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Variations in the rate of secretion of different glycosylated forms of rat α_1-acid glycoprotein

Anne DRECHOU, Jean-Denis ROUZEAU, Jeanne FEGER and Geneviève DURAND*
Laboratoire de Biochimie-URA CNRS 622, UFR des Sciences Pharmaceutiques et Biologiques, 5 rue J. B. Clément, 92296 Châtenay-Malabry Cédex, France

Various studies have shown that oligosaccharides play an important role in the intracellular transport and secretion of glycoproteins. We show here a difference in the rate of secretion of two mature glycoforms of a single protein, α_1-acid glycoprotein. This indicates the existence of kinetically different pathways for these two forms for transport from the medial Golgi to the extracellular medium.

INTRODUCTION

Various authors have detected variability in the rates of protein secretion in human [1–3], mouse [4] and rat [5] hepatoma cells. This variability is not due solely to differences in rates of transport from the rough endoplasmic reticulum (RER) to the Golgi apparatus, but also to different retention times within the Golgi cisternae and *trans*-Golgi network [2]. Parent *et al.* [3] have previously shown that newly synthesized secretory proteins are exported at distinct rates in human hepatoma cells. Lodish *et al.* [1] have postulated the existence of a receptor in the RER membrane which may regulate protein transport to the Golgi apparatus. Yeo *et al.* [2] suggest that the variability in the rate of transport reflects differential regulation by interaction with specific receptors in both the RER and the Golgi apparatus. Several studies using glycosidase inhibitors have shown that the glycosylation of certain proteins may affect the transit time through the cell (reviewed in [6]).

Rat α_1-acid glycoprotein (AGP) seems a good model with which to study the possible influence of the glycan structure of a secreted protein on its transit time through the cell, since, because of its affinity to concanavalin A (ConA), the different mature glycoforms in the secretion medium can easily be separated. The relative proportions of these forms are known to vary greatly in pathological states in man [7, 8] as well as in the rat [9].

The rate of secretion of the ConA-non-retained form of AGP was found to be higher than that of the ConA-retained form, not only in normal rats but also in rats suffering local inflammation for 40 h, in which the secretion of the retained form is predominantly increased [10]. This latter finding suggests that there is no direct correlation between the rate of secretion and the level of synthesis. Furthermore, these results point to the co-existence of two kinetically different intracellular pathways for two glycoforms of a single protein, AGP.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats were purchased from Charles River Laboratory (St. Aubin-les-Elbeuf, France). Local inflammation was induced by subcutaneous injection of 1 ml of turpentine 40 h before liver removal for hepatocyte isolation (referred to as 'inflamed' rats).

Preparation of hepatocyte monolayers

Hepatocytes from normal or inflamed rats were isolated using a collagenase perfusion technique [11] as modified by Davy *et al.* [12]. Hepatocyte suspension (10 ml) in Krebs buffer, pH 7.4 (1.8×10^6 cells/ml) was incubated on collagen-coated plates at 37 °C in a humidified atmosphere of CO_2/air (1:19).

Antibody purification

Antisera against rat AGP and rat albumin were raised in rabbits as previously described [13]. Specific immunoglobulins against rat AGP and albumin were purified by immunoaffinity chromatography according to the procedure of Ternynck [14].

Immunoenzymic assay

A 'sandwich' type e.l.i.s.a. was used to quantify AGP and albumin in secretion media and cell lysates, as previously described [15].

Radiolabelling experiments

Pretreatment. After adhesion to collagen, the medium containing unattached cells was discarded and cells were preincubated for 30 min in 9 ml of Eagle's minimum essential medium lacking L-methionine (Gibco–Biocult) supplemented with non-essential amino acids (Gibco–Biocult) and 10 mM-L-lactate.

Abbreviations used: RER, rough endoplasmic reticulum; AGP, α_1-acid glycoprotein; ConA, concanavalin A; PMSF, phenylmethanesulphonyl fluoride.

* To whom correspondence should be addressed.

Pulse labelling. After preincubation, the medium was removed and cells were further incubated in identical medium supplemented with L-[^{35}S]methionine (2 MBq/ml) for 10 min.

Chase. The pulse medium was removed and the cells were rapidly washed twice with 10 ml of Krebs buffer supplemented with 2 mM unlabelled L-methionine. To determine the zero point of chase, one plate was placed on ice and cells were lysed in lysis buffer [25 mM-Tris/HCl, pH 7.5, 20 mM-NaCl, 1% sodium deoxycholate, 1% Triton X-100, 1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM-benzamidine and 20 μM-leupeptin; 6 ml per plate]. The other plates received 9 ml of chase medium consisting of Eagle's minimum essential medium (Gibco–Biocult) supplemented with non-essential amino acids and 10 mM-L-lactate and containing an excess of L-methionine (2 mM). Following various chase times, plates were placed on ice. The medium was collected, immediately supplemented with 1 mM-PMSF, 1 mM-benzamidine and 20 μM-leupeptin, centrifuged for 10 min at 1000 *g* and stored at −20 °C until use. Cultures were carefully washed twice with Tris-buffered saline (at 4 °C) and then lysed in lysis buffer, centrifuged at 10000 *g* for 10 min and stored at −20 °C until use. Aliquots of media and cell lysates were processed for immunoprecipitation of AGP and albumin.

Immunoprecipitation

Preparation of the sample. Media were first adjusted to correspond to the immunoprecipitation buffer (20 mM-Tris/HCl, pH 8.0, 1% Triton X-100, 5 mM-EDTA, 1 mM-PMSF, 1 mM-benzamidine and 20 μM-leupeptin). Aliquots of media and cell lysates were made up to a volume of 3 ml with immunoprecipitation buffer.

Immunoprecipitation. Purified anti-(rat AGP) (20 μl) or purified anti-(rat albumin) antibody solution (30 μl) was added to each sample of cell lysate and medium. After 2 h of incubation at room temperature under constant rotation, 150 μl of a suspension of Protein A–Sepharose pre-equilibrated in immunoprecipitation buffer (50%, v/v) was added and samples were incubated overnight at 4 °C with constant rotation. The adsorbed immunocomplexes were pelleted by centrifugation for 5 min at 5000 *g*. The pellets were washed five times with immunoprecipitation buffer supplemented with 0.1% SDS and three times with 50 mM-sodium phosphate buffer, pH 7.5. Proteins were eluted by adding 200 μl of sample buffer [0.1 M-Tris/HCl, pH 6.8, 5% (v/v) 2-mercaptoethanol and 2% (w/v) SDS] at 95 °C for 5 min. The radioactivity in the eluted proteins (AGP or albumin) was counted in a liquid scintillation spectrometer. The purity of the immunoprecipitated AGP was checked by SDS/polyacrylamide-gel electrophoresis.

ConA–Sepharose chromatography of secreted AGP

Aliquots of media corresponding to the different chase times were dialysed, concentrated and adjusted to correspond to the starting buffer (0.01 M-sodium acetate, pH 6.0, 0.25 M-NaCl, 1 mM-$MgCl_2$ and 1 mM-$CaCl_2$). A small aliquot was used for AGP immunoprecipitation and quantification as described above, and the rest was applied to 1.5 ml columns of ConA–Sepharose (Pharmacia, 10 ml/h, room temperature). The first fraction (not retained by ConA) was eluted with the starting buffer, the second fraction (retained by ConA) was eluted with the starting buffer supplemented with 0.15 M-methyl-D-glucopyranoside and the third fraction (strongly retained by ConA) was eluted with the starting buffer supplemented with 0.5 M-methyl-α-D-mannopyranoside. Immunoprecipitation of AGP contained in the three fractions was performed as described above. The ConA–Sepharose chromatography recovery was 86±4%.

Peptide and protein quantification

Results of the chase experiments were expressed as the ratio of radioactivity incorporated into AGP or albumin to total (intra-+extra-cellular) protein-associated radioactivity for each plate. Radioactivity incorporated into total proteins was determined using phosphotungstic acid precipitation.

Data analysis

The results were analysed by approximating our system to the tricompartmental model:

$$A \rightarrow B \rightarrow C$$

where A and B represent the intracellular compartments, not accessible by experimental approaches. C represents the extracellular compartment. The experimental data were least-square fitted using a numerical iterative procedure. Then the curves were plotted and the half-times of secretion calculated using the set of best-fit parameters for each experiment.

Student's *t* test for paired samples was used to compare the half-times of secretion of ConA–non-retained forms with ConA-retained ones. The means are derived from at least four experiments.

RESULTS

In preliminary studies (results not shown), the secretion of AGP was found to be linear during the first 4 h of incubation (0.22 μg/4 h per 10^6 cells for hepatocytes from normal rats and 11 μg/4 h per 10^6 cells for hepatocytes from 40 h-inflamed rats). The two hepatocyte preparations also differed in the relative proportion of the ConA-retained form of AGP (13% for normal rats and 84% for inflamed rats).

Pulse-labelling experiments performed to determine the half-time of secretion of newly synthesized AGP (Fig. 1) gave values of 32 min for normal rats and 48 min for inflamed rats. This is consistent with values previously reported for other rapidly secreted glycoproteins, e.g. α_1 protease inhibitor [5] in Hep G2 cells.

The different chase-time media were applied to ConA–Sepharose and the AGP present in the three separated fractions was immunoprecipitated. No AGP was detected for normal or inflamed rats in the third fraction, which was eluted with the starting buffer supplemented with 0.5 M-methyl-α-D-mannopyranoside. The secretion of the two first labelled fractions eluted respectively with starting buffer (ConA-non-retained form) and with starting buffer supplemented with 0.1 M-methyl-α-D-glucopyranoside (ConA-retained form) were studied in normal and inflamed rats. Fig. 2(*a*) shows the secretion of the two labelled fractions obtained from normal rats, indicating that the ConA-non-retained fraction was secreted at a higher rate (half-time of secretion 25 min) than the ConA-retained form (59

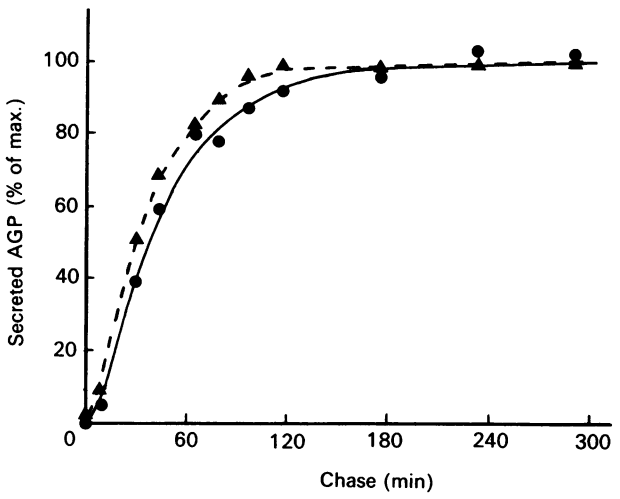

Fig. 1. Kinetics of secretion of pulse-labelled AGP in hepatocytes from normal and inflamed rats

Cells were pulse-labelled for 10 min with [^{35}S]methionine as described in the Materials and methods section. Extracellular radiolabelled AGP from normal (▲) and inflamed (●) rats was immunoprecipitated at the different chase times (10, 30, 45, 60, 80, 120, 180, 240 and 300 min) and the radioactivity was quantified in a liquid scintillation spectrometer. Radioactivity was expressed as a proportion of that contained in total proteins (intracellular + extracellular) of each plate. On the figure, data are expressed as percentage of the maximum secreted value during the chase. The curves shown are from a single representative experiment. Points are experimental data and the lines are the result of best-fit analyses. The mean of the secretion half-times obtained for at least four experiments is given in the text.

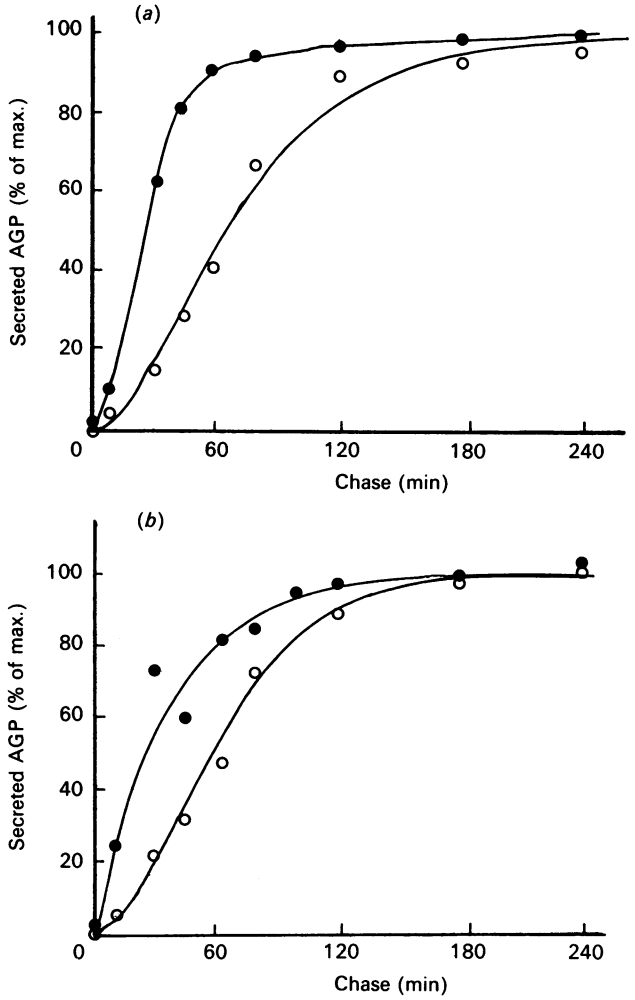

Fig. 2. Kinetics of secretion of pulse-labelled ConA-retained and ConA-non-retained forms of AGP

Cells isolated from normal (*a*) and inflamed (*b*) rats were pulse-labelled for 10 min with [^{35}S]methionine as described in the Materials and methods section. At the different chase times, extracellular AGP was separated into two fractions using ConA–Sepharose chromatography, i.e. ConA-retained (○) and ConA-non-retained (●), and then immunoprecipitated. Data are expressed as indicated in the legend to Fig. 1.

min). This difference was highly significant ($P < 0.001$). A similar significant difference between ConA-non-retained and ConA-retained forms was observed with AGP from inflamed rats (37 min and 50 min respectively; $P < 0.01$; Fig. 2*b*). The half-time of albumin secretion in normal rats (42.5 min) was similar to results obtained by other authors [1,16]. Although the synthesis of albumin is decreased during the inflammatory response [17,18], no significant modification of its half-time of secretion (39 min) was observed here after turpentine injection (Fig. 3).

DISCUSSION

Both forms of AGP (ConA-retained and -non-retained) appeared to be fully mature, i.e. bearing complex-type glycans, since no high-mannose AGP glycans were found in the secretion media (results not shown). Since both forms have the same peptide moiety and since the conversion to endoglycosidase-H resistance occurs in the medial Golgi, their structural differences can only result from processing events occurring later, either in the *trans*-Golgi or the *trans*-Golgi network. We have shown that ConA-non-retained and ConA-retained AGPs were secreted at different rates, in both normal and inflamed rats. Since the proportions of the two forms in the two hepatocyte culture media were different, there would appear to be no correlation between their respective levels of synthesis and rates of secretion. Moreover, this difference in the intracellular transport kinetics of two glycoforms of an identical protein would indicate the existence of two different pathways based only on glycan structure. Firestone *et al.* [19] suggest that late events in glycan maturation modify glycoprotein conformation and/or excretion. Analogous results have been obtained in studies of membrane-bound proteins. Haffar *et al.* [20] showed the existence of two different pathways for membrane-associated proteins, one involving a glucocorticoid-regulated site within the Golgi after the acquisition of the endoglycosidase H-resistant oligosaccharides; the second, which would be a non-regulated pathway, could be used for most membrane species. The same suggestion has been made by Clarke *et al.* [21] concerning the endocytosis of asialoglycoproteins, with the existence of two different vesicle systems in the *trans*-Golgi network which permit the return of the asialoglycoprotein receptor to the cell membrane. Similar

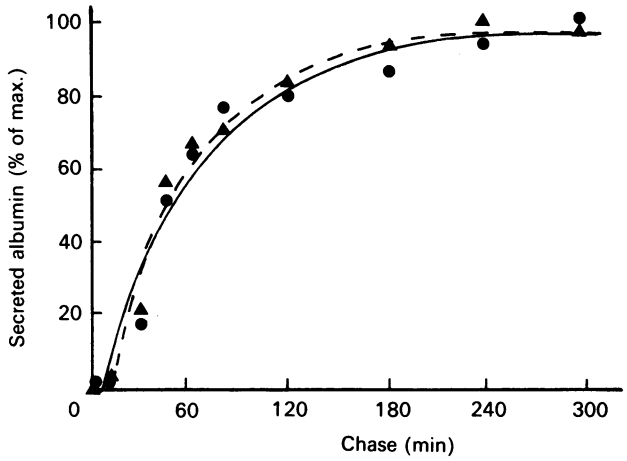

Fig. 3. Kinetics of secretion of pulse-labelled albumin in hepatocytes from normal and inflamed rats

Cells were labelled and secretory albumin was immunoprecipitated as described in the legend to Fig. 1. Data are expressed as indicated in Fig. 1. ▲, Normal rat; ●, inflamed rat.

findings have been reported with regard to the transferrin receptor.

The results presented here are consistent with the hypothesis that secreted proteins are transported from the Golgi in different vesicles which take different amounts of time to reach the cell membrane, depending not only on peptide structure but also on the nature of the glycan moieties.

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