A small G-protein involved in phosphatidylinositol-4-phosphate kinase activation

T. Urumow and O.H. Wieland

Institut für Diabetesforschung, Krankenhaus München-Schwabing, Kölner Platz 1, 8000 München 40, FRG

Received 8 February 1990

A guanine nucleotide binding protein tentatively designated G_s(PIPK) which activates purified phosphatidylinositol-4-phosphate kinase in vitro, has been partially purified from rat liver membranes and identified as a small G-protein with a molecular mass between 20 and 25 kDa.

G-protein, small; Phosphatidylinositol metabolism; Liver membrane; Lipid kinase regulation

1. INTRODUCTION

PIP kinase is a key enzyme in the PI cycle providing PIP_2 the substrate for the production of the two intracellular messengers inositol 1,4,5-trisphosphate and diacylglycerol by phospholipase C (for review see [1]). We have recently shown that stable GTP analogues stimulate ^{32}P incorporation from [γ-^{32}P]ATP into PIP_2 using plasma membranes from human placenta [2] and rat liver [3,4]. Similar results were obtained by others studying PIP kinase regulation of rat brain membranes [5]. This suggested the involvement of a stimulatory G-protein in the regulation of PIP kinase. This view was strengthened when we purified the enzyme from rat liver membranes and could separate GTPγS-insensitive PIP kinase from a protein fraction that, when added to the former, restored the susceptibility to stimulation by GTPγS [6]. In the present work we have partially purified the PIP kinase stimulating activity and have tentatively identified it as a small G-protein denoted G_s(PIPK).

2. MATERIALS AND METHODS

Materials were as indicated previously [4]. Ha-ras p21 protein [10] was kindly supplied by Dr I. Schlichting, Heidelberg.

2.1. *PIP kinase stimulation assay*

PIP kinase was purified from rat liver membranes and assayed as described in [6]. G-protein was tested by means of its capability to activate PIP kinase in the presence of GTPγS. The standard assay contained, in a final volume of 100 μl 0.2 mU of quercetin-Sepharose purified PIP-kinase [6], 10 μM GTPγS, 25 mM Tris-HCl, 25 mM $MgCl_2$, 0.5 mM EGTA-Na_2, 5 mM dithiothreitol, 1 mM [γ-^{32}P]ATP (10000 cpm/nmol) (NEN, Dreieich), 0.02 mM PIP added as liposomes [6] and a G-protein-containing fraction. The concentrations were chosen to give a linear response of the PIP kinase to stimulation by the G-protein fraction (see fig.1). The G-protein fraction was replaced by buffer in the controls. Samples were incubated for 30 min at 25°C and ^{32}P-labelled PIP_2 was measured as described [6].

Correspondence address: O.H. Wieland, Institut für Diabetesforschung, Krankenhaus München-Schwabing, Kölner Platz 1, 8000 München 40, FRG

Abbreviations: PIP kinase, phosphatidylinositol-4-phosphate kinase; PIP_2, phosphatidylinositol-4,5-bisphosphate; GTPγS, guanosine 5'-*O*-(3-thiotriphosphate); ATPγS, adenosine 5'-*O*-(3-thiotriphosphate); G_s(PIPK), PIP kinase stimulatory G-protein

2.2. *GTP-binding assay*

G-protein was determined by binding of [^{35}S]GTPγS essentially as described in [7]. Samples containing 0.1–3 μg of protein were incubated in 100 μl of a mixture composed of 20 mM Tris-HCl, pH 8, 1 mM EDTA-Na_2, 2.5 mM mercaptoethanol, 10 mM $MgCl_2$, 0.05% Triton X-100, and 10 pmol [^{35}S]GTPγS corresponding to 2.5 × 10^5 cpm. After 30 min incubation at 25°C the incubations were stopped as in [7], and were rapidly filtered through BA 85 nitrocellulose membranes (Schleicher and Schuell, Dassel, FRG). The filters were washed, dried, and counted as described [7]. Unspecific binding to bovine serum albumin amounted to less than 0.2% of the added radioactivity.

2.3. *Western blot analysis*

Proteins were separated on 12% SDS-polyacrylamide gels according to [8] and were transferred to nitrocellulose membranes (20 min at 4 mA/cm^2) using a semidry fast blot chamber (Biometra, Göttingen, FRG) with 25 mM Tris-HCl, 150 mM glycine, 10% methanol buffer, pH 8.3. GTP-binding of electroblotted proteins was measured as described in [9] by bathing the membranes in buffer containing 30 μCi (1 nmol/l) of [α-^{32}P]GTP (NEN, Dreieich) and 10 μmol/l unlabelled ATPγS (Sigma, München). Kodak X-Omat films were used for autoradiography.

3. RESULTS

In previous studies on the purification of PIP kinase from rat liver membranes we had observed that the enzyme was precipitated at 20–40% $(NH_4)_2SO_4$ saturation but that, in contrast to the original extract, the precipitated enzyme was no longer stimulatable by GTPγS. Interestingly, GTPγS sensitivity could be

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

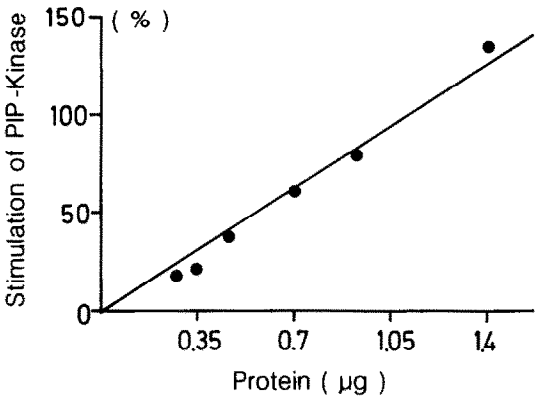

Fig.1. Dependency of the PIP kinase stimulation test on G_s(PIPK) concentration. Stimulation of quercetin Sepharose-purified PIP kinase, 60 ng/assay (step 4 in [6]) by fraction 14 from hydroxyapatite chromatography (fig.3) was assayed at the protein concentrations indicated on the abscissa. For assay conditions see section 2.1.

restored by a protein fraction collected at higher (40–70%) ammonium sulfate saturation (AS 40–70) which itself had no PIP kinase activity [6]. This fraction was used for the purification of a stimulatory putative G-protein.

For that purpose AS 40–70 prepared as described in [6] was dissolved in 20 mM Tris-HCl, pH 7.5 containing 2 mM EDTA-Na_2, 2 mM EGTA-Na_2, 10 mM mercaptoethanol, 0.05% Triton X-100, 1 mM benzamidine, 50 µg/ml PMSF, 10 µg/ml bacitracin, 0.25 µg/ml soybean trypsin inhibitor (buffer A). The preparation was applied to a Sephacryl S-200 column and eluted with buffer A. As shown in fig.2 PIP kinase

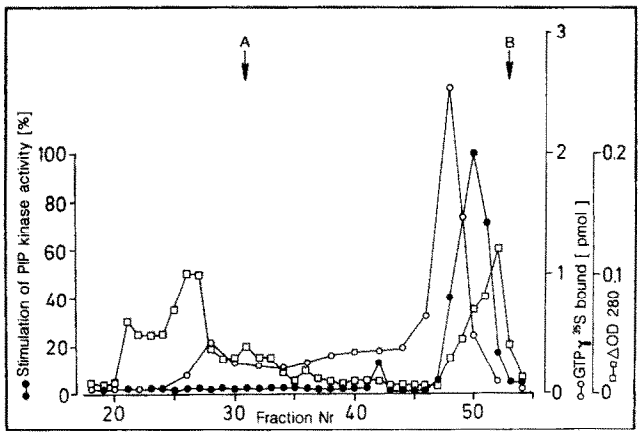

Fig.2. Gel partition chromatography of AS 40–70. 3.5 ml, corresponding to 10.8 mg protein, of AS 40–70 in buffer A, prepared as described in [6] were applied to a 2 × 80 cm Sephacryl S-200 column and equilibrated and eluted with buffer A at a rate of 10 ml/h. 4 ml fractions were collected and assayed for PIP kinase stimulating activity and [^{35}S]GTPγS binding as described in section 2. Marker albumin (M_r 60000) appeared in fractions 29–33 peaking at arrow A, cytochrome *c* (M_r 12500) in fractions 50–55 peaking at arrow B.

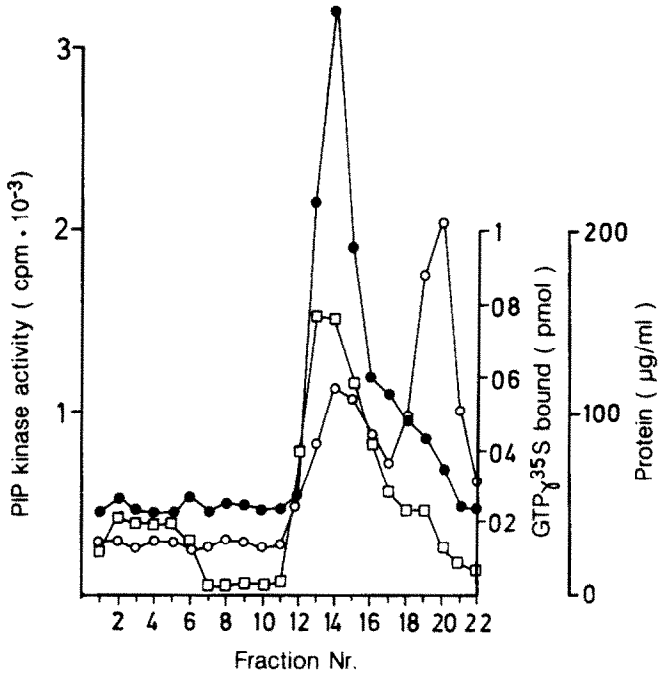

Fig.3. Purification of G_s(PIPK) by hydroxyapatite chromatography. 20 ml of the dialyzed AS 40–70 fraction corresponding to 6 mg protein were applied to a 10 × 1 cm hydroxyapatite column equilibrated with buffer A. The column was washed with 20 ml of the same buffer and eluted with 50 ml of a linear gradient of 0–250 mM phosphate in buffer A. 4.5 ml fractions were collected and assayed, for (□—□) Protein, (○—○) [^{35}S]GTPγS binding activity, (●—●) PIP kinase stimulating activity.

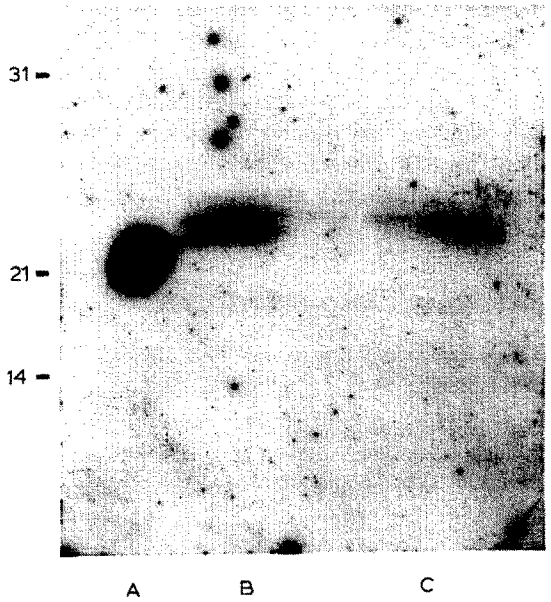

Fig.4. Western blot of G_s(PIPK). Autoradiograms of [α-^{32}P]GTP binding proteins are shown. Lane A: ras p21 0.1 µg; lane B: AS 40–70 fraction, 80 µg; lane C: G_s(PIPK) fraction 14, fig.3, 32 µg. Experimental details of SDS-PAGE and electroblotting are described in section 2.3.

Table 1

GTPγS dependency of PIP kinase stimulating G-protein

Compound (μmol/l)	PIP kinase activity (pmol/mg per min)	Change (%)
–	20.5 ± 0.6	–
GTPγS, 0.05	35.5 ± 5.0	+73
GTPγS, 0.2	43.4 ± 5.2	+112
GTPγS, 1	52.3 ± 4.4	+155
GTPγS, 5	58.5 ± 6.1	+185
GTPγS, 10	59.4 ± 5.8	+190
GTPγS, 10 + GDPβS, 1000	20.0 ± 3.1	–
GTP, 0.2	21.4 ± 2.6	–
GTP, 10	32.7 ± 4.6	+60
ATPγS, 10	16.7 ± 1.9	−18

Assays were performed as described in section 2.1 using DEAE-purified PIP kinase, 0.7 μg protein (step 3 in [6]) and 1.4 μg protein of purified G_s(PIPK) (fraction 14, fig.3). Averages of duplicate determinations ± absolute deviation (%) are given. PIP kinase activity in the absence of G_s(PIPK) was not changed by the addition of the compounds listed

stimulating activity and GTPγS binding activity appeared as two overlapping peaks eluting far behind albumin and just before cytochrome c. This indicated that our PIP kinase stimulatory G-protein is a small M_r G-protein.

The elution profile of AS 40–70 on hydroxyapatite chromatography is shown in fig.3. Here PIP kinase stimulating activity appeared as a distinct peak with the GTPγS binding activity. A second larger peak (fraction 20) eluted at higher phosphate concentrations, also exhibited GTPγS binding but did not stimulate the PIP kinase.

The peak fraction (fraction 14, fig.3) was characterized further by SDS-polyacrylamide gel electrophoresis and Western blot analysis. Fig.4, lane B represents an autoradiogram of the AS 40–70 starting material showing two prominent [α-^{32}P]GTP binding bands located in the 20–25 kDa region as judged from Ha ras p21 [10] as a marker (lane A). After hydroxyapatite chromatography (lane C), one of the prominent bands of lane B has almost disappeared suggesting that the other one may represent G_s(PIPK). Clearly, more extensive purification is needed to clarify the identity of G_s(PIPK). No [^{32}P]GTP binding was seen in the presence of 1 mM unlabelled GTP (data not shown).

G_s(PIPK) (fraction 14, fig.3) responded to GTPγS in a dose-dependent manner (table 1). GTP was also effective though to a lesser extent. No stimulation was seen with ATPγS. Specificity is further indicated by the fact that GDPβS abolished the effect of GTPγS. Similar results were obtained for the less pure AS 40–70 fraction as reported in [6].

4. DISCUSSION

Several small GTP-binding proteins of molecular mass around 20–30 kDa have been described including the ras proto-oncogenes. Although the exact functions of these proteins are not yet known, it is clear that they must have important roles in the control of cell differentiation and oncogenesis, and in the action of growth factors (for reviews see [11,12]). In the present work we have partially purified a small GTP-binding protein with an apparent molecular mass of about 23–25 kDa that stimulates PIP kinase in the presence of GTPγS. As far as we know this is the first report of a small G-protein that activates a soluble enzyme. Whether G_s(PIPK) is identical with one of the small G-proteins found in mammalian tissues remains to be clarified. Ras p21 was ineffectual in our system. The regulatory implications of the interaction of G_s(PIPK) with PIP kinase in vivo are now under study. In our previous studies [2] with intact membranes calcium mobilizing agonists did not stimulate PIP phosphorylation. This would suggest that G_s(PIPK) is not involved in calcium mobilization.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft, Bad Godesberg, FRG. We thank Miss W. Schmidmayr, C. Hackenberg and P. Drexler for skillful technical assistance, Mr P. Delly for preparing the graphs, and Mrs D. Lehmeier for typing the manuscript.

REFERENCES

[1] Cockroft, S. (1987) Trends Biochem. Sci. 12, 75–78.
[2] Urumow, T. and Wieland, O.H. (1986) FEBS Lett. 207, 253–257.
[3] Urumow, T. and Wieland, O.H. (1988) Biochim. Biophys. Acta 972, 232–238.
[4] Wieland, O.H., Urumow, T. and Hackenberg, C. (1990) Ann. NY Acad. Sci., in press.
[5] Smith, D. and Chang, K.-J. (1989) J. Biol. Chem. 264, 3206–3210.
[6] Urumow, T. and Wieland, O.H. (1990) Biochim. Biophys. Acta, in press.
[7] Northup, J.K., Smigel, M.D. and Gilman, A.S. (1982) J. Biol. Chem. 257, 11416–11423.
[8] Laemmli, U.K. (1970) Nature 227, 680–686.
[9] Bhullar, R.P. and Haslam, R.J. (1987) Biochem. J. 245, 617–620.
[10] John, J., Schlichting, I., Schlitz, E., Rösch, P. and Wittinghofer, A. (1989) J. Biol. Chem. 264, 13086–13092.
[11] Katsuhiko, Y., Tanimoto, T., Kim, S., Kikuchi, A. and Takai, Y. (1989) Clin. Chim. Acta 185, 347–356.
[12] Santos, E. and Nebrada, A.R. (1989) FASEB J. 3, 2151–2163.