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Affinity purification of GTPase proteins from oat root plasma membranes using biotinylated GTP

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Abstract

Biotinylated GTP was synthesized and it was demonstrated that this ligand was bi-functional: it competed with [^{3}H]Gpp(NH)p for binding to membrane proteins and it bound to immobilized avidin. Peripheral plasma membrane proteins were solubilized in a low-salt wash, incubated with GTP-biotin and biotinylated proteins were coupled to an avidin column. Elution with excess biotin yielded 10 polypeptides as seen with a silver stained SDS-PAGE gel. Antisera raised against *Ras*, a small GTPase, strongly interacted with three proteins with MW of 38, 27 and 25 kDa and also with 6 other proteins. G_α-common antibodies interacted with proteins of MW = 66 and 38 kDa. This method enables the rapid purification of GTP-binding proteins and opens the possibility to assign a role to specific GTPases in signal transduction pathways.

Key words: G-protein; GTPase; GTP-biotin; Monomeric avidin; Plant root; Plasma membrane; *Avena sativa*

1. Introduction

Plants respond to a variety of external stimuli like light (blue, red, far-red), hormones, touch, fungal elicitors, toxins, etc. Transduction of these signals is probably mediated by GTPase proteins [1]. GTPase proteins are proteins that bind and hydrolyse GTP, thereby switching between a GTP-bound ('on') state and a GDP-bound ('off') state. There are two main sub-families of GTPases: heterotrimeric G-proteins and small GTPases. Heterotrimeric G-proteins (G-proteins from here on) are activated by so-called seven helix receptors and they are made up by 3 subunits: α-subunit (MW around 40 kDa, containing the G-nucleotide binding site), a β-subunit (MW 35–36 kDa) and a γ-subunit (MW 7–10 kDa). The small GTPases are monomeric proteins with a MW between 20 and 30 kDa and their role is quite diverse: guiding vesicle transport, cytoskeleton organization and cell differentiation and proliferation.

The presence of members of these GTPase families in plants has been demonstrated with antibodies raised against mammalian G-proteins [2,3,4] and small GTPases [5,6]. Genes encoding GTPases were cloned by screening cDNA or genomic libraries using oligonucleotides deduced from mammalian genes as probe [7–9].

Despite the large number of reported putative G-proteins and small GTPases in plants, the number of studies assigning a functional role is still small. Warpeha et al. [4] demonstrated by means of photoaffinity labeling that the affinity of a 40 kDa polypeptide for GTP was modulated by blue light irradiation. The protein was recognized by polyclonal antisera directed against the α-subunit of transducin. Fairley-Grenot and Assmann [10] report that factors that influence G-proteins in non-plant systems (like GDP, GTP and cholera toxin) have an effect upon outward rectifying K^+-channels in guard cells of Fava bean. However, the G-protein involved was not identified nor was the external stimulus.

Attempts to demonstrate the involvement of G-proteins in hormonal signal transduction through negative cooperativity between receptor and G-protein have failed thusfar. This could be due to the high background caused by the abundance of GTP-binding proteins in the plasma membrane. Notably nucleoside diphosphate kinase (NDPK) can make up for 50% of [^{35}S]GTPγ binding to plasma membranes [11]. However, White et al. [12] demonstrated a stimulation of GTPγS binding to plasma membranes from *Pisum sativum* and *Zea mays* by mimicking an activated receptor with a mastoparan ana-

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Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; MEGA 9, nonoyl-*N*-methylglucamide; PM, plasma membrane.

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logue. The characteristic feature of the GTPase molecular switch (i.e. the increased affinity for GTP) could be used to identify the protein involved in a specific signal transduction pathway, if one could purify the GTPases based upon their affinity for GTP.

In this paper we describe the synthesis of a bi-functional GTP-derivative, GTP-Biotin and the one step isolation of a number of GTPases from the plasma membrane of oat root with affinity chromatography on a monomeric avidin column.

2. Material and methods

2.1. Materials

Immobilized tetrameric avidin, biotin-LC-hydrazide and BSA-biotin were from Pierce (Rockford, USA). GTP was from Aldrich and EDC was from Sigma (St. Louis, USA). All other chemicals were of the highest quality available.

2.2 Isolation of plasma membrane

Oat seeds (200 g) of *Avena sativa* L. were germinated on stainless steel screens and grown in the dark at 20°C for 6 days over a 1 mM $CaSO_4$ solution. From the roots, plasma membranes were purified as described earlier [13].

2.3 Synthesis of a biotinylated GTP

For the synthesis of biotinylated GTP, 3 μmol GTP and 5 μmol Biotin-LC-hydrazide were dissolved in 2 ml water. Next, 2 μmol 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were added to mediate the coupling between the terminal phosphate of GTP and the $-NHNH_2$ group of Biotin-LC-hydrazide. The mixture was incubated in the dark for 3 h at room temperature. G-nucleotides and GTP-Biotin were separated from EDC and Biotin-LC-hydrazide with HPLC on a Reverse Phase C-18 column (Phenomex, Hypersil-10, 250–10 mm) with as mobile phase 95% H_2O and 5% methanol. Subsequently, the G-nucleotide mixture was separated on a MemSep 1000 anion exchange filter (Millipore) which was equilibrated in 10 mM K^+-phosphate (pH 6.5); the newly synthesized GTP-Biotin eluted at 200 mM KCl.

2.4. Solubilization of peripheral plasma membrane proteins

Loosely bound peripheral proteins were released from the plasma membrane vesicles through one freeze/thaw cycle, five-fold dilution in 10 mM Tris-HCl (pH 7.5), followed by centrifugation at 100,000 × *g* (30 min).

2.5. Competition assay with [^{3}H]Gpp(NH)p

Solubilized proteins (from 0.3 mg total PM protein) were taken up in 2.5 ml Binding Buffer: 20 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 1 mM $CaCl_2$, 2.3 mM DTT, 1 mM PMSF and 10 mM nonoyl-*N*-methylglucamide (MEGA 9). The control treatment received [^{3}H]Gpp(NH)p (Amersham; specific activity 725 GBq/mmol) (2×10^{-9} M). The GTP-Biotin treatment received in addition GTP-Biotin (5×10^{-8} M). Binding was allowed to proceed for 4 h at room temperature. Free ligand and ligand bound to the protein fraction were separated by means of two PD-10 desalting columns (Pharmacia).

2.6. Monovalent avidin column

Tetrameric avidin agarose (50% slurry in 0.02% NaN_3) was packed into a 1 ml FPLC column (Pharmacia LKB, Uppsala Sweden) with 10 mM K^+-phosphate buffer (pH 7.0) + 0.02% NaN_3. After packing, the column was stripped to obtain monovalent avidin [14]. Non-exchangeable biotin binding sites were blocked by treating the column with 3 bedvolumes 2 mM biotin in the K^+-phosphate buffer. Exchangeable sites were regenerated by washing the column with 0.1 M glycine + 0.2 M KCl (pH 1.5). After regeneration the column was equilibrated in Binding Buffer. Column performance was tested with biotinylated BSA.

2.7. Purification and visualization of GTP-binding proteins

Solubilized proteins (from 5 mg PM proteins) were incubated in 2.5 ml Binding Buffer with 10^{-7} M GTP-biotin for 6 h at room temperature. Free ligand was removed using two PD-10 desalting columns. Thereafter the sample was cycled overnight through the avidin column at 4°C. After washing with Binding Buffer, bound proteins were eluted with 2 mM biotin in Binding Buffer. Fractions were freeze-dried and solubilized in 100 μl loading buffer (60 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 20 mM DTT and 0.002% Bromphenol blue), the mixture was heated for 10 min at 80°C and 20 μl samples were separated by SDS-PAGE (10.0% gel) using a Biorad Mini-Protean II System.

The proteins in the gels were transferred electrophoretically to a nitrocellulose membrane as described by [15]. The nitrocellulose membrane was blocked in Phosphate Buffered Saline containing 0.05% (v/v) Tween 20 (PBST) plus 1% (w/v) BSA for 1 h and incubated overnight at room temperature with 1:500 diluted rabbit antiserum against G_α-common peptide or monoclonal antibody against *Ras*, Y-13-259 (DuPont, NEN) and for 1 h with 1:7500 diluted goat anti-rabbit IgG or goat anti-mouse IgG conjugated to alkaline phosphatase (GARAP or GAMAP). The specific bands were visualized after incubation with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium as substrate.

3. Results

3.1. The newly synthesized ligand is bi-functional

In solution GTP is slowly hydrolyzed to GDP and GMP; during elution of the MemSep filter with a linear KCl gradient from 150 to 250 mM (flow-rate 4 ml/min) the G-nucleotides eluted with retention times of 3.65 (GTP), 2.65 (GDP) and 1.25 (GMP) min. Incubation of GTP with Biotin-LC-hydrazide and EDC resulted in a fourth peak (with an absorption spectrum identical to that of GTP) with a retention time of 2.00 min. The molecule present in this fraction contained biotin because the peak disappeared when the fraction was mixed with tetrameric (high-affinity) avidin-agarose beads before loading on the MemSep (Fig. 1).

If GTP-biotin is a bi-functional ligand, GTP-Biotin must retain its ability to bind to GTP-binding proteins. Fig. 2 shows that proteins solubilized from PM vesicles through a low salt wash bind [^{3}H]Gpp(NH)p. Addition of GTP-biotin (5×10^{-8}M) effectively competes with

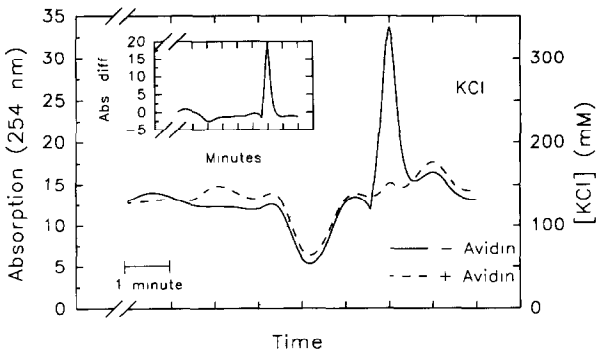

Fig. 1. Retention of purified GTP-Biotin by immobilized agarose, demonstrating the presence of a biotin moiety in the synthesized derivative. A MemSep 1000 anion exchange filter was used to bind the GTP-Biotin and elute it as a single peak with a KCl gradient. The inset shows the absorption difference between the two traces.

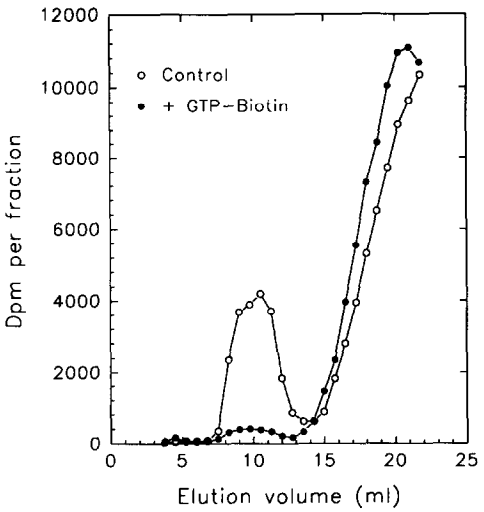

Fig. 2. Competition between [^{3}H]Gpp(NH)p (2×10^{-9}M) and GTP-Biotin (5×10^{-8} M) for binding to solubilized PM proteins, demonstrating the presence of a GTP moiety in the synthesized derivative. Proteins were separated from free ligands on two PD-10 desalting columns; fractions of 0.25 ml were collected.

[^{3}H]Gpp(NH)p showing that the newly synthesized molecule has good binding activity towards GTP-binding proteins.

3.2. *Affinity purification of GTP-binding proteins*

To test the performance of the avidin column GTP-biotin and BSA-biotin were pumped into the column; both compounds were retained by the column and could be eluted specifically with 2 mM biotin (data not shown). The 'reversible' binding capacity of the column was approximately 1 nmol/ml avidin-agarose.

Solubilized PM proteins were incubated with 10^{-7} M GTP-Biotin for 6 h, desalted and loaded overnight on the avidin-agarose column. The column was washed extensively and Fig. 3 shows a silver stained gel of the proteins that were eluted in consequetive (0.5 ml) fractions with 2 mM biotin. The first proteins elute after 1 ml and fraction 4 (after 1.5 ml) contains the peak fraction of eluted proteins. Two bands (66 and 60 kDa) were present in all fractions and might be artifacts from the silver staining technique [16]. Very pronounced is a 38 kDa band and other bands visible have apparent MWs of 54, 46, 35, 33, 30, 25, 22, 20 and 18 kDa.

3.3. *Identification of affinity purified proteins as GTPase proteins*

In order to assess that the proteins, purified through their ability to bind GTP, are related to known GTPase proteins, fraction 4 was run again on a 10% gel, blotted and immuno-decorated with *Ras* monoclonal antibody and G_α-common polyclonal (Fig. 4). *Ras* antibody cross-reacted strongly with bands with an apparent MW of 38, 27 and 25 kDa, besides 6 other bands: 67, 46, 33, 29, 22 and 18 kDa. G_α-common cross-reactivity was rather weak, but minor bands could be seen at 67 and 38 kDa. With asterisks in Fig. 3 we have indicated which silver stained bands were recognized by the Ras and G_α-common antibodies. Notably the 54 and 35 kDa silver stained band were not recognized. The 67 kDa band, present in all eluted fractions was recognized by both antibodies and it remains possible that there is a 67 kDa protein which elutes with biotin and underlies the all present 67 kDa protein.

Incubation of the blots with 10^{-7} M [^{35}S]GTPγS resulted in a few radioactively labelled (diffuse) bands between 30 and 45 kDa. This labeling was absent in the presence of cold GTPγS (10^{-6} and 10^{-4} M); data not shown.

4. Discussion

In this paper we report the synthesis of a novel GTP-derivative, with two functional groups: GTP and biotin. The new molecule has the same absorption characteristics as GTP (absorption maximum at 254 nm) and it competes effectively with [^{3}H]Gpp(NH)p for binding to GTP-binding proteins. When the molecule is mixed with avidin-agarose it is retained by the beads and when it is pumped into a monomeric avidin column it binds to the column and it can be recovered through column elution with excess biotin. From this we concluded that we have a bi-functional ligand that could be useful in for example the purification of GTP-binding proteins.

Binding of GTP-biotin to solubilized peripheral PM proteins, through the GTP-moiety, enabled the isolation of 10 polypeptides through reversible coupling of the biotin-moiety to the avidin column. Two of these proteins were recognized by both *Ras* and G_α-common antibodies, amongst these the most abundant 37–38 kDa protein (Fig. 3). A 54 and a 35 kDa protein were not

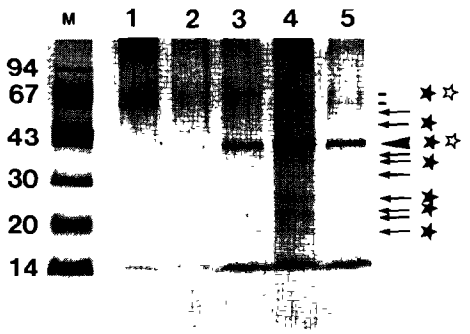

Fig. 3. Silver stained SDS-Page gel (10%) of GTP-Biotin pretreated proteins, eluted from a monomeric avidin column with excess biotin. Lane 1 shows the first fraction collected after starting the elution with biotin; fraction size is 0.5 ml. Fraction 3 (lane 3) is the first fraction containing GTP-binding proteins. M = molecular weight markers. Two proteins (indicated with −) were present in all fractions; and indicate the proteins recognized by Ras and G_α-common antibodies, respectively. The purification of a 38 kDa protein was most pronounced.

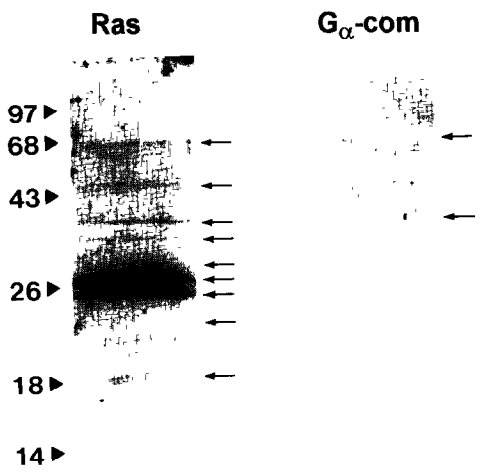

Fig. 4. Immunodection of GTPase proteins present in fraction 4 (lane 4, Fig. 3) with antiserum raised against *Ras* and G_α-common peptide. *Ras* antibody cross-reacts strongly with 38, 27 and 25 kDa peptides, whereas G_α-common antibody interacts weakly with 65 and 38 kDa proteins.

recognized by the antibodies. The 35 kDa protein might be a β-subunit of a heterotrimeric G-protein complex [17]. It must be born in mind that with this method of isolation (which also works for integral membrane proteins solubilized with detergents; unpublished results) β- and γ-subunits, effector and receptor proteins can co-purify with the GTP-binding peptide [18].

Plant GTPase proteins with a wide range of MW have been reported, using either antibody recognition or sequence homology. The proteins isolated with GTP-biotin fall well into the MW range reported, both for heterotrimeric G-proteins as small GTPases [4,6,19,20]. This indicates that oat root plasma membrane is a rich source for GTPases that transduce external signals and for GTPases that play a role in the regulation of diverse cellular processes.

Since binding of the ligand, GTP-Biotin (which can be added at any desired concentration), will depend upon the affinity of the GTPases, a shift in affinity due to an external stimulus will be detected in the bands appearing on SDS-PAGE gel. Using this approach, the possible role of specific GTPases in the signal transduction of fusicoccin and auxin (oat roots) and gibberillic acid (barley aleuron cells) is currently under investigation.

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References

[1] Terryn, N., Van Montagu, M. and Inzé, D. (1993) Plant Mol. Biol. 22, 143–152.
[2] Blum, W., Hirsch, K-D., Schultz, G. and Weiler E.W. (1988) Biochem. Biophys. Res. Commun. 156, 954–959.
[3] Bilushi, S.V., Shebunin, A.G. and Babakov, A.V (1991) FEBS Lett. 291, 219–221.
[4] Warpeha, K.M.F., Hamm, H.E., Rasenick, M.M. and Kaufman, L.S. (1991) Proc. Natl. Acad. Sci. USA 88, 8925–8929.
[5] Rodriguez-Rozales, M.P., Herrin, D.L. and Thompson Jr, G.A. (1992) Plant Physiol. 98, 446–451.
[6] Wang, M., Sedee, N.J.A., Heidekamp, F. and Snaar-Jagalska, B.E. (1993) FEBS Lett. 329: 245–248.
[7] Ma, H., Yanofsky, M.F. and Meyerowitz, E.M. (1990) Proc. Natl. Acad. Sci. USA 87, 3821–3825.
[8] Palme, K., Diefenthal, T., Vingron, M., Sander, C. and Schell, J. (1992) Proc. Natl. Acad. Sci. USA 89, 787 791.
[9] Terryn, N., Anuntalabhochai, S., Van Montagu, M. and Inzé, D. (1992) FEBS Lett 299, 287–290.
[10] Fairley-Grenot, K. and Assmann, S M. (1991) Plant Cell 3, 1037–1044.
[11] Perdue, D.O. and Hurley, J.B. (1993) Plant Physiol. Suppl. 102, 13.
[12] White, I.R., Wise, A. and Millner, P.A. (1993) Planta 191, 285–288.
[13] De Boer, A H., Watson, B.A. and Cleland, R.E. Plant Physiol. 89, 250–259.
[14] Kohanski, R.A. and Lane, M.D. (1990) Meth. Enzymol. 184, 194–200.
[15] Klein, P., Vaughan, B., Berleis, J. and Devreotes, P.N. (1987) J. Biol. Chem. 262, 358–364.
[16] Merrill, C.R., Goldman, D. and Van Kauren, M.L. (1984) Meth. Enzymol. 104, 441–447.
[17] Muschietti, J.P., Martinetto, H.E., Coso, O.A., Farber, M.D., Torres, H.N. and Flawia, M.M. (1993) Biochem. J. 21, 383–388.
[18] Lipson, K.E., Kolhatkar, A.A., Maki, R.G. and Donner, D.B. (1988) Biochemistry 27, 1111.
[19] Jacobs, M., Thelen, M.P., Farndale, R.W., Astle, M C. and Rubery, P.H. (1988) Biochem. Biophys. Res. Commun. 155, 1478–1484.
[20] Anuntalabhochai, S., Terryn, N., Van Montagu, M. and Inzé, D. (1991) Plant J. 1, 167–174.