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Complete Golgi passage of glycotripeptides generated in the endoplasmic reticulum of mammalian cells

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Abstract The tripeptide, *N*-octanoyl-Asn-[^{125}I]Tyr-Thr-NH_2, which contains the acceptor sequence for N-glycosylation, is readily taken up by cell culture cells, glycosylated in the endoplasmic reticulum (ER), and secreted into the medium. Therefore such glycosylated tripeptides have been used as markers for the vesicular flow from the endoplasmic reticulum to the plasma membrane [(1987) Cell 50, 289–300; (1990) J. Biol. Chem. 265, 20027–20032]. We have now studied the pathway taken by the glycotripeptides in mammals in more detail. In the perfused rat liver, the glycotripeptides secreted to the medium were analyzed by digestion with exoglycosidases, and a significant fraction was found to contain the terminating sequence -Gal-Sial, which is generated by processing enzymes that reside in the late Golgi apparatus. Thus we conclude that these glycotripeptides have passed through the complete Golgi complex on their way from the ER to the cell surface.

Key words: Vesicular transport; Golgi apparatus; Glycotripeptide

1. Introduction

Tripeptides with a hydrophobically modified amino terminus, a blocked carboxyl terminus and the sequence necessary for N-glycosylation, Asn-Xaa-Ser/Thr, are readily taken up by living cells and glycosylated in the endoplasmic reticulum (ER) [1,3]. Once glycosylation has occurred, however, they are no longer able to permeate membranes and must be actively transported out of the ER if they are to be secreted. This transport is presumed to occur by the constitutive secretory pathway, which proceeds in vesicles from the endoplasmic reticulum through the Golgi complex to the plasma membrane [1,4]. Glycosylated *N*-octanoyl-Asn-[^{125}I]Tyr-Thr-amides (glyco-OTP) are highly unlikely to contain any transport or retention signals, and therefore have been used to determine the rate at which bulk material travels along the secretory pathway. Resistance to endoglycosidase H (Endo H), typically acquired in the medial Golgi, of the glycotripeptides secreted from Chinese hamster ovary (CHO) cells [1] as well as dependence of secretion on ATP, cytosol and GTP hydrolysis in semi-intact CHO cells [2] were taken as indication that the glycotripeptides are transported through the Golgi in a vesicular fashion, indeed similar to newly synthesized secretory proteins.

Investigation of the [^{125}I]glycotripeptide species secreted by CHO and HepG2 cells had not given conclusive evidence for a passage through the complete Golgi apparatus, as modifications with galactose and sialic acid, typically acquired in the 'late' Golgi, were not detected [1]. The resistance observed to Endo H is acquired already in the medial Golgi [5], and consequently, complete passage of the glycotripeptides through the Golgi apparatus, including the *trans* Golgi, has been challenged [6,7]. In order to investigate the complete pathway taken by these glycopeptides, we therefore studied their secretion in perfused rat liver, a system that most closely resembles in vivo conditions. We report here a glycosylation structure typical of late Golgi processing of the carbohydrate chain of a major portion of the glycotripeptides secreted to the medium, implying that [^{125}I]glyco-OTP indeed passes through the complete Golgi apparatus on its way to the cell surface in rat liver.

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Abbreviations: ER, endoplasmic reticulum; OTP, *N*-octanoylated tripeptide Asn-Tyr-Thr-NH_2; Endo H, endoglycosidase H; CHO, Chinese hamster ovary; ConA, concanavalin A; TLC, thin-layer chromatography; VSV, vesicular stomatitis virus.

2. Experimental

2.1. Peptide synthesis and iodination

OTP was synthesized by solid phase peptide synthesis employing Fmoc amino acids according to [1]. ^{125}I iodination was performed as described [1] using the chloramine T procedure. Specific activity ranged from 1 to 2×10^8 cpm/nmol. The iodinated tripeptide was dissolved in dimethyl sulfoxide at 5×10^6 cpm/μl.

2.2. Liver perfusion

Liver perfusion experiments were carried out essentially as described [8]. The liver was removed from 200 g male Wistar rats anesthetized with Evipan (Bayer, Leverkusen, Germany). The perfusion medium consisted of RPMI 1640 (Seromed) supplemented with $NaHCO_3$ (2000 mg/l) and 1% (w/v) bovine serum albumin. After erythrocytes had been washed out, 50 ml medium was circulated through the liver, and 600 μCi (25 μg) [^{125}I]OTP in 100 μl dimethyl sulfoxide diluted 1:10 with perfusion medium was added. After 60 min, the medium was collected and [^{125}I]glyco-OTP was isolated from 1 ml aliquots by ConA-Sepharose chromatography as described [1]. The liver was cut into small pieces, suspended at 250 mg/ml in ice-cold buffer A (10 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.5% Triton X-100), shredded with an Ultra-Turrax (Ika, Staufen/Germany), filtered through cheesecloth, and homogenized in a Potter-Elvehjem homogenizer with motor-driven pistle at 4 °C. The homogenate was centrifuged at 1400 rpm ($400 \times g$) for 5 min in a Heraeus Omnifuge. The supernatant was heated to 95°C for 2 min, recentrifuged, and 1 ml aliquots of this supernatant were applied to ConA-Sepharose columns. [^{125}I]glyco-OTP was eluted with 0.5 M methyl-α-D-mannopyranoside as described [1].

2.3. Incubations with Brefeldin A

Wild-type CHO cells were grown in suspension cultures in α-MEM (Gibco) as described by Balch et al. [9]. For incubations with Brefeldin A, cells were preincubated in the presence of Brefeldin A (10 μg/ml), added from a 10 mg/ml stock solution in methanol. The same amount of methanol was added to control cells. After 30 min preincubation, cells were centrifuged ($400 \times g$, 5 min) and resuspended in α-MEM supplemented with Brefeldin A (10 μg/ml) or the corresponding volume of methanol (control) at 1×10^7 cells/ml. As a control for Brefeldin A action, an increase of sphingomyelin synthesis was measured according

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to Brüning et al. [10]. Further incubation procedures were as described by Wieland et al. [1].

Rat liver perfusion experiments with Brefeldin A were carried out by continuously pumping fresh RPMI 1640 medium with $NaHCO_3$ (2000 mg/l) either with or without 20 μg BFA/ ml through the liver at a rate of 7 ml/min. [^{125}I]OTP was added after a 15-min pulse with 1 mCi [^{35}S]methionine/cysteine. Chase medium contained 5 mM each of methionine and cysteine. Five minute medium fractions were collected, and [^{125}I]glyco-OTP was isolated from one third of the fractions by ConA-Sepharose chromatography. ^{35}S-Labeled secretory proteins were immunoprecipitated from another third of the fractions with an antibody against rat whole serum (Sigma).

2.4. Glycotripeptide analysis

Glycotripeptides eluted from ConA Sepharose columns in buffer A without Triton X-100 were applied to a SepPak C-18 cartridge (Waters), washed with 20 ml H_2O, eluted with 60% Acetonitrile and dried in a SpeedVac concentrator. About 1–5 μl of an aqueous solution of the concentrate was spotted onto a silica gel thin layer plate with grooved channels (Whatman). Chromatography was performed in butanol/acetic acid/water (5:2:2, v/v/v). The TLC plates were dried thoroughly and exposed to X-ray film (Kodak XAR5) at -70 °C, using an intensifying screen (DuPont Cronex Hi-plus). Enzymatic digestions of glycotripeptides were usually carried out over night in X-11 buffer, pH 5.5, supplied by Oxford Glycosystems, except for *N*-glycanase digestions, which were performed in a 50 mM sodium phosphate buffer, pH 7.5. Glycosidases were from Oxford Glycosystems, except for Endo H and *N*-glycanase (Boehringer). Digestions in which several enzymes were used were carried out either sequentially or simultaneously, both procedures giving the same results.

3. Results

3.1. Glycotripeptides secreted by the perfused rat liver contain sialic acid

In order to study [^{125}I]glycotripeptide secretion in mammals more closely with regard to the pathway taken, [^{125}I]OTP was added to the medium of a perfused rat liver. The intracellularly formed [^{125}I]glycotripeptides (4% of added [^{125}I]OTP) were isolated after one hour perfusion from the homogenized liver and the perfusion medium, respectively. Following enzymatic digestion with various glycosidases, the [^{125}I]glycotripeptides from liver extract and medium were analyzed by thin-layer chromatography (TLC) and autoradiography (Fig. 1A). [^{125}I]Glycotripeptides recovered from the liver were not susceptible to sialidase treatment (see Fig. 1A, lanes 1 and 2), and were

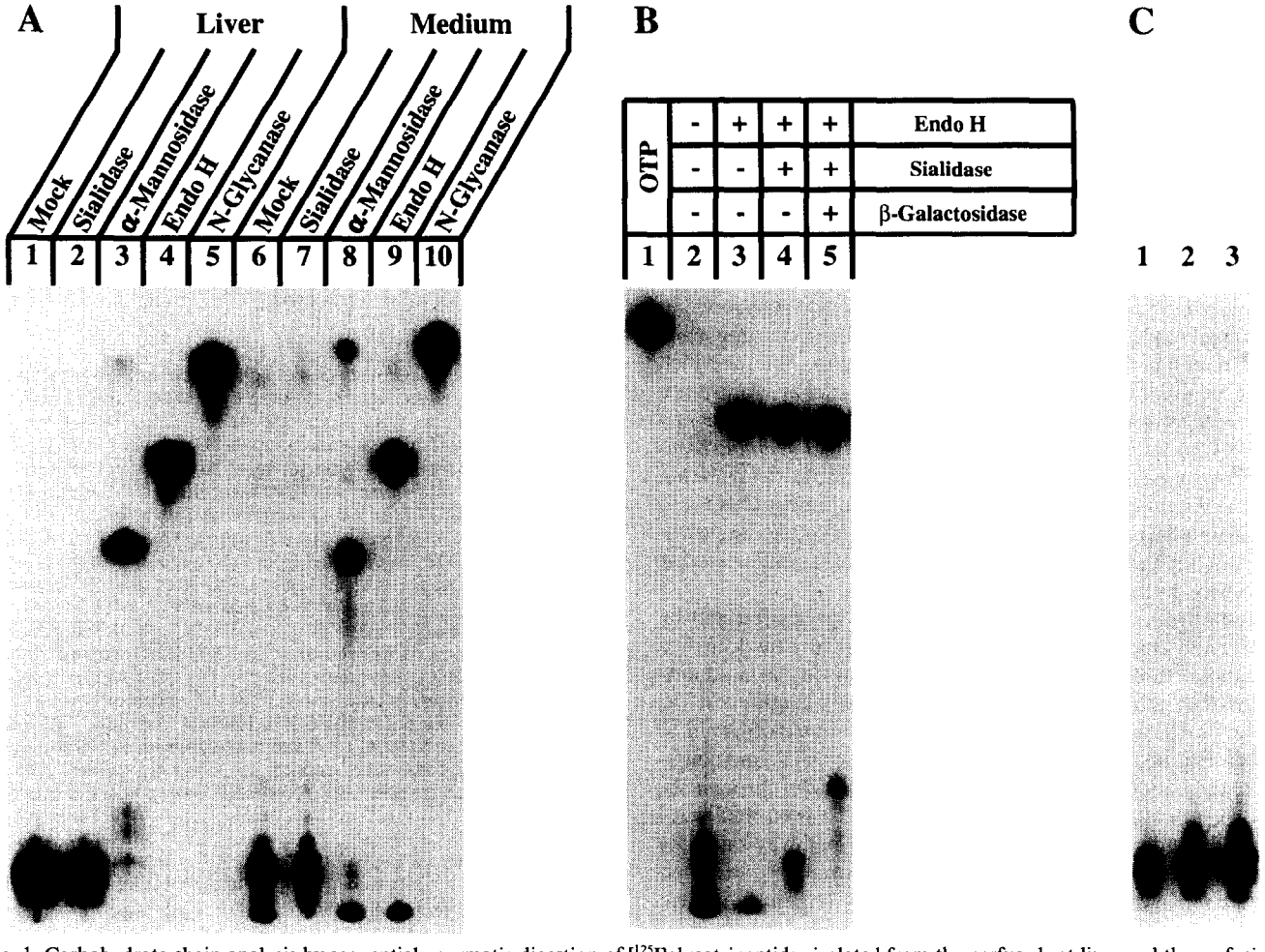
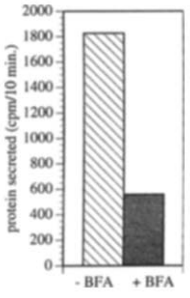
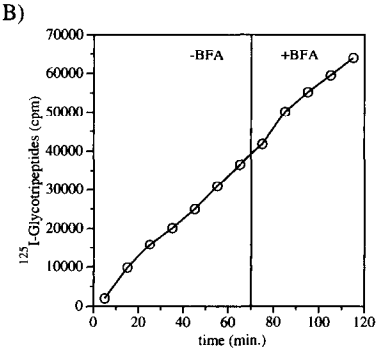


Fig. 1. Carbohydrate chain analysis by sequential enzymatic digestion of [^{125}I]glycotripeptides isolated from the perfused rat liver and the perfusion medium. Panel A, [^{125}I]glycotripeptides were isolated from a liver homogenate and from the perfusion medium after one hour of perfusion as described in section 2, and incubated for 16 h with the glycosidases indicated. Panel B, simultaneous digestion of [^{125}I]glycotripeptides from the perfusion medium with the glycosidases indicated. Panel C, [^{125}I]glycotripeptides before and after reperfusion (see text). Lane 1, liver extracted material before reperfusion (as in lane 1 of panel A); lane 2, [^{125}I]glycotripeptides reisolated from the perfusion medium; lane 3, the material from lane 2 after digestion with sialidase.

2 A)



2 B)



C

	- BFA					+ BFA					OTP
Endo H	-	+	+	+	-	-	+	+	+	-	
Sialidase	-	-	+	+	-	-	-	+	+	-	
β-Galactosidase	-	-	-	+	-	-	-	-	+	-	
N-Glycanase	-	-	-	-	+	-	-	-	-	+	
	1	2	3	4	5	6	7	8	9	10	11

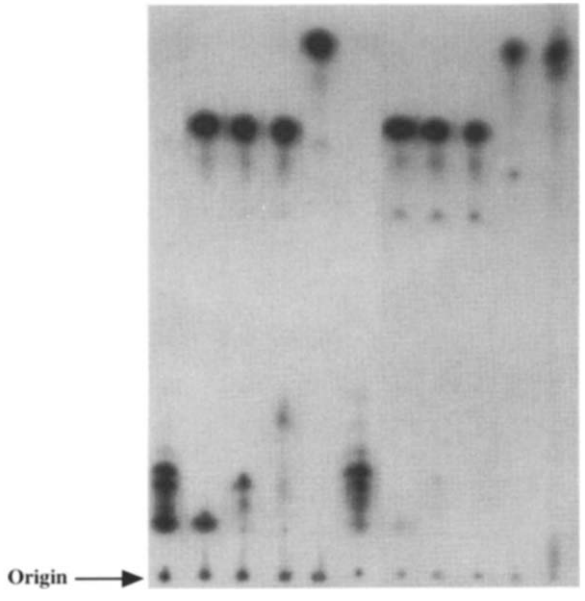


Fig. 2. Secretion by the perfused rat liver of ^{35}S-labeled proteins and [^{125}I]glycotripeptides in the absence and presence of Brefeldin A. Panel A, ^{35}S-labeled secretory proteins were immunoprecipitated from the medium of the perfused rat liver with an antibody against rat whole serum. The rate of secretion is given as immunoprecipitated ^{35}S-cpm/10 min fraction in the absence and presence of Brefeldin A. Panel B, secretion of [^{125}I]glycotripeptides in the absence and presence of Brefeldin A. cpm of combined 10 min fractions are given. Panel C, carbohydrate chain analysis of [^{125}I]glycotripeptides secreted by the perfused rat liver in the absence (lanes 1–5) and presence (lanes 6–10) of Brefeldin A.

completely hydrolyzed by Endo H (lane 4). This material was also cleaved by Jack Bean α-mannosidase (lane 3), an exoglycosidase which cleaves off terminal mannose residues bound in α-linkage. The TLC spot arising from this digestion should correspond to the peptide backbone connected to two *N*-acetylglucosamines and a β-bound mannose, while Endo H treatment leaves only the innermost *N*-acetyl-glucosamine attached to the peptide. From these digestions, high-mannose structures can be deduced for the carbohydrate chains present in the liver fraction [5].

[^{125}I]Glycotripeptides isolated from the perfusion medium, which accounted for 3.5% of the [^{125}I]glycotripeptides formed, showed a different glycosylation pattern (Fig. 1A, lanes 6–10). The slowest migrating spot in TLC, which comprised 30% of the radioactivity in lane 6, was no longer present after sialidase treatment, and at the same time completely resistant to Endo H. The remainder of the [^{125}I]labeled material in lane 6 was cleaved by Endo H as well as by Jack Bean α-mannosidase, and is therefore considered to represent high-mannose type oligosaccharide structures.

The Endo H resistant material was investigated in more detail by treating the secreted [^{125}I]glycotripeptides with exoglycosidases (in the presence of Endo H to eliminate the faster-migrating high-mannose species) and subsequent analysis by TLC and autoradiography (Fig. 1B). A sequentially higher mobility after sialidase and sialidase/β-galactosidase digestion was observed that indicates a glycosylation pattern typical of complex carbohydrates, which are formed by the sequential processing of the carbohydrate chain by glycosyl transferases residing in the *cis* (Golgi α-mannosidase I), *medial* (*N*-acetylglucosaminyltransferases I and II and Golgi α-mannosidase II), and *trans* Golgi (galactosyltransferase and sialyltransferase) [5,11–13].

Although this finding suggests that the secreted glycotripeptides have passed through the complete Golgi apparatus, it cannot be excluded that the galactosyl and sialic acid residues have been acquired by [^{125}I]glycotripeptides that had been secreted as high mannose types in the first place and subsequently taken up by the cells with reprocessing of their carbohydrate structures, as described for the cell-surface expressed dipeptidylpeptidase IV [14].

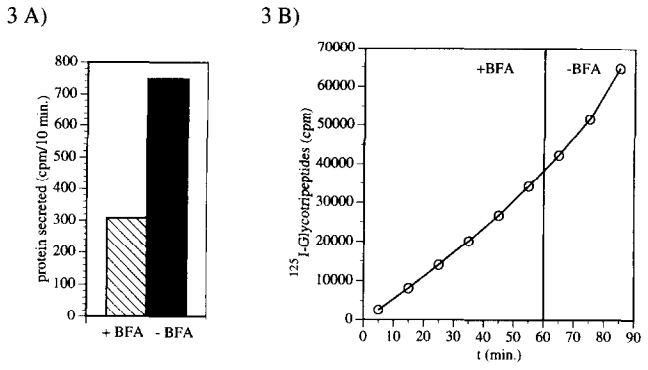
In order to investigate this possibility, a [^{125}I]glycotripeptide fraction isolated from the liver homogenate (that contained exclusively high-mannose chains, see above) was added to the medium of another perfused rat liver. After 1 h of reperfusion, [^{125}I]glycotripeptides were re-isolated by ConA sepharose column chromatography. While 10% of the recovered radioactivity was associated with the liver, this material no longer bound to Concanavalin A Sepharose, presumably because it was degraded. Likewise, 14% of the radioactivity recovered from the medium did not bind to Concanavalin A sepharose. The re-isolated material bound to ConA Sepharose was again analyzed by TLC and autoradiography. As shown in Fig. 1C, the glycosylation pattern had been altered only slightly by the reperfusion, with an additional, faster-migrating spot which, however, is not susceptible to sialidase digestion. The slower-migrating spot from Fig. 1A, lane 6, which we have shown to contain sialic acid, does not appear. Thus, sialylation is not due to reuptake by the liver and must therefore have occurred during passage of the secreted [^{125}I]glycotripeptides through the complete Golgi apparatus along the biosynthetic secretory pathway.

3.2. *Brefeldin A does not inhibit secretion of [^{125}I]glycotripeptides*

The fungal metabolite Brefeldin A is known to inhibit secretion of proteins in various cell lines by a mechanism which is

not yet fully understood but which results in dissociation of the Golgi complex and partial redistribution of Golgi markers to the ER [15,16]. Therefore, we expected this antibiotic to inhibit [^{125}I]glycotripeptide secretion in cells that are susceptible to Brefeldin A. Surprisingly, however, when the drug is added to CHO cells, the [^{125}I]glycotripeptides are secreted at the same rate as in the untreated control (not shown). The action of Brefeldin A under the conditions applied was monitored by measurement of an increase of truncated sphingomyelin synthesis according to Brüning et al. [10] (not shown).

This unexpected result prompted us to analyze the [^{125}I]glycotripeptides formed in rat liver perfused in the presence of BFA. To this end, BFA was added in a high concentration (20 μg/ml) to the perfusion medium. In order to maintain an efficient concentration of the antibiotic, which is degraded efficiently by glutathion-*S*-transferase [17], the perfusion medium was not recycled in these experiments. As a control for the action of BFA we monitored the secretion of ^{35}S-labeled proteins into the medium by immunoprecipitation with anti-rat serum antibodies. In a first experiment, perfusion after addition of [^{125}I]OTP was for 70 min in the absence of BFA, and subsequently for 50 min in the presence of BFA. The result is shown in Fig. 2. Although not blocked completely, protein secretion was reduced significantly by 70% (Fig. 2A). In contrast, secretion of [^{125}I]glycotripeptides was not affected at all (Fig. 2B). A comparison of the [^{125}I]glycotripeptide fractions obtained from the media in the absence and presence of BFA is given in Fig. 2C).Without BFA, the pattern shown in Fig. 1 was confirmed. The slowest migrating species again could be assigned to glycopeptides containing -Gal-Sial. However, this type of complex carbohydrate is missing in the fraction obtained in the presence of BFA. Instead, most of the material represents 'high-mannose' forms. This change of carbohydrate structures in the secreted [^{125}I]glycotripeptides is reversible, as shown in Fig. 3. In this experiment, [^{125}I]OTP was added to the medium after 15 min of perfusion with BFA. After additional 60 min in the presence of BFA, perfusion was continued for another 30 min in the absence of BFA. Protein secretion was reduced to 25% as compared to the secretion observed in the absence of BFA in a control experiment (see Fig. 2A). Switching from medium + BFA to medium −BFA resulted in an increase of protein secretion by 145% (Fig. 3A), indicating reversibility of the BFA effect on protein secretion in this system, whereas [^{125}I]glycotripeptide secretion was not significantly increased (Fig. 3B). Analysis of the glycotripeptide fractions isolated from the respective media clearly demonstrates that the effect of BFA on the carbohydrate structures was reversible, as depicted in lanes 6–10 of Fig. 3C).



C

	+ BFA					- BFA					OTP
Endo H	-	+	+	+	-	-	+	+	+	-	
Sialidase	-	-	+	+	-	-	-	+	+	-	
β-Galactosidase	-	-	-	+	-	-	-	-	+	-	
N-Glycanase	-	-	-	-	+	-	-	-	-	+	
	1	2	3	4	5	6	7	8	9	10	11

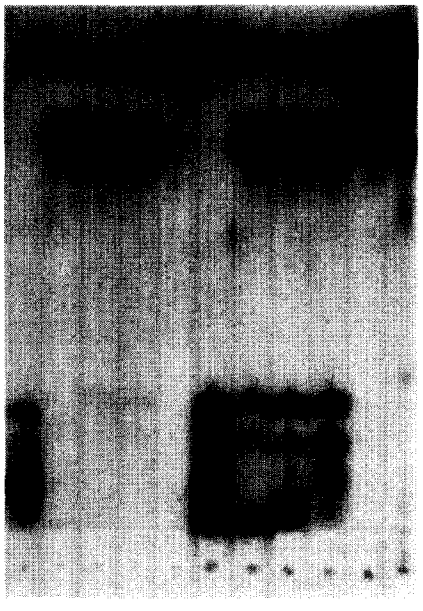


Fig. 3. Reversibility of the effects of Brefeldin A on protein secretion and [^{125}I]glycotripeptide glycosylation. Panel A, immunoprecipitated ^{35}S-labeled secretory proteins. Panel B, secretion of [^{125}I]glycotripeptides in the presence and absence of Brefeldin A. Panel C, carbohydrate chain analysis of [^{125}I]glycotripeptides secreted by the perfused rat liver in the presence (lanes 1–5) and absence (lanes 6–10) of Brefeldin A.

4. Discussion

In this paper, we have demonstrated that about 30% of the [^{125}I]glycotripeptides secreted by rat liver possess a complex carbohydrate chain that would require passage through the entire Golgi complex. Not all of the [^{125}I]glycotripeptides secreted have been processed, but it is surprising, anyway, that all of the sequential processing steps which are necessary for complex glycosylation can be performed on glyco-OTP with any degree of efficiency, as additional signals for glycoprotein processing are presumed to be needed, considering that secretory proteins can differ markedly with regard to their oligosaccharide structures. Also, most N-glycosylated sites in eukaryotic glycoproteins are associated with several different oligosaccharide structures [18]. These results for the first time provide evidence for passage of the glycotripeptides through the complete secretory pathway from the ER via the entire Golgi complex to the plasma membrane.

Brefeldin A does not inhibit secretion of [^{125}I]glycotripeptides, neither in CHO cells nor in the perfused rat liver. This

suggests a major difference in the transport properties of glycotripeptides as compared to proteins. One possible explanation that seems to be ruled out is the alternative secretory pathway recently reported for some secretory proteins (reviewed in [19]). These proteins seem to bypass the ER, since they lack cleavable signal sequences and are not glycosylated although some of them contain the consensus sequence for N-glycosylation. N-Glycosylation within the ER, however, is the prerequisite for formation of [^{125}I]glyco-OTP. This leaves two possibilities: (a) under physiological conditions, proteins and glycotripeptides employ the same set of transport vesicles, but under the non-physiological conditions imposed by Brefeldin A, a novel transport pathway is opened which cannot be taken by proteins, but is only available to small luminal molecules; or (b) proteins and small molecules such as the [^{125}I]glycotripeptides are secreted by different sets of transport vesicles, both types of vesicle passing the complete Golgi apparatus. In this case, Brefeldin A would not inhibit the pathway taken by small molecules. Evidence for this latter possibility can be found in the literature [20]. Newly synthesized cholesterol is present in vesicles that on sucrose density gradients comigrated with newly synthesized vesicular stomatitis virus (VSV) G protein, known to pass through the Golgi stack. However, cholesterol transport to the cell surface occurred with the same kinetics in the presence or absence of Brefeldin A, in agreement with our findings for the glycotripeptides. Similar observations have recently been published for sphingomyelin transport [21].

Regarding the application of the glycotripeptides as markers for vesicular bulk flow in mammalian cells, one important conclusion we had drawn previously [1] needs to be modified in light of data accumulated since. The glycopeptides were found to be transported to the cell surface as fast as the fastest known newly synthesized protein, and therefore we had concluded that cargo proteins are not concentrated during budding of an ER transport vesicle. However, since 1987 a major body of evidence has revealed that proteins require a substantial period of time after synthesis (5 min to over an hour [22–24]), during which they fold to assume a transport-competent structure. Therefore, the kinetics of glycotripeptide secretion do not rule out a concentration effect for proteins. In fact, in a recent publication [25], it was shown that VSV G protein is concentrated into ER transport vesicles. However, it should be noted that this concentration does not necessitate any signal-mediated uptake into vesicles. Newly synthesized (i. e. newly folded) proteins as a class may be directed into budding vesicles as they are released from chaperones, a mechanism that at the same time would explain the phenomenon of quality control. Another explanation for concentration of cargo membrane proteins into transport vesicles is that the ER membrane is densely populated by resident proteins, thus leaving only little space for secretory membrane proteins such as the VSV G protein, whereas transport vesicles, lacking the bulk of ER-resident proteins, can accomodate larger amounts of cargo per unit membrane area. These questions have been discussed in a recent review [26].

On the other hand, the rate of flow of glycopeptide and truncated lipid markers through the Golgi is about the same as that of proteins [27,28], and so the conclusion that cargo is not further concentrated during intra-Golgi vesicle budding stands, also consistent with direct measurement [29].

In any case, based on a variety of data ([1,2], and the results reported here), we conclude that at least the fraction of sialylated glycotripeptides represents a valid marker for vesicular bulk flow from the ER via the complete Golgi apparatus to the plasma membrane in mammalian cells.

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