effects of BFA on the Synthesis and Processing of Phosphorylated Sugar Chains. To study the effects of BFA on the synthesis and maturation of phosphorylated oligosaccharides, the anionic chains found in ConA fraction III were studied separately. The relative amount of [^{3}H]Man found in this fraction was not markedly different either with (4.0%) or without (6.0%) BFA (Tables I and II), but analysis on QAE-Sephadex showed pronounced changes (Figure 7). First, the great majority of the BFA sample (74-80%) contained only a single charge, while 75% of the control sample had 2-4 negative charges. Individual and combined sequential treatments with sialidase, mild acid and alkaline phosphatase showed that most of the negative charge in the BFA sample was due to sialic acid (68-83%) and not to phosphate, whereas in controls, only about 20% was due to sialic acids. These sialylated molecules that bind tightly to ConA are characterized below. Most of the oligosaccharides (64%) from the control samples contained 1 or 2 phosphomonoesters that were removed by phosphatase digestion. In contrast, the BFA samples contained mostly molecules with 1 phosphodiester and a small amount with 2 phosphodiesters. Only 3-5% of this fraction contained monoester and most of this was present on chains with only 1 Man-6-P residue. Thus, BFA substantially reduced overall phosphorylation, paticularly the addition of the second phosphate to those chains that already had 1 phosphate, and the conversion of the phosphodiesters to phosphomonoesters. The individual steps were reduced by 4-fold to >10-fold. Results are summarized in Table II and Figure 17.

Size and Nature of the Underlying Oligosaccharide of Anionic Molecules in ConA III Fractions. The reduced amount of phosphorylation seen in the presence of BFA could be due to excessive Man processing causing the loss of appropriate oligosaccharide acceptors for GlcNAc-phosphotransferase. To examine this, we analyzed the sialylated and phosphorylated chains by HPLC and exoglycosidase digestions (Figures 8 and 9). Material neutralized by mild acid treatment was derived from molecules that carried 1 sialic acid. The sole oligosaccharide in this fraction was an incomplete bi-antennary chain terminated by a single sialylated β-Gal attached to the GlcNAcβ1→2 Man linked α1→3 to the β-Man (Figure 8, and main text). This conclusion is based on the size, negative charge, exoglycosidase digestion pattern and characteristic elution in the ConA III fraction (42). Molecules with two terminal β-Gal residues or those with one β-Gal residue on the antenna α1→3 linked to the β-Man elute in ConA Fraction II (42).

The mild acid/alkaline phosphatase-neutralized phosphorylated molecules from the BFA samples were composed exclusively of high Man type chains, primarily Man$_5$-Man$_7$ (Figure 9). There was a particular enrichment of Man$_7$ (See Figure 9). Since the GlcNAc phosphotransferase requires terminal α1→2-linked Man residues, it is theoretically possible that the reduction of phosphorylation in BFA was caused by hyperactivity of α-mannosidase I, which cleaves these terminal residues. However, this is probably not the case because removal of the BFA restores much of the phosphorylation, including the formation of phosphomonoesters (Table II).

Effects of BFA on the Nature of the Underlying Oligosaccharide of Anionic Complex-type Chains in ConA I and II Fractions. Most glycosyltransferases are specific for particular acceptors. Thus, many of the results presented so far could be explained by the absence of the appropriate acceptors in the presence of BFA. To examine this question, we analyzed the size and monosaccharide sequence of the chains from desialylated ConA I + II samples, using a combination of exoglycosidase digestions and HPLC analysis. (Under these conditions, molecules that contain sulfate remain bound to the column and were not characterized further since they have been described in a previous study (39), and are present in very low amounts in the BFA samples).

CPAE cells normally synthesize abundant amounts of oligosaccharides with a size range larger than typical desialylated tri- and tetraantennary chains (Figure 10). The largest chains (18% of the total fraction) contained polylactosamine units as shown by endo-β-galactosidase digestion. Subsequent β-hexosaminidase digestion reduced the size of a portion of the chains further, but the digestion was incomplete and the pattern was complex, suggesting the presence of another blocking group. One possibility is that they contain terminal α-Gal residues. This was confirmed by their binding to *Griffonia simplicifolica* I-agarose which was eliminated by α-galactosidase treatment (Figure 12). A portion of the α-galactosylated chains contain polylactosamines, because they are sensitive to endo-β-galactosidase. Combined endo-β-galactosidase and α-galactosidase digestion produced molecules that contained three and four simple branches. Subsequent β-Gal and β-hexosaminidase digestions reduces the size of these oligosaccharides; however, none of the material was reduced to the size of the expected Man$_3$GlcNAc$_2$ core. The cause of the resistance is not completely resolved. If the oligosaccharides are hydrolyzed with 0.05 N HCl for 30 min at 100°C (MA50) prior to the digestions, a portion of the multiantennary material could now be converted to a trimannosyl core molecule. Longer hydrolysis times did not increase the amount of material running the tri-mannosyl core region (data not shown). These conditions should release Fuc residues from the outer branches which could prevent β-hexosaminidase digestion. The presence of such outer branch Fuc residues is supported by the detection of [^{3}H]Fuc in material that does not bind to *Pisum sativum* lectin (see main text). The cause of the remaining resistance to sequential digestion is unresolved (note that similar digestions of standard oligosaccharides give complete conversion to the trimannosyl core).

In contrast to the above finding, anionic oligosaccharides synthesized in the presence of BFA are much smaller and none have polylactosamines or terminal α-Gal residues (Figure 10). Very few are larger than a tetra-antennary chain with two terminal β-Gal residues. Individual β-Gal and β-hexosaminidase digestions partially reduce the size of this complex array of chains, but only when done sequentially in that order does any of material run in the position of the trimannosyl core. As in the case of the controls, some residual material remains undigestible by β-hexosaminidase. However, unlike the controls, prior mild acid treatment does not affect this distribution (Figure 10, Panel N), indicating the absence of Fuc on outer branches. The loss of outer branch Fuc residues is supported by the marked reduction of [^{3}H]Fuc in material that does not bind to *Pisum sativum* lectin (see main text). The nature of the residual resistance to sequential is unknown, but presumably is the same as that seen in the control.

Based on the size and the results of the digestion, about 70% of the chains in the BFA sample contain 1 β-Gal residue, and 30% contains 2 β-Gal residues, each with outer sialic acid residues. This corresponds well to the amounts of species with 1 and 2 charges. Taken together with the absence of terminal β-Gal residues in neutral oligosaccharides (see below), the data indicate that every time a β-Gal residue is added, it is immediately capped with a sialic acid residue. These results and those presented above for the major ConA III anionic species (Figure 8, and main text) suggest that addition of β-Gal is the key limiting step in further maturation of complex-type chains in the presence of BFA.

Effects of BFA on the Processing of Neutral Oligosaccharides.

Neutral ConA I Fraction - Oligosaccharides from control cells were resistant to β-galactosidase digestion, and bound to *Griffonia simplicfolica* -agarose columns via their terminal α-Gal residues. About 15% contained polylactosamine chains that terminated in an α-Gal residue. Sequential digestion with endo-β-Galactosidase followed by β-galactosidase and β-hexosaminidase digestion produced only a small portion of $Man_3GlcNAc_2$ core molecules (data not shown). When these digestions were preceded by α-galactosidase digestion, the chains no longer bound to *Griffonia* lectin (Figure 12), and subsequent digestion with β-Gal and β-hex reduced the majority of the material nearly to the size of the Man_3 $GlcNAc_2$ core structure (Figure 11). Thus, terminal α-Gal units were also abundant on non-repeating lactosamine units. Mild acid treatment (MA50) removed a major resistant peak around fraction 25 and shifted the remaining material exactly to the position of the trimannosyl core, suggesting the presence of outer branch and core α-fucosylation in the control cells. Thus, as with the anionic molecules (see above), the resistance to complete digestion could be explained only partly on the basis of outer Fuc residues. The corresponding molecules synthesized in the presence of BFA were much smaller and contained mostly 3 and 4 terminal GlcNAc residues. Treatment with α-galactosidase, endo-β-galactosidase, and β-galactosidase had no effect on the HPLC pattern, while β-hexosaminidase digestion alone reduced the great majority to the tri-mannosyl core structure. Prior mild acid treatment did not increase the amount of material running at the tri-mannosyl core nor reduce the amount of residual material resistant β-hexosaminidase A digestion, showing that outer branch fucosylation did not occur.

Neutral ConA II Fraction - Neutral molecules in this fraction synthesized in the presence of BFA consists of a mixture of Man_5 and Man_6 high mannose chains together with, biantennary chains terminated with 1 or 2 GlcNAc residues. Again, none had any terminal β-Gal residues. A combination of α-mannosidase and β-hexosaminidase digestions converted all of the material to free [^{3}H]Man or to [^{3}H]$ManGlcNAc_2$ (Figure 13). In the absence of BFA, this fraction contained bi-antennary molecules with terminal α-Gal residues and some high mannose-type chains. The large peak around Fraction 20 in Panel F is α-mannosidase resistant, and remains unidentified.

Neutral ConA III Fraction - In control cells without BFA, this fraction contains, in decreasing order, Man_9-Man_5 oligosaccharides that are all sensitive to α-mannosidase digestion. In the presence of BFA, this fraction contains two types of chains: a-mannosidase-sensitive chains of Man_{5-9} and biantennary GlcNAc terminated chains. Again, none of molecules in this latter group contained terminal β-Gal residues, and β-hexosaminidase digestion alone converts this material to the $Man_3GlcNAc_2$ core structure (Figure 14). The weighted average size of the high mannose-type chains in control cells is 7.6 Man residues and 6.6 Man residues in the BFA samples, indicating further mannose processing in the latter.

It is apparent that terminal β-Gal residues are not added to any of the neutral oligosaccharides in the presence of BFA; they are only found on anionic chains where they are always capped by sialic acids, but not by α-Gal residues.

Reversibility of the Effects of BFA on Oligosaccharide Structure. To determine whether normal oligosaccharide processing resumes after BFA is removed, cells were labeled with [^{3}H]Man for 30min in the presence of 1 μg/ml BFA and then chased for 3 h as before. BFA was then removed and the cells were maintained in culture for 12 h to allow secretion to resume as shown in Figure 2. At that point, the material in the medium and the cells was pooled and the *N*-linked oligosaccharides were released with PNGaseF, fractionated and analyzed as before. Table I shows that the proportions of the various ConA fractions and, therefore the amount of high mannose-type di-, tri-, and tetraantennary complex-type chains were the same in both control and recovered samples. The proportion of anionic oligosaccharides (50%), and their charge distribution (QAE-Sephadex) were also the same in control and BFA-recovered samples. Sialic acids accounted for most of the negative charge (in ConA fractions I+II) of both samples, although this was somewhat higher in the BFA sample (87%) than the control (75%). Sequential glycosidase digestion using β-hexosaminidase A at pH 3.5 confirmed that sulfate esters were found on molecules with 1, 2, and 3 charges (44% of control) (data not shown). Desialylated oligosaccharides also contained terminal α-Gal residues as shown by their binding to *Griffonia* lectin. The average number of α-Gal residues per chain was higher in the controls since they were more retarded than the BFA-recovered samples (Figure 15). α-Galactosidase digestion eliminated all binding to *Griffonia*. None of the HPLC patterns of the controls are shown, since their patterns were identical to those already presented for the earlier labelling. HPLC analysis of the BFA-recovered neutral ConA I fraction and desialylated anionic ConA fraction I+II (Figure 16) showed terminal α-Gal and β-Gal residues (while the controls had only α-Gal) showing that the addition of α-Gal was not complete when BFA was removed. Thus, during the recovery from BFA, not all of the newly added β-Gal residues are automatically capped by sialic acids even though α-galactosylation is limiting. This indicates that these two reactions are not truly competitive. Polylactosamine chains were also partially restored to the BFA samples (43% of control), but only on the sialylated molecules.

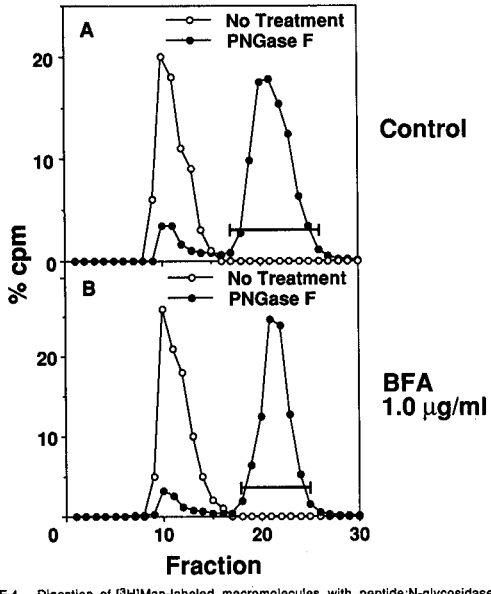

FIGURE 4. Digestion of [^{3}H]Man-labeled macromolecules with peptide:N-glycosidase F. The macromolecules were isolated from the void volume of an S-200 Sephacryl column and digested with PGNaseF as described under "Experimental Procedures". The upper panel shows control cells and the lower panel shows the cells incubated with 1.0μg/ml BFA with and without digestion. The areas indicated by the |——| were pooled for further study.

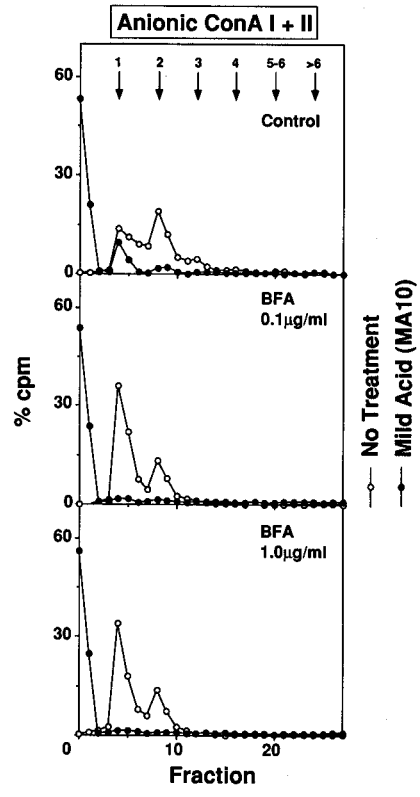

FIGURE 6. QAE-Sephadex analysis of ConA I+II anionic oligosaccharides formed in the presence and absence of BFA. Oligosaccharides synthesized in the absence (Upper panel) and presence of 0.1μg/ml (Middle panel) and 1.0μg/ml BFA (Lower panel) were analyzed by QAE-Sephadex chromatography before and after mild acid treatment (MA10) to remove sialic acids. The arrows show the beginning of elution with different NaCl concentrations needed to elute oligosaccharides with the indicated number of negative charges as described in (39).

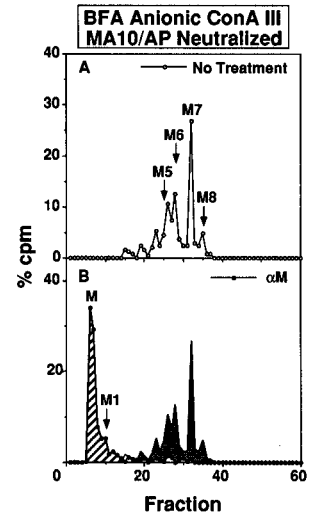

FIGURE 9. HPLC analysis of dephosphorylated chains from BFA ConA III. Fraction of the BFA sample. Anionic material that was neutralized by alkaline phosphatase digestion was analyzed by HPLC before and after α-mannosidase digestion.

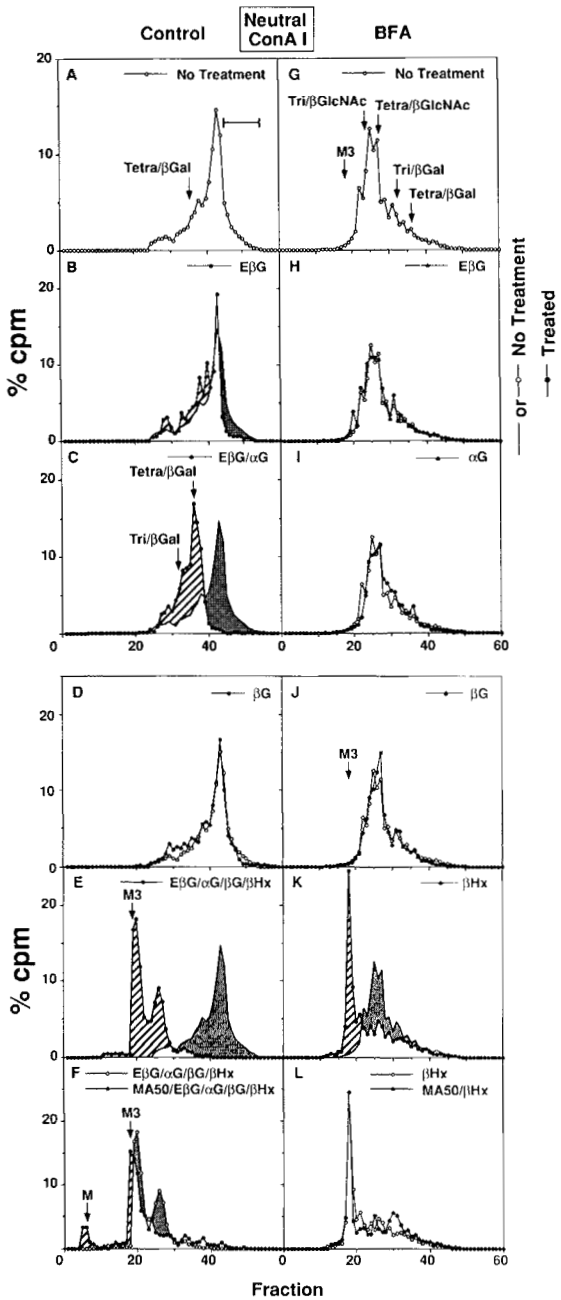

FIGURE 11. HPLC analysis of neutral ConA I fractions. Oligosaccharides from controls (Panels A-F) or BFA samples (Panels G-L) were analyzed without any treatment in panels A and G. The digestions were as shown in each panel with the appropriate standards are indicated. In panels F and L, the shaded and striped areas compare the effects of a prior acid treatment on the digestions indicated. These acid treatments (72) are designed to remove Fuc residues in the core region and on the branches of the chains shown in Panel E and to account for the shift. This type of change is not seen in the BFA samples (panel L), suggesting that the Fuc residues are not added to these chains on the outer branches.

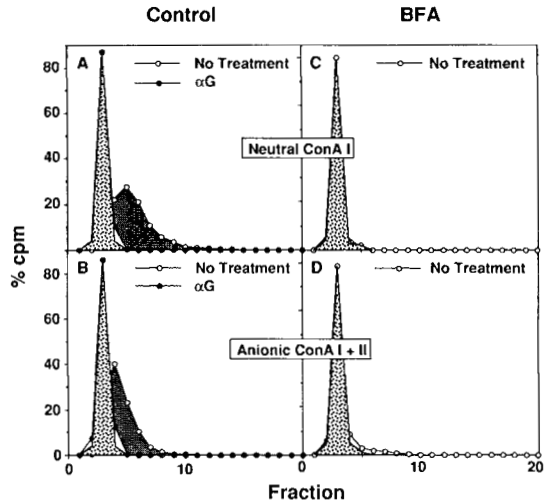

FIGURE 12. Analysis of neutral and anionic oligosaccharides on *Griffonia simplicifolia* lectin-agarose columns. Oligosaccharides were mixed with a non-binding ^{14}C-labeled glycopeptide and applied to a column containing *Griffonia simplicifolia* lectin as described in Experimental Procedures. At a concentration of 2 mg of lectin/ml of gel, oligosaccharides with increasing number of terminal α-Gal residues are progressively retarded on the column(74), but none are bound and require competitor for elution (not shown). In each case, 80-90% of the applied radioactivity was accounted for. The dotted area indicates the elution position of the unbound marker. In each case, α-galactosidase digestion caused all of the radioactivity to run exactly co-incident with the unbound marker. Panels are described on the figure.

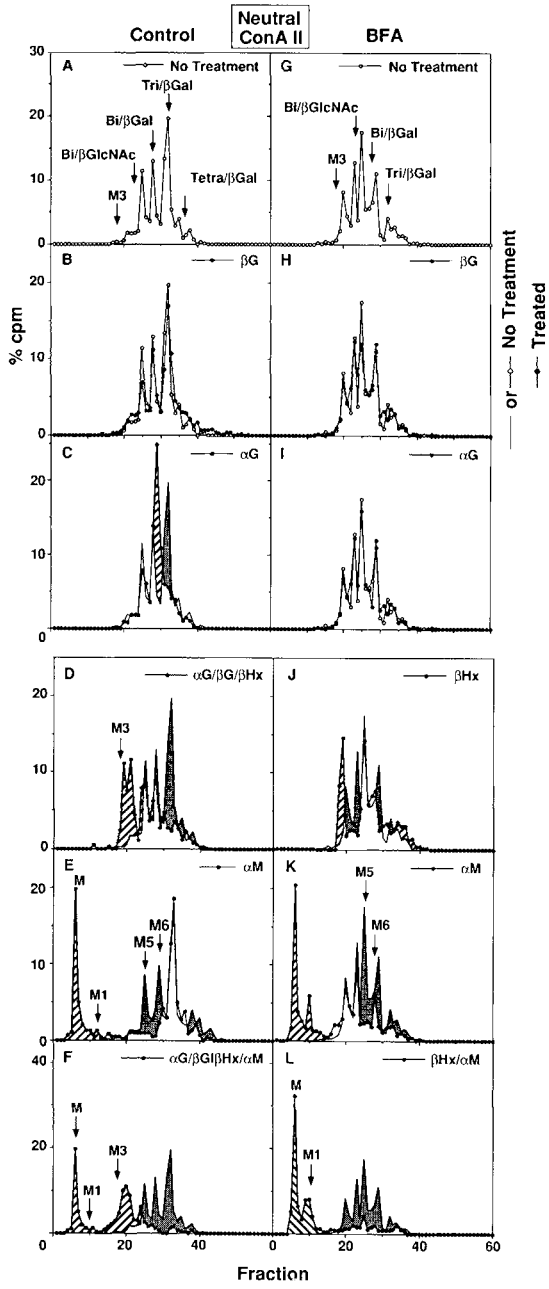

FIGURE 13. HPLC analysis of ConA II neutral oligosaccharides. The oligosaccharides were reduced and applied to the HPLC column without any treatment (Panels A and F) or following the indicated digestions. As in the other figures, the standards are as shown in Table III, the shaded area shows a loss of material compared to the control and the striped area indicates a gain. The large peak around fraction 20 in panel F is α-mannosidase resistant and remains unidentified.

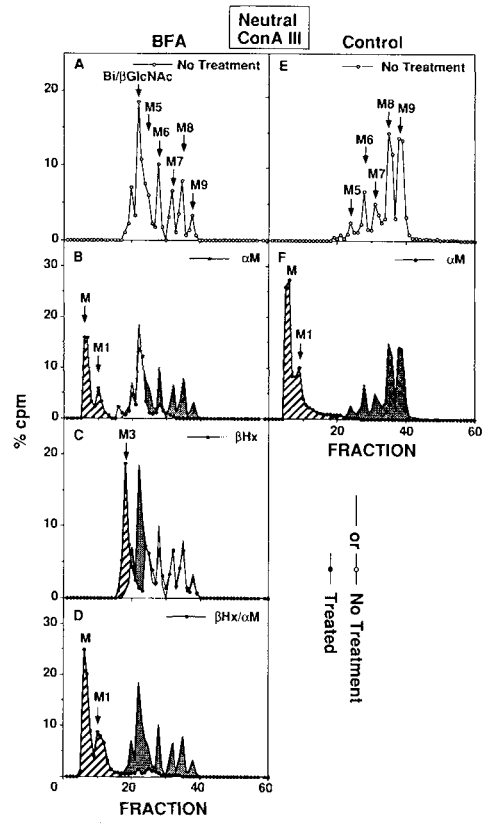

FIGURE 14. HPLC analysis of ConA III neutral oligosaccharides. The oligosaccharides were reduced and analyzed by HPLC. All panels are for BFA samples except for panels E and F (control). The digestions are indicated in each panel, and the standards are as presented in Table III.

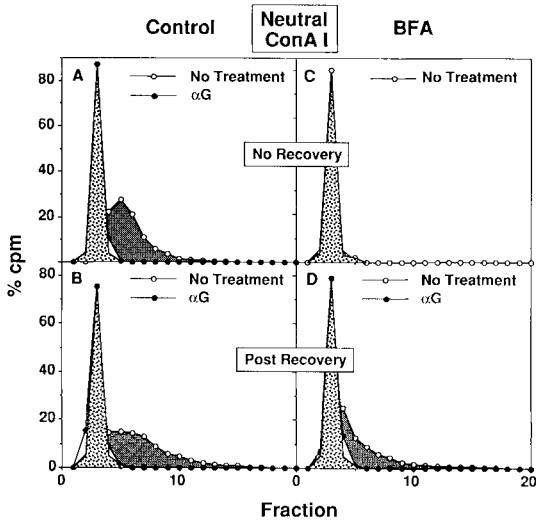

FIGURE 15. Analysis of recovered neutral oligosaccharides on *Griffonia simplicifolia* lectin-Agarose columns. Analysis was performed as described in Figure 12 and in "Experimental Procedures." Panels A and C show ConA I neutral fractions from control and BFA samples, while panels B and D show these respective fractions after the recovery period. The dotted area indicates the elution position of the unbound marker. In each case, α-galactosidase digestion caused all of the radioactivity to run exactly co-incident with the unbound marker.

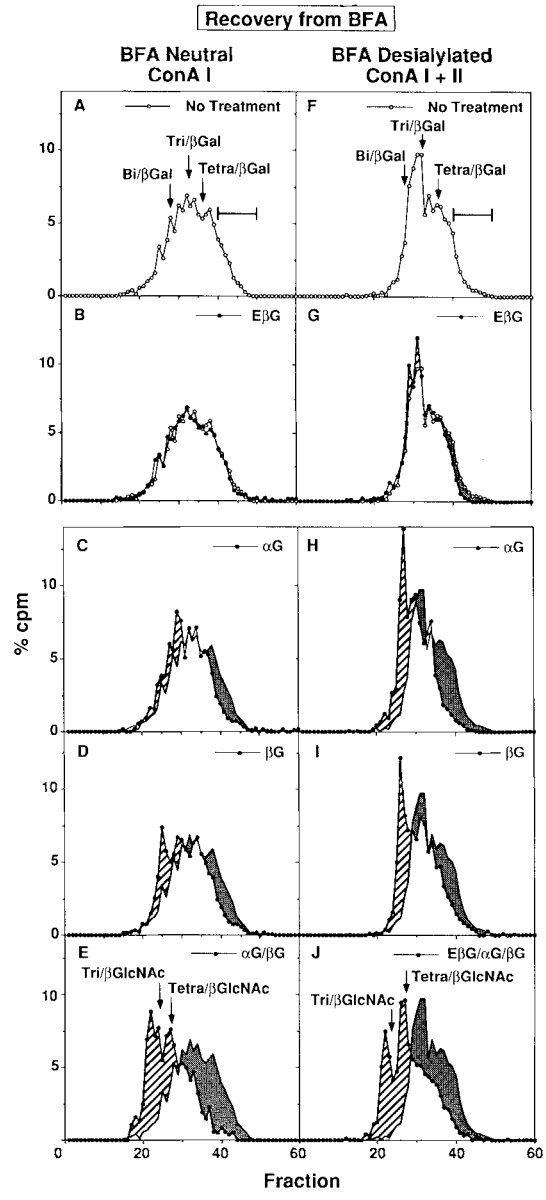

FIGURE 16. HPLC analysis of BFA neutral ConA I fraction and anionic ConA I+II fraction following recovery. Following recovery from the BFA block, neutral oligosaccharides from ConA fraction I (Panels A-E) and desialylated anionic oligosaccharides from ConA I+II were isolated and analyzed by HPLC. Treatments are indicated in each panel. Controls without BFA showed the same pattern and size-distribution as seen in the earlier labelling experiment and are hence not shown here.