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Glycotripeptides are released by yeast but not by mammalian microsomes

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Abstract Glycotripeptides generated in vivo in the endoplasmic reticulum (ER) have been used as markers to assess the rate of vesicular bulk flow from the ER via the Golgi apparatus to the plasma membrane in mammalian cells. The applicability of such glycotripeptides as markers for bulk flow along this pathway has been questioned by a report on non-vesicular release of glycotripeptides from yeast semi-intact spheroplasts. We have therefore investigated direct release of glycotripeptides from yeast and from mammalian microsomes and report here that such release is specific to the yeast system and cannot be detected in mammalian microsomes.

Key words: Vesicular transport; Endoplasmic reticulum; Glycopeptide

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, glycosylated in the endoplasmic reticulum (ER), and thereafter released into the medium [1]. During secretion their carbohydrate moieties are trimmed and processed in a Golgi-specific sequence of reactions, indicating their passage through the complete Golgi – the secretory pathway taken by newly synthesized proteins [2]. This experimental design has allowed us to measure the rate of vesicular bulk flow from the ER via the Golgi apparatus to the cell surface [1]. The resulting data have strongly contributed to our present view that newly synthesized proteins enter the constitutive transport pathway in an unsignalled manner, and that proteins destined for locations other than the cell surface are specifically retained at their residencies or directed to their destinations by signal–receptor interactions [3–6].

In order to correctly interpret kinetic data obtained in vivo in Chinese hamster ovary fibroblasts with these markers, their complete luminal inclusion during transport is a prerequisite. Such vesicular inclusion has been challenged by a recent publication which describes ATP-dependent translocation of glycotripeptides across the ER membrane in yeast semi-intact spheroplasts [7]. Previous studies have demonstrated glycopeptide retention within the mammalian ER [8,9], but they have not determined whether or not the glycopeptides are quantitatively retained; more importantly, not all of the factors reported necessary for glycopeptide translocation in yeast [7] were added in these studies. This has prompted us to investigate in an in vitro system whether or not mammalian microsomes show non-vesicular release as an alternative pathway for glycotripeptide secretion. Our reasoning for the use of microsomes was that, should there be a pump in the ER which translocates glycopeptides, this pump should be detectable not just in a semi-intact cell system but also in microsomes. This approach has allowed us to directly compare yeast and mammalian ER for glycotripeptide translocation. No evidence of non-vesicular release from mammalian microsomes was found, whereas such a release from yeast microsomal preparations was confirmed.

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Abbreviations: ER, endoplasmic reticulum; OTP, N-octanoylated tripeptide Asn-Tyr-Thr-NH_2; OTP(Asp), N-octanoylated tripeptide Asp-Tyr-Thr-NH_2; ConA, concanavalin A; TLC, thin layer chromatography; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); αF, yeast α factor.

2. Experimental procedures

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. Preparation of cell fractions

S. cerevisiae strain SKQ2N was used for the preparation of yeast semi-intact spheroplasts and yeast microsomes. Spheroplasting was performed according to Baker et al. [10]; yeast microsomes and cytosol were prepared essentially as described by Waters and Blobel [11]. Microsomes and cytosols from mammalian sources were prepared according to Walter and Blobel [12].

2.3. In vitro transport reactions

A standard 25 μl yeast incubation mixture contained 10 μl (100–150 μg) microsomes or semi-intact spheroplasts, 5 μl (80 μg) of yeast cytosol, 2 mM ATP, 10 mM creatine phosphate, 0.3 μl (8 mg/ml; Boehringer) creatine phosphokinase, and 50 μM each of UDP-glucose, UDP-*N*-acetylglucosamine and GDP-mannose in a 20 mM HEPES buffer, pH 6.8, containing 150 mM potassium acetate, 5 mM magnesium acetate, 1 mM dithiothreitol, 250 mM sorbitol. Incubations with mammalian microsomes were carried out in the same buffer at pH 7.5 and with mammalian cytosol. Incubations with yeast membranes were carried out at 20°C, mammalian microsomes were incubated at 30°C. At various time points, 25 μl aliquots were taken from upscaled samples and layered on top of a step gradient consisting of 50 μl 2.0 M sucrose and 100 μl 0.5 M sucrose, both in incubation buffer without sorbitol. Gradients were centrifuged for 10 min at 100,000 rpm and 4°C in a TLA-100 rotor (Beckman): the top half was taken as supernatant, the bottom half as pellet fraction. Each fraction was brought to 0.5% Triton X-100, 1 mM $CaCl_2$, 1 mM $MnCl_2$, heated for 2 min to 95°C and then transferred onto ice. After centrifugation for 5 min in an Eppendorf microfuge, samples were passed through ConA-Sepharose (Pharmacia) columns as described [1] for quantitation of the [^{125}I]glycotripeptides formed.

2.4. Glycotripeptide analysis

Glycotripeptides eluted from ConA-Sepharose columns in buffer A

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without Triton X-100 (10 mM Tris, pH 7.4, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.15 M NaCl) 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).

2.5. In vitro translation

Translation of the mRNA encoding for prepro-α-factor was performed according to Jackson and Hunt [13]. Translation took place for 30 min at 30°C in a reticulocyte lysate. After addition of cycloheximide to 1 mM, the incubation was continued for 20 min at 26°C in the presence or absence of yeast microsomal membranes. A further incubation for 10 min at 26°C followed in the presence or absence of proteinase K (150 μg/ml; Boehringer).

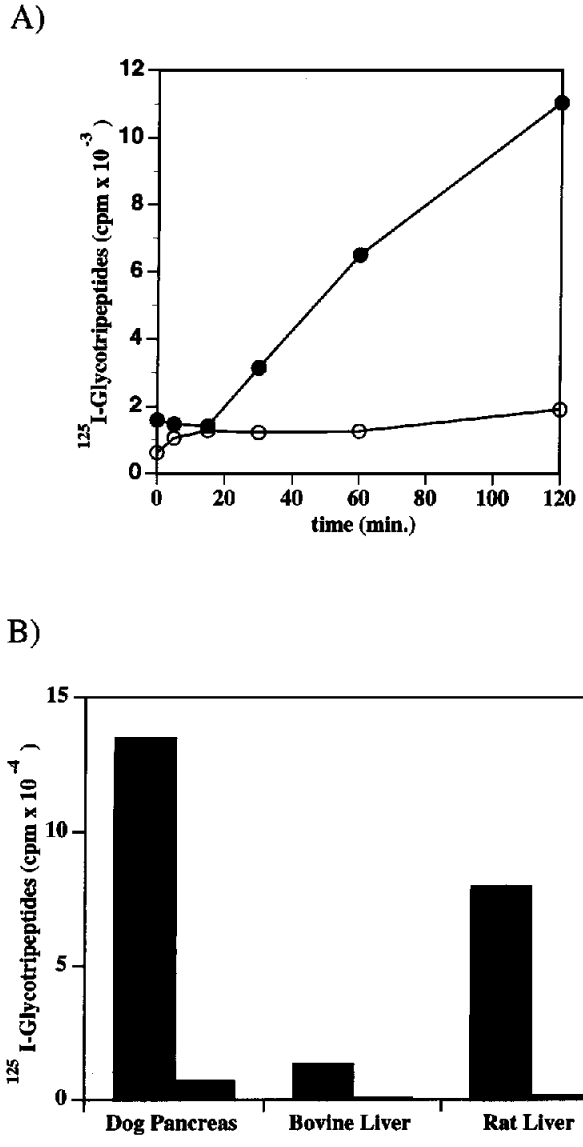

Fig. 1. Distribution of [^{125}I]glycotripeptides in mammalian microsomal fractions. (A) Time-course of an incubation of dog pancreas microsomes with 4×10^5 cpm of [^{125}I]OTP in the presence of rat liver cytosol and an ATP-regenerating system. ○, supernatant; ●, pellet. (B) Distribution of [^{125}I]glycotripeptides between pellet and supernatant fractions after 60 min incubation with 1.6×10^6 cpm of [^{125}I]OTP for different mammalian microsomal preparations. Dark bars indicate pellet fractions, light bars represent supernatant fractions. Values are averaged for separate incubations with rat liver cytosol and bovine brain cytosol. For dog pancreas microsomes, five individual preparations were averaged.

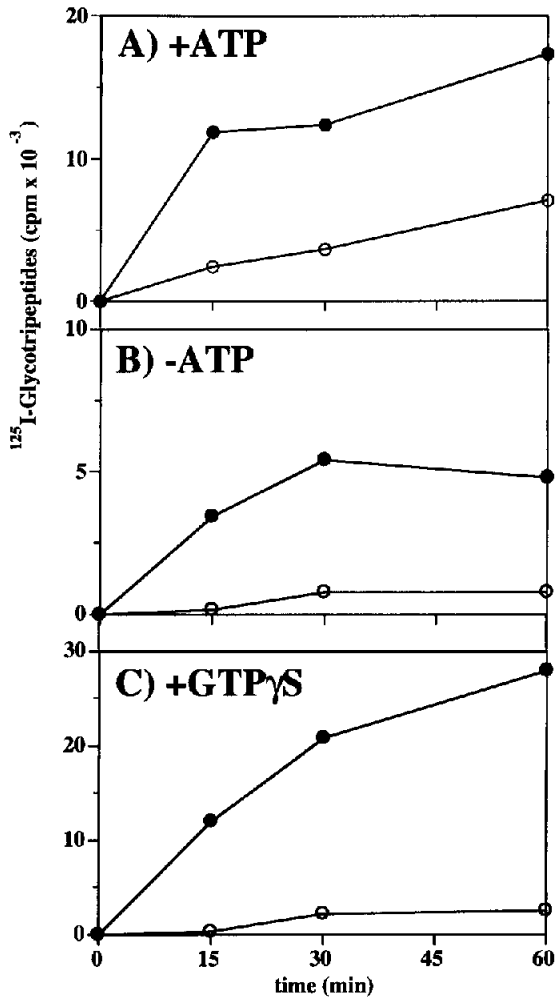

Fig. 2. Nucleotide dependence of the release of [^{125}I]glycotripeptides from yeast microsomes. (A) Microsomes prepared from *S. cerevisiae* were incubated in the presence of yeast cytosol, nucleotide sugar donors, and an ATP-regenerating system with 4×10^5 cpm of [^{125}I]OTP per sample. (B) Conditions as in A, but without the ATP-regenerating system. (C) Conditions as in A, but in the presence of 50 μM GTPγS. Vesicular and non-vesicular material was separated and quantified as described in section 2. Zero minute values, which typically accounted for about 1500 cpm in the pellet and 300 cpm in the supernatant fractions, were subtracted. ○, supernatant; ●, pellet.

3. Results

3.1. There is no time-dependent release of [^{125}I]glycotripeptides from mammalian microsomes

A recent report by Römisch and Schekman on glycotripeptide release from yeast semi-intact spheroplasts had implied a route of secretion for glycopeptides alternative to the constitutive secretory pathway, questioning their applicability as markers of vesicular bulk flow [7]. In order to determine whether [^{125}I]glycotripeptides can be released under physiological conditions from mammalian microsomes, we incubated dog pancreas

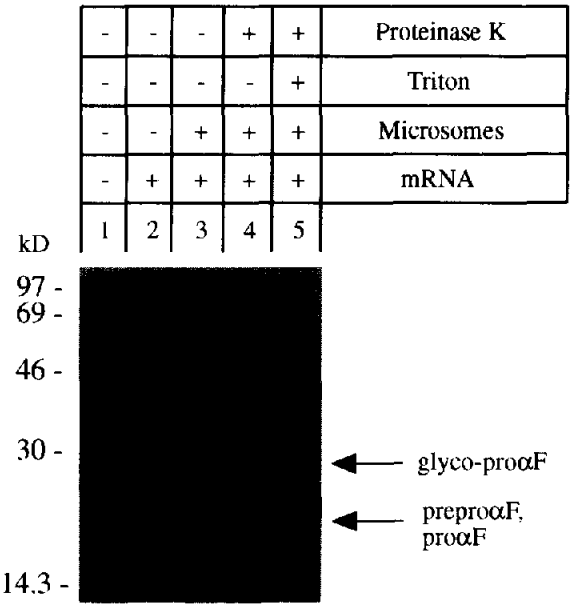

Fig. 3. Yeast microsomal membranes do not release glycosylated pro-α-factor. After translation in a reticulocyte lysate system, prepro-α-factor was incubated in the presence or absence of yeast microsomal membranes, proteinase K, and Triton X-100 as indicated. The lower band in lanes 3 and 4 corresponds to prepro-α-factor (preproαF) and pro-α-factor (where the signal sequence has been processed, proαF), the top band in these lanes corresponds to threefold glycosylated pro-α-factor (glyco-proαF).

microsomes with [^{125}I]OTP in the presence of rat liver cytosol, an ATP-regenerating system, and a mixture of sugar nucleotides (see section 2). [^{125}I]Glycotripeptides were isolated after high-speed centrifugation in order to separate vesicular from non-vesicular material (Fig. 1A). No accumulation with time of [^{125}I]glycotripeptides in the supernatant fractions could be detected. To investigate whether this result holds true for other mammalian species and tissues as well, we incubated microsomes of various origins with cytosols derived from bovine brain, bovine liver, and, as a control, yeast. The results are shown in Fig. 1B. The percentage of [^{125}I]glycotripeptides in the supernatant was as follows (mean ± S.D.): dog pancreas microsomes, 5.03 ± 1.98%; bovine liver microsomes, 4.63 ± 0.80%; rat liver microsomes, 1.45 ± 0.14%. Control incubations with yeast cytosol gave essentially the same results.

In no case was a significant amount of [^{125}I]glycotripeptides released into the supernatant fraction, indicating there is no measurable non-vesicular release of [^{125}I]glycotripeptides from the mammalian ER membrane.

3.2. *[^{125}I]Glycotripeptide release from yeast microsomes is blocked by GTPγS*

Microsomes prepared from the yeast *Saccharomyces cerevisiae* were incubated under the same conditions described above for dog pancreas microsomes (with yeast cytosol replacing the mammalian cytosol). The conditions used correspond to those employed by Römisch and Schekman for incubations with yeast semi-intact spheroplasts [7]. In this way, we confirmed the time-dependent release of [^{125}I]glycotripeptides into the supernatant upon addition of an ATP-regenerating system (Fig. 2A). This release was diminished when the ATP-regenerating system was omitted (Fig. 2B), although not to the same extent as had been reported for semi-intact spheroplasts. In the absence of the ATP-regenerating system there was also less overall glycosylation. Addition of ATPγS caused no further decrease in either glycosylation or release (not shown). However, this ATP-dependent release was blocked by GTPγS (Fig. 2C), a non-hydrolyzable analogue of GTP which is known to block vesicular transport. This had not been previously reported. Similar effects, although not as pronounced, were found when semi-intact spheroplasts were used (not shown).

To control whether this release of glycotripeptides is due to leakiness of the yeast membranes, prepro-α-factor was translated in vitro and yeast microsomes were added post-translationally (Fig. 3). The glycosylated pro-α-factor was protected from proteolysis by proteinase K in the absence but not in the presence of Triton X-100. Therefore, it is enclosed in the lumen of the microsomal membranes and we confirmed that release of [^{125}I]glycotripeptides is not due to leakiness of the yeast microsomal membranes.

3.3. *Characterization of [^{125}I]glycotripeptide release from yeast microsomes*

Based on their observations with yeast semi-intact spheroplasts, Römisch and Schekman suggested the existence of a peptide pump in the yeast ER to explain non-vesicular [^{125}I]gly-

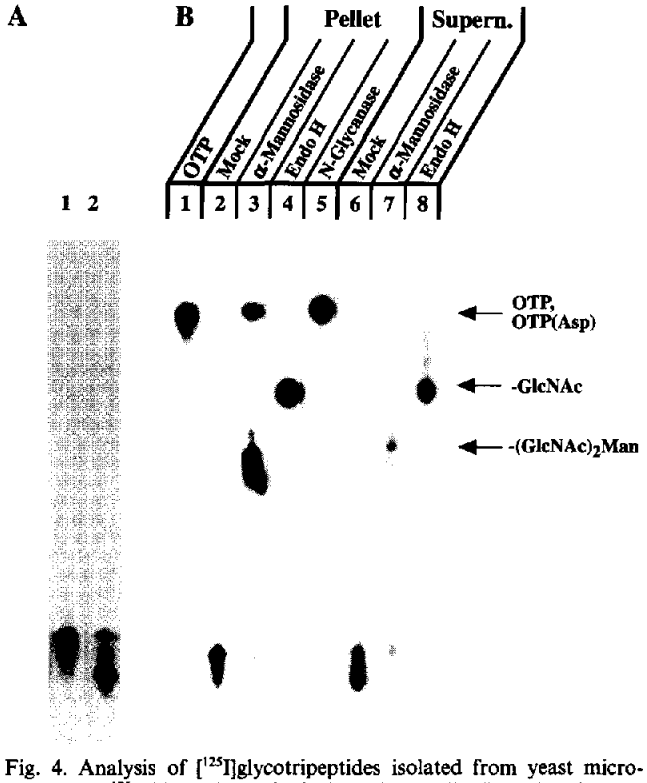

Fig. 4. Analysis of [^{125}I]glycotripeptides isolated from yeast microsomes. (A) [^{125}I]Glycotripeptides isolated from pellet (lane 1) and supernatant fractions (lane 2), respectively, of an incubation with yeast microsomes. Slower migration of the glycotripeptides in lane 2 indicates a larger carbohydrate chain as compared to the sample in lane 1. (B) The material from pellet and supernatant fractions was digested with jack bean α-mannosidase, endo H, or mock digested as indicated. Arrows show migration of OTP and OTP(Asp) (N-octanoylated tripeptide Asp-Tyr-Thr-NH_2 resulting from digestion with *N*-glycanase) as well as the deduced peptide-bound carbohydrate structures after digestion with endo H and jack bean α-mannosidase.

cotripeptide export. Therefore we decided to investigate the specificity of this putative pump regarding carbohydrate structure. To this end, [^{125}I]glycotripeptides were isolated from pellet and supernatant fractions of an incubation of yeast microsomes with [^{125}I]OTP. TLC analysis of the [^{125}I]glycotripeptides showed a markedly slower migration of a major portion of the supernatant fraction as compared to the pellet fraction (Fig. 4A), indicating a significantly larger carbohydrate chain. The percentage of slower-migrating material in the supernatant fraction varied, but in each case it constituted the most prominent spot in TLC, while it was hardly detectable in the pelleted fraction. Since digestion with α-mannosidase cleaved all of the [^{125}I]glycotripeptide species (Fig. 4B, lanes 3 and 7), leaving a single spot presumably representing the Manβ1-4GlcNAcβ1-4GlcNAc-trisaccharide connected to OTP, the presence of glucose residues, which could also be expected for ER-derived carbohydrate chains [14], can be ruled out. This modification of the carbohydrate chain is not due to a process taking place on the cytosolic side of the membranes, as the faster-migrating material isolated from the pellet fraction does not become modified during re-incubation with yeast microsomes and cytosol (not shown).

4. Discussion

The mechanism of non-vesicular release of glycotripeptides from yeast ER is unclear. In agreement with Römisch and Schekman we have found some stimulation by ATP of release of glycotripeptides from yeast microsomes, and in addition we found this release to be blocked by GTPγS. In our hands, release was not dependent on the addition of cytosol, as had been reported [7], but this may be due to the high membrane concentrations used and concomitant contamination with cytosolic factors in our assay system. Interestingly, the [^{125}I]glycotripeptides isolated from the supernatants of these incubations have a larger carbohydrate moiety than those found in the membrane-enclosed fraction. Previous investigations on the carbohydrate chains of glycoproteins isolated from the yeast ER had shown only minor variations in chain size, corresponding to $GlcNAc_2Man_8$ and $GlcNAc_2Man_9$ structures [14]. No mechanisms are known for the elongation of carbohydrate chains in the cytosol, and no modification of [^{125}I]glycotripeptides added exogenously to control incubations was observed (not shown). It should be considered that yeast microsomes represent a less well-defined membrane fraction than their mammalian counterparts. Thus, it cannot be excluded that the [^{125}I]glycotripeptides isolated from the supernatant had first been released from the ER in vesicular form and subsequently released to the medium.

Most importantly, however, a non-vesicular release of glycotripeptides from the mammalian ER seems highly unlikely, based on the results with mammalian microsomes presented here. Neither microsomes from cells containing a high percentage of rough ER (dog pancreas) nor those from cells enriched in smooth ER (rat liver) release significant amounts of [^{125}I]glycotripeptides under conditions where such a release is found from yeast microsomes. This is consistent with complete Golgi passage of glycotripeptides observed in vivo, and establishes the applicability of the glycotripeptides as markers for vesicular bulk flow from the ER to the plasma membrane in mammalian cells.

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