Short Communication

Posttranslational Processing of Proteins in Vacuoles and Protein Bodies Is Inhibited by Monensin

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ABSTRACT
A number of proteins that accumulate in vacuoles and protein bodies undergo posttranslational processing at these accumulation sites. These processing steps include proteolytic cleavage (e.g., pea lectin, soybean glycinin, and rice lectin) and the removal of some sugar residues from oligosaccharide side-chains (e.g., bean phytohemagglutinin). Treatment of immature rice embryos with the sodium ionophore monensin slows down the proteolytic processing of the rice lectin precursor (M, 23,000) to mature rice lectin (M, 10,000 and 8,000). Treatment of developing bean cotyledons with monensin slows down the removal of peripheral N-acetylglucosamine residues from the oligosaccharide side-chains of phytohemagglutinin. The results are consistent with the interpretation that these processing steps, which occur in vacuoles or protein bodies, are carried out by enzymes with an acidic pH optimum, and that monensin slows down processing by alkalization of the vacuoles or protein bodies.

Proteins such as storage proteins and lectins which accumulate in vacuoles and protein bodies are synthesized on the RER and transported via the endomembrane system to their sites of accumulation. The accumulation of these proteins in the vacuoles and protein bodies is accompanied by specific posttranslational processing steps such as proteolytic cleavage of the polypeptides (pro-proteins to proteins) or trimming of sugar residues from the oligosaccharide side-chains of glycoproteins (for review, see Ref. 4). Because of our interest in protein processing, we have studied the effect of the sodium ionophore monensin on processing. Work with animal cells has shown that monensin interferes with both exocytosis and endocytosis of proteins. Interference with exocytosis has been shown to be due to inhibition of protein transport from the Golgi cisternae to the transport vesicles. Interference with endocytosis is thought to be related to the alkalization of the prelysosomal and lysosomal compartments (for review, see Ref. 22). Such an alkalization effect has actually been described for the related ionophore nigericin (15). In this report, we show that monensin slows down the proteolytic processing of lectin in developing rice embryos, and the glycolytic processing (removal of GlcNAc residues) of phytohemagglutinin in developing bean (Phaseolus vulgaris) cotyledons. We postulate that this inhibition of protein processing is caused by the alkalization of the compartments (vacuoles or protein bodies) in which these proteins accumulate.

MATERIALS AND METHODS

Materials. Plants of Oryza sativa L. cv Koshihikari were grown in a greenhouse at 28 °C during the day (13 h) and 20 °C during the night. Experiments were carried out with developing embryos about 20 d postanthesis (weighing about 0.7 mg) when the rate of lectin accumulation is maximal (19). Plants of Phaseolus vulgaris L. cv Greensleeves were grown in a greenhouse. Organic chemicals were purchased from Sigma Chemical Co. unless otherwise indicated, and radiochemicals from Amersham Company.

Radioactive Labeling. Radioactive labeling of excised bean cotyledons was done as described by Spencer et al. (18) with L-[5,6-3H]fucose (25.6 Ci/mmol), or D-[6-3H]glucosamine-hydrochloride (24.8 Ci/mmol). Cotyledons were labeled with 5 μCi of precursor each, and the radioactive tissue was collected by cutting a thin slice from the cotyledon with a razor blade. The remainder of the cotyledon was discarded.

Fresh rice embryos weighing 0.7 mg each, were isolated with a scalpel blade, and lots of 40 embryos were incubated in a nutrient medium (4% [w/v] sucrose, 150 mM glutamine, 5 mM KCl, 4 mM CaCl₂, and 0.05% [w/v] ampicillin [pH 6.1]) containing 10 μCi of [35S]cysteine (1270 Ci/mmol) at 30 °C for 1.5 h. Thereafter, the embryos were washed free of labeled precursor and further incubated for different times in the presence of 10 mM (freshly made) unlabeled cysteine in nutrient medium (pH 6.1). Labeled cysteine was chosen as precursor because of the unusually high cysteine content (about 24%) of rice lectin (23). Monensin was added to a final concentration of 50 μM (during pulse and chase periods) by taking 1/10 of a stock solution of 500 μM monensin in 1% ethanol. Ethanol was also added to the controls to a final concentration of 0.1%.

Homogenization of the Rice Embryos, Fractionation of the Extracts, and Isolation of Rice Lectin. The embryos were homogenized with a mortar and pestle in 1 ml of buffer (100 mM Tris-HCl [pH 7.8], 1 mM EDTA, and 12% [w/w] sucrose) at 4°C, (using a ratio of 30:1 for medium: tissue) and the homogenate was centrifuged for 1 min at 1000g to remove nuclei and cell wall debris. Then the radioactivity incorporated into protein (TCA-precipitable material) was determined. The organelles were separated from soluble proteins and small molecules by Sepharose-4B (Pharmacia, Uppsala, Sweden) chromatography on 10 x 1 cm columns (Econo-column, Bio-Rad) in the same buffer. To visualize the peak of the soluble proteins, 500 μg of Cyt c was
added. The light scattering organelle peak can easily be identified. Organelle and soluble fractions were brought to 0.5% Triton X-100, prior to isolation of the lectin. Rice lectin was isolated from all fractions by affinity chromatography on immobilized N-acetylglucosamine (Selectin 1, Pierce Chemical Company, IL) as described previously (19).

Homogenization of the Bean Cotyledons and Isolation of PHA. The three to five slices of radioactive tissue were homogenized with a mortar and pestle in 3 ml of PBS (10 mM K2HPO4-HCl [pH 7.4] with 150 mM NaCl) containing 1% (w/w) Tween 20, when intact organelles were not needed, or in the same way as the rice embryos when intact organelles were desired. The latter method of homogenization disrupts the protein bodies resulting in the admixture of cytosol and protein body content. The affinity procedure of Felsted et al. (7) was used to isolate PHA from soluble or organelle fractions, after the addition of 1% (w/w) Tween 20. The PHA eluted from the thyroglobulin affinity column was dialyzed against distilled H2O, lyophilized, and used for proteolytic digestion to obtain the glycopeptides.

Isolation of PHA Glycopeptides and Gel-Filtration Chromatography. The radioactive, affinity-purified, lyophilized PHA was resuspended in 500 μl of 50 mM Tris-HCl (pH 8.5) and 1 mg of proteinase K (Merck, Darmstadt, F.R.G.) was added; one drop of toluene was added and the solution incubated at 37°C. After 24 h, 1 mg of pronase (CB grade, Calbiochem) was added and incubation carried out for another 24 h. The reaction was stopped by adding 15 μl of glacial acetic acid and the solution was loaded on a column (20 x 1 cm) of Bio-Gel P-4 (minus 400 mesh) (Bio-Rad) equilibrated with 0.1 N acetic acid. Chromatography was performed at room temperature. Fractions (1.0 ml) were collected and 20 μl counted for radioactivity and the fractions combined and freeze-dried. Digestion with β-N-acetylglucosaminidase (from Jack Bean, Sigma Chemical Co.) was performed by incubation at 37°C for 48 h in 500 μl of 50 mM Na-citrate (pH 4.6) with 5 units of enzyme or without enzyme as a control.

Analysis of the Glycopeptides. To determine the size of the glycopeptides (before and after enzyme treatment) they were chromatographed on a Bio-Gel P-4 column as described above, except that the column was 100 cm long. To determine the proportion of radioactivity in terminal GlcNAc, the enzyme-treated mixture was fractionated on a 20 cm column and the glycopeptide peak separated from the free GlcNAc. The radioactivity in each peak was determined and totaled.

SDS-PAGE and Fluorography. SDS-PAGE was performed on 12.5% polyacrylamide gradient gels using a discontinuous system (10). Fluorography was done with Amplify (Amersham, UK) as a scintillator. The relative intensity of labeling of bands on gels was determined by cutting the gels and counting the radioactivity, in the polypeptides.

RESULTS

Monensin Slows Down Proteolytic Processing of Rice Lectins. Rice lectin is present in the endomembrane system of rice embryos as a high mol wt precursor of Mr, 23,000. During or shortly after its transport from the ER to its site of accumulation, this pro-protein is converted into an Mr, 18,000 lectin polypeptide (20, 21). After this first processing event, this polypeptide is further cleaved into two smaller pieces of Mr, 10,000 and 8,000, respectively (20, 21). To find out how monensin affects these processing steps, we carried out a pulse-chase experiment with [35S]cysteine as described. The homogenates were fractionated into a soluble (i.e. cytosol + vacuolar contents) fraction and an organelle fraction, and lectin isolated by affinity chromatography from the soluble fraction. The lectin polypeptides were fractionated by SDS-PAGE and a fluorograph prepared (Fig. 1A). The results show that at the end of the labeling period of 1.5 h most of the radioactivity was in the Mr, 23,000 polypeptide, although some processing to the Mr, 18,000 polypeptide had already occurred in the control sample (Fig. 1A, lane a). At the end of the 8 h chase, radioactivity was present in the precursor (Mr, 23,000), the intermediate (Mr, 18,000), and the final products (Mr, 10,000 and 8,000) in the control (Fig. 1A, lane c), but the final processing step was severely inhibited by monensin (Fig. 1A, lane d). The radioactive bands were cut out and the radioactivity determined and expressed as percentage in each polypeptide (Mr, 23,000, 18,000, and 10,000 + 8,000).

After the 8 h chase, 1550 and 1479 cpm [35S]cysteine was incorporated in the lectin fractions from control and monensin-treated embryos, respectively. In the lectin in control embryos, 378, 525, and 576 cpm were recovered in the Mr, 23,000, Mr, 18,000, and Mr, 10,000 + 8,000 polypeptides, respectively. In the lectin from monensin-treated embryos, 849, 553, and 148 cpm were recovered in the Mr, 23,000, Mr, 18,000, and Mr, 10,000 + 8,000 polypeptides, respectively.

The total amount of radioactivity in the soluble fraction was set at 100% for each time-point. The results (Fig. 1, B, C, and D) show that the conversion of the Mr, 23,000 precursor proceeds considerably slower in the presence of monensin than in its absence. Consequently, less of the Mr, 18,000 polypeptide was present in the embryos treated with monensin.

The most striking difference, however, was observed for the Mr, 10,000 and 8,000 polypeptides which consisted only 10% of the lectin after the 8 h chase time in the presence of monensin, whereas in the controls they constituted up to 40%. In addition to this effect on processing, monensin also slowed down the transport of lectin out of the endomembrane system. The Mr, 18,000 lectin in the endomembrane system was always 23,000 (data not shown).

Monensin Inhibits Glycolytic Processing of PHA in Developing Bean Cotyledons. The accumulation of PHA in protein bodies of bean cotyledons is accompanied by a complex processing step in which terminal GlcNAc residues are attached to a modified oligosaccharide side-chain in the Golgi complex, and slowly removed again in the protein bodies (25). This glycolytic processing step results in a reduction in size of the oligosaccharide side-chains, and of the entire glycopolyptide (26). The processing step can be mimicked in vitro by treatment of isolated glycopolyptides with β-N-acetylglucosaminidase, an enzyme which removes peripheral GlcNAc residues. The modified oligosaccharide side-chain contains fucose (26) and can be readily labeled by incubating the cotyledons with [3H]fucose. To determine how monensin affects this processing step of PHA, bean cotyledons were labeled for 6, 12, or 24 h with [3H]GlcN in the presence or absence of monensin. The cotyledons were homogenized, the unbroken organelles fraction removed, and PHA isolated from the soluble fraction which contains the contents of the protein bodies. The glycopeptide fraction obtained after proteolysis of PHA was treated with β-N-acetylglucosaminidase and the percentage of GlcNAc released was calculated. The GlcNAc which is released by this enzyme is in a terminal position on the oligosaccharide, while the GlcNAc which is not released is in the chitobiose portion of the oligosaccharide. After a short labeling period (2.5 h) about 40% of the radioactivity in GlcNAc in PHA can be removed by enzyme treatment (25). The results in Table I show that this percentage declines rapidly as the labeling period is extended. We have shown that this is due to the removal of terminal GlcNAc in the protein bodies (25). In the presence of monensin, this decline is considerably slowed indicating that the in vivo removal of GlcNAc has been slowed by the ionophore.

In a second experiment, bean cotyledons were labeled with

2 Abbreviation: PHA, phytohemagglutinin.
Table 1. Percentage of Radioactivity in GlcNAc Removed from the Glycopeptides of [3H]GlcN-Labeled PHA, by Treatment with β-N-Acetylglucosaminidase

<table>
<thead>
<tr>
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<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
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<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Monensin</td>
<td>34</td>
<td>25</td>
<td>12</td>
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FIG. 2. Cotyledons of P. vulgaris were labeled with [3H]fucose for 20 h in the presence of monensin (50 μM) or its absence. PHA was isolated from the homogenate, and digested with protease. The glycopeptides were fractionated on Bio-gel P4 (A, control; B, monensin), and the major peaks pooled (horizontal bars), treated with β-N-acetylglucosaminidase, and reanalyzed on Bio-gel P4 (C, control; D, monensin). Monensin (panel B) retards the in vivo processing of the oligosaccharide side-chains compared to control (panel A). Processing can be brought about by incubation with β-N-acetylglucosaminidase.

[3H]fucose for 20 h either in the absence or presence of monensin, and PHA isolated by affinity chromatography. The PHA was digested with protease and the radioactive glycopeptides analyzed on a Bio-Gel P-4 column. After 20 h of labeling the glycopeptides from control PHA formed a single peak around fraction 39 (Fig. 2A) representing the mature, totally processed form of the modified (i.e. fucosylated) glycopeptide of PHA. A shorter labeling time (e.g. 6 h) results in the presence of two peaks: one around fraction 34 (the precursor) and one around fraction 39 (the mature glycopeptide) (see Ref. 24). When cotyledons were labeled for 20 h in the presence of monensin, the fucose-containing glycopeptides of PHA formed two peaks (Fig. 2B): a large peak (fraction 34) where the precursor is normally found, and a small peak (fraction 39) in the position of the mature glycopeptide. Such a pattern is similar to the one obtained when control samples are labeled for 2 h only, and relatively little processing has taken place (see Fig. 7 in Ref. 24). If the large peak in Figure 2B represents unprocessed glycopeptide, then treatment with β-N-acetylglucosaminidase should cause in vitro processing. The fractions of the large peaks shown in Figure 2, A and B, were pooled, and digested with β-N-acetylglucosaminidase. The products were analyzed by chromatography on Bio-Gel P-4 in the same way (Fig. 2, C and D). The control glycopeptide was not affected by the enzyme treatment, while the glycopeptide synthesized in the presence of monensin moved to the position of the mature glycopeptide. The treatment with β-N-acetylglucosaminidase removes peripheral GlcNAc residues (25) and constitutes in vitro processing, by mimicking the in vivo removal of peripheral GlcNAc residues. This result therefore shows in a different way (independent of labeling with [3H]glucosamine) that monensin slows down the removal of GlcNAc in the protein bodies.

**DISCUSSION**

Monensin is a sodium ionophore which interferes with two aspects of cellular metabolism. It disrupts Golgi function by causing the Golgi cisternae to swell and this results in an inhibition of protein transport from the cisternae stack to the transport vesicles (reviewed in Ref. 21). Monensin disrupts the normal structure of the Golgi apparatus of plant cells (13). We have recently shown that the transport of PHA from its site of synthesis (the RER) to the protein bodies is mediated by the Golgi complex, and that monensin inhibits the transport step between the cisternae and the dense transport vesicles (3). Similarly, monensin prevents the incorporation of sugar residues into the hemi-cellulose fraction of the cell wall, and this is a convenient way to assess the effectiveness of monensin in a plant system (3). In the rice embryos used here, 50 μM monensin caused a 40% inhibition...
of the uptake of [3H]galactose, and an 82% inhibition of its incorporation into cell wall macromolecules (Stinissen, unpublished results). Monensin also affects the acidity of the prelysosomal and lysosomal compartments of animal cells, thereby interfering with endocytosis (review in Ref. 22). Raising the lysosomal pH may also interfere with the processing of lysosomal proteins, if the processing steps are carried out by enzymes with an acidic pH optimum. This happens when lysosomal pH is raised with the lysomotropic amine chloroquine (16). Monensin has recently been shown to slow down the degradative processing of proteins in animal cells (16). The evidence presented here indicates that the processing of proteins in plant vacuoles and protein bodies, which have an acidic pH (11, 14), is inhibited by monensin.

We studied two different processing steps in two different systems. Monensin inhibits the processing of rice lectin in developing rice embryos. Rice lectin and its homolog wheat germ agglutinin are synthesized as pro-proteins with \( M_r \) 23,000, and converted to polypeptides with \( M_r \) 18,000. Rice lectin then undergoes a second processing step in which the polypeptide with \( M_r \) 18,000 is split into polypeptides with \( M_r \) 10,000 and 8,000 (20, 21). The delayed posttranslational processing takes several hours to come to completion and probably occurs in the vacuoles where cereal lectins accumulate (12). Monensin has its most profound effect on the processing of the \( M_r \) 18,000 polypeptide to polypeptide with \( M_r \) 10,000 and 8,000. There is a much smaller effect on the processing of the \( M_r \) 23,000 pro-protein to the \( M_r \) 18,000 intermediate polypeptide. The subcellular site of accumulation of rice lectin is not known for certain, but wheat germ agglutinin has been shown to accumulate in the small vacuoles of meristematic cells of the wheat embryo (12). We postulated by analogy, because of the similarity of the biological and structural properties of the two molecules, that rice lectin accumulates in similar vacuoles of the rice embryo (21). Similar posttranslational proteolytic processing in vacuoles and protein bodies occurs in legume cotyledons. In developing pea cotyledons, legumin and vicilin (5) as well as lectin (9) are proteolytically processed in the protein bodies. Other proteins such as soybean glycinin (2, 17) undergo similar posttranslational proteolytic processing. These processing steps probably also occur at the sites of accumulation (see Ref. 4 for review).

Monensin also inhibits the removal of terminal GlcNAc residues from the oligosaccharide side-chains of PHA. Three residues can be removed in vitro by \( \beta-N \)-acyethylglucosaminidase, an enzyme with a pH optimum of 4.6 (1). We assume that the same enzyme which occurs in protein bodies (8) normally removes these residues in vivo. We postulate that in each case the effect of monensin on processing is due to its effect on vacuolar or protein body pH. Raising the pH of the compartment would slow down the action of enzymes with an acidic pH optimum.

**LITERATURE CITED**

2. BARTON, KA, JF THOMPSON, JT MADISON, R ROBENTHAL, NP JARVIS, RN BEACHY 1982 The biosynthesis and processing of high molecular weight precursors of soybean glycinin subunits. J Biol Chem 257: 6089-6095
3. CHRISPEELS MJ 1983 The Golgi apparatus mediates the transport of phytohemagglutinin to the protein bodies in bean cotyledons. Plant Cell 158: 140-151
7. FELSTED RL, RD LEAVITT, NR BACHUR 1975 Purification of the phytohemagglutinin family of proteins from red kidney beans (Phaseolus vulgaris) by affinity chromatography. Biochim Biophys Acta 405: 72-81
11. MATILE P 1975 The Lytic Compartment of Plant Cells. Springer-Verlag, Heidelberg
13. MOLLENHAUER HH, DJ MORRÉ, JO NORMAN 1982 Ultrastructural observations of maize root tips following exposure to monensin. Protoplasma 112: 177-126
15. OHKUMA S, B POOLE 1978 Fluorescence probe measurements of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc Natl Acad Sci USA 75: 3327-3331
22. TARTAKOFF AM 1983 Perturbation of vesicular traffic with the carboxylic ionophore monensin. Cell 32: 1026-1028