Sugar-nucleotide-binding and autoglycosylating polypeptide(s) from nasturtium fruit: biochemical capacities and potential functions

Ahmed FAIK*^{†1}, Darrell DESVEAUX*[‡] and Gordon MACLACHLAN*

*McGill University, Department of Biology, 1025 Av. Dr. Penfield, Montreal, QC, H3A 1B1, Canada, [†]Michigan State University, DOE-Plant Research Laboratory, East Lansing, MI 48824, U.S.A., and [‡]Université de Montréal, Department of Biochemistry, P.O. Box 6128, Station Centre Ville, Montreal, QC, H3C 3J7, Canada

Polypeptide assemblies cross-linked by S–S bonds (molecular mass > 200 kDa) and single polypeptides folded with internal S–S cross-links (< 41 kDa) have been detected by SDS/PAGE in particulate membranes and soluble extracts of developing cotyledons of nasturtium (*Tropaeolum majus* L.). When first prepared from fruit homogenates, these polypeptides were found to bind reversibly to UDP-Gal (labelled with [¹⁴C]Gal or [³H]uridine), and to co-precipitate specifically with added xyloglucan from solutions made with 67% ethanol. Initially, the bound UDP-[¹⁴C]Gal could be replaced (bumped) by adding excess UDP, or exchanged (chased) with UDP-Gal, -Glc, -Man or -Xyl. However, this capacity for turnover was lost during incubation in reaction media, or during SDS/PAGE under reducing conditions, even as the glycone moiety was conserved by autoglycosylation to form a stable 41 kDa polypeptide.

Polyclonal antibodies raised to a similar product purified from *Arabidopsis* bound to all the labelled nasturtium polypeptides in immunoblotting tests. The antibodies also inhibited the binding of nasturtium polypeptides to UDP-Gal, the uptake of UDP-[¹⁴C]Gal into intact nasturtium membrane vesicles and the incorporation of [¹⁴C]Gal into nascent xyloglucan within these vesicles. This is the first direct evidence that these polypeptides facilitate the channelling of UDP-activated sugars from the cytoplasm through Golgi vesicle membranes to lumenal sites, where they can be used as substrates for glycosyltransferases to synthesize products such as xyloglucan.

Key words: autoglycosylation, reversibly glycosylated polypeptide, UDP-Gal-binding polypeptide, xyloglucan biosynthesis.

INTRODUCTION

Polypeptides that become labelled upon incubation with UDP-[¹⁴C]Glc have been reported recently in membrane and soluble enzyme preparations from pea epicotyl [1,2], *Arabidopsis*, maize and tobacco [3]. The products fractionate after electrophoresis as a single band with a molecular mass of 41 kDa, and cDNA analysis shows substantial sequence homology between them. Despite the evident stability of these labelled polypeptides, when they are first prepared they appear to be capable of transferring label to certain nucleotide acceptors. Thus the label was said to be ‘chased’ from the product by addition of high concentrations of unlabelled UDP-Glc, or ‘bumped’ by addition of UDP. These observations led to the naming of the product as a ‘reversibly glycosylated polypeptide’ (RGP). However, there are problems with the biochemical implications of such a title. To date, no covalent sugar–amino acid bond has been identified that has a high-energy linkage comparable with that of the sugar–nucleotide bond. No polypeptides are known that bind UDP-Glc so tightly that they will not dissociate during fractionation in SDS/PAGE. No direct evidence has been presented to demonstrate that purified glycosylated polypeptides are indeed capable of acting as sugar donors to any other acceptor. Thus, the legitimacy of the title RGP is not established, and their function is unknown.

The present study has re-investigated the putative turnover of sugar from RGPs using developing fruits of nasturtium (*Tropaeolum majus* L.) as an enzyme source and UDP-[¹⁴C]Gal as a sugar donor. It is concluded from time-course measurements

that the term RGP is a misnomer. The glycosylated 41 kDa polypeptide is an unreactive glycoprotein formed relatively slowly by glycosyl transfer from a rapidly formed UDP-sugar-binding polypeptide. It is the evanescent bound sugar nucleotide that is capable of acting as a sugar donor, not the final stable glycoprotein.

In a prescient early (1986) report [4] of UDP-sugar-binding polypeptide from mung bean, it was suggested that its observed turnover may indicate a role in the biosynthesis of polysaccharides like xyloglucan (XG). A potential relationship as an intermediate or precursor for XG biosynthesis in pea [1,2] and *Arabidopsis* [3] was surmised from the facts that UDP-[³H]Xyl and UDP-[³H]Gal could chase [¹⁴C]Glc from association with their polypeptides, and [¹⁴C]Glc was replaced by the tritiated sugars. Immunogold labelling with antibody raised to *Arabidopsis* RGP localized it to trans-Golgi dictyosomal cisternae, where XG biosynthesis occurs [5,6], but not to plastids, plasma membrane or endoplasmic reticulum. This argues against a role for RGP in biosynthesis of starch, cellulose or other glycoproteins. New evidence is presented here for a specific interaction between the nasturtium UDP-Gal-binding polypeptide and XG biosynthesis by the demonstration that XG binds in a complex with this polypeptide that precipitates selectively from alcoholic solution, a phenomenon not duplicated by other related polysaccharides.

Nasturtium fruit and UDP-Gal were used in this study as enzyme and substrate sources because mature nasturtium seeds contain about 100 times as much ‘storage’ XG, deposited as a temporary reserve in periplasmic spaces of cotyledon cells, as

Abbreviations used: RGP, reversibly glycosylated polypeptide; AtRGP1, *Arabidopsis thaliana* reversibly glycosylated polypeptide 1; TCA, trichloroacetic acid; XG, xyloglucan.

¹ To whom correspondence should be addressed, at Michigan State University (e-mail faik@pilot.msu.edu).

'structural' XG, which is incorporated into primary cell walls [7,8]. The mass of storage XG is much more heavily galactosylated (22 % Gal, w/w) than wall XG (6 % Gal). It was anticipated, therefore, that developing nasturtium cotyledons should be a relatively rich source of whatever polypeptides are needed in the Golgi for mobilizing and transferring galactose from UDP-Gal to XG. Polyclonal antibodies to purified RGP from *Arabidopsis* were used as a probe to detect homologues in extracts of nasturtium fruits. These antibodies not only bound to typical RGP bands in electrophoresis profiles of nasturtium protein, they also inhibited galactosylation of nasturtium RGP and incorporation of [14 C]Gal into nascent XG within nasturtium Golgi vesicles.

Transporter proteins with high affinity for UDP-sugar-binding polypeptides, including UDP-Gal, have been described in membranes of many animals and yeast, where they are thought to have regulatory roles in channelling substrate from the cytoplasm to glycosyltransferases in the Golgi lumen [9,10]. Transporter protein for UDP-sugars has also been described in association with the outer membrane of pea Golgi vesicles [11]. All of the studies of plant RGPs to date speculate that they may function as an intermediate sugar-channelling polypeptide, but no evidence for this possibility has been published. The present study demonstrates that antibodies to *Arabidopsis* RGP do indeed inhibit the uptake of UDP-[14 C]Gal into intact nasturtium vesicles. This constitutes the first direct indication that the RGP can act as a sugar-nucleotide-transporting polypeptide in vesicles that form polysaccharides like XG, at least for the time during which it binds sugar nucleotide and before it autoglycosylates to a stable glycoprotein.

MATERIALS AND METHODS

Enzyme assays

Nasturtium fruits or excised cotyledons were harvested at 23 days post-anthesis, when they were about half developed and actively synthesizing reserves like XG [8]. Tissues were homogenized with a high-speed blender in a standard extraction medium [12]. Particulate fractions (100 000 g) extracted with CHAPS detergent and supernatants were used as enzyme sources as described earlier [12] in studies of XG-dependent galactosyltransferase activity in pea epicotyl. Assay conditions were similar to those that were effective in demonstrating the incorporation of [14 C]Gal from UDP-[14 C]Gal into pea XG subunits. The XG that was employed as potential galactosyl acceptor was derived from nasturtium seed storage XG [8] and partially degalactosylated [12] with nasturtium seed β -galactosidase [13]. Other polysaccharides that were added to reaction mixtures as potential galactosyl acceptors were tamarind seed XG, rye arabinogalactan and potato β -galactan (all from Megazyme, Bray, Ireland), carboxymethyl cellulose (CM-cellulose; Hercules Powder Co., Wilmington, DE, U.S.A.) and laminarin (Calbiochem). Radioactive substrates were all manufactured by New England Nuclear (DuPont). Specific radioactivities were as follows: UDP-[U- 14 C]Gal, 12 GBq/mmol; UDP-[6- 3 H]Glc, 156 GBq/mmol; UDP-[1- 3 H]Xyl, 329 GBq/mmol. Uridine-labelled UDP-Glc was prepared from [5,6- 3 H]UTP, 1483 GBq/mmol, by incubation with Glc-1-P, UDP-Glc pyrophosphorylase and pyrophosphatase (Sigma) as described in [11].

Standard reaction mixtures for generating labelled protein or polysaccharide were assembled in centrifuge tubes (1.5 ml) containing 20 μ l of assay medium (0.1 M Hepes/KOH, pH 6.8, 25 mM $MgCl_2$, 25 mM $MnCl_2$, 5 mM dithiothreitol and 0.5 M sucrose), 10 μ l of UDP-[14 C]Gal (0.232 nmol, 167 000 d.p.m.), plus either 10 μ l of water (controls) or 10 μ l of 1 % (w/v) XG.

Reaction was initiated by adding 10 μ l of enzyme extract (10–20 μ g of protein). Mixtures were incubated at room temperature and routinely terminated at 30 min by adding 1 ml of cold 67 % ethanol (-20°C). Alcoholic mixtures were immediately centrifuged (18 000 g, 10 min) and pellets were washed three times with 67 % ethanol to remove unused substrate. Radioactivity was measured in pellets by liquid-scintillation spectroscopy using a counter (Beckman, model LS-5000 TD) that could discriminate between ^3H and ^{14}C . In some tests, reaction mixtures were terminated by adding 10 % trichloroacetic acid (TCA; 16 h, 4°C) and centrifuging to remove TCA-insoluble protein and salts of the substrate. The precipitated protein was washed free of substrate by mixing and centrifuging in 67 % ethanol. The TCA-soluble supernatant containing endogenous and added XG was precipitated and washed with 67 % ethanol. Pulse-chase experiments were conducted routinely by incubating reaction mixtures with 3 μ M UDP-[14 C]Gal as above for various times (pulse) followed by addition of relatively high concentrations (50–100 μ M) of alternative substrate or binding agent (10–15 min chase) before adding 67 % ethanol.

Product analysis

Ethanol-insoluble products of reaction mixtures were analysed for composition and size distribution by gel filtration through columns (1.1 cm $\times$ 126 cm) of Sepharose CL-6B (Pharmacia), before or after digestion with 0.01 % (w/v) Pronase (Sigma) in 10 mM phosphate buffer, pH 7.5, and 0.01 % (w/v) NaN_3 for 16 h at 35°C , or with 0.05 % (w/v) *Trichoderma* cellulase (Megazyme) in 50 mM sodium acetate buffer, pH 5.8, for 16 h at 40°C . Products were eluted in 0.1 M NaOH/0.01 % NaBH_4 and radioactivity in fractions was determined after neutralization with HCl. Total carbohydrate was measured with phenol sulphuric acid [14] using tamarind XG as standard, and protein with an assay kit (Bio-Rad) using BSA as standard.

^{14}C -Labelled protein that was insoluble in 10 % TCA and 67 % ethanol and digested to labelled glycopeptides by Pronase was analysed by SDS/PAGE (10 % separating gel) according to standard methods, in the presence or absence of β -mercaptoethanol (5 %, v/v). Protein bands were stained with Coomassie Brilliant Blue and the gel soaked in EN3HANCE (DuPont) for 30 min and then dried for autoradiography. Size was estimated by comparison with the mobilities of marker proteins after electrophoresis. Gel chromatography was conducted through columns (1.1 cm $\times$ 126 cm) of Sephacryl S-200 (Pharmacia) using 20 mM Tris/HCl, pH 7, and 0.01 % NaN_3 as eluant.

Labelled products that were soluble in 10 % TCA but insoluble in 67 % ethanol and digested to oligosaccharides by cellulase treatment were fractionated on columns (1.1 cm $\times$ 126 cm) of Bio-Gel P₂ (Bio-Rad) with 0.01 % NaN_3 as eluant. Labelled oligosaccharides were injected into a CarboPac PA-100 column (Dionex) and eluted under pressure (Beckman HPLC). The positions of labelled oligosaccharides were compared with those of authentic XG subunits (Dionex pulsed amperometric detector) as described in [12].

UDP-[14 C]Gal uptake

In experiments to examine the absorption of supplied UDP-[14 C]Gal into relatively intact nasturtium vesicles, procedures were similar to those employed for assaying membrane transporters for UDP-[14 C]Glc in pea Golgi cisternae [11]. Homogenization was performed with a minimum of abrasion by chopping cotyledons in the above extraction buffer (5 ml/g of tissue) with razor blades, followed by grinding briefly in a mortar

with pestle, filtration through fine nylon mesh, and centrifugation of the filtrate at 100 000 *g*. Aliquots of the pellet [15 μ l, from about 10 mg (fresh weight) of tissue] were incubated with the standard assay medium (20 μ l) plus substrate UDP-[14 C]Gal (10 μ l) for various times up to 35 min. Uptake and retention of 14 C into vesicles was measured by diluting the incubation medium and vesicles with 1 ml of cold assay medium and filtering through a Gelman membrane (2.5 cm diameter, 0.45 μ m pores) with a Millipore syringe. Retained vesicles were washed thoroughly with another 1 ml of assay medium, neutralized and dried for 14 C measurement. Separation of vesicles from incubation medium and washing took approx. 90 s.

In order to estimate the proportion of total [14 C]Gal that was recovered from washed vesicles which had been incorporated into protein and polysaccharide, duplicate reaction mixtures were terminated by adding 1 vol. of cold 20% TCA. The precipitate (protein) and supernatant (polysaccharide) were pelleted and washed with 67% ethanol in order to remove absorbed but unused UDP-[14 C]Gal and to determine 14 C incorporated into products.

Use of antibodies to RGP

Polyclonal antibodies raised by Ivan Delgado (Michigan State University) against a fusion protein containing most of the amino acid sequence of *Arabidopsis thaliana* RGP1 (AtRGP1) were a gift from Professor Natasha Raikhel (Michigan State University). These antibodies detected two bands on electrophoresis gels (41 and 64 kDa) in immunoblots of extracts of pea, tobacco and corn, but not in extracts of yeast or mammalian sources [3]. The smaller polypeptide corresponded to the size of subunit RGP; the larger may have had a uridine-binding domain but its function was not established.

Antibodies to AtRGP1 were employed in present tests as probes to detect homologues of *Arabidopsis* RGP in SDS/PAGE profiles of nasturtium protein. SDS/PAGE was performed with separating gels (12%) with or without added β -mercaptoethanol. Polypeptides were transferred to a 0.45 μ m nitrocellulose membrane (Bio-Rad) using a semi-dry apparatus at 100 mA for 45 min. Membranes were stained with Ponceau and blocked in 3% (w/v) milk powder in 20 mM Tris/HCl, pH 7.4, and 150 mM NaCl for 1 h at 23 °C. Anti-AtRGP1 was used at a dilution of 1:2000 in the above buffer containing 0.1% Tween 20 for a 45 min incubation with the filters. After washes (3 $\times$ 10 min) to remove unbound antibodies, the membranes were incubated in a 1:5000 dilution of goat anti-IgG linked to horseradish peroxidase (Bio-Rad) for 50 min. A Western-blot detection system (ECL[®], Amersham) was used to autograph the immunoreactive polypeptide bands. Anti-AtRGP1 was also tested as potential inhibitor of nasturtium RGP galactosylation in standard assay systems, and as inhibitors of uptake of UDP-[14 C]Gal into intact nasturtium vesicles, prepared as above [11].

RESULTS

[14 C]Gal-labelled nasturtium protein binds to XG specifically

Table 1 records typical data showing that nasturtium fruit enzyme extracted from membranes by detergent, or soluble in buffer supernatants, was capable of incorporating 14 C from UDP-[14 C]Gal into ethanol-insoluble product(s), provided that incubation was conducted in the presence of added tamarind or nasturtium seed XG. Recovery of 14 C was enhanced at least 15-fold in the presence of XG over values in controls, and other potential polysaccharide acceptors had no such effect. However, partially degalactosylated nasturtium XG was no more effective

Table 1 Incorporation of 14 C from UDP-[14 C]Gal into fractions of nasturtium homogenate: co-precipitation of 14 C product(s) and XG

Extracts of nasturtium fruits, at a stage where they were generating XG at a high rate (average fresh weight $\approx$ 0.5 g/fruit), were incubated for 30 min with UDP-[14 C]Gal (3 μ M) with or without various polysaccharides (0.2%, w/v) as described in the Materials and methods section. Reactions were terminated and products were precipitated immediately in 67% ethanol and collected by centrifugation. Washed pellets were assayed for 14 C. Values given are averages of two estimations that differed by less than 10%. CM-cellulose, carboxymethyl cellulose.

Additive to reaction mix	14 C Incorporation (d.p.m./h per mg of fresh weight)	
	CHAPS extract of 100 000 <i>g</i> pellet	100 000 <i>g</i> Supernatant
None (control)	0.6	3.4
Tamarind XG	14.0	62.0
Nasturtium XG	11.0	54.0
Degalactosylated XG	14.0	65.0
CM-cellulose	1.8	5.7
Arabinoxylan	1.4	4.0
1,4- β -Galactan	1.0	4.2
Laminarin	0.6	3.2

at enhancing 14 C recovery in precipitates than fully galactosylated XG, which implies that supplied XG was not recognized preferentially as a galactosyl acceptor in this system, as it was using pea enzyme [12]. In fact, yields of label in pellets were identical when XG was added to reaction mixtures after incubation with enzyme, just before flocculation in 67% ethanol, which means that XG was not affecting the reaction progress, but the precipitation. On investigation, 14 C in the product collected by centrifugation was equal in the absence and presence of XG when precipitation in 67% ethanol was conducted overnight at -20 °C, rather than immediately after mixing. Accordingly, results in Table 1 mean that XG specifically and rapidly co-precipitated with the [14 C]Gal-labelled products, indicating strong binding to XG in the early stages of dehydration in alcoholic solution.

The 14 C products that co-precipitated with added XG (Table 1) were [14 C]galactosylated protein(s). The evidence may be summarized as follows. The 14 C products all precipitated in cold 10% TCA and eluted from columns of Sepharose CL-6B with a broad apparent size range equivalent to standard proteins with molecular masses at or above 200 kDa. Very little 14 C eluted with XG peaks at a much higher molecular mass, and no detectable digestion occurred in cellulase. The 14 C products were depolymerized by Pronase to labelled glycopeptides that eluted near the total volume of the columns. They were stable for hours during either electrophoresis or elution from columns of Sepharose CL-6B in 0.1 M NaOH at 20 °C. They were degraded in 2 M trifluoroacetic acid (120 °C, 1 h) to free [14 C]Gal. No differences were detected in the size of 14 C products recovered from reaction mixtures containing CHAPS extracts of membrane fractions or from the buffer-soluble supernatant.

Sizes of [14 C]galactosylated protein

Figure 1 shows SDS/PAGE profiles of Coomassie Brilliant Blue-stained polypeptides and the corresponding autoradiographs of 67% ethanol-insoluble products generated by reaction mixtures containing UDP-[14 C]Gal, added XG and CHAPS extracts of nasturtium membranes. All of the RGPs for which sequence analyses are available contain seven cysteine amino acids in loci

Figure 1 SDS/PAGE of ethanol-insoluble products from reaction mixtures containing UDP-[^{14}C]Gal, storage XG and CHAPS extract of nasturtium membranes

After a 30 min incubation as described in the Materials and methods section and Table 1, the reaction was terminated by adding cold 67% ethanol and immediately centrifuging to precipitate products. Precipitates were washed in 67% ethanol and 10% TCA before electrophoresis was conducted in the absence or presence of reducing agent (5% β -mercaptoethanol). Similar profiles were obtained using the 100 000 g supernatant of nasturtium homogenate as enzyme. Molecular-mass markers are indicated in kDa.

that are conserved [1,3] and potentially available for cross-linking within a single polypeptide chain or between discrete chains. Accordingly, electrophoresis was conducted in the absence of reducing agent (β -mercaptoethanol), or in its presence under conditions where all S-S linkages would be reduced to free SH terminals. As expected, under non-reducing conditions, multiple bands of heavily stained polypeptides were present, and label was concentrated in bands with a size equivalent to markers > 200 kDa and < 41 kDa. The addition of β -mercaptoethanol resulted in the disappearance of many of the largest and smallest stained bands, with most of the polypeptides now clustered in bands between 40 and 65 kDa. The label was confined to one band at 41 kDa, which is the molecular mass reported for RGP from peas [1,2], *Arabidopsis* [3] and mung bean [4].

We interpret these observations as indicating that nasturtium fruits have the capacity to galactosylate a polypeptide with a size comparable to RGPs in other tissues, but here it exists mainly in forms that are cross-linked by S-S bonds to other polypeptide chains and possibly to itself in complexes with molecular masses > 200 kDa. Single polypeptide chains may also fold into relatively compact structures held together by internal S-S linkages. These could account for the glycosylated polypeptides that are observed at an apparently low molecular mass (< 41 kDa) until reduced and denatured to form the extended chain (Figure 1).

Similar conclusions were reached from the results of fractionating nasturtium extracts through columns of Sephacryl S-200 with eluant in the presence or absence of 10 mM dithiothreitol. The capacity for galactosyl transfer to ethanol-insoluble products peaked in aliquots that eluted at or near the void volume of the column (> 200 kDa), but in the presence of reducing agent they eluted near the lower end of the broad profile

Table 2 Pulse-chase experiments with sugar nucleotides

CHAPS-soluble enzyme from nasturtium fruit (average fresh weight ≈ 0.4 g/fruit) was pulsed with UDP-[^{14}C]Gal (3 μM , 10 min) in the presence of nasturtium XG (0.2%, w/v), followed by chase (10 min) with water (control) or unlabelled sugar nucleotides (100 μM). Label in the product precipitated by 67% ethanol was determined after chase. In controls, conditions were such that $\approx 2000 \pm 100$ d.p.m. (S.E.M.) (≈ 2 pmol) of ^{14}C were recovered in the ethanol-insoluble product. When Glc or Xyl were tritiated, ^3H replaced ^{14}C in the final product.

Chase	Percentage of control
None	100
GDP-Man	109
GDP-Glc	108
GDP-Fuc	88
UDP-GlcNAc	88
UDP-GalNAc	89
UDP-Glc	24
UDP-Man	27
UDP-Xyl	33
UDP-Gal	34
UDP-GlcA	58
UDP-GalA	68

of total protein (results not shown). These results implied that the polypeptide equivalent to RGP catalysed its own galactosylation, whether combined with other polypeptides or not. Autoglycosylation has also been described [15] in carrot extracts fractionated by gel filtration and PAGE in the presence or absence of dithiothreitol.

Reversibility of [^{14}C]Gal labelling

In freshly prepared reaction mixtures from nasturtium, when a 10 min pulse in 3 μM UDP-[^{14}C]Gal was followed by the addition of 100 μM UDP-Gal, -Glc or -Xyl in a 10 min chase, at least two-thirds of the label normally recovered in polypeptide precipitated by 67% ethanol was lost during the chase (Table 2). When the chase was conducted with these sugar-nucleotides tritiated in the sugar moiety, ^{14}C was replaced in the polypeptide product by ^3H . This indicates unambiguously that the UDP-[^{14}C]Gal could exchange with other UDP-sugars in the presence of fresh nasturtium extract. GDP-sugars were ineffective as chase reagents. None of the nucleosides of RNA or DNA showed any capacity to chase UDP-[^{14}C]Gal from nasturtium polypeptide, but all of the pyrimidine nucleotides were at least partially effective, with diphosphates $>$ triphosphates $>$ monophosphates, and UDP $>$ CDP $>$ TDP (results not shown). As noted for pea RGP [1], these nucleotides, and especially UDP and UMP, also inhibited the initial incorporation of label from UDP-[^{14}C]sugars into nasturtium polypeptide. In all of these respects, the [^{14}C]Gal-labelled polypeptide from nasturtium resembled RGPs from pea and *Arabidopsis*.

One difference in the results of pulse-chase reactions in the present tests versus earlier experiments was that UDP-Man from various suppliers was as effective as the UDP-sugars, which are precursors for XG at displacing UDP-[^{14}C]Gal from nasturtium polypeptide (Table 2), although UDP-Man was ineffective at chasing UDP-[^{14}C]Glc from pea and *Arabidopsis* polypeptide [1–3]. Another difference is that with nasturtium enzyme, UDP-uronic acids were partially effective chase reagents, although UDP-*N*-acetyl sugar amines were not (Table 2). Thus, if the

Figure 2 Reversibility of ^{14}C incorporation from UDP- ^{14}C Gal (pulse) by UDP (chase)

Reaction tubes contained buffer-soluble supernatant from homogenates of nasturtium fruits (mean fresh weight ≈ 0.5 g/fruit, $5 \mu\text{g}$ of protein/reaction mixture), tamarind XG and UDP- ^{14}C Gal for the times indicated (pulse, $\bullet$), followed by chase (15 min) with UDP in duplicate tubes ($\circ$). Reactions were terminated by centrifuging in 67% ethanol and d.p.m. were determined in washed precipitates.

nasturtium 41 kDa polypeptide is a potential intermediate for polysaccharide biosynthesis, the end product is not necessarily confined to XG.

An important property of the nasturtium 41 kDa polypeptide, which could also apply to RGP from pea and *Arabidopsis*, but was not recorded for them, is that the capacity for the ^{14}C Gal moiety to be chased did not last long. As shown in Figure 2, after a few minutes of pulse in UDP- ^{14}C Gal, the label that normally precipitated with polypeptide in 67% ethanol could be eliminated almost completely from the reaction mixture by a 15 min chase in excess UDP just before centrifugation. This reversibility is what gave rise to the term RGP. But after about 3 h pulse, only half of the ^{14}C associated with polypeptide was chasable by UDP, and by 24 h no label was dislodged. By that time, it was clearly inappropriate to refer to the labelling of nasturtium 41 kDa polypeptide as 'reversible'.

The apparent stabilization or loss of reactivity of the galactosylated polypeptide is not due to denaturation of the polypeptide during incubation. When the nasturtium enzyme preparation used in Figure 2 was incubated alone for 24 h under the same conditions, it retained fully the ability to be labelled by a pulse of UDP- ^{14}C Gal and 'bumped' by a chase with UDP. Evidently, it was the reactivity of the bond linking ^{14}C Gal to the polypeptide that decayed with time, from an initial high-energy state comparable with that of the linkage between sugar and pyrophosphate in sugar nucleotides, to the relatively low energy of a standard glycoprotein.

Reversible UDP-Gal-polypeptide complex eventually autoglycosylates *in vitro*

The above results raise a key question that has not yet been clearly addressed about the biochemical capacity of RGPs, namely whether the initially reversible linkage between sugar and polypeptide is due to direct glycosylation of polypeptide via an unusual activated covalent bond, as the term RGP implies, or to a UDP-sugar-polypeptide complex, as the observed binding

Figure 3 Reversibility of a pulse in ^3H uridine-labelled UDP-Glc by chase in UDP or UDP- ^{14}C Gal

Reaction tubes contained buffer-soluble supernatant enzyme ($2 \mu\text{g}$ of protein), tamarind XG ($100 \mu\text{g}$), ^3H UDP-Glc ($0.04 \mu\text{M}$) for the times indicated (pulse, $\blacksquare$), followed by chase (15 min) in UDP- ^{14}C Gal ($3 \mu\text{M}$; $\circ$) or UDP ($100 \mu\text{M}$; $\square$). Incorporation of ^3H uridine (A) or ^{14}C Gal (B) was measured in product insoluble in 67% ethanol immediately after pulse, or pulse plus chase.

preference for uridine nucleotides implies. The following direct test shows that the latter interpretation is correct. When nasturtium protein was pulsed with ^3H uridine-labelled UDP-Glc, the polypeptide which co-precipitated with XG in 67% ethanol was initially tritiated. Bound ^3H in reaction mixtures could be evicted by adding UDP or UDP- ^{14}C Gal (Figure 3A), just as the initial binding of ^{14}C in UDP- ^{14}C Gal was displaceable by UDP (Figure 2) or UDP- ^3H Glc (Table 2). After the first hour of pulse in UDP- ^3H Glc, the tritium began to decorporate from the product and the proportion which was chasable diminished (Figure 3). This is in contrast to the retention of ^{14}C from UDP- ^{14}C Gal in the polypeptide, even as the reversibility of labelling decayed (Figure 2, cf. Figure 3B). The incorporation of labelled sugar into a stable product during pulse (Figure 2) reached a maximum after a few hours, long before the available substrate had been consumed (98% remained unused).

These data indicate that UDP-sugars as such are initially bound reversibly to polypeptide in a complex from which they are readily displaced by UDP or any of several UDP-sugars, but that the sugar moiety gradually transfers to a stable linkage in polypeptide. The process must be autocatalytic and nearly stoichiometric since the glycosylated polypeptide becomes saturated at a value close to the maximum that is initially chasable

Figure 4 Immunoblot of SDS/PAGE profiles under reducing conditions of nasturtium extracts using anti-AtRGP1 to detect homologues of *Arabidopsis* RGP

Lane 1, protein (30 µg) in particulate membranes of nasturtium cotyledons. Lane 2, partially purified protein (5 µg) from the 100 000 g supernatant of homogenates of whole nasturtium fruit. The latter was derived from a peak in fractions from columns of Sephacryl S-200 that actively incorporated ^{14}C into RGP in the presence of dithiothreitol. Molecular-mass markers are indicated in kDa. The bands that bound these antibodies were the only Coomassie Brilliant Blue-positive components in the profiles that showed any reaction.

(Figure 2), and then is unable to bind more sugar nucleotide. Since glycosylation inhibits binding, the implication is that the glycosylation and binding sites are on the same polypeptide.

Antibodies to RGP bind to nasturtium polypeptides and inhibit autogalactosylation

Figure 4 shows immunoblots of SDS/PAGE profiles of particulate (lane 1) and soluble (lane 2) enzyme fractions from developing nasturtium fruits, using as probe the polyclonal antibodies raised against RGP1 from *Arabidopsis*. These antibodies react with polypeptides of 41 and 66 kDa in extracts of *Arabidopsis*, pea, tobacco and maize [3]. In nasturtium profiles, anti-AtRGP1 recognized under reducing conditions a polypeptide with a molecular mass of 41 kDa, which is the size of the [^{14}C]Gal-labelled polypeptide (Figure 1) and the [^{14}C]Glc-labelled polypeptides from other plant sources [1–4]. It also reacted with a family of at least three nasturtium polypeptides with molecular masses between 62 and 70 kDa, which correspond to several of the bands that stain heavily with Coomassie Brilliant Blue but are not labelled with [^{14}C]Gal (Figure 1). Under non-reducing conditions, anti-AtRGP1 did not detect any of the nasturtium 41 or 62–70 kDa bands in separating gel regions. They were probably linked with reducible S–S bonds into a larger complex (see Figure 1), which would have been retained by the stacking gel.

Anti-AtRGP1 was also tested for its ability to inhibit galactosyl transfer to partially purified RGP isolated under reducing conditions from nasturtium fruit. Supernatant extracts were fractionated by gel filtration, as in Figure 4, and assayed for [^{14}C]Gal transfer to 67% ethanol-insoluble products in the presence and absence of antibodies. At antibody concentrations above 1:50, ^{14}C incorporation was inhibited by about 40% (results not shown).

Anti-RGP inhibits UDP-[^{14}C]Gal uptake and XG biosynthesis in intact nasturtium vesicles

The progress of uptake of ^{14}C from 3 µM UDP-[^{14}C]Gal with or without anti-AtRGP1 (1:50 dilution) into washed nasturtium vesicles is shown in Figure 5(A). Particulate fractions of nas-

Figure 5 (A) Uptake of UDP-[^{14}C]Gal into relatively intact nasturtium membrane vesicles, in the presence (●) or absence (○) of anti-AtRGP1 and (B) incorporation of [^{14}C]Gal into TCA-insoluble products

Particulate fractions containing intact vesicles were obtained by centrifugation (100 000 g) of homogenate of 10 mg (fresh weight) of nasturtium cotyledons. They were incubated with 3 µM UDP-[^{14}C]Gal (160 000 d.p.m./reaction) for the times indicated, when membranes were rapidly filtered and washed with buffer. Total uptake of ^{14}C was measured in the washed membranes retained by a filter device (A). Incorporation of ^{14}C into protein was measured in parallel reaction mixtures by adding TCA and washing the precipitate with 67% ethanol (B). Incorporation into XG (TCA-soluble product) was detectable but at a low level (about 10% of incorporation into protein).

turtium cotyledons had been isolated gently with methods described earlier [11] for measuring sugar-nucleotide transporter activity in intact pea Golgi vesicles. The uptake into the nasturtium fractions was saturated within 10 min and the antibodies suppressed this accumulation by about 50%. This was due to inhibition of the UDP-[^{14}C]Gal-channelling mechanism, not the incorporation of ^{14}C into TCA-insoluble protein, which was unaffected by the added antibodies (Figure 5B). Since the antibodies do inhibit galactosylation of solubilized 41 kDa polypeptide (see above), this component in intact vesicles was evidently located at an internal vesicle site that was not accessible to antibody. Thus the observed inhibition of substrate channelling (Figure 5A) may be due to interaction of anti-RGP with the only other nasturtium polypeptides to which they bind, namely those with a size of 62–70 kDa (Figure 4). These larger polypeptides also bind to the 41 kDa RGP-like polypeptide(s) but do not themselves become labelled when incubated with UDP-[^{14}C]Gal (Figure 1). This is the first tangible evidence for a link between a UDP-sugar channelling mechanism and the RGP-like polypeptide.

Table 3 Effects of anti-RGP on galactosyl transfer from UDP-[¹⁴C]Gal by intact nasturtium vesicles with or without the detergent BRIJ 58

Reaction mixtures contained substrate + degalactosylated nasturtium seed XG + vesicles from 10 mg (fresh weight) of cotyledons dissected from nasturtium fruit (average fresh weight $\approx$ 0.5 g/fruit) with or without anti-ATRGP1 (1:50 dilution) in the presence or absence of 0.5% BRIJ 58. Reactions were terminated (30 min) by adding TCA (10%, w/v, final concentration) and centrifuging before washing the pellet and precipitating the supernatant with 67% ethanol. TCA-soluble fractions contained the added XG and TCA-insoluble fractions contained all of the enzymic protein. Values given are averages of two estimations which differed by less than 10%.

Membranes/detergent	Galactosyl transfer (d.p.m./h per mg of fresh weight)	
	TCA-soluble	TCA-insoluble
Control	17.0	160.5
+ BRIJ 58	20.2	331.0
Δ	+3.2	+170.5
Anti-ATRGP1	7.4	135.0
+ BRIJ 58	8.2	141.6
Δ	+0.8	+6.2

Measurements of incorporation of ¹⁴C from UDP-[¹⁴C]Gal into stable products by intact nasturtium vesicles with or without anti-RGP were repeated in the presence and absence of the detergent BRIJ 58. This is a polyoxyethylene acyl ether that permeates plant membrane vesicles and turns them 'inside out' [16]. The intent of adding it here was to examine by a more direct approach the topology of the reactions controlling [¹⁴C]Gal incorporation by nasturtium membranes. BRIJ 58 doubled the incorporation of [¹⁴C]Gal into TCA-insoluble protein, indicating a major increase in glycosyl transfer when the inner surface of membranes was exposed to substrate. In the presence of anti-RGP, however, no significant increase in TCA-insoluble label was evoked by BRIJ 58, which supports the conclusion that RGP in nasturtium vesicles is normally buried at the luminal surface.

A small fraction (7–12%) of the total UDP-[¹⁴C]Gal that was incorporated into stable products by intact nasturtium vesicles was TCA-soluble, and the yield of this product was inhibited by anti-RGP (Table 3). Since the degree of inhibition was the same in the absence (–56%) and presence (–59%) of BRIJ 58, and comparable with the observed inhibition of UDP-[¹⁴C]Gal uptake (Figure 5A), it appears to have been generated by galactosyl-transferase activity that was limited by the flux of substrate. Separate tests showed that the labelled TCA-soluble product in this experiment was not depolymerized by Pronase, but was digested by cellulase to fragments that fractionated on columns of Bio-Gel P2 to positions equivalent to XG subunits. HPLC of these fragments through Dionex columns indicated that ¹⁴C was confined to the same two oligosaccharides observed in TCA-soluble products formed from UDP-[¹⁴C]Gal by pea enzyme [12], which are different from the subunits found in storage XG [8]. We are obliged to conclude that present tests with nasturtium vesicles only recovered galactosyltransferase activity that was responsible for biosynthesis of structural (wall) XG, not the great bulk of reserve XG.

DISCUSSION

Results from this study show that nasturtium fruit contains proteins with properties similar to those known as RGPs in many other plant tissues, most specifically: (i) they become transiently labelled with several UDP-[¹⁴C]sugars which can

exchange or donate the sugar moieties with one other and UDP (Table 2); (ii) they fractionate in SDS/PAGE to a labelled polypeptide with a molecular mass of 41 kDa (Figure 1); and (iii) they bind to antibodies prepared to purified *Arabidopsis* RGP (Figure 5).

However, further properties have been documented for the nasturtium RGP that change the perception of its biochemical capacities to such a degree that the term should probably be abandoned. In particular, double-labelling experiments (Figure 3) prove that the 41 kDa polypeptide initially binds to both sugar and uridine in sugar nucleotide and it is this activated form that acts as a sugar donor. In time, the sugar-nucleotide-polypeptide complex autoglycosylates to form the stable 41 kDa polypeptide observed after SDS/PAGE. Despite numerous attempts, we and other researchers have never found this glycoprotein to be capable of transferring any of its glycone to another polymer in any plant enzyme preparation. In fact, the 41 kDa polypeptide may be a preparation artefact that never exists as such *in vivo*. Thus the term RGP is a misnomer, since the substrate-binding polypeptide that is chasable is not directly glycosylated, and the terminally glycosylated polypeptide is not formed reversibly. We suggest that an appropriate name for these polypeptides which accurately reflects their properties would be sugar-nucleotide-binding and autoglycosylating polypeptide(s), abbreviated to SNB-AP.

It has been speculated [1–4] that these polypeptides may act as sugar-transporter polypeptides, which channel UDP-sugars from the cytoplasm where they are synthesized to Golgi lumen [11,17]. They could be used there as sugar donors to glycosyltransferases for biosynthesis of polysaccharides like XG [5,6]. The present study provides the first direct evidence for this hypothesis in the observations that anti-RGP inhibits the uptake of UDP-[¹⁴C]Gal into nasturtium vesicles (Figure 5A) and, at the same time, inhibits incorporation of ¹⁴C from UDP-[¹⁴C]Gal into TCA-soluble XG (Table 3). A close connection between XG and the complex of polypeptides that bind UDP-sugars was evident from the first experimental results obtained in this study (Table 1, Figure 1), where XG unilaterally promoted the precipitation of 41 kDa polypeptides and other polypeptides that bound to them by S–S linkages. Moreover, topological studies of intact nasturtium vesicles (Table 3) indicate that assemblies of UDP-sugar-binding polypeptides are concentrated on the luminal side where the XG is formed.

To be sure, a substantial yield of the 41 kDa polypeptide was recovered from nasturtium homogenate in buffer-soluble washes (Table 1), as it was in pea and *Arabidopsis* [1–3]. If these components originated as peripheral polypeptides loosely bound to the cytoplasmic surfaces of Golgi membranes, they could have acted *in vivo* as the first scavengers for sequestering and transporting cytoplasmic UDP-sugars to the luminal UDP-sugar-binding polypeptides. It is also possible that the soluble form of the 41 kDa polypeptide functions *in vitro* as a catalyst for generating UDP-sugars. Sucrose synthase, for example, is isolated from plant tissue homogenates partly soluble in buffer and partly bound to particulate fractions (plasma membrane), where it may donate glucose from sucrose to cellulose synthase via UDP-Glc [18]. The feasibility for such a collaboration between synthases was demonstrated earlier by direct measurement of glucosyl transfer from sucrose to β -glucan by soluble sucrose synthase coupled to particulate β -glucan synthase in pea preparations [19]. Other soluble enzymes from plants that can generate UDP-sugars include UDP-Gal pyrophosphorylase [20] and Gal-1-P: UDP-Glc uridylyltransferase [21]. The latter purifies under reducing conditions by SDS/PAGE to a size close to 41 kDa. There are clearly multiple forms and potential functions

for the UDP-sugar-binding domains in plant polypeptides, and further biochemical and cytological studies are needed to sort out which are active in any particular tissue *in vivo*.

We wish to thank Professor Natasha Raikhel and Mr Ivan Delgado (Michigan State University-DOE/PRL) for preparing polyclonal antibodies to AtRGP1 and for useful discussions, and Dr R. Subramaniam (University de Montréal) for assistance in immunoblot experiments. These studies were supported by a graduate fellowship (to D.D.) and research operating grant (to G.M.) from the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

- 1 Dhugga, K. S., Ulvskov, P., Gallagher, S. R. and Ray, P. M. (1991) *J. Biol. Chem.* **266**, 21977–21984
- 2 Dhugga, K. S., Tiwari, S. C. and Ray, P. M. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 7679–7684
- 3 Delgado, I. J., Wang, Z., deRocher, A., Keegstra, K. and Raikhel, N. V. (1998) *Plant Physiol.* **116**, 1339–1349
- 4 Read, S. M., Thelen, M. and Delmer, D. P. (1986) in *Proceedings of the 4th Cell Wall Meeting* (Vian, B., Reis, D. and Goldberg, R., eds.), pp. 308–311, University Pierre et Marie Curie Press, Paris
- 5 Brummell, D. A., Camirand, A. and Maclachlan, G. (1990) *J. Cell Sci.* **96**, 705–710
- 6 Zhang, G. F. and Staehelin, L. A. (1992) *Plant Physiol.* **99**, 1070–1080
- 7 Hoth, A., Blaschek, W. and Franz, G. (1986) *Plant Cell Reports* **5**, 9–12
- 8 Desveaux, D., Faik, A. and Maclachlan, G. (1998) *Plant Physiol.* **118**, 885–894
- 9 Abeijon, C., Mandon, E. C. and Hirschberg, C. B. (1997) *Trends Biochem. Sci.* **22**, 203–207
- 10 Toma, L., Pinhal, M. A. S., Dietrich, C. P., Nader, H. B. and Hirschberg, C. B. (1996) *J. Biol. Chem.* **271**, 3897–3901
- 11 Muñoz, P., Norambuena, L. and Orellana, A. (1996) *Plant Physiol.* **112**, 1585–1594
- 12 Faik, A., Chileshe, C., Sterling, J. and Maclachlan, G. (1997) *Plant Physiol.* **114**, 245–254
- 13 Edwards, M., Bowman, Y. J. L., Dea, I. C. M. and Reid, J. S. G. (1988) *J. Biol. Chem.* **263**, 4333–4337
- 14 Dubois, M., Giles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.* **28**, 350–356
- 15 Quentmeier, H., Ingold, E. and Seitz, H. U. (1987) *Planta* **171**, 483–488
- 16 Johansson, F., Olbe, M., Sommarin, M. and Larsson, C. (1995) *Plant J.* **7**, 165–173
- 17 Orellana, A., Neckelmann, G. and Norambuena, L. (1997) *Plant Physiol.* **114**, 99–107
- 18 Delmer, D. and Armor, Y. (1995) *Plant Cell* **7**, 987–1000
- 19 Rollit, J. and Maclachlan, G. (1974) *Phytochemistry* **13**, 367–374
- 20 Gross, K. C. and Pharr, D. M. (1982) *Plant Physiol.* **69**, 117–121
- 21 Gross, W. and Schnarrenberger, C. (1995) *Eur. J. Biochem.* **234**, 258–263

Received 10 November 1999/8 February 2000; accepted 29 February 2000