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Inactivation of Raf-1 by a Protein-tyrosine Phosphatase Stimulated by GTP and Reconstituted by G\(_{ai/o}\) Subunits*

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A membrane-associated form of Raf-1 in v-Ras transformed NIH 3T3 cells can be inactivated by protein phosphatases regulated by GTP. Herein, a distinct protein-tyrosine phosphatase (PTPase) in membrane preparations from v-Ras transformed NIH 3T3 cells was found to be activated by guanyl-5’-yliimidodiphosphate (GMPNP) and was identified as an effector for pertussis toxin (PTx)-sensitive G-protein \(G_{ai/o}\) subunits. PTPase activation was blocked by prior treatment of cells with Ptx. PTPase activation by GTP, but not GMPNP, was transient. A GMPNP-stimulated PTPase (PTPase-G) co-purified with \(G_{ai/o}\) subunits during Superose 6 and Mono Q chromatography. PTPase-G activity in Superose 6 fractions from GDP-treated membranes was reconstituted by activated \(G_{ai/o}\) but not \(G_{bg}\) subunits. PTPase-G may contribute to GMPNP-stimulated inactivation of Raf-1 in v-Ras cell membranes because Raf-1 inactivation was PTx-sensitive and PTPase-G inactivated exogenous Raf-1.

Raf-1 is a protein serine/threonine kinase that functions downstream of Ras in the MAP kinase cascade, phosphorylating and activating mitogen-activated protein kinase kinase (MKK or MEK) (reviewed in Ref. 1). The mechanism of Raf-1 activation and inactivation is incompletely understood. Raf-1 activation occurs at the plasma membrane following binding to Ras-GTP (2, 3). Activation of Raf-1 in vitro by a mixture of membranes from v-Ras transformed and v-Src transformed fibroblasts requires ATP/Mg, compatible with a requirement for phosphorylation of Raf-1 for enzymatic activation (4). Raf-1 becomes phosphorylated on tyrosine and serine residues and activated upon co-expression with Ras and Src in Sf9 cells (reviewed in Ref. 4) or NIH 3T3 cells (5). Both tyrosine and serine phosphorylation of Raf-1 appear to be required for Raf-1 activation because Raf-1 isolated from Sf9 cells also expressing Ras alone* or Ras and Src\(_{527F}\) (6) can be inactivated in vitro by treatment with either protein-serine/threonine or -tyrosine phosphatases.

Membranes from mammalian cells transformed with oncogenic Ras contain a portion of cellular Raf-1 in a constitutively active form (7). This membrane-associated form of Raf-1 can be inactivated by protein phosphatases regulated directly or indirectly by GTP (6). We performed experiments to characterize the protein phosphatase responsible for GTP-stimulated inactivation of Raf-1 and to define the mechanism of its regulation.

EXPERIMENTAL PROCEDURES

Materials—Guanine nucleotides were purchased from Boehringer Mannheim. NIH 3T3 cells transformed with Ha-Ras (G12V) were obtained from Dr. L. Feig (Tufts University, Boston, MA). Antibodies to G-protein subunits were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PTx was a generous gift from Dr. E. Hewlett (University of Virginia). The sources of other reagents have been described (4, 6).

Membrane Preparations—Membranes were isolated (4 °C) from cells using the procedures described (4) and resuspended to 1 mg/ml total protein in buffer A (25 mM HEPES, pH 7.6, 10 mM EDTA, 10 mM EGTA, 0.2% (v/v) 2-ME, 0.2% (v/v) 2-mercaptoethanol; MEK, MAP kinase/ERK kinase; BSA, bovine serum albumin; K52R, kinase-defective p42 MAPK; RCM, reduced, carboxymethylated, and maleylated; Ptx, pertussis toxin; PTPase-G, GMPNP-stimulated PTPase.

Activation of PTPase in Membranes—Membranes (100 µg in 100 µl of resuspension buffer) were incubated (100,000 x g, 10 min, 4 °C) in an Airfuge (Beckman) and resuspended with 100 µl of buffer C (25 mM HEPES, pH 7.4, 0.1 mM EGTA, 1 mM MgCl\(_2\), 0.2% (v/v) 2-ME, 1 mM BSA). PTPase activity was assayed by incubating (30 °C) for 5 min (in a final volume of 0.1 ml total assay volume).

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1 The abbreviations used are: MAP kinase, mitogen-activated protein kinase (also known as ERK (extracellular signal-regulated kinase)); PTPase, protein-tyrosine phosphatase; GMPNP, guanylyl-5’-yl imidodiphosphate; GT5-S, guanosine 5’-O-(3-thiotriphosphate); 2-ME, 2-mercaptoethanol; MEK, MAP kinase/ERK kinase; BSA, bovine serum albumin; K52R, kinase-defective p42 MAPK; RCM, reduced, carboxymethylated, and maleylated; Ptx, pertussis toxin; PTPase-G, GMPNP-stimulated PTPase.

2 Jelinek, T., Dent, P., Sturgill, T. W., and Weber, M. J., J. Biol. Chem., 271, 151, 15115 (1996).

3 Conditions for loading membranes with guanine nucleotides used herein were modeled after conditions for loading Ras with GTP and have not been optimized for heterotrimeric G-proteins.
PTx blocks guanine nucleotide-stimulated inactivation of Raf-1 in membranes from v-Ras cells

| PPase inhibitors | Without PTx treatment | With PTx treatment |
|------------------|-----------------------|-------------------|
| C                | 100                   | 93 ± 12.7         |
| GTP              | 115 ± 7.8a            | 87 ± 7.0          |
| GMPPNP           | 16.5 ± 10.1b          | 87 ± 2.8          |
| GDP              | 97 ± 11.3             | 87.5 ± 9.2        |

a NIH 3T3 cells transformed with Ha-Ras (G12V) were treated with PTx (100 ng/ml, 20 h) or left untreated (control) prior to isolation of membranes. Portions of membranes were incubated in the presence or absence of protein phosphatase (PPase) inhibitors, and Raf activity was determined by MEK-stimulated phosphorylation of p42 MAP kinase (see “Experimental Procedures”). Data are expressed as the percentage of incorporation for samples incubated without guanine nucleotides and PPase inhibitors (controls 100%): untreated membranes 17,016 ± 318 cpm; PTx-treated membranes 16,729 ± 243 cpm; n = 4; (mean ± S.D.), b p < 0.01.

Reactions were terminated by sequential additions of 200 μl of cold 20% (v/v) trichloroacetic acid and 10 μl of 10 mg/ml BSA with mixing. Samples were centrifuged, and 210-μl supernatants were counted in scintillation. Data were expressed as percentage release of total 32P in the assay.

Characterization of GMPPNP-stimulated PTPase after Solubilization and Chromatography—Membranes (200 μg of total protein) were incubated for 30 min on ice with 2 mM (final concentration) GMPPNP or GDP, adjusted to 2 mM MgCl2 in excess of chelators with a concentrated stock, and then solubilized by addition of 25 μl of 10% (v/v) Triton X-100 with gentle trituration. Supernatant was recovered after centrifugation (as above), and 200 μl (representing ~80–90% of the sample) was chromatographed (4 °C, 0.25 ml/min, 0.25-m fractions) on a Superose 6 HR 10/30 column (Pharmacia Biotech Inc.) equilibrated in buffer D (25 mM HEPES, pH 7.4, 2 mM MgCl2, 0.1 mM EDTA, 10% (v/v) glycerol, 0.01% (v/v) Triton X-100, 0.2% (v/v) 2-ME, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride). Raf-1 deactivation was assayed (6) by incubation (30 °C, 15 min) of 0.1 μg of active FLAG-Raf-1 (co-expressed with Ras and SrcCΔ727) with 2 μl of fractions in buffer C containing 2.4 μM microcystin-LR; Raf-1 activity was measured by MEK1 phosphorylation. Proteins were precipitated with trichloroacetic acid (10% w/v, final) for SDS-polyacrylamide gel electrophoresis (11% gel) and immunoblotting with ECL (Amersham). Where indicated, pooled fractions were rechromatographed (4 °C, 0.5 ml/min, 1 ml fractions) on a Mono Q HR 5/5 column (Pharmacia) equilibrated in buffer D. The column was developed with a linear gradient from 0–500 mM NaCl in buffer D.

Reversal of GMPPNP Activation by GDP—Portions (4 μl) of Superase 6 fractions from GMPPNP-treated membranes were treated sequentially on ice by addition of 4 μl of buffer A (15 min) to chelate magnesium and promote dissociation (9) of GMPPNP followed by addition of 4 μl of either 6 mM GMPPNP or GDP containing 30 mM MgCl2 (30 min) and then assayed for PTPase activity.

Reconstitution with G12V/Subunits—A mixture of Gα proteins (principally Gαiα, Gαiλ, and Gαi 2) were purified from bovine brain and separated into Gαiα, Gαiλ, and Gαi 2 oligomers by phenyl-Sepharose chromatography essentially as described (10). The resolved Gαiα, Gαiλ, and Gαi 2 subunits were pooled separately and concentrated (50 μg/ml) and were >85% pure. The Gαiα subunit preparation bound GTP-γS and was free of Gαiλ as assessed by immunoblotting; the Gαiλ preparation was free of Gαi 2 by the absence of GTP-γS binding.4 Baculovirus-expressed Gαi 2 subunits were expressed and purified to near homogeneity essentially as described (11). Portions of each preparation were incubated on ice (15 min) with an equal volume of 12 mM guanine nucleotide, 60 mM MgCl2 before use.

RESULTS AND DISCUSSION

Incubation of membranes from v-Ras cells with an antibody that binds the Ras effector domain did not prevent GMPPNP-stimulated inactivation of membrane-associated Raf-1 (6), suggesting involvement of G-proteins other than Ras in Raf-1 inactivation (data not shown). To test for involvement of PTx- sensitive heterotrimeric G-proteins, we compared the abilities of guanine nucleotides to stimulate inactivation of endogenous Raf-1 in membranes from untreated v-Ras transformed cells and membranes from cells that were treated overnight with PTx (Table I). GTP or GMPPNP, but not GDP, stimulated inactivation of Raf-1 in membranes prepared from untreated cells, and inactivation was blocked by inclusion of microcystin-LR and vanadate to inhibit protein-serine/threonine and -tyrosine phosphatases. Raf-1 in membranes prepared from PTx-treated cells was not susceptible to guanine nucleotide-stimulated inactivation (Table I). PTx specifically catalyzes ADP-ribosylation of α subunits of heterotrimeric G-proteins of the G1 family (except G11) (reviewed in Ref. 12). Thus, these results strongly suggested that GTP-stimulated inactivation of Raf-1 in the mixture of membranes from v-Ras cells was mediated by activation, directly or indirectly, of protein phosphatases by heterotrimeric G-proteins of the G1 family.

Schally and co-workers (13) previously demonstrated that addition of somatostatin to membranes of pancreatic cancer cells promoted dephosphorylation of epidermal growth factor

4 M. A. Lindorfer and J. C. Garrison, unpublished data.
receptors autophosphorylated on tyrosine, implying activation of a PTPase by G-protein-coupled receptors. Stork and co-workers (14) extended this observation by demonstrating that GMPPNP addition to membranes could stimulate a PTPase activity, assayed using p-nitrophenyl phosphate as substrate, and that activation of PTPase activity by somatostatin was PTx-sensitive. Since Raf-1 deactivation was PTx-sensitive (Table I) and a purified PTPase can inactivate Raf-1 in vitro (6), we hypothesized that a G-protein-activated PTPase might be responsible for guanine nucleotide-stimulated inactivation of Raf-1.

To test this hypothesis, we determined whether loading of membranes isolated from v-Ras cells with GTP or GMPPNP stimulated PTPase activity, using as substrate [[32P]Tyr]RCM-lysozyme (Fig. 1). GTP significantly increased the rate of 32P release in comparison to GDP (Fig. 1A). When membranes were reasayed after 30 min, the increase in PTPase activity due to GTP was absent (Fig. 1B, O–—–O). Transient activation of PTPase activity by GTP is consistent with the transient activation of a G-protein a subunit due to timed GTP hydrolysis (12). Reloading these same membranes with GTP partially recovered enhanced activity (Fig. 1B, O–—–O), proving that the decrease in activity was not due simply to protein lability. GMPPNP, which is not hydrolyzed by G a subunits, caused a persistent elevation of PTPase activity (Fig. 1B). Activation also occurred when 100 μM concentrations of guanine nucleotide were used (data not shown). Neither GTP nor GMPPNP caused an increase in PTPase activity in membranes from PTx-treated cells (data not shown). Together, these data demonstrate activation of a [[32P]Tyr]RCM-lysozyme phosphatase by a PTx-sensitive G-protein.

In parallel experiments, GMPPNP or GTP stimulated PTPase activity in membranes isolated from parental NIH3T3 cells with results similar to those in Fig. 1 (data not shown). Membranes from parental cells, however, contained 5-fold less GMPPNP-stimulated PTPase activity than membranes from v-Ras cells (data not shown). Membranes from cells transformed with a different oncogenic Ras, RasQL61, also showed significantly increased levels of GMPPNP-stimulated PTPase activity relative to membranes from parental cells. Thus, the greater specific activity of GMPPNP-stimulated PTPase is a consequence of Ras transformation.

The GMPPNP-stimulated PTPase was characterized by gel permeation chromatography in buffer containing 0.01% (v/v) Triton X-100 following solubilization of guanine nucleotide-treated membranes with 1% (v/v) detergent. Solubilization per se increased total PTPase activity 4–5-fold, but a 2–3-fold stimulation of activity in GMPPNP-treated versus GDP-treated membranes was preserved (data not shown). The principal peak of PTPase activity observed with GDP-treated membranes from v-Ras cells eluted between the positions of the standards bovine γ-globulin (150 kDa) and BSA (67 kDa) with an apparent mass of ~70 kDa (Fig. 2A). Treatment of membranes with GMPPNP reproducibly caused the appearance of a peak of PTPase activity that eluted earlier, at ~100 kDa (Fig.

![Fig. 2. Characterization of PTPase-G by Superose 6 and Mono Q chromatographies.](image-url)

Membranes from v-Ras transformed NIH 3T3 cells were incubated with GMPPNP (A) or GDP (C), solubilized with Triton X-100, and fractionated for analyses (see "Experimental Procedures"). A and B, portions (2 μl) of fractions were assayed for [[32P]Tyr]RCM-lysozyme phosphatase activity. B, Raf-1 was treated with portions (2 μl) of fractions from GMPPNP-treated membranes and assayed (●) by MEK phosphorylation. Arrows a and b and bar c, markers for elution of bovine γ-globulin, BSA, and soybean trypsin inhibitor, respectively. C, Immunoblot with anti-G a sub antibody C-20 of proteins from GMPPNP-treated membranes in indicated fractions from Superose 6 chromatography. D, fractions 33 to 39 inclusive were pooled, subjected to Mono Q chromatography, and assayed for PTPase activity. E, Immunoblot with anti-G a sub antibody of proteins from GMPPNP-treated membranes in indicated pairs of pooled fractions from Mono Q chromatography (D). Data (A–E) are representative of 3 experiments.
2A). We refer herein to this GMPPNP-stimulated PTPase as PTPase-G. An additional species of GMPPNP-stimulated PTPase of higher $M_\text{r}$ (−150,000) was occasionally observed as a smaller peak or leading shoulder (data not shown; see also Fig. 3B). The latter may correspond to a labile PTPase.

We tested whether PTPase-G could deactivate active Raf-1, using FLAG-Raf-1 purified from Sf9 cells co-expressing Ras and SrcT527F. A single Gaussian-shaped peak of activity causing deactivation of Raf-1 was detected and corresponded to PTPase-G (Fig. 2B). The deactivation of Raf-1 occurred in the presence of 2.4 $\mu$M microcystin-LR and was abolished by 0.1 mM vanadate, consistent with the action of a PTPase (data not shown). Raf-1 deactivating activity was increased 5–7-fold by GMPPNP in comparison with GDP (data not shown). Thus, activation of PTPase-G can explain, at least in part, the ability of GMPPNP to cause inactivation of endogenous Raf-1 in membranes from v-Ras cells (6).

Our membrane preparations contain membranes derived from the endoplasmic reticulum in addition to plasma membranes and thus contain PTP1B (15). PTP1B was detected by immunoblotting (data not shown), and its elution corresponded to the major peak of [$^{32}$P]Tyr]RCM-lysozyme phosphatase activity migrating with an apparent mass of ∼70 kDa (centered at fraction 42) and not to the peak of GMPPNP-stimulated PTPase (centered at fraction 39). The peak of [$^{32}$P]Tyr]RCM-lysozyme phosphatase containing 50-kDa PTP1B (Fig. 2B) did not coincide with deactivation of exogenous Raf-1, suggesting that PTP1B is not PTPase-G. The COOH-truncated, 37-kDa form of PTP1B deactivated Raf-1 (6), but this may be due to its reactivity in vitro at high concentrations or the absence of regulatory sequences (15).

Using an antibody that recognizes G$_{\alpha_{i1-3}}$, we detected G$_{\alpha}$ subunit(s) in fractions containing GMPPNP-stimulated PTPase (Fig. 2C). Antibodies C-10 and K-20 to G$_{\alpha_{i1-3}}$ and G$_{\alpha_{o}}$ also detected a protein of 41 kDa in these fractions (data not shown). No G$_{\alpha_{i1}}$ subunits were detected in these fractions by immunoblotting with antibody T-20 that recognizes G$_{\alpha_{i1-4}}$ (data not shown). The profile of the elution of immunoreactive 41-kDa protein correlated well with the profile of GMPPNP-stimulated PTPase activity (Fig. 2, compare A and C). G$_{\alpha_{i1}}$ subunits were absent from corresponding fractions in profiles from GDP-treated membranes, indicating that co-elution of G$_{\alpha}$ and PTPase in these fractions required G-protein activation (data not shown). These results are consistent with activation of a PTPase by binding of G$_{\alpha}$ subunits and not G$_{\alpha_{i1}}$ subunits.

Superose 6 fractions encompassing the leading edge of the PTPase-G peak were pooled to reduce cross-contamination with the peak of PTP1B and subjected to ion exchange chromatography on Mono Q. PTPase-G was resolved into multiple forms that eluted in the middle of the gradient (Fig. 2D). The latter may correspond to a labile PTPase. Reasons for this heterogeneity are unknown. Each of these forms co-eluted with 41-kDa proteins recognized by antibodies to G$_{\alpha}$ (Fig. 2E) and also by antibodies to G$_{\alpha_{i1-4}}$ (data not shown). PTP1B eluted in fractions 17–19 and did not co-elute with a GMPPNP-stimulated peak of PTPase activity or with G$_{\alpha}$ subunits. Recovery of PTPase-G activity after Mono Q chromatography was low, 20–30%, which may be indicative of disruption of a PTPase-G complex.

Reversibility of activation was demonstrated by substitution of GDP for bound GMPPNP in PTPase-G in fractions from Superose 6 chromatography (Fig. 3A). Portions of column fractions from GMPPNP-treated membranes were incubated with or buffer/GMPPNP/Mg (●). Data for G$_{\alpha_{i1}}$ superimposed upon the control profile and were not plotted. Data are Representative of 2 (C) or 3 (A and B) experiments.
GDP or GMPPNP in the presence of Mg$_{2+}$ chelators to promote nucleotide dissociation (9) prior to readd to Mg$_{2+}$ and assay. Replacement of GMPPNP with GMPPNP preserved the PTase-G activity. Replacement of GMPPNP with GDP abolished the peak of PTase-G, resulting in a profile after reassay nearly identical with the profile obtained by assay of fractions from GDP-treated membranes. Since G$_{i2}$ was not detected in the fractions containing PTase activity, reversal of activation by GDP strongly supports regulation by G$_{i2}$ subunits.

To definitively test this hypothesis, we examined the ability of purified G-protein subunits to activate PTase in fractions from GDP-treated membranes (Fig. 3, B and C). Preparations of brain G$_{i1}$ and G$_{o}$ subunits were utilized to provide a range of subunits principally derived from members of the G$_{i/o}$ family (10). Addition of brain G$_{i}$-GMPPNP, but not brain G$_{i}$-GDP, increased activity of PTase that eluted in the leading shoulder of the constitutive ~70-kDa peak of PTase activity (Fig. 3B). Brain G$_{i}$-GMPPNP also activated a species of M$_{r}$ ~150,000 that may correspond to the occasionally observed species of GMPPNP-stimulated PTase activity noted above. Addition of a mixture of brain G$_{i2}$ subunits in the presence of GDP or GMPPNP did not alter PTase activity (data not shown). Importantly, addition of activated recombinant G$_{o}$, G$_{i1}$, and G$_{i2}$ also stimulated PTase activity (Fig. 3C). G$_{o}$ appeared to cause a greater activation than G$_{i1}$ and G$_{i2}$ suggesting specificity in the interaction with the effector PTase, but detailed titration studies will be required to determine the detailed potency and efficacy of various PTX-sensitive G$_{i/o}$ subunits.

G-protein-coupled receptors may initiate both positive signals for MAP kinase activation via G$_{i/o}$ subunits and negative signals via G$_{o}$ subunits, depending on context and the specific G protein-coupled. For example, epitope-tagged MAP kinase expressed in Cos-7 cells is transiently activated by isoproterenol via endogenously expressed β-adrenergic receptors (16). The stimulatory signal is provided by G$_{i/o}$, and is Ras-dependent. An inhibitory signal is provided by G$_{o}$-mediated elevation of cAMP concentration; elevation of cAMP in cells prior to stimulation blocks MAP kinase activation. In pheromone signaling in Saccharomyces cerevisiae, G$_{o}$ stimulates an adaptive pathway that antagonizes G$_{i/o}$-mediated activation of the MAP kinase-related enzyme FUS3 (17). The signaling mechanisms for MAP kinase activation by mammalian G-coupled receptors are complex (18); it appears that specific G$_{i/o}$ subunits may promote, inhibit, or possibly be indifferent to activation of MAP kinase by G$_{i/o}$. Complexity is also indicated by reports that overexpression of GTPase-impaired G$_{i/o}$ caused transformation and activation of GTPase kinase in Rat-1 but not NIH 3T3 cells (19), and that overexpression of GTPase-impaired G$_{o}$ transformed NIH 3T3 cells (20).

Thus, a balance of positive and negative signals determines the extent of MAP kinase activation by G-protein-coupled receptors. PTase-G may deliver a negative signal from receptors that couple to responsible subtypes of G$_{i/o}$ to modulate the timing or extent of activation of MAP kinase. While our experiments reveal a negative modulation by PTase-G of membrane-associated Raf-1 that has been already activated by v-Ras, we cannot exclude the possibility that PTase-G may also act positively in other contexts or temporal sequences. Identification of unambiguous effectors for G$_{i/o}$, as well as G$_{o}$ subunits has been elusive (reviewed in Ref. 21). Our findings strongly implicate PTases as effectors for activated PTX-sensitive G$_{i/o}$ protein(s), fulfilling each of the classical biochemical criteria utilized to establish adenylate cyclase as an effector for G$_{i/o}$. Our findings also show that reconstitution with G$_{i/o}$ subunits can serve as an assay for purification and identification of PTase-G. Elucidation of the pathways regulated by G$_{i/o}$-regulated PTases should provide insight into the mechanisms of mitogenic signaling and cell cycle control.

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