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Regulation of self-glycosylation of reversibly glycosylated polypeptides from *Solanum tuberosum*

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Reversibly glycosylated polypeptides (RGPs) belong to a family of self-glycosylating proteins believed to be involved in plant polysaccharide synthesis. The precise function of these enzymes remains to be elucidated. Our results showed that the RGP 38-kDa subunit is phosphorylated in potato extracts (*Solanum tuberosum* L.). An increase in the self-glycosylation of *Solanum tuberosum* RGP (StRGP) 38-kDa subunit was observed after alkaline phosphatase (AP) treatment. Our results suggest that phosphorylation of StRGP appears to regulate its self-glycosylation. It was determined that when the StRGP reaction was carried out in the presence of UDP-[^{14}C]Glc as the sugar donor and then 1 m*M* UDP was added in a chase-out experiment, radioactive UDP-Glc was obtained indicating that StRGP reaction seems to be reversible. The anomeric configuration of transferred sugars to StRGP protein was also studied.

Introduction

Glycosyltransferases are particularly important in plants since they convert the product of photosynthesis into disaccharides, oligosaccharides and polysaccharides. These enzymes can be classified into different families based on the activated sugar-donor molecule, kind of transferred sugar, kind of sugar acceptor molecule and whether the enzyme forms an α- or β-glycosyl linkage. In general, uridine diphosphate (UDP) glycosyltransferases mediate the transfer of glycosyl residues from activated nucleotide sugars to acceptor molecules and a superfamily of over 100 genes has been identified in *Arabidopsis thaliana* (Ross et al. 2001). Out of these glycosyltransferase genes, the function of the enzymes encoded by them has been confirmed for only a handful on account of their extreme substrate specificity, presence of different isoforms and their association to membranes. UDP-Glc is used in various major metabolic pathways such as in the synthesis of starch, cellulose, callose and xyloglucan (Odegard et al. 1996). Therefore, to modulate those metabolic pathways it is critical to understand the distribution of UDP-Glc within a plant cell.

Plant cells, unlike animal cells, are surrounded by a cell wall. The cell wall is a dynamic structure that plays an important role in plant development (Carpita and Gibeaut 1993). Studies aimed at identifying proteins responsible for the biosynthesis of cell wall polysaccharides have revealed the presence of specific polypeptides, called reversibly glycosylated polypeptides (RGPs) (Dhugga et al. 1997). After UDP-Glc pulse labelling the radioglucosylation of RGP protein from pea was chased-out by the addition of 1 m*M* of UDP-Glc, UDP-Gal, UDP-Xyl or UDP, indicating that glucosylation is reversible (Dhugga et al. 1991).

The presence of RGPs has been reported in membrane and soluble preparations of pea epicotyls (Dhugga et al. 1991, 1997), maize (Rothschild and Tandecarz 1994), Arabidopsis (Delgado et al. 1998), potato (Bocca et al. 1999), wheat and rice (Langeveld et al. 2002). The RGP function is still unknown, however, there is some evidence suggesting this protein as a possible intermediary in polysaccharides metabolism such as xyloglucans, which are mainly constituted by Glc, Gal and Xyl.

Abbreviations – AP, alkaline phosphatase; P-ser, phosphoserine; RGP, reversibly glycosylated polypeptide; UDP, uridine diphosphate; UPTG, UDP-Glc: protein transglucosylase.

Indeed, RGP1 has been implicated in xyloglucan biosynthesis in *Pisum sativum* (Dhugga et al. 1997). Recently, it was suggested that RGP proteins might function in cell wall synthesis and/or in starch synthesis (Langeveld et al. 2002).

RGP proteins have partial homology with cellulose synthases and other β-glycosyltransferases suggesting that RGPs may be non-processive β-glycosyltransferases functioning as intermediates in the transfer of a single sugar residue from a nucleotide sugar to an acceptor molecule (Saxena and Brown 1999). Likewise, Faik et al. (2000) suggested that in Nasturtium fruit an autoglycosylating polypeptide(s) could facilitate the channelling of UDP-activated sugars from the cytoplasm through Golgi vesicle membranes to luminal sites, where they can be used for xyloglucan synthesis. In animal tissues another autocatalytic glucosyltransferase, glycogenin, performs the first steps of glycogen synthesis (Alonso et al. 1995).

Phylogenetic analysis of the enzyme catalysing the Glc-protein linkage in potato tuber formerly termed UDP-glucose: protein transglucosylase (UPTG) revealed that this protein is another member of the RGP subfamilies from dicots (Wald et al. 2003). Cloning of two cDNAs, encoding StRGP1 and StRGP2 proteins and their expression in *E. coli* have been previously reported (Bocca et al. 1999). The addition of unlabelled UDP-sugars completely chased-out the radioactivity when purified potato tuber RGP was used, although this effect was less marked when recombinant StRGP1 or a partially purified protein from potato were used (Moreno et al. 1987, Bocca et al. 1999). Therefore, StRGP glucosylation reversibility may vary considerably, depending on the protein purification. Another interesting point is that the proportion of StRGP protein molecule that becomes glucosylated during the reaction could be influenced by the presence of factor(s) in the potato tuber fraction (Bocca et al. 1999).

A protein-inhibiting glucosylating activity of RGP present in developing maize endosperm was identified as the SS1 isozyme of sucrose synthase (Wald et al. 1998). Traditionally, it has been assumed that sucrose synthase is essential for starch biosynthesis. However, SS1 has been shown to be required for the maintenance of cell wall integrity in maize kernels, whereas the SS2 isozyme is needed mainly for generating precursors for starch biosynthesis (Chourey et al. 1998).

Although substrate specificity and localization support a role for RGP in hemicellulosic polysaccharide synthesis, many questions remain unanswered regarding the precise function of self-glycosylating proteins in plant tissues. The objective of this study was to further analyse its self-glycosylation reaction and to search for factor/s that could modify StRGP glycosylation.

Materials and methods

Plant materials

Potato (*Solanum tuberosum* L. cv. Spunta) plants were grown in a greenhouse under natural light supplemented with fluorescent light (60 μmol photon $m^{-2} s^{-1}$) provided by cool white 40 W fluorescent tubes (OSRAM, Danvers, MA) under a photoperiod of 8-h light/16-h dark.

We previously isolated cDNAs from a library of potato stolon tips (clone E11 encoding for StRGP1 and clone E2 encoding for StRGP2) (Bocca et al. 1999). The expression of recombinant proteins in *E. coli* was performed as described (Bocca et al. 1999). 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).

Potato tuber enzymes

Potato tuber enzymes were obtained as described (Lavintman et al. 1974). The supernatant after centrifugation at 25 000 *g* was used as a source of enzyme (S25) or centrifuged again at 100 000 *g*. This supernatant was brought to 50% saturation with $(NH_4)_2SO_4$. The precipitate dissolved in 10 ml of buffer A (50 m*M* Tris-HCl, pH 7.4, 10 m*M* 2-mercaptoethanol) was used as a source of a soluble enzyme potato preparation (S140). The 100 000 *g* pellet was re-suspended in buffer A and used as a membrane potato preparation (P140). The S140 was applied to a Mono Q HR 10/10 column (Pharmacia, Uppsala, Sweden) previously equilibrated with buffer A. The enzyme activity was eluted with a KCl gradient from 0 to 0.5 *M* in the same buffer. Fractions containing StRGP activity eluting between 0.17 and 0.2 *M* KCl were pooled and used as a source of StRGP enzyme (StRGP Mono Q).

RGP glycosylation assays

For glycosylation assays, 30 μg of the Mono Q purified StRGP (StRGP Mono Q) or 50–100 μg of recombinant RGP protein were incubated with UDP-[^{14}C]Glc, UDP-[^{14}C]Gal or UDP-[^{14}C]Xyl (0.2 nmol, 100 000 cpm) and radioactivity incorporated to the protein was measured into a 10% (w/v) trichloroacetic acid (TCA) pellet as described (Bocca et al. 1999). Alternatively, incubation was stopped with 10% (w/v) trichloroacetic acid and precipitated proteins were separated by SDS-PAGE and subjected to autoradiography for 2–3 weeks.

For the chase-out experiments, after glucosylation of the enzyme the reaction mixture was subjected to chromatography on a Sephadex G25 column (40 × 9 mm). Fractions of 250 μl were collected and radiolabelled RGP protein (RGP[^{14}C]-Glc) was pooled and incubated with 1 m*M* of UDP for 30 min at 30°C. TCA was added to precipitate proteins and the soluble radioactive products were subjected to a mixed bed ion exchange (Dowex, USA, 50 W–X4). Thin-layer chromatography (TLC) on silica gel 60 plates (Merck, Munich, Germany, 4 × 20 cm) developed in aceto- nitrile/acetic acid/ethanol/water (65 : 5 : 10 : 20), along with UDP-[^{14}C]Glc (100 000 cpm) and [^{14}C]Glc (40 000 cpm) as standards was performed. Autoradiography was carried out at −70°C using Biomax MR film (Kodak, USA). Alternatively, radioactive components

were analysed by HPLC using an Ultrasphere 5 µm ODS (4.6 × 250 mm) (Beckman, USA) using a diode array detector module (Beckman). Compounds were eluted in an isocratic mode with 40 m*M*-triethylamine-phosphate buffer (pH 6.5) at a flow rate of 1.0 ml min^{-1} at room temperature and radioactivity determined in 200 µl aliquots as described. UDP-[^{14}C]Glc and [^{14}C]Glc were used as standards.

StRGP Mono Q protein was radioglycosylated for 30 min at 30°C with UDP-[^{14}C]Glc, UDP-[^{14}C]Xyl or UDP-[^{14}C]Gal. Radioactivity values in the TCA precipitate were 15 300, 10 900 and 7500 cpm, respectively. Five units of β- and α-glucosidase (Sigma, St Louis, MO, USA) in 100 m*M* sodium citrate buffer (pH 6) and in 100 m*M* sodium acetate buffer (pH 5), respectively; 1 U of β-xylosidase in 50 m*M* sodium acetate buffer (pH 5) and α- and β-galactosidase (Sigma) in 50 m*M* sodium acetate buffer (pH 4) were used. Digestions were carried out at 37°C for 48 h, except for galactosidases (at room temperature). HPLC analysis using HPX 87C and HPX 42C (Aminex, USA) columns, eluted with water at a flow rate of 0.6 ml min^{-1} at 85°C were performed. A carbohydrate analysis (Waters, USA) column was also used, eluted with acetonitrile/water (70 : 30, v/v) at a flow rate of 1 ml min^{-1} at room temperature. An LKB Bromma System (Sweden; Model 2142) was employed. Radioactivity was determined in 300 µl aliquots. Reference sugars were detected by the alkaline silver nitrate technique (Trevelyan et al. 1950).

β elimination treatments

The RGP protein was self-xylosylated or self-galactosylated as described above and alkaline borohydride reduction was performed as described by Tandecarz and Cardini (1978). Proteins were dissolved in 0.5 *M* NaOH at 50°C for 5 h in the presence of 1 *M* BH_4Na. The reaction mixture was acidified with acetic acid. The proteins were then methanol precipitated and the resulting supernatant was passed through a Dowex 50 W-X8 resin (H^+ form) to remove the cations. The eluate was brought to dryness and the boric acid excess was removed as methyl borate. The soluble radioactive product was dissolved in a small volume of water and analysed by paper electrophoresis in 100 m*M* sodium molybdate pH 5. The radioactive peaks were detected on a 7201 Packard radiochromatogram scanner. Reference sugars were detected as described above.

Phosphorylation–dephosphorylation assays

For the in-vitro StRGP phosphorylation, S25 enzyme was incubated for 30 min at 30°C with a buffer containing 50 m*M* Tris-HCl pH 8, 10 m*M* $MgCl_2$, 1 µCi [γ-^{32}P]ATP, 1 m*M* ATP, 0.25 m*M* $CaCl_2$ and 150 n*M* okadaic acid. RGP protein was immunoprecipitated using antipotato tuber RGP antibody and submitted to SDS-PAGE and autoradiography for 7 days at −70°C using Kodak BIOMAX MR film.

For the alkaline phosphatase treatment, StRGP Mono Q, recombinant RGP1 or RGP2 enzymes were incubated for 30 min at 30°C in 50 m*M* Tris-HCl buffer (pH 7.4), 10 m*M* $MnCl_2$, 10 m*M* $MgCl_2$ in the presence or absence of 5–20 U calf intestine alkaline phosphatase (AP) (Promega, Madison, WI). Radioactive sugar nucleotides (approximately 0.2 nmol; 80 000 cpm) were added to the reaction mixture and incubation was carried out for another 30 min at 30°C. Radioactive material incorporated in the TCA-precipitated protein was measured or subjected to SDS-PAGE as described.

SDS-PAGE and immunoblots

SDS-PAGE analysis was performed on 10% acrylamide gels according to Laemmli (1970). Immunoblot analysis using antipotato tuber RGP antiserum was performed as described (Towbin et al 1979; Bocca et al. 1999). Immunoblots using P-serine and P-tyrosine antibodies were carried out according to the supplier's instructions.

Results

Analysis of the StRGP reaction

In order to examine the reversibility mechanism of the reaction catalysed by RGP, a chase-out experiment was performed. After glucosylation of the protein with micromolar concentration of radioactive UDP-Glc, 1 m*M* of UDP was added and the incubation continued for another 30 min. Then, we analysed the protein radioglucosylation before and after the chase-out experiment (Fig. 1A). It was found that UDP was capable of chasing-out the radiolabel of RGP 38-kDa subunit since a clear decrease in radiolabel intensity of RGP protein 38-kDa subunit was observed after incubation with UDP (Fig. 1A). Quantitative analysis of the radioactivity incorporated to the protein showed that 70% of the radioactivity in the TCA precipitate decreased when UDP was added (2158 versus 725 cpm) (Table 1).

It seems that after the chase-out experiment the radioactivity moved from radioglucosylated protein to the reaction solution. Therefore, in order to identify the radioactive product present in the solution it was subjected to HPLC and TLC analysis. Prior to UDP addition, the incubation mixture was subjected to filtration on a Sephadex G25 column in order to eliminate UDP-Glc excess present after radioglucosylation of the enzyme. Figure 1B, inset, shows that residual radioactive sugar nucleotide was totally separated from radiolabelled RGP protein (RGP-[^{14}C]Glc). Finally, the RGP-[^{14}C]Glc, essentially free of UDP-[^{14}C]Glc, was incubated in the presence or absence of 1 m*M* of UDP and TCA supernatant radioactivity was subjected to HPLC and TLC analysis (Fig. 1B and C). After HPLC analysis, a main peak corresponding to UDP-Glc mobility was observed (Fig. 1B). The small amount of glucose recorded may be due to sugar nucleotide hydrolysis. To confirm the presence of radioactive sugar nucleotide, TLC analysis was performed and a radioactive component migrating as UDP-Glc was obtained (Fig. 1C). The formation of radioactive UDP-Glc after incubation of

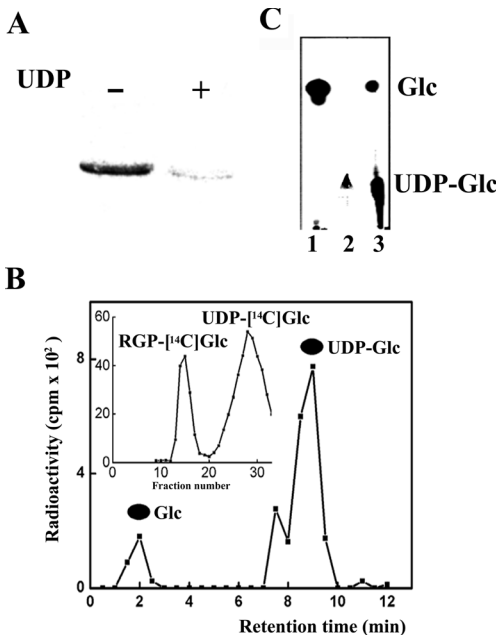

Fig. 1. (A) StRGP purified by Mono Q, incubated during 30 min with UDP-[^{14}C]Glc and then for another 30 min in the presence (+) or absence (–) of 1 m*M* of UDP. Proteins were TCA precipitated as described in Table 1 and analysed by SDS-PAGE, followed by autoradiography. After the chase-out experiment, the soluble radioactive components contained in the TCA supernatant were subjected to HPLC chromatography on the Ultrasphere ODS column (B) or to TLC analysis (C). Arrow in (A) indicates the 38-kDa subunit of the RGP protein. After enzyme glucosylation the incubation mixture was subjected to Sephadex G25 to remove excess UDP-[^{14}C]Glc (B, inset). Numbers 1 and 3 in (C) correspond to [^{14}C]Glc and UDP-[^{14}C]Glc as standard, and 2 corresponds to radioactive components discharged from the protein in the presence of 1 m*M* of UDP.

RGP-[^{14}C]Glc with UDP suggested that the RGP reaction is reversible.

It has been suggested that purified potato and recombinant StRGP1 enzyme is capable of self-galactosylation from UDP-Gal in addition to self-glucosylation and self-xylosylation from UDP-Glc and or UDP-Xyl (Bocca et al. 1999). Therefore, we analysed the question if self-xylosylated or self-galactosylated StRGP is chaseable by UDP. We observed that the addition of 1 m*M* UDP was able to decrease radioactivity from the TCA precipitate in 63–78% (Table 1) after protein self-xylosylation or self-galactosylation with micromolar concentration of radioactive UDP-Xyl or UDP-Gal, respectively.

Table 1. Ability of UDP to chase-out the radioactivity incorporated into StRGP from UDP-[^{14}C]Glc, UDP-[^{14}C]Xyl or UDP-[^{14}C]Gal. After incubation for 30 min with 2 μ*M* of sugar nucleotides, 1 m*M* of UDP was added and the incubation was continued for 30 min.

UDP-[^{14}C]sugar	UDP	Radioactivity in TCA precipitate (cpm)
UDP-Glc	–	2158 ± 123
	+	725 ± 69
UDP-Xyl	–	1672 ± 97
	+	660 ± 86
UDP-Gal	–	1229 ± 109
	+	438 ± 38

Glycosidase treatments

There is scant information about the sugar residue linked to the RGP protein or its glycosyl linkage type, thus the radioglucosylated, radioxylosylated or radiogalactosylated StRGP protein was subjected to α- or β-glucosidase, α- or β-galactosidase or β-xylosidase treatment. We found that the linkage is sensitive to β-glucosidase and β-xylosidase treatments as 82–93% of the radioactivity linked to the protein was released to the trichloroacetic acid (TCA) supernatant (Table 2). Although a significant amount of radioactivity was also sensitive to β-galactosidase (65%), the amount of radioactivity released by α-galactosidase is probably due to a contamination of commercial enzyme used in the experiment (Table 2).

HPLC analysis indicated that a significant radioactivity present in the TCA supernatant migrated identically as galactose after β-galactosidase treatment (Fig. 2A); as glucose after β-glucosidase treatment (Fig. 2C), and a main radioactive peak eluted as xylose after β-xylosidase treatment (Fig. 2B). Although the nature of the minor peaks observed (Fig. 2A and B) is unknown, their migrations are unrelated to disaccharides (Fig. 2A). Using a 42C, Aminex column (Fig. 2B), a satisfactory separation between xylose and glucose was obtained which, however, failed to separate glucose from galactose.

β-elimination treatment

πTandecarz and Cardini (1978) reported that the [^{14}C]Glc incorporated in the TCA precipitate after the incubation of particulate StRGP with micromolar of UDP-[^{14}C]Glc was alkali labile and after sodium borohydride reduction a labelled compound identified as sorbitol was obtained. Similarly, the radioactivity incorporated in the TCA-insoluble product after the radioxylosylation or radiogalactosylation of the RGP protein was alkali labile and after β-elimination treatment, the radioactive product released from the protein migrated as xylitol or galactitol, respectively (Fig. 2D and E).

Table 2. Glycosidase treatments after radioglycosylation of StRGP protein from UDP-[^{14}C]Glc, UDP-[^{14}C]Gal or UDP-[^{14}C]Xyl. Results are expressed as the percentage of radioactivity present in the TCA precipitate relative to the amount of radioactivity present in the absence of the respective glycosidases as control (100%). Values are means ± SE, n = 3.

Treatment	Radioactivity in TCA precipitate (% control)		
	UDP-[^{14}C]Glc	UDP-[^{14}C]Xyl	UDP-[^{14}C]Gal
β-glucosidase	18 ± 2	–	–
α-glucosidase	82 ± 6	–	–
β-xylosidase	–	7 ± 0.4	–
α-galactosidase	–	–	70 ± 8
β-galactosidase	–	–	35 ± 4

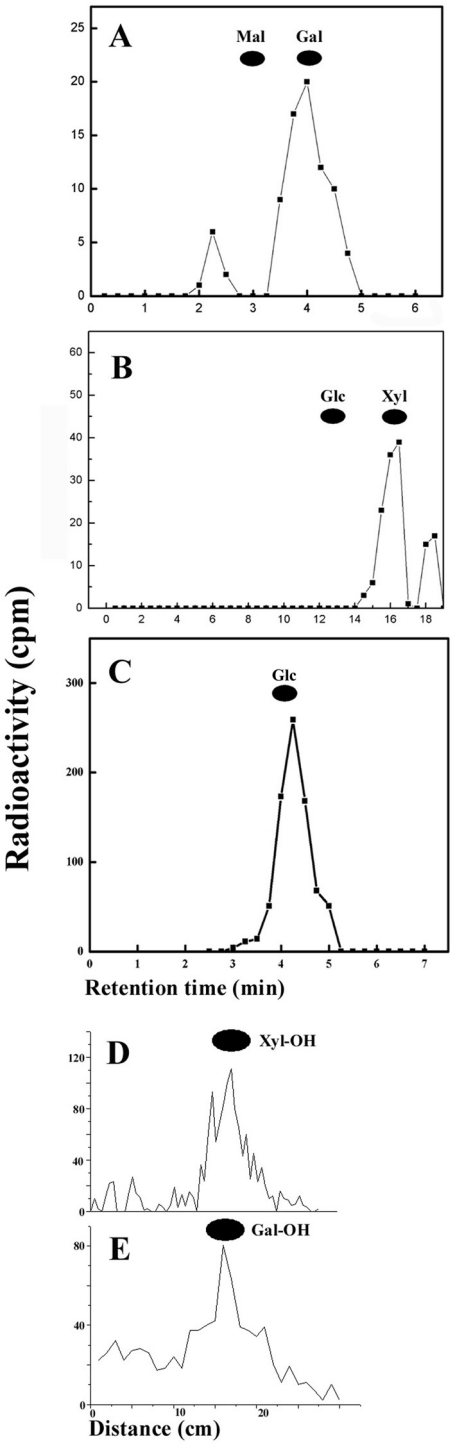

Fig. 2. Analysis by HPLC chromatography of radioactive products released from RGP protein after β-galactosidase treatment (87C column). (A), β-xylosidase treatment (42C column) (B) and β-glucosidase treatment (carbohydrate analysis column). (C). Radioscanning of paper electrophoresis after borohydride reduction of radiolabel products released from the radioxylosylated (D) or radiogalactosylated (E) RGP protein.

Phosphorylation of StRGP protein

Herein we have investigated whether StRGP may be phosphorylated in an in-vitro system by incubating the enzyme with [^{32}P]ATP. Radiophosphorylation of the 38-kDa subunit was observed by inmunoprecipitation (Fig. 3A). Immunoblot analysis using P-serine or P-tyrosineantibodies was performed to analyse which protein amino acid is phosphorylated (Fig. 3B). A membrane and a soluble protein preparation were assayed since StRGP is present in both (Moreno and Tandecarz 1982, Bocca et al. 1999). A 38-kDa band was reactive using the P-serine antibody in both preparations, although higher band intensity was observed in the soluble preparation as compared to the membrane one (Fig. 3B, upper). No reactive bands were obtained using the P-tyrosine antibody. In contrast, by using the anti-StRGP antibody a lower intensity of the 38-kDa band in the soluble preparation in comparison with the membrane one was found (Fig. 3B, lower).

Alkaline phosphatase treatment of StRGP protein

As an alternative strategy to study the possible effects of phosphorylation on the RGP activity, an attempt was made to discern any significant effect in RGP activity upon changes in protein phosphorylation. It was found that pre-incubation of the partially purified StRGP and StRGP1 and StRGP2 recombinant proteins with alkaline phosphatase (AP) led to an increase in [^{14}C]Glucose

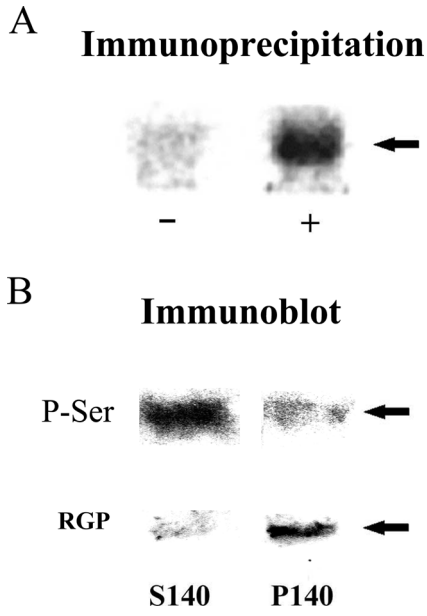

Fig. 3. (A) Autoradiography after in-vitro phosphorylation of potato tuber fraction (S25) incubated with ^{32}[P]ãATP before (–) and after (+) immunoprecipitation with anti-StRGP antibody. (B) Immunoblotting of soluble (S140) or membrane (P140) proteins from potato tuber using a P-serine antibody (upper) and anti-StRGP antibody (bottom). Arrows indicate the 38-kDa subunit of StRGP protein.

Table 3. AP treatment increased radioactivity incorporation to TCA precipitate of StRGP Mono Q and recombinant StRGP1 and StRGP2. Incubation with UDP-[^{14}C]Glc was performed and radioactivity in TCA precipitate was measured. Values are mean ± SE, n = 3.

Enzyme	AP	Radioactivity (cpm)
StRGP Mono Q	–	3100 ± 153
	+	4480 ± 218
StRGP1	–	1005 ± 63
	+	6782 ± 280
StRGP2	–	2100 ± 98
	+	17 200 ± 1300

incorporation to the TCA precipitate (Table 3). To confirm a direct effect on RGP protein self-glucosylation after AP treatment, glucosylation of the protein was analysed by autoradiography after SDS-PAGE. We observed that an increase in radioactivity incorporated in the TCA precipitate correlated with an increase in radioglucosylation of the StRGP1 and StRGP2 38-kDa subunit after AP treatment (Fig. 4A and B). Figure 4A shows that the radioxylosylation and radiogalactosylation of the StRGP1 also increased after AP treatment. Phosphatase inhibitors such as okadaic acid (Fig. 4B) mainly block the effect of AP on the radioglucosylation of the RGP enzyme (Table 4).

AP treatment also produced a marked increase in the radioglucosylation of high molecular size bands (80 and 116 kDa) from both recombinant protein extracts (Fig. 4A and B, left). The nature of these high molecular radioglucosylated polypeptides remains unknown. However, Western blot analysis revealed that they reacted with the anti-StRGP antibody (Fig. 4B, right).

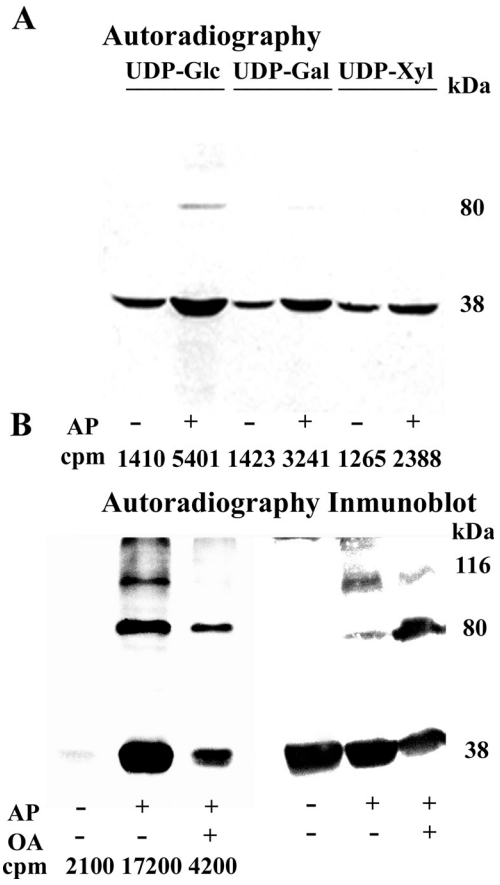

Fig. 4. (A) Autoradiography after SDS-PAGE of recombinant StRGP1 after its pre-incubation in the presence (+) or absence (–) of AP. Incubations with UDP-[^{14}C]Glc, UDP-[^{14}C]Xyl or UDP-[^{14}C]Gal were assayed as described in Methods. (B left) Autoradiography after SDS-PAGE or (B right) immunoblot analysis of recombinant StRGP2 pre-incubated with AP in the absence or presence of okadaic acid (OA) using an anti-StRGP antibody. Glycosylation with UDP-[^{14}C]Glc was performed as described in 'Materials and Methods'.

Discussion

The RGPs proteins have been suggested to be involved in polysaccharide synthesis, although many issues should be addressed about the physiological role of these glycosyltranferases. Previous studies have provided evidence that several sugar nucleotides and UDP were capable of chasing-out the radioactivity incorporated into self-glycosylated proteins, although the underlying mechanism is not fully understood (Bocca et al. 1999, Saxena and Brown 1999). Our results indicate, for the first time, that in the presence of UDP the radioglucosylated StRGP is capable of synthesizing radioactive UDP-Glc (Fig. 1). Thus, RGP may catalyse a reversible reaction in which UDP discharges the label from the polypeptide. Consistent with this, it was previously reported that UDP-Glc, UDP-Xyl and UDP-Gal at millimolar concentrations were capable of chasing-out the label, whereas UDP-Man, UDP-GlcNac, UDP-GlcUA and UDP-GalUA were ineffective (Bocca et al. 1999).

In Nasturtium fruits it has been reported that an autoglycosylating polypeptide(s), which is initially chaseable, is not directly glycosylated. It is initially bound to UDP-Glc and finally becomes glucosylated (Faik et al. 2000). However, our results indicate that the radiolabelled StRGP protein obtained after incubation with UDP-[^{14}C]Glc for 30 min, which is chaseable (Fig. 1A), is glycosylated, since β-glucosidase treatment renders a radioactive product migrating as glucose (Fig. 2C).

Earlier in-vitro studies have shown that StRGP catalyses the linkage of one glucose residue from UDP-Glc to become self-glucosylated (Tandecarz and Cardini 1978, Tandecarz et al. 1995). Here, we observed that when the

Table 4. Phosphatase inhibitors blocked the increase in radioactivity incorporation to TCA precipitate by EPF. Recombinant StRGP1 was pre-incubated with EPF eluting at 0.3 *M* KCl with phosphatase inhibitors. Values are means ± SE, n = 3.

Phosphatase inhibitors	EPF	Radioactivity (cpm)
–	–	1005 ± 70
–	+	6782 ± 255
NaF 40 m*M*	+	1200 ± 55
Okadaic acid 0.1 μ*M*	+	1325 ± 63

radioxylosylated or radiogalactosylated protein was submitted to β-elimination treatment, xylitol and galactitol were produced, respectively (Fig. 2D and E). This suggests that one residue of these sugars is also linked to the protein. These results demonstrate that one residue of glucose, xylose and galactose were linked to the protein in a β-configuration (Fig. 2, Table 2).

Although the RGP glucosylation reversibility was extensively studied, here we describe for the first time that xylose and galactose residues linked to the protein are also chaseable by UDP (Table 1).

A partial glycosylation reversibility of the soluble RGP purify by Mono Q (Table 1; Fig. 1) was observed. This result is in agreement to earlier observations indicating that using recombinant RGP or partially purified StRGP preparations a partial chase-out of the radioactivity was obtained (Moreno et al. 1987, Bocca et al. 1999). On the other hand, using the purified potato tuber protein, complete reversibility of StRGP glucosylation was documented (Bocca et al. 1999). The existence of a factor/s able to modify the self-glycosylation of the enzyme was previously suggested in potato preparations (Bocca et al. 1999). In agreement with these suggestions preliminary results of our laboratory indicate that a plant factor, possible a putative phosphatase, might regulate the RGP self-glycosylation. The presence of such a factor could be responsible for the partial chase-out glycosylation.

Here, an increase in RGP protein self-glycosylation was achieved after AP treatment (Table 3, Fig. 4). After AP treatment, a good correlation between the increment in the radioactivity incorporated to the TCA precipitate and the radioglycosylation of the protein was obtained. This result together with the fact that StRGP was found phosphorylated in vitro (Fig. 3) suggested that phosphorylation is able to modulate RGP glycosylation.

Table 3 and Fig. 4 show that potato as well as recombinant RGP proteins increased its self-glycosylation after AP treatment. A greater effect on glucosylation using recombinant proteins in comparison with RGP potato protein was found. The nature of the different behaviour of the RGP proteins after AP treatment is not known. However, differences at the protein level, perhaps related to their phosphorylation state, are now under investigation. In this respect, we observed different kinetic parameters and differential expression of mRNA in potato tissues for the recombinant RGP proteins (Wald et al. 2003).

We demonstrated that StRGP subunit was phosphorylated in potato in an in-vitro experiment using endogenous protein kinase/s (Fig. 3A). Furthermore, in-vivo labelling of phosphoproteins studied in *E. coli* strain XL1Blue, transformed with the pBluescript harbouring the E11 or E2 clone, revealed that recombinant RGPs are phosphorylated (Moreno, unpublished result). It is not yet clear which site on the enzyme phosphorylates, however, immunoblot results using a P-serine antibody indicated that a serine residue of the protein is implicated (Fig. 3B). Such results are not unexpected since StRGP1 and StRGP2 amino acid sequences contain 20 (6 Ser, 6 Thr and 8 Tyr) and 19 (7 Ser, 5 Thr and 7 Tyr) possible phosphorylation sites, respectively, as determined by the NETPHOS prediction program (Blom et al. 1999). The 38-kDa subunit of the recombinants StRGP1 and StRGP2 proteins were visualized as a doublet band with slightly different electrophoretic mobility after SDS-PAGE (Fig. 4). In potato extracts, StRGP subunit protein also showed a doublet band with slightly different electrophoretic mobility after electrophoresis in SDS-PAGE (Bocca et al. 1997). Recently, we suggested that the doublet might arise from a post-translational modification since it is present after protein translation from a single RGP mRNA (Wald et al. 2003).

Currently, StRGP protein phosphorylation cannot be interpreted in terms of a physiological function. It remains to be shown which protein interactions are affected by phosphorylation. In this connection, StRGP protein was detectable as both, soluble and membrane-associated forms (Moreno and Tandecarz 1982, Bocca et al. 1997), and immunoblot analysis (Fig. 3) suggests that soluble StRGP subunit is in a more phosphorylated form with respect to membrane-associated enzyme.

RGP has also been localized associated to membranes presumably of the Golgi apparatus in Arabidopsis and pea (Dhugga et al. 1997, Delgado et al. 1998). It is quite likely that RGP protein in vivo may exist in both phosphorylated and unphosphorylated forms liable to modulate its glycosylation. It may be assumed that glycosylation of the RGP protein regulates its association with membranes working as part of a mechanism controlling RGP localization depending on the cell's metabolic stage. Dephosphorylation of the RGP protein may promote its association to the Golgi apparatus in an active glycosylating form correlating with xyloglucan synthesis. On the other hand, phosphorylation should promote an enzyme found to be soluble in cell cytoplasm.

We have previously described that native StRGP from potato tuber preparations is associated with membranes as an oligomeric protein (Moreno et al. 1986, Ardila and Tandecarz 1992). Bocca et al. (1997) reported that the RGP complex in potato is a pentamer or a hexamer. Recently, complex formation of two classes of RGP from wheat endosperm and rice was also described (Langeveld et al. 2002). Reversible phosphorylation of sucrose synthase, another enzyme linked to biosynthetic processes such as cell wall or storage products, provides a potent regulatory mechanism of sugar metabolism in response to developmental and environmental signals (Huber and Huber 1992, Subbaiah and Sachs 2001). It was reported that dephosphorylation of sucrose synthase promotes a conformational change in which hydrophobic residues are more exposed to the solvent favouring its membrane association (Amor et al. 1995, Winter et al. 1997, Winter and Huber 2000). In all likelihood, StRGP dephosphorylation stimulates its glycosylation, which in turn may favour its association to other proteins or itself.

Whereas most proteomic studies focus on differential expression levels, post-translational modifications such as phosphorylation, glycosylation and acetylation provide additional levels of functional complexity to the

proteome of the cell. Although phosphorylation is recognized as an essential mechanism for controlling protein function, less is known about the specific roles of single monosaccharide modification (Vosseller et al. 2001).

A major goal for future research is to isolate the participants in the regulation mechanism of StRGP self-glycosylation, as valuable candidates to discern the first stage in polysaccharide synthesis.

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