Release of soluble resident as well as secretory proteins from HepG2 cells by partial permeabilization of rough-endoplasmic-reticulum membranes

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Secretory proteins migrate from the rough endoplasmic reticulum (ER) to the Golgi complex at different rates. Selective retention of specific proteins to rough-ER membrane constituents could explain this phenomenon. We have permeabilized HepG2 cells with low concentrations of saponin. Release of newly synthesized proteins was studied after brief labelling in the presence of [³⁵S]methionine. The efflux of several secretory proteins was studied at various saponin concentrations; a 2-fold higher saponin concentration was required to release transferrin compared with that required to release albumin and orosomucoid. Glucosidase II, a soluble resident protein of the ER, is released at the same saponin concentration as albumin. Saponin did not destroy the membrane skeleton structure; at the concentrations used, the integral membrane protein G of vesicular-stomatitis virus remained fully associated with the cells.

INTRODUCTION

The endoplasmic reticulum (ER) is the primary site for synthesis of proteins and lipids destined for multiple locations in the cell. Some proteins remain in the rough ER as residents, whereas others are transported to the Golgi complex for cellular or extracellular destinations. The export of membrane and secretory proteins from the rough ER is a selective process (reviewed in [1]). This phenomenon was initially recognized for secretory proteins from pancreas and liver. Pancreatic amylase remains longer in the rough ER than trypsinogen [2]. In hepatoma cells different secretory proteins arrive in the Golgi complex at specific time periods after their synthesis; albumin and α_1 -antitrypsin leave the rough ER at much higher rates than does transferrin [3–6]. In addition to the transient ER proteins, two categories of resident proteins can be distinguished: integral membrane and luminal (soluble) proteins. The newly made secretory proteins must somehow be distinguished from the luminal resident proteins during their transport out of the rough ER. Recently, Munro & Pelham [7] discovered that three luminal rough-ER proteins share the C-terminal sequence Lys-Asp-Glu-Leu [7]. They conclude that this oligopeptide is a retention signal for soluble resident rough-ER proteins. However, it is not known with which (rough-) ER component this oligopeptide interacts.

If newly synthesized secretory proteins are initially associated with resident proteins or lipids of the rough ER, possibly via a specific C-terminal tetrapeptide, this can explain why some proteins leave the rough ER faster than others. Assuming a non-selective vesicle transport between the rough ER and the Golgi complex, it is likely that typical 'slow' proteins (like transferrin in hepatoma cells) interact relatively strongly with these resident components of the rough ER, whereas 'fast' proteins, like albumin, do not interact. This concept is strongly

suggested by experiments of Wieland *et al.* [8], who showed that oligosaccharide-linked tripeptides were transported from the rough ER to the cell surface at the rate of bulk flow. In the present study we have made an attempt to examine the nature of the retention by which soluble rough-ER proteins are kept in the lumen of the ER. We have permeabilized cellular membranes of human hepatoma cells (HepG2) such that soluble cytoplasmic and compartmentalized proteins can escape while integral membrane proteins remain in place. We have measured the retention of different secretory proteins and of glucosidase II, a soluble rough-ER resident protein that is synthesized in the rough ER as a glycoprotein with one or two high-mannose oligosaccharide chains [9]. Permeabilization of rough-ER membranes results in selective release of secretory proteins; transferrin (a slow-moving protein) escapes from the ER membranes at a higher detergent concentration than do fast-moving proteins like albumin and orosomucoid. The rough-ER enzyme glucosidase II behaves like albumin.

MATERIALS AND METHODS

Cells

The human hepatoma cell line HepG2 (clone a16) was cultured in monolayer in Eagle's minimal essential medium containing 10% (v/v) 'decomplemented' fetal-bovine serum [10]. Infection with vesicular-stomatitis virus was done for 4 h before the start of the metabolic labelling, as previously described [3].

Metabolic labelling and solubilization of cells

Nearly confluent HepG2 cells grown on 35 mm-diameter Petri dishes were labelled for 10 min with [³⁵S]methionine (60 μ Ci/ml) (800–1200 Ci/nmol; The Radiochemical Centre, Amersham, Bucks., U.K.). After

Abbreviations used: ER, endoplasmic reticulum; VSV-G, integral membrane protein G of vesicular-stomatitis virus; PBS, phosphate-buffered saline (0.05 M-sodium phosphate/0.15 M-NaCl, pH 7.4) without Ca²⁺; $t_{1/2}$, half-time (of transport); SDS/PAGE, SDS/polyacrylamide-gel electrophoresis.

metabolic labelling the cells were washed in phosphate-buffered saline without Ca^{2+} (PBS) at 0°C and then placed on a rocking platform in 1 ml of PBS containing various concentrations of saponin (Merck, Darmstadt, Germany) for 30 min. In some experiments 0.25 M-sucrose in 20 mM-Hepes, pH 7.4, was used instead of PBS. Thereafter, the permeabilization medium was removed and the cells were washed with PBS and lysed in 1% Triton X-100/1 mM-phenylmethanesulphonyl fluoride in PBS. Portions of the cell extracts and the permeabilization media were used for immunoprecipitation.

Immunoprecipitation and SDS/polyacrylamide-gel electrophoresis

Immunoprecipitations were carried out as previously described [3]. Portions of the Triton X-100-soluble material were immunoprecipitated with either normal rabbit IgG (control) or rabbit anti-(human albumin), anti-(human transferrin), anti-(human total serum proteins), or anti-(human orosomucoid) antisera (Nordic, Tilburg, The Netherlands). The antiserum against pig kidney glucosidase II, prepared as reported previously [11], was used. IGSORB (New England Enzyme Center, Boston, MA, U.S.A.) was used to isolate immune complexes. To test the immune assays for completeness, increasing amounts of specific antiserum were added to aliquots of the labelled extract and the immune precipitates were analysed in 10%-(w/v)-polyacrylamide gels in the presence of SDS (SDS/PAGE). In all instances a sufficient amount of antiserum was added to immunoprecipitate the antigen quantitatively. After electrophoresis the gels were fluorographed and the fluorograms were scanned with a microdensitometer (E.C. Apparatus, St. Petersburg, FL, U.S.A.) within the linear range of the film and the microdensitometer.

RESULTS

The rough ER is the primary site for synthesis of proteins on membrane-bound polyribosomes and the starting point of their transport to multiple destinations. Thus proteins present in the lumen of the rough ER are either newly synthesized and destined for transport to other organelles (mostly to be secreted) or they belong functionally to the rough ER. To study the mechanisms underlying discrimination of these proteins, we solubilized HepG2 cells with low concentrations of saponin. Cells were first incubated for 10 min in the presence of [^{35}S]methionine to label the total complement of rough-ER proteins. At this time most of the [^{35}S]methionine-labelled secretory proteins are still present in the rough ER. As Fig. 1 shows, newly synthesized albumin was released at saponin concentrations higher than 0.2 mg/ml; at 1 mg/ml all labelled albumin could be removed from the cells. Albumin is transported from the rough ER to the Golgi with a half-time ($t_{1/2}$) comparable with that of α_1 -antitrypsin (20 min), whereas transferrin has a $t_{1/2}$ of 150 min [5]. To understand the reason for this difference, a model was proposed in which export from the rough ER is signal-dependent, i.e. slow-moving proteins would leave the compartment at the bulk rate, whereas fast-moving proteins would be accelerated by binding to special carriers [6]. In this concept fast-moving albumin is expected to be washed out at saponin concentrations higher than those required to wash out slow-moving transferrin. Fig. 1 shows that this is not the

Fig. 1. Saponin titration of release of rough ER proteins

HepG2 cells, infected with vesicular-stomatitis virus for 4 h, were preincubated in minimal Eagle's medium without methionine for 20 min and then labelled in the presence of $60 \mu\text{Ci}$ of [^{35}S]methionine/ml for 10 min. Thereafter the cells were washed and permeabilized in the presence of various concentrations of saponin. The cells were completely solubilized in 1% Triton X-100. The amounts of specific proteins present in the cells were determined after quantitative immunoprecipitation, analysis on SDS/PAGE and quantification of the fluorograms. The release is the amount of protein expressed as a percentage of that present in the cells not treated with saponin. Each point is the mean result for two independent experiments. $\circ$, Albumin; $\bullet$, glucosidase II; $\blacksquare$, transferrin; $\square$, VSV-G.

case. Transferrin can only be washed out of the rough-ER lumen at detergent concentrations higher than 0.5 mg/ml, which is clearly different from that required to wash out albumin. To elaborate this further we tested the release of another 'fast-moving' secretory protein, namely orosomucoid. This glycoprotein behaved exactly the same as albumin (results not shown). Our conclusion is that 'fast-moving' proteins like albumin and orosomucoid interact less with ER constituents than does a 'slow-moving' protein like transferrin. To exclude the possibility that saponin destroyed the membrane skeleton structure, vesicular-stomatitis-virus-infected HepG2 cells were used, labelled for 10 min and treated with the same concentration of saponin. Fig. 1 shows that, at the detergent concentrations used, the integral membrane glycoprotein (VSV-G) remained fully associated with the cells.

In addition to integral membrane proteins, the rough ER also contains water-soluble resident proteins. An example of such a protein is glucosidase II [9]. This 100 kDa enzyme is regarded a functional protein of the ER. It removes α 1,3-linked glucose from high-mannose oligosaccharides *N*-linked to asparagine residues of glycoproteins. It itself is a glycoprotein with high-mannose (Asn-GlcNAc₂-Man₉-Glc₁) oligosaccharide chain(s). We therefore determined the effect of saponin on the release of this enzyme. Fig. 1 shows that glucosidase II is washed out at exactly the same saponin concentrations as those at which 'fast-moving' secretory proteins are washed

Fig. 2. Effect of Ca²⁺ on saponin permeabilization

Vesicular-stomatitis-virus-infected cells were labelled for 10 min and thereafter the saponin-induced efflux of labelled proteins was determined at various Ca²⁺ concentrations. An intermediate saponin concentration ([S]) (0.85 mg/ml) was used in the presence of 0.25 M-sucrose. At this concentration only 10 % of the rough-ER albumin is released in the absence of Ca²⁺. As a control, the release of VSV-G was determined (left panel). The right panel shows immunoprecipitated serum proteins released from the cells. The lanes 6, 12 and 18 show the effect of 1.8 mg of saponin/ml; at this high concentration no VSV-G and almost all albumin was released from the cells, whereas in the medium maximal release of total serum proteins was obtained.

out. If the release was measured in cells after a chase period of 1 h, no difference was noted, indicating that both newly and previously synthesized glucosidase II behave identically. If the release was measured in the presence of 0.25 M-sucrose and 20 mM-Hepes, pH 7.4, instead of PBS, all release patterns shifted to higher concentrations of saponin, reflecting the membrane-stabilizing effect of sucrose; however, differences in release between albumin, glucosidase II and transferrin remain the same.

The ER is a Ca²⁺-storing compartment (reviewed in [12]). Therefore we explored the possibility that Ca²⁺ plays a role in rough-ER sorting processes. Fig. 2 shows the effect of increasing concentrations of Ca²⁺ on the release of [³⁵S]methionine-labelled proteins from the rough ER. The cells were infected with vesicular-stomatitis virus to monitor the behaviour of integral membrane proteins (VSV-G) as well. The release was measured at an intermediate saponin concentration (0.85 mg/ml in sucrose/Hepes). Under these conditions limited amounts of newly synthesized proteins are released from the rough ER (Fig. 2). When the same permeabilization was done in the presence of increasing concentrations of Ca²⁺, almost all albumin left the cells, resulting in a 2–10-fold increase. VSV-G remained fully associated with the cells at all concentrations, indicating that no cells were lost during the wash procedure and no membranes were destroyed (Fig. 2, lanes 1–6). To investigate the effect of Ca²⁺ on other secretory proteins, we used the wash-out media to immunoprecipitate total serum proteins. As

Fig. 2 (lanes 13–18) shows, the amount of albumin reflects the amount of complement compared with that present in the cells at different saponin concentrations. Several other proteins efflux from the cells in comparable quantities. Among them are proteins of lower, as well as higher, molecular mass compared with albumin. Thus it is unlikely that release of proteins from semi-permeabilized rough ER is dependent on molecular mass in the range between 30 and 150 kDa. If, instead of Ca²⁺, Mg²⁺ was present in the saponin wash buffer, only a slight increase (less than 10 % of the Ca²⁺ effect) of release was observed. Also, in the presence of EDTA, or if the wash procedure was carried out at different pH values (5.7–8.2), no increase was noted.

To measure the kinetics of protein release from the rough ER in the presence of low concentration of detergent, it is essential that these proteins, once released from the ER, can pass the cell surface membrane without restriction. To rule out the possibility that saponin acts differently on rough ER and cell membranes, we tested the effect of various saponin concentrations on release of total cellular proteins. Fig. 3 shows that cellular proteins, mostly cytosolic, are washed out at the same, or somewhat lower, saponin concentration as 'fast-moving' secretory proteins. The extent of release of acid-precipitable radioactivity was comparable with protein loss determined by a total protein assay (results not shown). This implies that appearance of secretory proteins in the wash-out medium reflects the release from the ER content.

Fig. 3. Release of total proteins from saponin-treated cells

Pulse-labelled vesicular-stomatitis-virus-infected cells were incubated with various concentrations of saponin [S] (in PBS) for 30 min on ice. The cells were then washed in PBS and lysed in 1% Triton X-100. Aliquots (20 μ l) of the solubilized cells were analysed by SDS/PAGE.

DISCUSSION

In the present study we show that saponin can be used to make rough ER membranes permeable to proteins. Previous studies have shown that, in addition to the plasma membrane, endomembranes are also permeabilized in the presence of saponin [13,14]. Saponin-induced protein release is primary due to complex-formation with cholesterol [15,16]. Membranes containing high concentrations of cholesterol (plasma membrane) release their soluble protein content at lower saponin concentrations than do membranes with little cholesterol (rough ER and Golgi complex) [17–19]. We have shown that saponin is able to permeabilize rough-ER membranes such that integral membrane proteins are completely retained in the cell, while at the same time soluble proteins are fully released. Moreover, the protein size is not a critical factor, as released of both high- and low-molecular-mass proteins (in a range between 30 and 150 kDa) is the same at a certain saponin concentration. As release of proteins from permeabilized cells is dependent on both saponin concentration and time, we have measured release at various saponin concentrations at a fixed time (30 min) at 0 °C. This time was chosen as maximal release was obtained at about 20 min at all saponin concentrations tested. This has also been reported for the release of albumin and haptoglobin from rat hepatocytes [20] and for tyrosine hydrolase and phenylethanolamine *N*-methyltransferase from chromaffin cells [21].

The first sorting event after translocation of proteins synthesized on membrane-bound polyribosomes occurs in the lumen of rough-ER cisternae. Sorting is accomplished between resident proteins of rough ER and others. Thus secretory proteins are continuously sorted from soluble ER proteins. Different proteins are transported to the Golgi complex at different rates [3,5]. If transport

delay is due to interaction of the 'slow' secretory proteins with ER membrane constituents, partial permeabilization of the ER membrane would release those proteins first which had no, or a weak, interaction with these constituents. In this concept transferrin, a protein slowly transported between the rough ER and the Golgi complex, is released at a higher saponin concentration than are albumin and orosomucoid. The nature of this interaction is unknown; there is presumably involvement of neither Ca^{2+} ions nor of pH differences in the range of 5.7–8.2. However, all soluble proteins tested can escape from the rough ER at saponin concentrations well below that required to release intrinsic membrane proteins. This observation could be taken as evidence that protein-lipid interactions are involved in the retention of 'slow-moving' secretory proteins.

For soluble resident proteins like glucosidase II, the situation is different. Munro & Pelham [7] noted that a C-terminal tetrapeptide (KDEL in the one-letter notation) is necessary for retention of a number of resident rough-ER proteins. It is not known whether this peptide is the only retention mechanism for soluble ER proteins, but this model suggests interaction between a specific oligopeptide and some other constituent in order to obtain retention. At present it is unknown whether glucosidase II contains this 'retention' signal peptide. Recently, Pelham [22] attached the tetrapeptide to the lysosomal enzyme cathepsin D, and his experiments support the idea that soluble ER proteins are transported to a post-ER compartment. In this context it is tempting to believe that soluble ER resident proteins exit the rough ER as 'fast-moving' secretory proteins, but are selectively and actively sorted in the *cis*-Golgi to be returned to the rough ER. This complies with our data showing that glucosidase II is barely retained in the rough ER in the presence of low concentrations of saponin. If glucosidase contains the KDEL sequence, our data show that this does not result in a membrane interaction strong enough to prevent rapid release. Taken together our results suggest that luminal ER proteins are retained by more than one retention and/or egress mechanism. This is further substantiated by the possibility of the ER-degradation pathway suggested by Lippincott-Schwartz *et al.* [23].

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