Planta (2007) 226:335–345
DOI 10.1007/s00425-007-0485-3

ORIGINAL ARTICLE

Complex formation regulates the glycosylation of the reversibly glycosylated polypeptide

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Received: 10 October 2006 / Accepted: 26 January 2007 / Published online: 1 March 2007

Abstract Reversible glycosylated polypeptides (RGPs) are highly conserved plant-specific proteins, which can perform self-glycosylation. These proteins have been shown essential in plants yet its precise function remains unknown. In order to understand the function of this self-glycosylating polypeptide, it is important to establish what factors are involved in the regulation of the RGP activity. Here we show that incubation at high ionic strength produced a high self-glycosylation level and a high glycosylation reversibility of RGP from *Solanum tuberosum* L. In contrast, incubation at low ionic strength led to a low level of glycosylation and a low glycosylation reversibility of RGP. The incubation at low ionic strength favored the formation of high molecular weight RGP-containing forms, whereas incubation at high ionic strength produced active RGP with a molecular weight similar to the one expected for the monomer. Our data also showed that glycosylation of RGP, in its monomeric form, was highly reversible, whereas, a low reversibility of the protein glycosylation was observed when RGP was part of high molecular weight structures. In addition, glycosylation of RGP increased the occurrence of non-monomeric RGP-containing forms, suggesting that glycosylation may favor multimer formation. Finally, our results indicated that RGP from *Arabidopsis thaliana* and *Pisum sativum* are associated to golgi membranes, as part of protein complexes. A model for the regulation of the RGP activity and its binding to golgi membranes based on the glycosylation of the protein is proposed where the sugars linked to oligomeric form of RGP in the golgi may be transferred to acceptors involved in polysaccharide biosynthesis.

Keywords Golgi · Plant polysaccharides · Reversibly glycosylated polypeptide · Self-glycosylation · Xyloglucan

Abbreviations

BNGE	Blue native gel electrophoresis
LIS	Low ionic strength
HIS	High ionic strength
RGP	Reversibly glycosylated polypeptide
TCA	Trichloroacetic acid
UDP	Uridine diphosphate

Introduction

A number of glycoconjugates and polysaccharides are synthesized by a multiplicity of glycosyltransferases (Leloir and Cardini 1962; Ross et al. 2001; Coutinho

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et al. 2003). Currently there are more than 7,000 genes encoding for putative glycosyltransferases (E.C.2.4.x.y) in the databases, however, only a few of them have been functionally characterized. In addition, many of them encode for membrane bound proteins, making difficult their expression in heterologous systems (Delmer 1999; Edwards et al. 1999; Perrin et al. 1999; Doblin et al. 2002).

Biochemical studies indicate that only a few glycosyltransferases are able to perform self-glycosylation (Mühlenhoff et al. 1996; El-Battari et al. 2003). In yeast and mammalian cells, a self-glycosylated protein named glycogenin is thought to be required for initiation of α(1,4)-linked glucan (Cao et al. 1995). In plants, proteins capable of self-glycosylation have also been described (Moreno and Tandecarz 1982; Dhugga et al. 1991; Tandecarz et al. 1995). The protein named reversibly glycosylated polypeptide (RGP) is capable to self-glycosylate using radiolabeled UDP-Glc, UDP-Xyl and UDP-Gal (Dhugga et al. 1991). In the presence of these radiolabeled UDP-sugars, the protein can reversibly link the sugar to itself. Upon the addition of unlabeled UDP-sugars or UDP, the radioactive sugar is chased-out from the protein (Dhugga et al. 1991). Recently, Testasecca et al. (2004) reported that the reaction catalyzed by RGP isolated from potato tuber is indeed reversible, since the radioglucosylated-RGP was capable of re-synthesizing radioactive UDP-Glc in the presence of UDP.

Immunolocalization by electronmicroscopy studies showed that RGP is located in the golgi apparatus, however, subcellular fractionation analysis also showed a significant amount of the protein in the soluble fraction (Dhugga et al. 1991, 1997; Wald et al. 1998, 2003; Testasecca et al. 2004). Recently, it was described that RGP1 from *Arabidopsis thaliana* and *Zea mays*, fused to green fluorescent protein and transiently expressed in tobacco (*Nicotiana tabacum*), are plasmodesmal-associated proteins delivered to plasmodesmata via the golgi apparatus (Sagi et al. 2005).

Dhugga et al. (1997) reported the cloning of a *Pisum sativum* RGP and then orthologs genes were described in *Arabidopsis thaliana* (Delgado et al. 1998), *Solanum tuberosum* L. (Bocca et al. 1999), *Triticum aestivum* and *Oryza sativa* (Langeveld et al. 2002).

Substrate specificity and localization support a role for RGP in hemicellulosic polysaccharide synthesis (Dhugga et al. 1997), however the precise function of this protein remains to be elucidated. It was proposed that different classes of RGPs, termed RGP1 and RGP2, might be involved in the biosynthesis of different plant polysaccharides such as hemicelullose and starch (Langeveld et al. 2002). In addition, Selth et al. (2006) provide new insight into the role of RGP from *Solanum lycopersicum* (tomato) since they reported its interaction with the *Tomato leaf curl virus* (TLCV) V1 protein.

Post-translational modifications such as oligomerization, phosphorylation and proteolytic cleavage are factors that have been found to modify the activity of different glycosyltransferases (Geigenberger et al. 1994; Sasai et al. 2001; Pozueta-Romero et al. 2004). The presence of oligomeric forms of the RGP protein was earlier reported (Ardila and Tandecarz 1992; Bocca et al. 1997). Langeveld et al. (2002) found by yeast two-hybrid analysis that the wheat RGP2 interacts with both wheat RGP1 and RGP2, and by affinity purification of the complex they revealed that no other protein was part of the complex suggesting that the protein complexes containing RGP could be created by self-association.

Different degrees of reversibility in the glycosylation of RGP were reported (Moreno et al. 1986, 1987; Bocca et al. 1999); however, no correlation between these two findings was established.

Therefore, many questions remain unanswered regarding the precise function of self-glycosylating proteins in plant tissues and thus a further biochemical characterization of the protein is imperative. The knowledge of factors involved in the regulation of the activity is an important issue to take into account in order to understand the role of RGP. Here, we study whether oligomerization regulates the self-glycosylation and glycosylation reversibility of RGP. In addition, the protein was found associated to the golgi membranes as part of high molecular weight oligomers.

Materials and methods

Radioactive material

UDP-[^{14}C]Glc (11,100 GBq mol^{-1}), UDP-[^{14}C]Gal (11,100 GBq mol^{-1}) and UDP-[^{14}C]Xyl (11,100 GBq mol^{-1}) were obtained according to Thomas et al. (1968). Biochemical reagents were purchased from Sigma (St Louis, MO, USA) unless otherwise noted.

Source of RGP

Potato tuber enzymes were obtained as described (Testasecca et al. 2004). The supernatant of 100,000*g* was precipitated by adding 50% (w/v) ammonium sulfate. The protein was dissolved in 10 ml of buffer A (50 mM Tris–HCl, pH 7.4, 10 mM 2-mercaptoethanol). This soluble potato preparation was applied to a Mono Q

HR 10/10 column (GE Healthcare, Bucks, UK) previously equilibrated with buffer A and eluted with a KCl gradient (0–0.5 M). An asymmetrical peak fraction containing RGP activity was obtained eluting between 0.17 and 0.24 M KCl with a maximum active peak eluting at 0.2 M KCl. Two identical aliquots of the active fraction (350 µl) were dialyzed overnight against 50 mM buffer Tris–HCl (low ionic strength, LIS) or against 100 mM buffer Tris–HCl containing 200 mM KCl (high ionic strength, HIS), in a dialysis tubing of 12-14 MWCO. The recombinant StRGP proteins were obtained as earlier described (Bocca et al. 1999; Wald et al. 2003) and dialyzed against 50 mM buffer Tris–HCl. H6 clone showing RGP activity was isolated from a potato leaf library as described (Bocca et al. 1999).

RGP glycosylation assay

For RGP glycosylation assays, proteins were incubated with UDP-[^{14}C]Glc, UDP-[^{14}C]Gal or UDP-[^{14}C]Xyl (0.2 nmol, 100,000 cpm) for 30 min and the radioactive sugar incorporated into the protein was measured into a 10% trichloroacetic acid (TCA) pellet as described (Wald et al. 2003). Alternatively, precipitated proteins were separated by SDS-PAGE and subjected to autoradiography for 2–3 weeks at −70°C using Biomax MR film (Kodak) as described (Bocca et al. 1999). Images from radioactive RGP separated by SDS-PAGE were also obtained using a phosphoimager (Molecular Dynamics STORM 840, GE Healthcare).

For the chase-out experiments, the radiolabeled RGP protein was incubated with 1 mM of UDP for 30 min at 30°C as reported by Testasecca et al. (2004). The glucosylation reversibility (%R) was calculated as the sugar released from the protein by the ratio between the radioactivity chased-out from RGP after incubation with UDP, and the radioactivity associated to the protein in the absence of UDP. The radioactivity was quantified by phosphoimaging. Liquid scintillation counting was carried out with Ultima Gold mixture (Perkin-Elmer Life Sciences, Wellesley, MA, USA) in a Beckman LS 6500 scintillation counter.

Size-exclusion chromatography on Superose 12

Aliquots of the RGP protein eluted from the Mono Q column (200 µl) were dialyzed at LIS or HIS as described above and applied to a Superose 12 column HR 10/30 (GE Healthcare). The column was equilibrated with LIS or HIS buffer and eluted at a flow rate of 0.5 ml min^{-1}. Fractions of 500 µl were taken and RGP activity was determined in 250 µl of each fraction as described. The column was calibrated with molecular mass standard proteins trypsinogen (29 kDa), carbonic anhydrase (48 kDa), and lactate dehydrogenase (176 kDa).

SDS-PAGE and immunoblot

SDS-PAGE analysis was performed on 10% (w/v) acrylamide gels according to Laemmli (1970). An aliquot of 50 µg of total protein was precipitated with 10% TCA, centrifuged and resuspended in 20 µl 1× Laemmli buffer and heated for 1 min. For Western-blot analysis, proteins were transferred to polyvinylidene fluoride membranes (Hybond-P, Amersham Pharmacia Biotech, Piscataway, NJ, USA) using a mini Trans-Blot cell (BioRad, Hercules, CA, USA). Membranes were blocked with 3% (w/v) non-fat milk in TBS. The blots were incubated with anti-potato tuber RGP antibody (1:1,000 dilution) for 90 min as described (Bocca et al. 1999) or with anti-pea RGP antibody as described by Dhugga et al. (1991). Immunoreactive bands were visualized by the ECL system (Amersham Pharmacia Biotech).

Extraction of golgi vesicles and separation of proteins by sedimentation coefficient

Pea golgi membranes were obtained as described by Muñoz et al. (1996), similar procedure was performed to obtain golgi vesicles from *Arabidopsis thaliana*. Sterile seeds from *Arabidopsis thaliana* (ecotype Columbia) were grown in liquid Murashige and Skoog medium (Murashigue and Skoog 1962), containing 2% sucrose (w/v) in an Erlenmeyer flask at 70 rpm. Growth was allowed in the dark at 23°C for 14 days. For membrane solubilization, the plant material was incubated for 15 min at 4°C in a buffer containing 10 mM Hepes pH 7.0, 5 mM EDTA, 600 mM NaCl, 1.5% Triton X-100 and 1 mM phenylmethylsulphonyl fluoride (PMSF). For protein separation, 5 ml of a 5–25% (w/v) sucrose linear gradient was used as described by Wang et al. (1998). The proteins obtained after solubilization were loaded onto this gradient and centrifuged for 14 h at 100,000*g* using an AH650 Sorvall rotor. After the centrifugation, 11 fractions were collected. The pellet obtained at the bottom of the tube was resuspended in 500 µl of 25% (w/v) sucrose. To detect RGP, each fraction was denaturated in Laemmli buffer, separated by SDS-PAGE followed by immunoblot and revealed with anti-pea RGP antibody made as described by Dhugga et al. (1991). The same procedure was used for molecular weight markers (ovoalbumin, LDH, β-galactosidase and catalase). Ovoalbumin and

catalase were detected using silver staining. The distribution of LDH and β-galactosidase was assessed enzymatically as described by Zewe and Fromm (1962).

Isolation of the RGP protein on 2D blue native gels (BNGE)

A golgi-enriched fraction from *Arabidopsis thaliana* grown etiolated in liquid culture was obtained using the same procedure applied for etiolated peas (Muñoz et al. 1996). A protein extract from the golgi vesicles-enriched fraction was obtained. Aliquots of 182 µg of extracted proteins were added in a buffer containing 750 mM amino caproic acid, 50 mM Bis–Tris pH 7.0 and 40% (w/v) glycerol. Protein complexes were solubilized by adding 1.5% (v/v) Triton X-100 and incubating for 30 min on ice. The solubilized proteins were centrifuged at 100,000*g* for 30 min at 4°C. Serva blue solution G250 (Serva Electrophoresis, Heidelberg, Germany) was added to the supernatant [100 mM Bis–Tris, 500 mM amino caproic acid, 5% (w/v) Serva Blue G250]. Protein extracts (about 36 µg of proteins) were separated on 5.5–12% (w/v) acrylamide gradient gels in the first dimension. For the second dimension, a gel lane was cut from the native gel and loaded into a 10% (w/v) SDS-PAGE (Schagger and von Jagow 1991; Schagger et al. 1994); except for the SDS-PAGE, denaturing buffer for the second dimension was 10% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris/HCl, pH 6.8, 0.002% (w/v) bromophenol blue and 50 mM dithiothreitol. The immunoblot procedure was performed as described above. RGP was detected using an anti-pea RGP antibody kindly donated by K. Dhugga.

Results

The glycosylation level and the glycosylation reversibility of RGP depend on the ionic strength

The glucosylation properties of RGP were studied under different ionic strengths. To perform these experiments, a soluble potato tuber protein was purified as described in "Materials and methods". The peak eluting at 0.2 M KCl after Mono Q chromatography contained the majority of the activity and was used as a source of RGP enzyme. Two similar aliquots were dialyzed at LIS and HIS, and then the protein glucosylation was performed using micromolar concentrations of radioactive UDP-[^{14}C]Glc. The radioactive glucose incorporated to the protein was assessed in the TCA precipitate (Table 1) and the RGP glucosylation level was determined by autoradiography or by phosphoimaging after SDS-PAGE (Fig. 1a). Results showed that a high amount of radioactive glucose (8.25 pmol) was linked to the protein, when RGP previously dialyzed at HIS was incubated with UDP-[^{14}C]Glc (Fig. 1a, Table 1). In contrast, a relative low level of glucose (3.1 pmol) was linked to the protein when the glucosylation reaction was carried out using RGP previously dialyzed at LIS (Fig. 1a, Table 1). To determine the reversibility of RGP glucosylation, the glucosylated protein was incubated with 1 mM of UDP for 30 min as described (Testasecca et al. 2004). Low glucosylation reversibility of glucosylated RGP previously dialyzed at LIS was obtained when it was challenged with UDP. In

Table 1 Effect of dialysis and protein concentration on radioactivity incorporated to the RGP protein

Condition	Protein (µg µl^{-1})	Glc incorporation (pmol)		
		−UDP	+UDP	% R[a]
LIS	1.0	3.09 ± 0.15	2.38 ± 0.12	23
HIS	1.0	8.25 ± 0.41	1.26 ± 0.06	85
Control	0.5	1.37 ± 0.07	0.01 ± 0.00	99
Control	1.0	2.37 ± 0.12	0.60 ± 0.03	78
Control	2.5	3.83 ± 0.19	1.85 ± 0.09	52

Aliquots of RGP active peaks eluting at 0.2 M KCl after Mono Q chromatography without dialysis (control) or after dialysis at LIS or HIS, were incubated with UDP-[^{14}C]Glc in the presence or absence of UDP. Representative data of three independent experiments

[a] Percentage of glycosylation reversibility ± SD

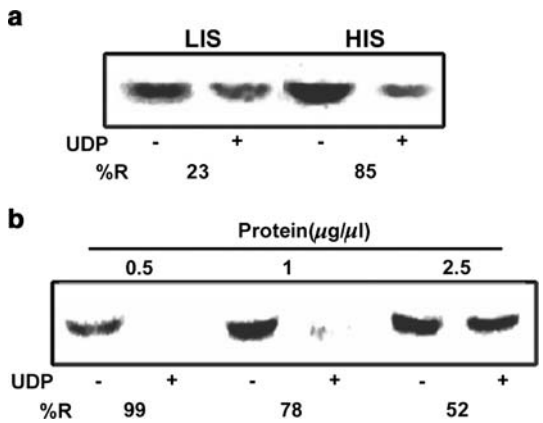

Fig. 1 **a**, **b** RGP glucosylation and glucosylation reversibility depend on the ionic strength and on the protein concentration. **a** Autoradiography after SDS-PAGE on a 10% (w/v) polyacrylamide gel of RGP active peak eluting at 0.2 M KCl after Mono Q chromatography and dialyzed at LIS or HIS (1.0 µg µl^{-1} protein concentration). Protein was incubated with UDP-[^{14}C]Glc at similar ionic strength of the dialysis. **b** Autoradiography after SDS-PAGE of different protein concentration (0.5, 1 and 2.5 µg µl^{-1} protein concentrations) of RGP active peak. Protein was incubated with UDP-[^{14}C]Glc maintaining the ionic strength condition of the Mono Q peak. ± indicates the absence or the presence of UDP in the chase out experiments

contrast, glucosylated RGP previously dialyzed under HIS exhibited high glucosylation reversibility upon challenge with UDP (Table 1, Fig. 1a). We defined the percentage of glucosylation reversibility (% R) as the ratio between the radioactivity chased-out from RGP after incubation with UDP and the radioactivity associated to the protein in the absence of UDP. Thus, a low glucosylation level and a low % R (23%) were obtained when RGP was previously dialyzed at LIS. On the other hand, a high glucosylation level and a high % R of RGP (85%) were obtained when the protein was previously dialyzed at HIS (Fig. 1a). The radioactivity associated to RGP was analyzed in a polyacrylamide gel and quantified using a phosphoimager. The % R obtained using this procedure was in good agreement with the values obtained by TCA precipitation (Table 1). Similar results were obtained when UDP-Gal and UDP-Xyl were used as substrates (data not shown).

Previous studies showed that upon separation on SDS-PAGE, the RGP could be found as a 40-kDa protein, which is the molecular weight reported for RGP (Dhugga et al. 1991). However, under certain circumstances the protein has a lower electrophoretic mobility and can be found as 80 or 116 kDa protein suggesting that RGP is part of a stable high molecular weight complex (Wald et al. 1998, 2003). To analyze whether the incubation under LIS or HIS had any effect on the electrophoretic mobility of RGP, the protein was subjected to incubation under LIS or HIS, separated by SDS-PAGE and analyzed by Western-blot using an anti-potato RGP antibody (Fig. 2). The results showed that upon incubation under HIS, the protein migrated around 40 kDa, whereas incubation under LIS produced a decrease in the electrophoretic mobility and RGP was present as a 116-kDa protein. This result suggests that RGP can be part of protein complexes sensitive to ionic strength.

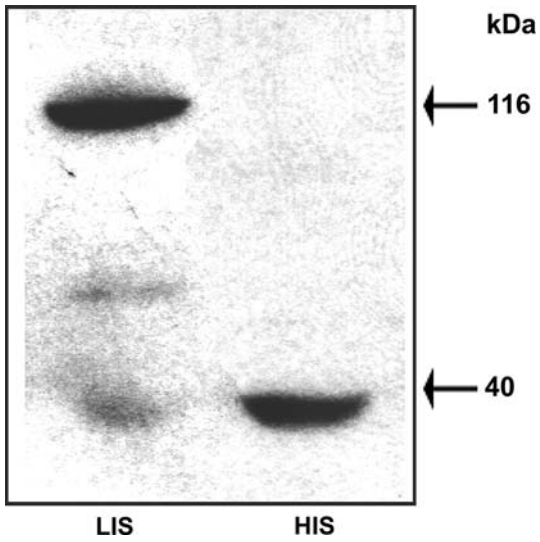

Fig. 2 Western-blot analysis of RGP active peak eluting at 0.2 M KCl after Mono Q chromatography dialyzed at LIS or HIS. Fractions of 350 µl of the 0.2 M KCl Mono Q peak was dialyzed at LIS (*left lane*) or HIS (*right lane*), as described in Fig. 1 and "Materials and methods". This aliquot was precipitated by TCA 10% (w/v) and analyzed by SDS-PAGE and Western-blot using an anti-potato tuber RGP antibody. *Arrows* correspond to the molecular mass of monomer and RGP-containing proteins

Protein concentration has an effect on RGP glycosylation

It is known that both LIS and high protein concentrations favors protein–protein interaction. To study whether protein concentration has any effect on the glycosylation of RGP, different concentrations of the enzyme were used to measure protein glucosylation. RGP showed an increase in glucosylation between 0.5 and 1 µg µl^{-1} of protein in the assay, however a fivefold increment in the protein concentration only produced a twofold increase in the protein radiolabeling (1.37 vs. 3.83 pmol Glc incorporated). The kinetics of the glucosylation reaction was analyzed by time course experiments using different protein concentrations. It was found that UDP-sugar incorporation depends on the protein concentration in a range of 0.5–1 µg µl^{-1} with a linear phase slope, while at higher protein concentrations the ability of RGP to be glycosylated reaching a plateau, is probably due to protein oligomerization (data not shown). The addition of bovine serum albumin, incubation for 30 s or 30 min did not make any difference in the pattern of RGP glucosylation at different protein concentrations. Regarding the reversibility in glycosylation, the results showed a decrease in the % R when the protein concentration increased (Table 1). At 0.5 µg µl^{-1} almost a 100% R was observed, whilst using 1 and 2.5 µg µl^{-1} protein a lower % R was observed (78 and 52%, respectively; Fig. 1b). Similar results were obtained when chase-out experiments were performed adding 10 mM UDP instead of 1 mM UDP ruling out any loss of % R due to UDP hydrolysis.

The data presented above suggest that conditions that favor protein–protein interaction produce a decrease both in RGP glucosylation and reversibility. In contrast, conditions that disturb protein–protein interaction favor both RGP glucosylation and reversibility.

The RGP protein can be part of complexes that change upon its glycosylation

We investigated the molecular size of the RGP protein using FPLC size-exclusion chromatography after dialysis

at LIS or HIS (Fig. 3). Separation of RGP by Superose 12 chromatography following dialysis at LIS showed a heterogeneous profile of elution and three peaks containing active RGP were detected (Fig. 3a). The first peak corresponded to a high molecular weight form that eluted in the exclusion limit size (MW $\geq 3 \times 10^5$). The second and the third peaks corresponded to active forms migrating between 230 and 176 kDa (Fig. 3a). In contrast, a sharp peak eluting in fractions corresponding to a molecular size of 76–40 kDa was observed after dialysis of RGP at HIS (Fig. 3b, filled triangles). These results indicated that dialysis at different ionic strengths produced changes in the molecular weight, suggesting that RGP is forming part of high molecular weight structures. Figure 3 also shows that RGP presented a higher activity when the protein was subjected to dialysis at HIS compared to LIS (a maximum of 6 pmol vs. 1.5 pmol of Glc incorporated to the protein, respectively).

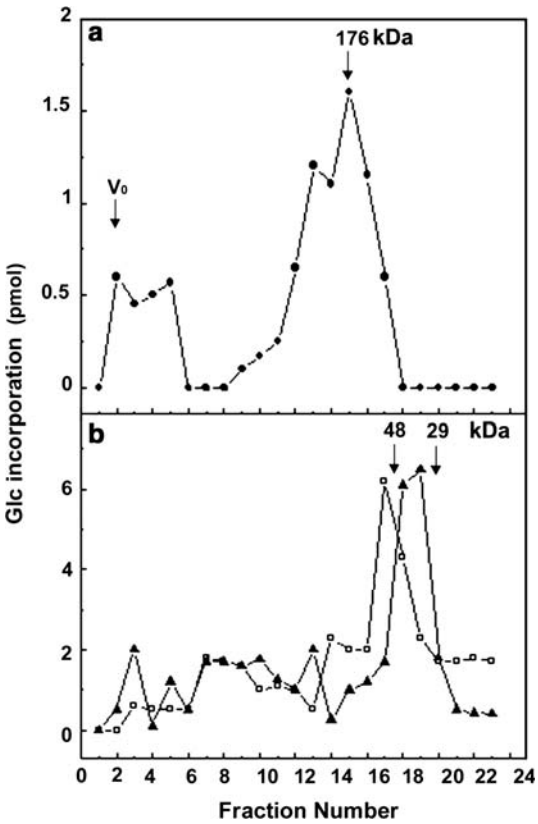

Fig. 3 **a**, **b** Effect of the ionic strength in the RGP complex formation. Superose 12 chromatography was performed on fractions dialyzed at LIS (**a**, *close circles*), on fractions dialyzed at HIS (**b**, *close triangles*) or first dialyzed at LIS followed by dialysis at HIS (**b**, *open squares*). Aliquots were incubated with UDP-[^{14}C]Glc during 2 h and the radioactive glucose incorporated to the RGP protein in the TCA precipitate was determined. *Vo* corresponds to the dead volume, *arrows* correspond to the elution volume of protein markers and *numbers* to their molecular weights

To analyze whether the high molecular weight RGP-containing forms obtained after LIS dialysis can be interconverted into low molecular weight forms, another RGP aliquot was dialyzed under LIS and re-dialyzed under HIS and subjected to similar separation by size exclusion chromatography, as described in Fig. 3. These results showed the appearance of a predominant low molecular weight active RGP after re-dialysis from LIS to HIS (Fig. 3b, open squares). Moreover, no loss of activity was observed despite the change in molecular weight. In addition, the re-dialysis of RGP also changed the reversibility and an increased in % R was observed. Therefore, an increase both in glycosylation activity and reversibility took place upon re-dialysis from LIS to HIS. These results led us to conclude that RGP monomers are more active and exhibit higher glycosylation and reversibility than RGP present in higher molecular weight forms.

To study the effect of glycosylation on the formation of protein complexes we investigated whether the incubation of RGP with UDP-glucose, UDP-galactose and UDP-xylose, under conditions that promote glycosylation had any effect on the formation of RGP-multimers. The results shown above indicated that changes in the electrophoretic mobility of RGP on SDS-PAGE were a hint of the formation of protein complexes. Therefore, after incubation of a recombinant version of RGP with any of the three radiolabeled nucleotide sugars that have been shown to glycosylate RGP, the protein was separated by SDS-PAGE and further analyzed by Western-blot using an anti-potato RGP antibody (Fig. 4a) or autoradiography (Fig. 4b), as previously described (Wald et al. 1998). Before the glycosylation reaction, the protein showed the 40-kDa band as the prominent one (Fig. 4a, lane 1) however, upon incubation with any of the nucleotide sugars the amount of the 40-kDa form decreased. The quantification of the monomer shown in Fig. 4a, lane 2–4, revealed a significantly decrease after glycosylation with UDP-Glc, UDP-Xyl and UDP-Gal (43, 27 and 21%, respectively). In contrast, high molecular weight bands clearly increased after glycosylation.

The autoradiography analysis shows a more intense 80-kDa band but also strong signals for the monomer form. On the other hand, at protein level the monomer almost disappeared. This apparent discrepancy may be explained by the fact that protein detection by autoradiography is an extremely sensible technique.

Hence, these results strongly suggest that RGP glycosylation promotes the formation of glycosylated protein complexes, because a sort of ladder with increments in the range of 40 kDa was observed, indicating

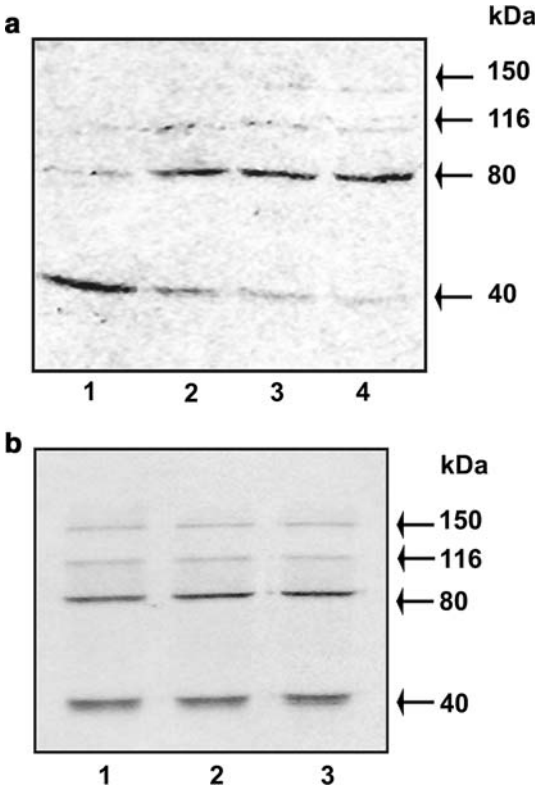

Fig. 4 **a**, **b** Complex formation regulates glycosylation of RGP. **a** Western-blot analysis of recombinant StRGP protein incubated in the absence of any UDP-nucleotide (*lane 1*) or incubated with radioactive UDP-Glc (*lane 2*), UDP-Xyl (*lane 3*) and UDP-Gal (*lane 4*) under LIS conditions. RGP was detected using an anti-potato tuber RGP antibody. **b** Autoradiography after SDS-PAGE of recombinant StRGP protein incubated with radioactive UDP-Glc (*lane 1*), UDP-Xyl (*lane 2*) and UDP-Gal (*lane 3*). *Arrows* correspond to the molecular mass of monomer and RGP-containing proteins expressed in kDa

that glycosylated RGP was part of multimers likely formed by RGP molecules.

The RGP protein associated to golgi vesicles is part of high molecular weight protein complexes

Studies performed by Dhugga et al. (1997) using immunogold combined with transmission electron microscopy indicated that RGP is localized in the golgi apparatus. To analyze whether RGP is present in this organelle as a monomer or as part of a protein complex, we isolated golgi vesicles from *Arabidopsis thaliana* or etiolated *Pisum sativum* (pea) epicotyls and analyzed the molecular weight of the native RGP using sedimentation gradients and blue native gel electrophoresis (BNGE). RGP extracted from the membranes using Triton X-100, a non-ionic detergent, was separated by sedimentation in a 5–20% (w/v) sucrose gradient. The distribution of RGP in the gradient was analyzed by Western-blot using an anti-pea RGP antibody. The result shows that RGP was distributed throughout the gradient (Fig. 5) although a slightly increment was observed in the zone corresponding to a molecular size of 400 kDa suggesting that RGP located in the golgi apparatus may be mainly present in high molecular weight structures.

The previous result suggested that RGP could be part of multiple protein complexes with increasing molecular weights. To analyze this possibility we used blue native gel electrophoresis (BNGE), a procedure that has been broadly used to study the formation of protein complexes in organelle membranes (Schagger and von Jagow 1991). Extraction of proteins from the membrane with Triton X-100 (0.1, 0.5 and 1.5%, v/v) was performed, followed by BNGE and Western-blot analysis (Fig. 6). A series of bands recognized by the anti-pea RGP antibody improved their sharpness at higher concentration of detergent (Fig. 6a), these bands ranged in size from below 400 kDa to above 880 kDa. Interestingly, the pattern of the complexes containing RGP behaved as a ladder that seems to have stepwise increases in size. To confirm that these bands indeed contained monomeric RGP, we took one of the lanes from the BNGE and loaded onto SDS-PAGE second dimension. The results showed that each of the bands observed in the first dimension contained a protein that upon separation in the second

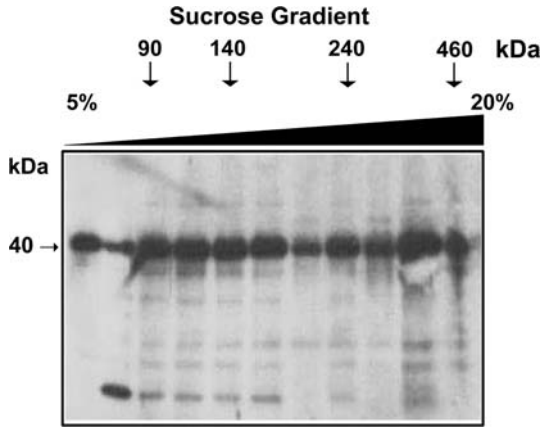

Fig. 5 Pea RGP protein associated to golgi membranes exhibits an heterogeneous sedimentation coefficient. Golgi membranes were solubilized using 1% (v/v) Triton X-100 and sedimented through a 5–20% (w/v) sucrose gradient, fractions were collected and 30 µl of each were submitted to SDS-PAGE. RGP protein was identified by Western-blot analysis using an anti-pea specific antibody as described in "Materials and methods". The molecular weight markers (*arrowheads*) ovoalbumin (90 kDa), LDH (140 kDa), catalase (240 kDa) and β-galactosidase (460 kDa) were separated by sedimentation. Ovoalbumin and catalase were detected using silver staining. The distribution of LDH and β-galactosidase were assessed enzymatically in each gradient fraction

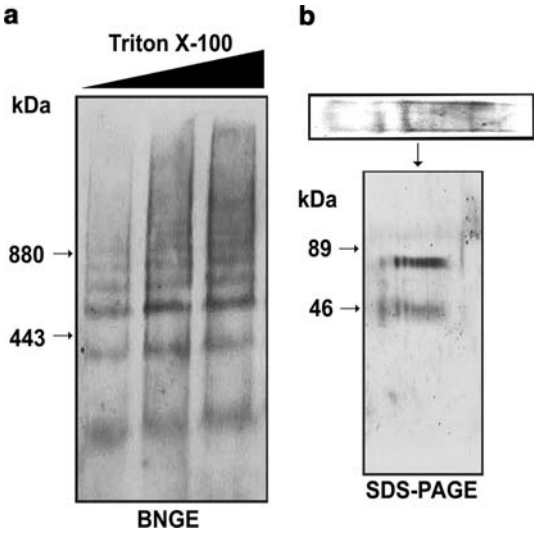

Fig. 6 **a**, **b** The RGP protein is part of multiple protein complexes. **a** Extraction of proteins from golgi membranes of *Arabidopsis thaliana* was performed at different Triton X-100 concentrations (0.1, 0.5 and 1.5%, v/v) followed by separation in BNGE and Western-blot analysis. **b** BNGE was done using protein obtained from increasing detergent concentration. The lane obtained from the 1.5% (v/v) Triton X-100 solubilization condition was loaded in a SDS-PAGE second dimension, and RGP was detected using an anti-pea RGP antibody. *Arrows* correspond to the pre-stained molecular weight marker proteins in kDa

dimension produced two spots (40 and 80 kDa) that were detected by the antibody (Fig. 6b). Unexpectedly, the relative abundance of the two spots indicated that the bigger one (80 kDa) was more abundant than the smallest one (40 kDa). Based on the results presented above (Fig. 4), these findings suggest that the majority of the RGP associated to the golgi may be glycosylated. Finally, these results suggest again, that RGP bound to the golgi apparatus is part of high molecular weight protein complexes.

Discussion

The present study provides compelling evidence that RGP is incorporated into protein complexes that likely correspond to homo-multimers. Our results also indicate that the glycosylation of the monomeric form plays a role in the incorporation of RGP into protein complexes. In addition, there is a correlation between the self-glycosylating activity of RGP and its presence in protein complexes, resulting in a high activity when it is present as a monomer and in a low activity when it is present in a multimeric form. Furthermore, the reversibility of the glycosylation also depends on the association level of RGP, while the monomeric form is highly reversibly; the multimeric form is less susceptible to release the glycosyl residue in the presence of UDP. Finally, RGP bound to golgi membranes is part of protein complexes of different molecular weight. Overall, the behavior exhibited by RGP is quite unique and it can provide us a clue regarding to the function of RGP in vivo.

It is unlikely that the described properties exhibited by RGP are the result of modifications such as proteolysis or denaturation of the protein. First, the changes in both enzymatic activity and reversibility upon incubation under different ionic strength were completely reversible and no change in the total activity was detected, arguing against a proteolytic effect that may alter the functionality of RGP. Second, the results presented in this paper were obtained using both the purified protein from potato as well as a recombinant version from the same specie. Third, the separation on SDS-PAGE did not show evidence of proteolytic degradation and when protein complexes were observed under these conditions, their migration corresponded to the addition of subunits of around 40 kDa, the molecular weight of the monomeric form of RGP. Fourth, the evidence that RGP is part of protein complexes came from experiments using different sources of the enzyme (*Solanum tuberosum*, *Pisum sativum* and *Arabidopsis thaliana*) as well as different experimental approaches (size exclusion chromatography, SDS-PAGE, sedimentation and blue native gel electrophoresis), then we are convinced that the evidence supporting the presence of RGP in protein complexes is quite strong.

Previous data suggested that potato RGP might be part of protein complexes (Ardila and Tandecarz 1992; Bocca et al. 1997). Moreover, Langeveld et al. (2002) showed that wheat RGP1 and RGP2 were part of protein complexes. Here, we confirmed that RGP from different species is present in protein complexes but, in addition, we described that complex formation modulates the reversibility and the glycosylation capability of RGP. This behavior may also explain the different degrees of reversibility in the glycosylation of RGP that has been previously reported (Moreno et al. 1986, 1987; Bocca et al. 1999).

It was earlier shown by yeast two-hybrid analysis that the wheat RGP2 interacts with both RGP1 and RGP2 and by affinity purification of the complex no other protein was found (Langeveld et al. 2002). Therefore, RGP containing complexes could be created by self-association. According to this, the potato protein separated on Superose 12 (Fig. 3) appears in different molecular weight forms that are larger in size compared to the monomer. These structures disappeared after incubation at HIS (Fig. 3b) and active

RGP as a monomer was recovered. Incubation of RGP under conditions that favored complex formation followed by SDS-PAGE (Fig. 2), showed stepwise increments in size with a separation around 40 kDa between the different forms, which coincide with the size of the monomer. These data show an unexpected biological complexity involving a reversion at the RGP monomer stage when we dialyzed at HIS (Fig. 3), while RGP containing complexes were maintained during SDS-PAGE conditions (Fig. 2). Although, in general protein complexes are disrupted and the proteins migrate as monomer after electrophoretic experiments, we were unable to solubilize the SDS-resistant RGP oligomers using a wide variety of harsh conditions (heating at 100°C, chemical denaturing treatments as 1% SDS during 1 h, 3 M urea, 5 M guanidinium HCl, 4 M LiBr or 1% Triton). Other researchers also described oligomeric proteins, which are resistant to dissociation by heat and SDS (Schagger et al. 1994; Membré et al. 2000; Norambuena et al. 2002; Castelli and Vitale 2005). In addition, Castelli and Vitale (2005) reported a pea protein that acquires a complex structure and forms SDS-resistant proteins after its association to membranes. All these data indicate that RGP from different species is able to self-associate to form oligomers.

Our results indicate that glycosylation of RGP with UDP-glucose, UDP-galactose and UDP-xylose led to a shift of RGP from the monomer stage to structures with higher molecular weights, suggesting that glycosylation change the ability of RGP to become part of a complex (Fig. 4). The fact that oligomers can be disabled to the monomer by dialysis under HIS but not by SDS-PAGE conditions suggests that oligomeric protein could be sensitive to a specific change in microenvironment, which modifies its tertiary or quaternary structure. The kind of association involved in the oligomerization process is not known. Probably, the metabolic context inside the plant cell such as a high dynamic pool of UDP-sugars and/or specific membrane microenvironment could be mimicked by changes in ionic strength in our experimental model. According to this hypothesis, we found that upon glycosylation, the electrophoretic mobility of RGP on SDS-PAGE diminished. So, glycosylation may alter the hydrophobicity/hydrophylicity status of the protein, producing the exposure of domains favoring stronger protein–protein interaction leading to the formation of stable SDS-resistant oligomers. Interestingly, no radiolabeled-glycosylation of high molecular weight bands was observed when the 0.2 M peak of RGP after Mono Q chromatography was dialyzed at LIS (Fig. 1a). This result may be explained by two alternatives. The first is the saturation by unlabeled glycosyl residues, linked already to the protein. The other possibility is that RGP present in high molecular weight forms become less susceptible to be glycosylated, maybe due to a different accessibility of the sugar residue within the protein complex. Glucosylation may be occurring by an inter-glycosylation reaction between different RGP molecules, then, if an inter-sugar transference takes place between two RGP molecules, in the oligomeric form the self-glycosylation could be impaired. A similar mechanism has been postulated for glycogenin, a proposed initiator of mammalian glycogen biosynthesis, which transfers glucose residues from UDP-glucose in a self-glycosylation reaction (Cao et al. 1995; Gibbons et al. 2002).

We described that monomeric RGPs (Fig. 3, Table 1) have biochemical properties that are different from oligomeric RGPs, (at least from the putative tetramers of 176 kDa, Fig. 3a). However, complexes are also found not only as tetramers but also as a putative trimer (116 kDa; Fig. 2, lane1) and as a putative dimer (80 kDa; Fig. 4a, b, 6b). In addition, the protein was found distributed almost evenly on sucrose gradient fractionation, between 90 and 460 kDa, with a prominent peak at 240 kDa in golgi membranes of *Arabidopsis thaliana*. The functionality of different complex sizes as well as their stoichiometry are under investigation. Our data point out that subsequent RGP oligomerization would not only depend on specific metabolic context, but also on plant developmental stage. In this sense, experiments performed in rice plantlets after 3 days of culture showed high amount of SDS-resistant proteins as putative trimer as the prominent protein, while in ungerminated seeds the monomer is the predominant one (data not shown).

RGP has been detected both soluble and bound to the golgi membrane. Under an increment in the ionic strength potato as well as maize, RGP proteins were released from membrane or plasmodesmata (Moreno and Tandecarz 1982; Epel et al. 1996; Bocca et al. 1997). It is possible that the oligomeric RGP becomes more hydrophobic increasing its affinity by membranes. Certainly, RGP in golgi membranes isolated from *Pisum sativum* etiolated epicotyls as well as *Arabidopsis thaliana* etiolated plants, were present in different degrees of oligomerization. Thus, we hypothesize that glycosylation of RGP leads to the formation of oligomers that may target soluble RGP into the golgi membrane. This process would also avoid the transfer of the sugar unit back to UDP. Yet it could not completely be ruled out that other nucleotide sugars

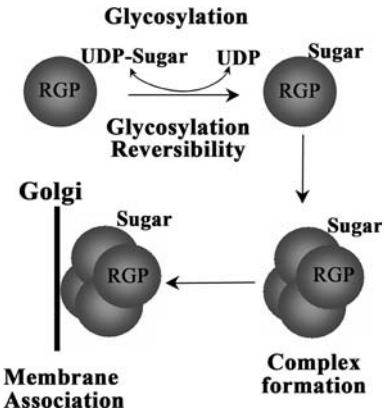

Fig. 7 Schematic representation of RGP complexes occurrence, their correlation with a decreased ability to release glucose to UDP and their association with golgi. The reaction catalyzed by monomeric soluble RGP is reversible. Glycosylation of the monomeric soluble RGP led to the formation of high molecular weight RGP-containing structures that increase their affinity for golgi membrane. The glycosylation reversibility by UDP is lower at the oligomeric stage

would interact with RGP. In order to come to a better comprehension of both complexes, formation and organelles association, we tested others nucleotide sugars than those shown here, such as ADP-glucose, UDP-mannose, UDP-galacturonic acid, UDP-glucuronic acid, however, no binding to RGP was found (Bocca 1998). Recently, Sagi et al. (2005) also found the RGP associated to golgi protein as well as plasmodesmata suggesting a new role for the protein exporting nucleotide sugars.

The occurrence of RGP complexes, the correlation with a decreased ability of those complexes to release glucose to UDP, as well as the confirmation that RGP is associated with golgi, are schematized in Fig. 7. Glycosylation seems to promote self-association and likely a change in RGP structure, given the fact that RGP associated to the golgi membrane is present in an oligomeric state. Although the exact role of the RGP must be elucidated, many experimental data involve the protein in xyloglucan biosynthesis (Perrin et al. 1999; Faik et al. 2000; Madson et al. 2003). Here, we propose that glycosylation of the RGP monomer leads to the formation of high molecular weight RGP-containing structures, increasing their affinity for the golgi membranes.

Acknowledgments We thank Susana Raffo and Marta L. Bravo for excellent technical assistance. The Andes-Antorchas Foundations (grant C-13680/3), by the Secyt (grant PICT 01-06986), by the CONICET (grant PIP 6076), by the Fondecyt (grant 1030551) and MNPCB (P02-009-F) supported this work. S.M. is Career Investigator of the CONICET and V.D.P. was supported by CONICET fellowships, Argentina.

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