C-Mannosylation of Human RNase 2 Is an Intracellular Process Performed by a Variety of Cultured Cells*

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C2-α-Mannosyltryptophan was discovered in RNase 2 from human urine, representing a novel way of attaching carbohydrate to a protein. Here, we have addressed two questions related to the biosynthesis of this modification: (i) is C-mannosylation part of the normal intracellular biosynthetic route, and (ii) how general is it, i.e. which organisms perform this kind of glycosylation? To answer the first question, RNase 2, which is identical to the eosinophil-derived neurotoxin, was isolated from intracellular stores of cultured human HL-60 cells. The enzyme was C-mannosylated at Trp-7, showing that the modification occurs intracellularly, before secretion of the protein. The second question was investigated by immunological and chemical analysis of RNase 2 purified from the supernatant of transiently transformed cells from different organisms. This revealed that C-mannosylation occurs in cells from man, green monkey, pig, mouse, and hamster. The observation that pig kidney cells contain the machinery for C-mannosylation of Trp-7 of human RNase 2 but that the homologous RNase from porcine kidney is not a substrate, since it does not contain a tryptophan at position 7, strongly suggests that C-mannosylated proteins other than RNase 2 exist. Recombinant RNase 2 isolated from insect cells, plant protoplasts, and Escherichia coli was not C-mannosylated. These results not only form the basis for further studies on the biochemical aspects of C-mannosylation but also have implications for the choice of cells for production of recombinant glycoproteins.

Post-translational modification of proteins by covalent attachment of carbohydrate is a common and widespread phenomenon. Two kinds of glycosylation have been known for a long time: N-glycosylation, where the sugar residues are linked to N\(^\text{O}\) of Asn, and O-glycosylation, where the linkage is to O\(^\text{D}\) of Thr or Ser. Both have been extensively characterized with respect to their biosynthetic pathways as well as their distribution in nature. They are found in many organisms, ranging from bacteria to man, and occur in a variety of proteins (1). Recently, a new kind of linkage between a carbohydrate and a protein was discovered in human ribonuclease 2 (RNase 2), namely a C-glycosidically linked mannose residue (2, 3). In this case the C-1’ atom of the mannose residue is directly linked to the C-2 atom of the indole moiety of Trp-7 (Scheme 1).

RNase 2 from urine is completely identical in primary structure to eosinophil-derived neurotoxin (EDN), which is located in the cytotoxic granules of the eosinophil and may play a role in the anti-parasitic action of these cells (4). EDN is a potent neurotoxin, causing muscle stiffness and ataxia when injected intracerebrally into experimental animals (Gordon phenomenon, see Refs. 5 and 6). This is associated with the loss of Purkinje cells and vacuolation of white matter in the cerebellum, brain stem, and spinal cord (6).

The putative glycosyltransferase that carries out the modification of RNase 2 must have a considerable degree of specificity as it transfers a mannosyl residue to Trp-7 while fully ignoring the tryptophan at position 10 (2). Originally, (C\(^2\)-Man-)Trp was found in peptides obtained from RNase 2 from human urine. Subsequently, using NMR it was shown in the entire, intact protein (7). Since the modification was also found in RNase 2 isolated from human erythrocytes, it was concluded that C-mannosylated RNase 2 from urine does not represent a metabolized form of the excreted protein but that it constitutes a genuine post-translational modification (7). It could be argued, however, that human erythrocytes are not representative for other types of cells, because they lack a nucleus and other major cell organelles. As a result of the absence of protein synthesis, erythrocyte proteins are not replaced and age. This raises the possibility that C-mannosylation of RNase 2 in erythrocytes results from an aging process, in contrast to protein N- and O-glycosylation, which is part of the biosynthetic route of newly made proteins. We have addressed this question by isolating RNase 2 from cultured human promyelocytic cells (HL-60) and examining its C-mannosylation status.

In contrast to N- and O-glycosylation, knowledge about the organisms in which C-mannosylation occurs is lacking, because so far only RNase 2 from human sources has been analyzed. This issue is of importance for further studies on the biosynthesis of (C\(^2\)-Man-)Trp and in the search for other proteins containing this modification. In addition, it may also have practical implications for the choice of appropriate cells for the production of recombinant proteins with or without this modification. Therefore, we have expressed human RNase 2 in cells from a variety of organisms. The secreted proteins were purified to near homogeneity and analyzed with an antibody specific for (C\(^2\)-Man-)Trp of RNase 2, as well as by mass spectrometry and Edman degradation of purified peptides.

MATERIALS AND METHODS

Human RNase 1 and 2 were purified as described (2, 7). Bovine pancreatic RNase B and keyhole limpet hemocyanin were obtained

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1 The abbreviations used are: EDN, eosinophil-derived neurotoxin; (C\(^2\)-Man-)Trp, C\(^2\)-α-mannopyranosyltryptophan; ESIMS, electrospray ionization-mass spectrometry; FCS, fetal calf serum; HPLC, high-performance liquid chromatography; LC-ESIMS, liquid chromatography interfaced with ESIMS; PAA, polyacrylamide; r-RNase 2, recombinant RNase 2; Bis-Tris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxymethyl)propane-1,3-diol.
from Sigma. The protease from *Staphylococcus aureus* was purchased from Promega, Madison, WI, and thermolysin and N-glycosidase F were from Boehringer Mannheim, Germany, and CH-activated and protein A-Sepharose were from Pharmacia, Uppsala, Sweden. Cell culture media and FCS were from Life Technologies, Inc.

**Production of Antibodies and Western Analysis—Antibodies against human RNase 2 have been described previously (7).** Antibodies specific for RNase 2 containing (C^-Man^-Trp at position 7 were obtained by immunizing New Zealand White rabbits with the thermolytic peptide (5-10) (2) coupled with glutaraldehyde to keyhole limpet hemocyanin (350 µg peptide/mg hemocyanin). An initial injection of 430 µg of conjugate in Freund’s complete adjuvant was given, followed by 2 booster injections with 430 µg of conjugate in Freund’s incomplete adjuvant after 1 and 2.5 months. Specific antibodies were purified as described (7).

**SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described (7).**

**Purification of RNase 2 from HL-60 Cells—**HL-60 cells (ATCC 240-CCl) were grown in RPMI 1640 medium, containing 15% FCS. All isolation procedures were performed at 4 °C, and buffers for extraction and chromatography contained a mixture of protease inhibitors, leupeptin (0.2 µg/ml), benzamidine HCl (2 µg/ml), pepstatin A (0.2 µg/ml), p-methanesulfonyl fluoride (0.2 mM), unless indicated otherwise. Harvested cells (7.5 × 10^9) were washed twice with phosphate-buffered saline, and RNase 2 was extracted with 380 ml of 0.1% trifluoroacetic acid for 2 h with stirring. After centrifugation at 2000 × rpm in a table-top centrifuge for 4 min at room temperature, the supernatant was dialyzed against 20 mM Tris-HCl, pH 7.5, and loaded onto a column of SP-Sepharose (1.5 × 11 cm) equilibrated in the same buffer. The column was washed, and proteins were eluted with 0.5 mM NaCl in the same buffer. The fractions containing RNase 2 were diluted 10-fold with 20 mM Bis-Tris-HCl, pH 7.5, and loaded onto a column of heparin-Sepharose (1 × 7.5 cm) equilibrated in the same buffer. The column was washed, and RNase 2 was eluted with 0.6 mM NaCl in the same buffer. After 5-fold dilution with 20 mM Tris-HCl, pH 7.5, the RNase 2 containing fractions were purified by immunoaffinity chromatography. An immunoaffinity column was prepared by covalently cross-linking 

**Protein C-Mannosylation—**A synthetic gene coding for the RNase 2 signal sequence for secretion (MVPKLFTSQI-)

**Figure 1**

**A synthetic gene coding for the RNase 2 signal sequence for secretion (MVPKLFTSQI-)**

**Expression and Purification of Recombinant RNase 2—**HEK293 (ATCC CRL 1573), COS7, LLC-PK1 (ATCC CRL 1392), and CHO cells were grown in Dulbecco’s modified Eagle’s medium containing 10% FCS or 10% newborn calf serum. Conditioned media were collected 2 and 6 days post-transfection and stored at −80 °C. The medium was passed over a Sepharose Q column (2.5 × 10 cm) equilibrated in 20 mM Tris-HCl, pH 7.5. r-RNase 2 appeared in the flow-through and was purified by immunoaffinity chromatography and C4 reversed phase HPLC (gradient 0–80% solvent B in 75 min) as described for RNase 2/HL-60, except that no protease inhibitors were added to the buffers. 

**Drosophila melanogaster Schneider 2 cells (14) in Schneider II medium (Life Technologies, Inc.) containing 10% fetal calf serum were grown in 10-cm tissue culture plates at 25 °C and transfected with 5 µg of plasmid pVL1392edn or pAcMP2edn and 0.5 µg of linearized BaculoGold™ DNA (Pharmingen). Cells were incubated at 27 °C for 5 days, and the recombinant virus was amplified 3 times. For large scale protein production, virus-infected SF9 cells were grown in serum-free medium (SF 900 II, Life Technologies, Inc.). Conditioned medium was harvested after 48 h at 37 °C and stored at −80 °C. The medium was centrifuged and dialyzed against 20 mM Bis-Tris-HCl, pH 6.0, and the RNase was purified essentially as described for RNase 2/HL-60. However, the heparin-Sepharose column was omitted; a 0.25–1M NaCl gradient was used to elute the enzyme from the SP-Sepharose column, and no protease inhibitors were added to the buffers.

**Escherichia coli strain BL21 (DE3) (Stratagene, La Jolla, CA) harboring plasmid pET11dedn was grown in LB medium containing ampicillin (100 µg/ml) at 37 °C. Expression of RNase 2 was induced with 0.1% isopropyl-β-d-thiogalactoside, and bacteria were harvested after 3 h. Cells were resuspended in 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 100 mM NaCl, and lysed in a French pressure cell at 10,000 p.s.i. Inclusion bodies were collected by centrifugation and cleaned up by two sequential wash/centrifugation steps with 1% Nonidet P-40 and 1 mM urea.

Ten mg of RNase 2 in inclusion bodies were incubated for 2 h at room temperature in 10 ml of 0.1 mM Tris-HCl, pH 8.0, containing 2 mM EDTA, 6 µg guanidine hydrochloride, 200 µg diethiothreitol. Insoluble material was removed by centrifugation, and the supernatant was diluted 100-fold into a stirred solution of 0.1 mM Tris-HCl, pH 8.0, containing 2 mM EDTA, 0.5 mM 1-arginine, 5.6 mM oxidized glutathione. Refolding pro-
ceeded for at least 48 h at 25 °C. The refolding solution was dialyzed against 20 mM Bis-Tris-HCl, pH 6.0, and chromatographed on heparin-Sepharose as described for RNase 2/HL-60, except that no protease inhibitors were added to the buffers. The column was washed with 250 mM NaCl and eluted with 1 M NaCl in the same buffer. RNase 2 containing fractions were dialyzed against 20 mM Bis-Tris-HCl, pH 6.0, and applied to a 1-ml Mono S column (Pharmacia) with a flow of 0.5 m/min. RNase 2 was eluted using a linear NaCl gradient (150–600 mM). RNase 2 containing fractions were pooled, dialyzed against 50 mM NH4HCO3, and stored at −80 °C.

E. coli strain KS474 (16) harboring plasmid pASKedlyn was grown in LB medium containing ampicillin (100 µg/ml) at 37 °C, and expression of RNase 2 was induced with 160 µM isopropyl-β-D-thiogalactoside. After 3 h cells were harvested and lysed as described above. Insoluble material was removed by centrifugation, and r-RNase 2/E. coli2 was purified as described for r-RNase 2/Sf9. All RNases were active in an assay using yeast RNA as the substrate (17).

Characterization of Recombinant RNase 2—Lyophilized RNase 2 (0.5–1.0 µg) in 25 µl of 50 mM Hepes-NaOH, pH 7.8, containing 10 mM CaCl2 was digested with thermolysin (0.1% w/w) at 75 °C for 60 min. Peptides were fractionated by reversed phase HPLC on a 2.1-mm diameter C18 column (solvent A, 0.1% trifluoroacetic acid). A linear gradient of 20–41% solvent B (0.08% trifluoroacetic acid, 70% CH3CN) over 50 min at a flow rate of 0.2 ml/min was used. Peptides were detected at 214 nm. The percentage modification of a particular RNase 2 was calculated from the ratio of the peaks of the modified and unmodified peptides, using a calibration curve obtained by digesting and fractionating mixtures containing different ratios of RNase 2/urine and r-RNase 2/E. coli1.

LC-ESIMS was performed using an Applied Biosystems model 140 chromatograph equipped with a 0.3-mm diameter C8 column and interfaced with a Sciex API III mass spectrometer (18) operating in the multi-ion-monitoring mode at m/z = 691.5, 838.5, and 1000.5. Methods for protein reduction, carboxymethylation, proteolytic cleavage, solid phase Edman degradation, and ESIMS have been described (2, 18).

RESULTS

Modification-specific Antibodies and Quantitation of the Degree of C-Mannosylation—To be able to analyze the state of C-mannosylation of small amounts of RNase 2 (1–2 µg) from cultured cells, two analytical tools were established. First, modification-specific antibodies were raised against the peptide comprising residues 5–10 of RNase 2 (FT(C2-Man-)WAQW). The affinity purified α-(5–10) antibodies recognized RNase 2 from human urine (RNase 2/urine; Fig. 1A, lane 1) but not recombinant RNase 2 (r-RNase 2; Fig. 1A, lane 2) isolated from the inclusion bodies of E. coli with unmodified Thrp at position 7 (r-RNase 2/E. coli1, see below). To exclude that the α-(5–10) antibodies cross-reacted with mannosyl residues in one of the N-glycans of RNase 2/urine, the fragment containing these glycans (residues 13–134) was produced by proteolytic cleavage at Glu-12 and examined by Western analysis. As cleavage of RNase 2/urine proceeded, the signal produced by the α-(5–10) antibodies was lost (Fig. 1B, lower panel) but that by the α-RNase 2 antibodies remained (Fig. 1B, upper panel). These experiments showed that the α-(5–10) antibodies recognized an epitope located in peptide-(5–10). Because RNase 2/urine and r-RNase 2/E. coli1 differ in this region only with respect to C2-Mannosylation of Trp-7 and therefore do not necessarily occur adjacent to C-mannosylated Trp in other proteins (26), a micro-method was developed for quantitating the degree of C-mannosylation, using RNase 2/urine (fully C-mannosylated at Trp-7) and fully unmodified r-RNase 2/E. coli1. Thermolytic digestion of RNase 2/urine and fractionation of the peptides by C18 reversed phase HPLC yielded C-mannosylated peptide-(5–10) (Fig. 2A, peak b). Cleavage of r-RNase 2/E. coli1 resulted in the formation of two peptides (Fig. 2B, peaks a and c), which were examined by LC-ESIMS. Peak a ((M + H)+ = 691.5) was assigned to residues 6–10 (TWAQW), whereas peak c ((M + H)+ = 838.5) contained residues 5–10 (FTWAQW). Digestion of mixtures containing RNase 2/urine and r-RNase 2/E. coli1 resulted in all three peptides (not shown). By varying the molar ratios of the two RNases, a calibration curve was obtained that related the mole fraction of (C2-Mann-)Trp in the protein mixture to the relative area of peak b (Fig. 2C). The hyperbolic shape of this curve resulted mainly from the difference in extinction coefficient between (C2-Mann-)Trp and Trp (18).

Endogenous RNase 2 from HL-60 Cells—To examine whether cells that actively divide and synthesize proteins carry out C-mannosylation, RNase 2 was purified from the human promyelocytic cell line HL-60, which yielded 10 µg of RNase 2 from 7.5 × 109 cells (21% recovery). The protein migrated on SDS-PAA gels as a broad smear (Fig. 3A, lane 1), which in a subclone of HL-60 cells has been attributed to heterogeneity of N-linked glycans (21). This was confirmed for the cells used

[2] J. Krieg, unpublished results.

[3] In this publication RNase 2 has been indicated with eosinophil-derived neurotoxin.
here by treatment with N-glycosidase F, which resulted in RNase 2/HL-60 that co-migrated with unglycosylated r-RNase 2/E. coli1 (Fig. 3A, lanes 2 and 3). Western analysis of N-glycosidase F-treated RNase 2/HL-60 using the α-(5–10) antibodies gave a positive result (Fig. 3A, lane 5), with RNase 2/urine and r-RNase 2/E. coli1 serving as the positive and negative controls, respectively (Fig. 3A, lanes 4 and 6). This demonstrated the presence of (C²-Man-)Trp in RNase 2/HL-60. The position of the mannosylated Trp in the protein was established by chemical analyses. Comparison of the thermolytic peptide maps of RNase 2/HL.60 and RNase 2/urine by LC-ESIMS in the single ion-monitoring mode at m/z = 1000.5 demonstrated the presence of modified peptide-(5–10) (Fig. 3, B and C, upper traces). The sequence of this peptide was determined by Edman degradation to be FT(C²-Man-)WAQW. In addition, a small amount of unmodified peptides-(5–10) and -(6–10) was detected by LC-ESIMS at m/z = 838.5 and 691.5 (Fig. 3, B and C, lower and middle traces). Quantitation by the method described above showed that 90% of the RNase 2/HL-60 molecules contained (C²-Man-/Trp at position 7 (Table I).

Treatment of HL-60 cells with butyric acid leads to differentiation toward eosinophils (22, 23) and increases the expression of RNase 2 (21). RNase 2 from cells treated this way was also found to be C-mannosylated at Trp-7 (data not shown).

**Ectopic Expression of r-RNase 2 in Mammalian Cells**

Transfection of HEK293 (human embryonal kidney) cells, LLC-PK₁ (porcine kidney epithelial) cells, CHO (Chinese hamster ovary) cells, NIH 3T3 (mouse) fibroblasts, or COS7 (transformed green monkey kidney) cells with an expression vector for human pre-RNase 2 resulted in the secretion of RNase activity and RNase 2 antigen into the culture medium. No activity could be detected in cells transfected with a control plasmid. The proteins were purified to near homogeneity and examined immunologically and chemically. In all cases, the proteins appeared heterogeneous on SDS-PAA gels (Fig. 4, A and B, lanes 3–7) with an apparent molecular mass substantially higher than that of RNase 2/urine (Fig. 4, A and B, lane 1). Because N-glycosidase F treatment resulted in a single band that co-migrated with r-RNase/E. coli1 (data not shown), it was concluded that the increased mass and heterogeneity were due to the N-linked glycans.

r-RNase 2 isolated from all of these cells gave a positive signal in Western analysis using the α-(5–10) antibodies (Fig. 4C, lanes 3–7). This indicated that C-mannosylation had occurred. Chemical analyses established the position of (C²-Man-)Trp in the protein. Thermolytic digests of each of the r-RNase 2s were fractionated by reversed phase HPLC, resulting in three peptides originating from the region containing the two tryptophans. The results obtained with r-RNase 2/HEK293 are shown in Fig. 2D as a representative example. In all cases, the three peaks were assigned by LC-ESIMS as described above, and peptide b was subjected to Edman degradation, yielding the sequence FT(C²-Man-)WAQW. These results show that in the mammalian cells tested, Trp-7 but not Trp-10 became C-mannosylated.

The degree of modification of the different r-RNase 2s was determined from the thermolytic peptide maps and is given in Table I, together with a summary of all the evidence for the presence of (C²-Man-/Trp).

**Fig. 2. Quantitation of the degree of C-mannosylation.** Purified r-RNase was digested with thermolysin at 75 °C, and peptides were fractionated by reversed phase HPLC on a 2.1-mm diameter C₁₈ column. The eluate was monitored at 214 nm. Only the portion of the chromatogram containing peptides from the region 5–10 has been plotted against the molar fraction of (Man-)Trp giving the sequence FT(C²-Man-)WAQW. The degree of modification of the different r-RNase 2s was determined from the thermolytic peptide maps and is given in Table 1, together with a summary of all the evidence for the presence of (C²-Man-/Trp).

**Fig. 3. C-Mannosylation of RNase 2 isolated from HL-60 cells.** A, purified RNase 2 from HL-60 cells was electrophoresed on a 12.5% SDS-PAA gel. Western analysis was performed using the αRNase 2 antibodies (left-hand panel) or the α-(5–10) antibodies (right-hand panel). Lane 1, RNase 2/HL-60; lanes 2 and 5, RNase 2/HL-60 treated with N-glycosidase F; lanes 3 and 6, r-RNase 2/E. coli1 (negative control); lane 4, RNase 2/urine (positive control). The high molecular weight species in lane 5 represents an aggregate peculiar to this preparation. B, LC-ESIMS in the single ion monitoring mode of thermolytic peptides obtained from RNase 2/urine (upper trace) and r-RNase 2/E. coli1 (middle and lower trace).
was confirmed by the results presented here (Fig. 2 and Fig. 4, lane 2). We also directed the protein into the periplasm using a plasmid containing the ompA signal sequence for secretion and purified the protein (r-RNase 2/E. coli2). Evidence for actual translocation was obtained from Edman degradation, which demonstrated that the signal peptide had been cleaved.

No binding of the α-(5–10) antibodies was observed, and only the two unmodified peptides were observed in the thermolytic peptide map (Table I).

Attempts to purify r-RNase 2 from Xenopus laevis oocytes and Saccharomyces cerevisiae failed, because the amounts of enzyme secreted were too small for reliable analyses.

DISCUSSION

We conclude from the results presented that in mammalian cells protein C-mannosylation of Trp is part of the normal intracellular biosynthetic route of a secreted protein. The observation that RNase 2/HL-60, which was isolated from the secretory granules in HL-60 cells, is 90% C-mannosylated indicates that this modification actually occurs before secretion.

At present, it is not known in which cellular compartment this happens, but given that the known mannose precursors for glycoprotein synthesis in mammals, GDP-mannose and dolichol phosphomannose, occur in the cytoplasm and in the lumen of the endoplasmic reticulum, respectively (reviewed in Ref. 25), it seems likely that C-mannosylation occurs in either one of these compartments.

RNase 2 from urine is completely identical in primary structure to EDN. The question has been raised whether C-mannosylation is tissue-specific and whether EDN is also C-mannosylated (4). The results obtained here with EDN from HL-60 cells, which are regarded to be a model for eosinophil differentiation (22), demonstrated that (C²-Man-)Trp also occurs in EDN. (C²-Man-)Trp does not seem to be essential for the neurotoxic activity of EDN, however, since also the related RNase eosinophil cationic protein, which contains an arginine at position 7, is toxic (4).

C-Mannosylation of RNase 2 took place in the five cell lines of mammalian origin tested, albeit with different degrees of efficiency (Table I). The reason for these differences is unclear, but it does not seem to depend on the phylogenetic distance of the cell donor species, because human RNase 2 was C-mannosylated better in mouse 3T3 cells than in either cells from man, pig, or monkey (Table I). Probably, the differences stem from a difference in the amount of substrate or transferase present in each cell type. The specificity of the C-mannosylation reaction was the same in all cases. As in RNase 2 isolated from urine (2) or erythrocytes (7), Trp-7 but never Trp-10 was modified in r-RNase 2 from the different cell lines. This indicates that the
transferase involved has similar properties in these species.

It is of particular interest to note that porcine kidney cells (LLC-PK1) human RNase 2 was C-mannosylated at Trp-7 but that the homologous enzyme from porcine kidney contains an unmodified, basic amino acid at this position (26). Furthermore, Trp-10 in the latter RNases remains unmodified. These observations lead to the conclusion that pig kidney cells contain the C-mannosylation machinery, but not the appropriate RNase substrate, suggesting that C-mannosylated proteins other than RNase 2 may be found.

The results obtained here with cultured cells form the basis for the elucidation of the biochemical aspects of C-mannosylation. Since a number of mutants that affect the synthesis of sugar substrates used in protein N-glycosylation are available for the CHO cell line (27), it will now be possible to investigate which sugar precursors are involved in C-mannosylation. Furthermore, the structural details of RNase 2 that govern the specificity with respect to Trp-7 may be addressed by site-directed mutagenesis.

At present it is unclear why the cells from plants and insects used here do not carry out C-mannosylation of human RNase 2. We examined the expression of RNase 2 in Sf9 cells using baculovirus, either under the control of the late basic protein or the very late polyhedrin promoter, as well as by calcium phosphate-mediated DNA transfection in the very late polyhedrin promoter, as well as by calcium phosphate-mediated DNA transfection in D. melanogaster Schneider 2 cells. With neither of these approaches was C-mannosylation of RNase 2 observed (Table I). Insect and plant cells contain both GDP-mannose and dolichol phosphomannose (28, 29), and r-RNase 2 in these cells was indeed N-glycosylated as suggested by its high apparent molecular mass (Fig. 4, lanes 8–10). It seems likely that these cells either do not contain a transferase for C-glycosylation or that the enzyme has a specificity that does not allow modification of Trp-7 in the context of human RNase 2. The latter explanation is reasonable, because glycosylation of Trp in a structurally unrelated neuroepithelium from the stick insect Carausius morosus has been reported (30). Although, the sugar and its mode of attachment have not been established, the published mass spectrometry data are consistent with a C-glycosidic linkage (18).

The results presented here also have practical implications. The production of secreted, recombinant proteins is common practice in some of the cells used in this study (31). Since CHO and COS cells can C-mannosylate Trp, this may add another possible source of variability to recombinant glycoproteins produced in these cells. Mass differences of 162 Da of such proteins, compared with the theoretical values, should not be attributed to microheterogeneity in the N- or O-linked glycans, without examining the state of modification of the Trp residues. Furthermore, our results provide examples of cells that may be used if C-mannosylation is to be avoided.

In conclusion, we have shown that C-mannosylation of Trp is not restricted to cells from man but that it is widespread in mammalian species. The process appears to take place intracellularly, before secretion of the protein.

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