Improved methods to detect GTP-binding proteins from plants

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Improved methods are described for the detection of GTP-binding proteins (G-proteins) in the protonema of moss *Funaria hygrometrica* and coleoptiles of corn (*Zea mays*) and sorghum (*Sorghum vulgare*). We optimized conditions for the transfer of proteins to nitrocellulose, production of high titer polyclonal anti-Gα (common) antibodies and finally the detection of G-proteins by amplification. In addition to the α-subunit of heterotrimeric G-proteins (M_r 41–43 kDa), a small molecular weight class (< 30 kDa) was also detected by anti-Gα (common) antibodies. An easy, reliable and efficient filter assay is also described to quantify the toxin catalyzed ADP-ribosylation. The apparent K_m of the NAD has been determined to be approximately 1·5 μM for the microsomal fraction of moss. Inclusion of GTP stimulated ADP-ribosylation by 2–27-fold. One to three polypeptides representing the α-subunit of heterotrimeric G-proteins of (M_r 37–43 kDa) were ADP-ribosylated in all three plants. The anti-Gβ (C-terminus) antibody cross-reacted strongly with 39 and 34 kDa polypeptide in moss and corn respectively. By employing improved methods two classes of G-proteins have been shown to be present in three plant species.

1. Introduction

Plants respond to a variety of intracellular and extracellular signals. The molecular mechanism of signal perception and transduction is not yet clear and is an area of intense research activity. Plants and animals seem to share common signalling molecules but the regulatory circuits could be different. GTP-binding proteins (G-proteins) have been implicated in many signal transduction events in animals, yeast and slime moulds (Gillman 1987; Kaziro *et al* 1991). Based on the molecular weight and subunit composition, the G-protein super-family is broadly classified into two major classes, the heterotrimeric and small molecular weight (Kaziro *et al* 1991). Heterotrimeric G-proteins consist of three non-identical α-, β- and γ-subunits with molecular masses being typically 35–55 kDa, 35–36 kDa and 8–10 kDa respectively.

The small M_r G-proteins are monomers of 18-32 kDa in mammalian system. These can be broadly classified into six subfamilies, the Ras, Rho, Rab/Ypt, Ran, Arf and Sar based on their sequence homology (Simon *et al* 1991). This small M_r class of G-protein has been implicated in diverse cell processes such as growth factor signalling, cell secretion, protein import to nucleus and restructuring of cytoskeleton (Simon *et al* 1991). So far only the G-proteins of Rho class have been reported to be ADP-ribosylated by botulinum toxin (Didsbury *et al* 1989; Sekine *et al* 1989).

Several reports indicate the presence of G-proteins in plants but in most cases only small M_r G-proteins of Ypt, Rab and Arf class have been found (Ma 1994). Much remains to be learnt about the diversity and existence of heterotrimeric Gα and other small M_r G-proteins. It has been suggested that, a part of the problem could be due to the inadequate techniques that were originally designed for animal cells (Ma 1994) and have been directly applied to plants without ascertaining their suitability. There is thus a need to improve the existing biochemical techniques to detect G-proteins in plants. We have optimized and modified the immunological methods and toxin catalyzed ADP-ribosylation and used them to detect G-proteins in the protonema of

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moss *Funaria* and etiolated coleoptiles of corn (*Zea mays* L.) and sorghum (*Sorghum vulgare* Pers.). Using improved methods, our studies show the presence of two classes of G-proteins in the plants tested. In the present study we also show that the Gα (common) antibody can detect small M_r G-proteins.

Materials and Methods

2. *Plant materials and growth conditions*

The experiments were carried out using the protonema of moss *Funaria hygrometrica* Hedw. (cell line J-2) and coleoptiles of corn (*Z. mays*) and sorghum (*S. vulgare*). Chloronema cells of moss were cultured in continuous light in liquid suspension cultures (Johri 1974) using the minimal medium supplemented with glucose (MMG), harvested at a cell density of 4–5 mg per ml and kept frozen until used. Hybrid corn (variety MMH 65) and sorghum (variety MSH 51) seeds were soaked for 6 h in autoclaved tap water and germinated on sterile moist vermiculite at 30°C in dark. Approximately 1 cm long coleoptile tips were excised from 2-day old (sorghum) or 4-day old (corn) seedlings and used immediately or frozen in liquid nitrogen.

2.2 *Sub-cellular fractionation for ADP-ribosylation*

Five g of frozen protonema or coleoptiles were ground in liquid nitrogen and the powdered material was resuspended in a buffer containing 100 mM sucrose, 100 mM sodium HEPES pH 8·0, 0·5 mM EDTA, 40 mM 2-mercaptoethanol, 1% polypolyvinyl-pyrillidone, 1 mM PMSF, 75 μM Antipain and 75 μM Leupeptin (2·5 vol/g tissue). The homogenate was filtered through two layers of nylon cloth and the filtrate was centrifuged successively at 5,000 *g*, 14,000 *g*, 35,000 *g* using a Kubota RA 400 rotor and at 110,000 *g* using a Sorvall SW41 rotor. The resulting pellets have been referred as Pa1, Pa2 Pa3 and Pa4 respectively. The pellets were resuspended in a buffer (0·5 ml/g tissue for Pa1 and Pa2 and 0·2 ml/g tissue for Pa3 and Pa4) containing 100 mM potassium phosphate pH 7·5, 200 mM NaCl, 2 mM EDTA, 30 mM DTT, frozen as 100 μl aliquots in liquid nitrogen and stored at −70°C until used.

2.3 *Sub-cellular fractionation for immuno-detection of G-proteins*

Frozen protonema or coleoptiles were ground to a fine powder as described above and suspended in grinding buffer (2·5 vol/g) containing 250 mM sucrose, 20 mM Tris-HCl, pH 8·0, 1 mM EDTA, 1 mM DTT, 1% sodium cholate and protease inhibitors (final 75 μM each of Antipain, Leupeptin, Chymostatin, Pepstatin and TPCK). After keeping in ice for 15 min, the homogenate was filtered through two layers of nylon cloth and the proteins in the filtrate were separated by SDS-PAGE. In order to obtain the microsomal fractions, the cell powder was suspended in grinding buffer devoid of sodium cholate and processed as described above. The filtrate was centrifuged at 6,000 *g* for 30 min and the resulting supernatant was spun at 110,000 *g* for 45 min at 4°C. The microsomal pellet was either suspended in grinding buffer containing 1% sodium cholate or extracted with chilled 10% TCA in acetone. In the later case, the precipitated proteins were washed 5–7 times with ether, dried and stored at 4°C until used.

2.4 *Protein estimation*

The proteins were solubilized using 300 mM KOH for 60 min at 60°C and quantified using the dye binding method (Bradford 1976). All determinations were done in duplicate and the amount of protein was calculated using BSA as standard.

2.5 *SDS-PAGE and Western blotting*

The solubilized proteins (125–150 μg) were separated using 12% denaturing gel (Laemmli 1970). The bis-acrylamide concentration was reduced from 0·26% to 0·0625% to achieve an optimum resolution of various classes of Gα subunits (Mitchell *et al* 1989). The pre-stained Rainbow™ coloured molecular weight markers contained myosine (220 kDa), phosphorylase b (97·4 kDa), bovine serum albumin (66 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21·5 kDa) and lysozyme (14·3 kDa). The proteins were transferred to nitrocellulose membrane using the published procedure (Towbin *et al* 1989) with some modifications as described in the § 3.1. The gels were equilibrated for 30 min in transfer buffer containing SDS at room temperature and the wet transfer was done at 4°C using 100 mAmp current for 2 h and then 200–250 mAmp for 14–15 h.

2.6 *Antisera*

The polyclonal rabbit antiserum, against the 15-mer peptide CGAGESGKSTVKQMK corresponding to the conserved region of Gα, was raised and affinity purified as described by Mumby and Gilman (1991). The peptide with an amino-terminal cysteine was coupled to keyhole limpet hemocyanin (KLH) by m-maleimidobenzoyl-N-hydroxysuccinamide ester (MBS). Peptide synthesis, purification and conjugation to KLH were done by Bio-synthesis, Inc. (Lewisville, TX, USA). The KLH coupled peptide was resuspended in phosphate buffer and sonicated briefly to make a homogenous suspension.

The method and schedule of immunization of the rabbits were as described by Green *et al* (1982) with some modifications. Rabbits were injected subcutaneously with antigen mixed with methylated BSA (Kitajima *et al* 1983). Equal amount of antigen and methylated BSA (500 µg each) were mixed in a final volume of 250 µl and the first injection was with Freunds complete adjuvant followed by four boosters (250 µg of each of peptide and methylated BSA) made with incomplete adjuvant. Rabbits were bled on the fifth day after the last injection and the specific antibodies were affinity purified using the peptide-affinity column (Mumby and Gilman 1991). Affinity purified antibodies were stored in 0·02% sodium azide at 4°C.

2.7 *Immuno-detection of G-proteins*

The blots prepared as described in § 2.5, were kept at 41°C for 15 min. This treatment was found to improve the retention of proteins on the nitrocellulose. The non-specific binding sites were blocked with 5% fat free casein in a buffer containing 10 mM Tris-HCl pH 7·5, 50 mM NaCl and 2 mM $CaCl_2$ and 0·1% Tween 20 (milk-TBS-T), for 2 h at room temperature. The blots were then treated with affinity purified anti-G (common) antibodies at a dilution of 1/300 in milk-TBS-T for 2 h at room temperature and overnight at 4°C. Blots were washed four times, with milk-TBS-T and incubated with biotinylated anti-rabbit IgG at a dilution of 1/500 for 2 h at room temperature. The blots were successively washed with milk-TBS-T, TBS-T and treated with Avidin-Biotin solution according to manufacturer's instructions. The blots were again washed with TBS-T followed by TBS and developed with DAB (0·5 mg/ml) using H_2O_2 as reductant.

2.8 *ADP-ribosylation assay*

A rapid, reliable and sensitive filter assay was developed to quantify ADP-ribosylated G-proteins. The cholera toxin (9 µg) was activated by incubation in 40 mM DTT and 50 mM potassium phosphate buffer pH 7·5 for 30 min at 30°C (final volume 15 µl). All subsequent steps until the addition of [^{32}P]-β NAD were done at 0°C in ice. Various sub-cellular fractions were thawed and kept on ice. ADP-ribosylation was done in a final volume of 62 µl containing 10 mM thymidine, 100 µM GTP, 30 µl of sample (100–150 µg protein) and 15 µl of activated cholera toxin (CT). The control reactions contained only the buffer used for activating the CT. The reactants were gently mixed and left on ice. After 20 min, the reactions were started by adding 3 µCi (6 µl) of [^{32}P]-β NAD and tubes were incubated at 32°C for 30 min. After incubation, duplicate samples of 10 µl each of the reaction mixture were applied to Wattman No 3 filter discs pre-coated with 3 mM NAD. Filters were dropped in 300 ml of chilled 5% TCA containing 20 mM tetra-sodium-pyrophosphate (TCA-PP) solution. After 30 min the TCA-PP solution was heated to boiling for 5 min and then cooled in ice. The TCA-PP was discarded and the filters were washed twice with TCA-PP (250 ml per wash, 30 min each wash). The filters were dried and the radioactivity determined. In order to analyse the ADP-ribosylated polypeptides, the remaining 42 µl of the reaction mixture was denatured with SDS sample buffer, boiled for 5 min, centrifuged and analysed using 12% SDS-PAGE. After electrophoresis, the gels were treated with 150 ml of TCA-PP for 1–2 h with continuous shaking. The gels were then washed extensively with 5% methanol and 7% acetic acid till no radioactivity was released, equilibrated with a mixture of 3% glycerol 20% methanol for 1 h, dried and exposed to X-ray film without any intensifying screen at –80°C for 2 days.

2.9 *Seeds and chemicals*

Hybrid corn and sorghum seeds were from Maharashtra Hybrid Seeds Co., Mumbai. KLH coupled Gα (common) peptide, CGAGESGKSTVKQMK was synthesized by Bio-synthesis Inc., USA. Anti-Gβ (C-terminal) antibody was purchased from Du Pont NEN. [^{32}P]-β NAD and pre-stained protein molecular weight markers were from Amersham Inc. Thymidine, NAD, cholera toxin, trichloroacetic acid (TCA), protease inhibitors, sodium salt of cholic acid, DTT and GTP were from Sigma Chemical Co. Victastain ABC kit was from Vector Laboratories, TX, USA. Other chemicals were of highest purity available.

3. Results

3.1 *Effect of SDS on transfer of sodium cholate solubilized proteins*

The sodium cholate solubilized protein fractions showed several polypeptides in moss, corn and sorghum (figure 1, lanes 1–3). It was observed that the low molecular weight polypeptides (< 40 kDa) from these fractions were only partially transferred to the nitrocellulose using the procedure of Towbin *et al* (1989). This problem of partial transfer was finally solved by adding SDS to the transfer buffer. The inclusion of 0·04% (w/v) SDS resulted in complete transfer of the polypeptides (figure 1, lanes 7–9). This SDS concentration was found to be optimal and a reduction led to a poor transfer of the low M_r polypeptides (figure 1, lanes 4–6).

3.2 *Immuno-detection of G-proteins on blots*

We first established the specificity of the affinity-purified antibodies for recognizing G-proteins across the species.

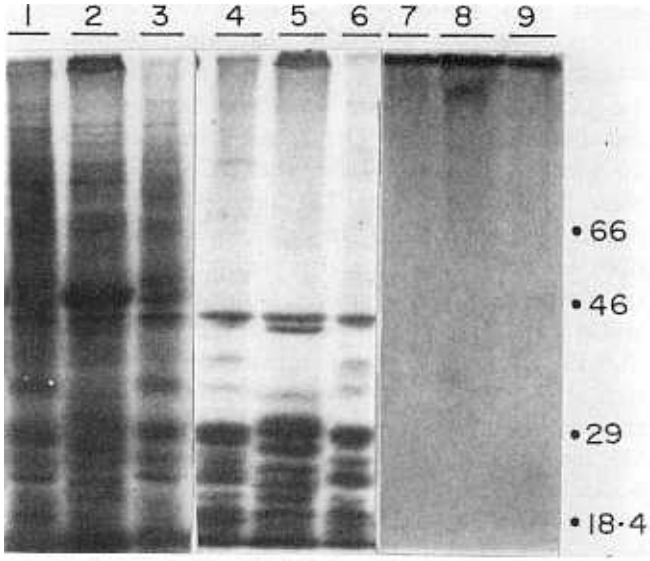

Figure 1. Effect of SDS concentration on the transfer of low molecular weight polypeptides. 150 µg of sodium cholate solubilized proteins from moss (lanes 2, 5, and 8), corn (lanes 3, 6 and 9) and sorghum (lanes 1, 4 and 7) were separated using 10% SDS-PAGE and transferred to nitrocellulose filter. Commassie blue stained gels before transfer (lanes 1–3), after transfer in the presence of 0·03% SDS (lanes 4–6) and 0·04% SDS (lanes 7–9) in the buffer.

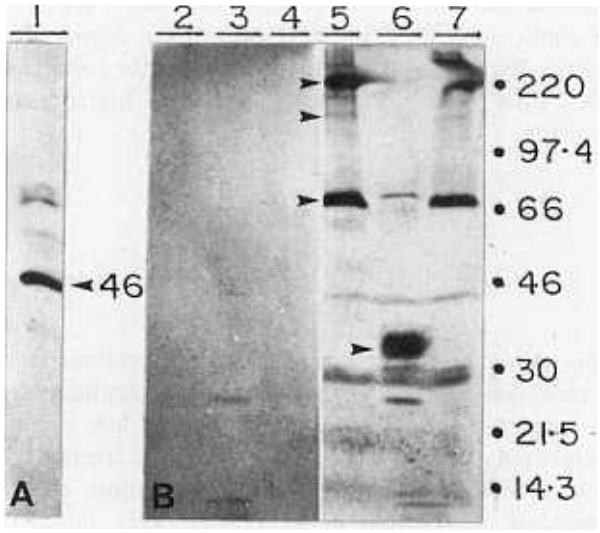

Figure 2. Effect of amplification on the detection of G-proteins. The affinity purified anti-G (common) antiserum was used to detect G-proteins in moss (lanes 3, 6), corn (lanes 4, 7), sorghum (lanes 2, 5) and in the crude extract of *P. falciparum* (lane 1). Western blots were analysed either without amplification (lanes 2–4) or after amplification (lanes 5–7) using Avidin-Biotin conjugate. 150 µg of sodium cholate solubilized proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose filter and probed with antibodies. Polypeptides non-specifically cross-reacting to the biotinylated anti-rabbit IgG are marked by arrows.

A single major polypeptide of 46 kDa was detected in the crude extract of malaria parasite, *Plasmodium falciparum* (figure 2A). This observation is consistent with the reported size of Gα from malaria parasite (Thelu *et al* 1994). The immuno-detection of plant G-proteins was greatly improved upon amplification of signal using Avidin-Biotin system (ABC kit) from vector laboratories (figure 2B, lanes 5–7), however, the commercially available biotinylated anti-rabbit IgG alone cross-reacted with one to three polypeptides non-specifically (arrows in lane 5, figure 2). After amplification a polypeptide of 43 kDa, comparable to α-subunit of heterotrimeric G-proteins was detected in the three plant species. Small M_r G-proteins of M_r 28 or 28 and 30 kDa were also observed in the three species. A 25 kDa polypeptide was found specifically in the protonema of moss *Funaria* even without amplification (figure 2, lane 3). Thus, the antibodies developed by us detected two classes of G-proteins, the Gα-subunits and the low molecular weight ones.

3.3 *Cholera toxin stimulated ADP-ribosylation*

The filter assay as described in § 2.8 was used to quantify the ADP-ribosylated products. Using the assay conditions as described, the incorporation of radioactive NAD continued up to 30 min at 4°C (data not shown) and the apparent K_m of the NAD was determined to be approximately 1·5 µM for the pellet 3 (Pa3) of moss (figure 3). A 46 kDa ADP-ribosylated polypeptides was detected in

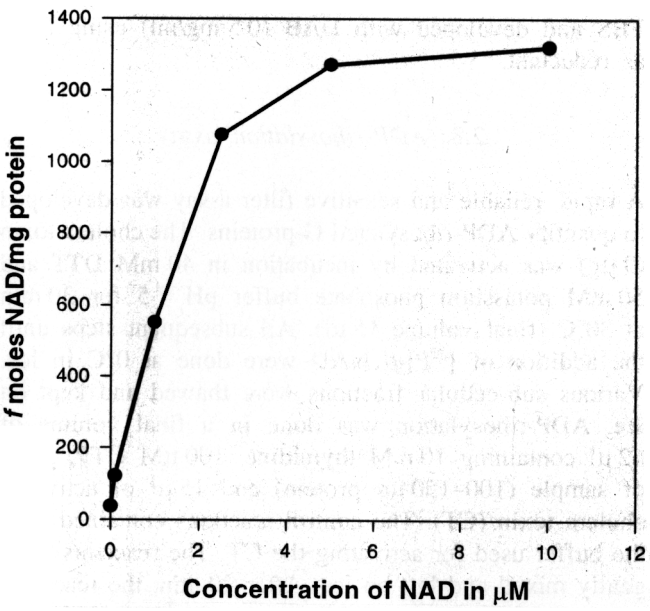

Figure 3. Apparent K_m of NAD using the microsomal fraction of moss. The NAD incorporated was calculated based on triplicate determinations.

all three plants while additional polypeptide of 43 kDa in corn (figure 4, lane 4) and a 37 kDa in both sorghum and corn were also detected (figure 4, lanes 2 and 4). Sorghum seems to contain an additional 16 kDa ADP-ribosylated polypeptide (figure 4, lane 2). We are unable to comment about the nature of this polypeptide.

3.4 *Effect of GTP and protease inhibitors on ADP-ribosylation*

The fractions Pa3, showed maximum incorporation of NAD in the three plants. The ADP-ribosylation was enhanced by several-fold in the presence of GTP in the reaction mixture (table 1). The toxin catalyzed ADP-ribosylation in the presence of GTP was found to be highest in corn as compared to moss and sorghum (table 1). Inclusion of water-soluble protease inhibitors such as Leupeptin and Antipain stimulated ADP-ribosylation 2–3-folds over control (table 2). As the maximum incorporation of NAD was observed in the presence of these two inhibitors, both of them were used routinely in ADP-ribosylation reaction. Increasing the concentration of these inhibitors from 16–150 μM did not affect ADP-ribosylation. Protease inhibitors dissolved in ethanol such as TPCK also improved NAD incorporation but the solvents such as ethanol or DMSO themselves also inhibited ADP-ribosylation partially (table 2). Despite the inhibition by solvents, TPCK at 150 μM concentration restored the NAD incorporation to the level of control. These results show that the ADP-ribosylation site is susceptible to proteolytic action.

3.5 *Polypeptides recognized by anti-G antibodies*

In order to identify the β-subunits, the anti-C-terminal antibody was used. This antibody cross-reacted with several polypeptides in the sodium cholate solubilized crude extracts and TCA-acetone extracted microsomal fractions of moss, corn and sorghum (figure 5). In moss and corn we observed 39 and 34 kDa polypeptides respectively (figure 5, lanes 2, 3, 5 and 6). Very low cross-reactivity to a 36 kDa was also observed in the sodium-cholate extracted fractions of moss (figure 5, lane 2, marked by dot). The molecular weights of 35–39 kDa are comparable to known β-subunits of heterotrimeric G-proteins from other systems. Besides these,

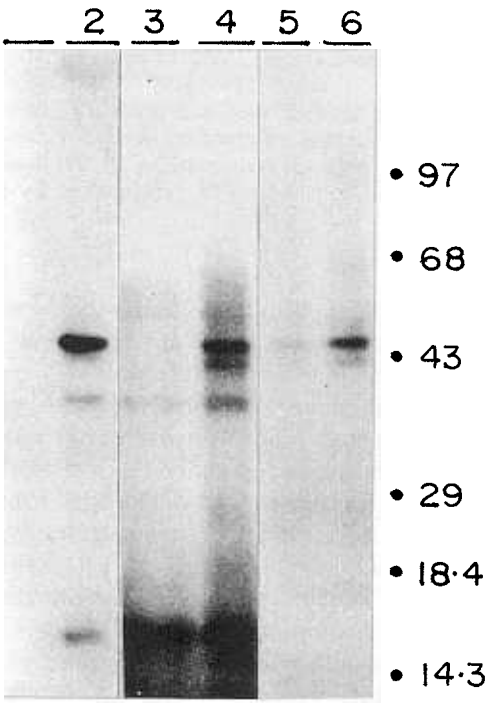

Figure 4. Effect of GTP on the ADP-ribosylation of polypeptides in moss, corn and sorghum. The post-microsomal supernatant (S_4) fractions were ADP-ribosylated in the absence of GTP (lanes 1, 3, 5) or in the presence of GTP (lanes 2, 4, 6). 100 μg of ADP-ribosylated proteins from sorghum (lanes 1, 2), corn (lanes 3, 4) and moss (lanes 5–6) were separated using 12% SDS-PAGE, dried and autoradiographed.

Table 1. Cholera toxin catalyzed ADP-ribosylation of sub-cellular fractions.

Plant	Sub-cellular fraction[a]	CT[b]	CT + GTP[c]	Fold stimulation with GTP[d]
Moss	F	3·8	6·6	1·8
	Pa_1	10·2	51·3	5·0
	Pa_2	13·5	93·9	6·9
	Pa_3	17·4	123·3	7·0
	Pa_4	12·3	31·2	2·5
	S_4	3·0	3·6	1·2
Corn	F	137	507·3	3·7
	Pa_1	11·2	268·2	23·7
	Pa_2	10·8	292·2	27·0
	Pa_3	29·1	713·4	24·5
	Pa_4	13·9	264·3	19·1
	S_4	20·8	27·7	1·3
Sorghum	F	17·1	30·1	1·8
	Pa_1	41·7	578·5	13·8
	Pa_2	12·6	318·0	25·2
	Pa_3	18·5	422·7	22·8
	Pa_4	30·2	216·1	7·2
	S_4	12·7	28·2	2·2

[a]Sub-cellular fractions were obtained by differential centrifugation of cell free extract as described in § 2. Filtrate (F); Pellets at 5,000 *g* (Pa_1); 10,500 *g* (Pa_2); 35,000 *g* (Pa_3); 110,000 *g* (Pa_4) and post microsomal supernatant (S4) and ADP-ribosylation was carried out in the presence of protease inhibitors (75 μM each of Antipain and Leupeptin dissolved in water).
[b]The values are expressed as fmol of NAD incorporated/30 min/mg protein. Stimulation by cholera toxin (CT) only.
[c]ADP-ribosylation in the presence of CT and GTP (CT + GTP).
[d]Fold stimulation of ADP-ribosylation with GTP is the ratio of CT + GTP/CT.

Table 2. Effect of solvents and protease inhibitors on ADP-ribosylation.

Treatments		fmols NAD/mg protein
Control (H_2O)		65·1
Control (Ethanol)*		26·0
Control (DMSO)*		45·9
Leupeptin	A (16)	138·5
(in H_2O)	B (66)	131·9
	C (150)	134·2
Antipain	A (16)	150·4
(in H_2O)	B (66)	154·1
	C (150)	174·6
TPCK	A (16)	37·9
(in ethanol)	B (66)	43·7
	C (150)	61·5

*The ADP-ribosylation was done with the microsomal proteins of moss (pellet Pa3) as described in § 2. Ethanol or DMSO (4% v/v) were included in the ADP-ribosylation reaction mixture. The protease inhibitors were dissolved in solvents as shown above to obtain a stock solution. 2 µl from the stock solution was added to a reaction mixture of 40 µl so as to obtain final concentration of A, 16 µM; B, 66 µM and C, 150 µM. The reactions were carried out for 30 min at 32°C and NAD incorporated was determined as described in § 2.

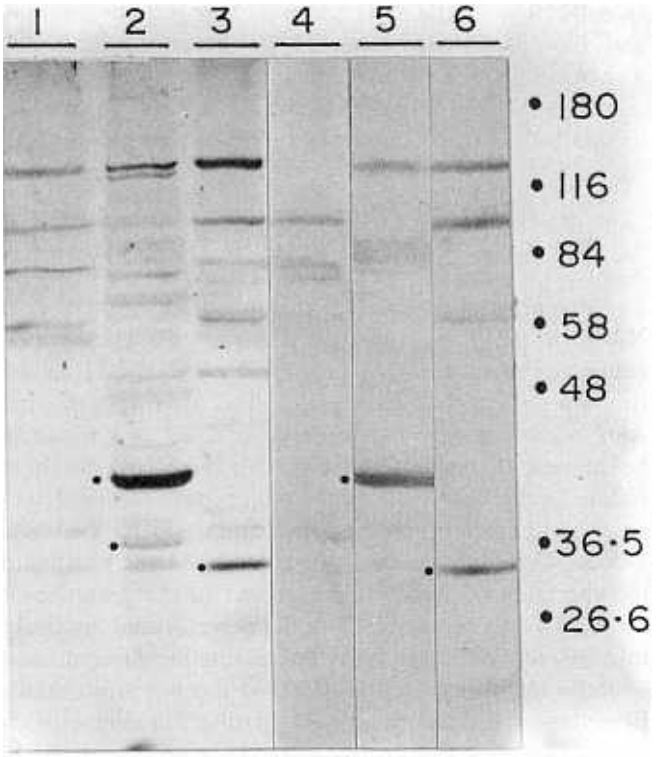

Figure 5. Polypeptides recognized by C-terminus anti-Gβ antibodies. Sodium cholate solubilized crude extract (lanes 1–3) and TCA-acetone extracted samples (lanes 4–6) from sorghum (lanes 1 and 4), moss (lanes 2 and 5) and corn (lanes 3 and 6) were fractionated using 12% SDS-PAGE, transferred to nitrocellulose filter and incubated with anti-Gβ C-terminus antibodies. The cross-reacting polypeptides were detectable without amplification. Polypeptides corresponding to the β-subunits of mammalian G-protein, based on M_r, are marked by dots.

several high molecular weight polypeptides were also detected with this antibody in the three plant species. Far fewer polypeptides were observed specifically in high molecular weight range, if the microsomes had been washed with TCA-acetone.

4. Discussion

There are various methods to detect the G-proteins. Most of the commonly used methods are based on the binding of non-hydrolyzable GTP analogue such as [γ-^{35}S]GTP in solution, binding of radioactive GTP on the filters (Drobak *et al* 1988), GTPase assay, toxin catalyzed ADP-ribosylation and immunological cross-reactivity. The GTP-binding assay using non-hydrolyzable GTP analogue and GTPase assays suffer from limitations, such as, high background caused by the non-signal transducing G-proteins (Perdue and Hurley 1993) or lack of specificity for particular type of G-proteins (Ma 1994). Assays based on toxin catalyzed ADP-ribosylation and immunological cross-reactivity have proved invaluable for the detection of heterotrimeric and small molecular weight G-proteins. Conventionally, the antibodies against the conserved domains e.g., Gα (common) have been used. The commercially available Gα (common) antibodies were found to be unsuitable for detecting plant homologues (present work, result not shown) and therefore it became necessary to raise high titer polyclonal anti-peptide antibodies. As expected, amplifying the signal using ABC kit led to a far improved detection of G-proteins in plants. Similar to many other regulatory proteins, G-proteins seem to be present in low amounts in the plants and thus an amplification was considered worthwhile to improve the sensitivity. The specificity and higher detection limits were achieved respectively by using the affinity purified antibodies and amplification steps. As the biotinylated anti-rabbit IgG itself cross-reacted to a few polypeptides non-specifically, it was essential to include suitable controls in each experiments.

In principle, antibodies directed against the highly conserved GTP-binding domain should detect all classes of G-proteins including the small M_r ones. There are only a few reports where both small molecular weight and G subunits have been reported (Ricart *et al* 1995; Zaina *et al* 1994). We have used a 15-mer peptide coupled to KLH to raise a high titer of polyclonal antibodies (Mumby and Gilman 1991). As reported by Kitajima *et al* (1983), inclusion of methylated BSA with the antigen greatly improved the antibody titer. The proteins from particulate fractions were solubilized by

sodium cholate in the presence of higher concentration (75 μM) of protease inhibitors. The solubilized proteins of $M_r < 40$ kDa were transferred efficiently to nitrocellulose only in the presence of SDS in the transfer buffer. One or two polypeptides of 41–44 kDa and several low M_r polypeptides were not transferred at a SDS concentration lesser than 0·04% in the buffer (figure 1, lanes 4–6). The position of the Gα-subunit coincides exactly with the un-transferred polypeptide region of the gel and therefore could be the reason for the failure to detect the Gα-subunits in many plants.

While identifying the ADP-ribosylated G-proteins, it was observed that the ADP-ribosylated polypeptides were of a higher M_r than the ones identified by antibodies. As it is known that the mobility of ADP-ribosylated Gα-subunits is relatively slower in non-gradient gel systems (Gill and Woolkalis 1991), this discrepancy can be explained. We observed a 37 kDa ADP-ribosylated polypeptide in both corn and sorghum in the present study and wish to suggest that this could be the ADP-ribosylated product of the reported 33 and 34 kDa polypeptide from sorghum and corn respectively (Ricart *et al* 1995; Bilushi *et al* 1991). These G-proteins were undetectable by antibodies presumably because they are present in low amounts. Gα-subunits of 40 and 41 kDa have also been identified from corn (Clarkson *et al* 1991) and sorghum (Ricart *et al* 1995). Consistent with these findings, we detected a 43 kDa polypeptide using the anti-Gα (common) antibodies in the three plant species. Polypeptides of similar M_r of 43–46 kDa were also found to be ADP-ribosylated. Unlike the immunological method, the ADP-ribosylation assay indicated the presence of two or three isoforms of α-subunits. This can be due to greater sensitivity of the ADP-ribosylation.

As expected, addition of GTP stimulated several-fold ADP-ribosylation by particulate fraction in three plants, as the GTP bound form of α-subunit is a substrate of CT. Since the CT can ADP-ribosylate many non-signal transducing membrane bound G-proteins, such as tubulin, poly (ADP-ribose) polymerase, nuclear proteins (Gill and Woolkalis 1991), the quantitative data in the membrane fractions represents essentially the ADP-ribosylation by all these proteins. Although, the supernatant fractions showed reduced NAD incorporation and barely 20–100% stimulation by GTP, most of the ADP-ribosylation seems to be due to G-proteins and a far cleaner autoradiogram was obtained. All pellets also showed these ADP-ribosylated polypeptides and a lot of background (data not presented). The proteins in the particulate fractions were ADP-ribosylated to a far higher extent in corn and sorghum than in moss. In corn, the ADP-ribosylation without GTP was maximum in the cell-free homogenate (F), while in its presence, it was highest in the Pa3 fraction. The presence of adequate endogenous GTP in the fraction F can explain the above result.

The anti-Gβ C-terminal antibodies detected several polypeptides in the crude extracts solubilized by sodium cholate in three species. The most prominent polypeptides were the 39 kDa in moss and 34 kDa in corn. These seem to be the β-subunit of G-proteins (figure 3) and their molecular masses are in good agreement with the reported M_r of 34–36 kDa from yeast, slime moulds and mammalian systems. Based on the isolation of c-DNA clones, the predicted protein encoded by the longest open-reading frame of maize (*ZGB1*) and *Arabidopsis* (*AGB1*) would be about 41·7 and 41 kDa respectively (Weiss *et al* 1994).

The high M_r polypeptides observed in the present work (figure 5) could be the proteins which share epitopes with Gβ subunit. The COP1 protein (constitutive photomorphogenic locus) is an example of such a protein, having an N-terminal zinc finger for DNA binding and a C-terminal Gβ homology domain present in a single polypeptide first identified in *Arabidopsis*. The calculated molecular weight of this protein is 74·5 kDa and it has been proposed to play the role of a transcriptional repressor in photomorphogenesis (Deng *et al* 1992). Two other plant proteins, Cblp in *Chlamydomonas* (Schloss 1990) and *arc1* gene product in tobacco (Ischda *et al* 1993) also have been shown to have the fused Gβ, WD-40 motifs. It is possible that the antibody detects several of these polypeptides of higher molecular weight, however, non-specific crossreactivity of the primary antibody is not ruled out.

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