RESEARCH COMMUNICATION

Activation of a protein tyrosine phosphatase and inactivation of Raf-1 by somatostatin

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Human somatostatin receptor 3 ('hsstr$_3$') was transiently expressed in NIH 3T3 cells stably transformed with Ha-Ras (G12V). Somatostatin activated a protein tyrosine phosphatase, and inactivated the constitutively active, membrane-associated form of the Raf-1 serine kinase present in these cells *in vivo* and *in vitro*.

INTRODUCTION

Somatostatin is a widely distributed, neuroendocrine peptide that mediates diverse physiological actions in the central nervous system and in peripheral tissues. These physiological functions include neurotransmission and inhibition of secretion by endocrine and exocrine cells. Somatostatin also inhibits the growth of a variety of cancers in animals, as well as the growth of a variety of transformed cell lines in culture (reviewed in [1]). The mechanisms involved in growth inhibition by somatostatin are incompletely understood.

The ability of somatostatin and somatostatin-related peptides to inhibit growth of cells in culture suggested to others [2] that somatostatin might activate effector(s) which negatively regulate mitogenic signalling. Schally and co-workers demonstrated [2] that somatostatin stimulated dephosphorylation of tyrosine-phosphorylated epidermal-growth-factor (EGF) receptors in membranes isolated from pancreatic cancer cells (Mia PaCa-2). This result implied activation of a protein tyrosine phosphatase (PTPase) by somatostatin. Stork and co-workers implicated G-proteins in the PTPase activation mechanism [3] by showing that the GTP analogue guanosine 5′-[β,γ-imido]triphosphate (p[NH]ppG) stimulated PTPase activity, assayed with *p*-nitrophenyl phosphate as exogenous substrate, and that pertussis toxin (PTx) blocked stimulation of PTPase activity by p[NH]ppG or somatostatin. Preparations of activated somatostatin receptors (sstrs), partially purified by immunoaffinity chromatography, contain PTPase activity and a 66 kDa protein detected by immunoblotting with a polyclonal anti-peptide antibody to a sequence in Src homology domain 2-containing PTPase (SHPTP)1 [4]. Despite these reports and others, modulation of mitogenic signalling by G-protein-coupled receptors via stimulation of PTPase activity has not been widely studied or appreciated.

Raf-1 is a serine/threonine kinase which functions downstream of Ras in a mitogenic signalling cascade (reviewed in [5]). A portion of Raf-1 in v-Ras-transformed cells is membrane-associated and constitutively active [6]. Raf-1 immunoprecipitated from membranes isolated from v-Ras-transformed cells is recognized by anti-phosphotyrosine antibodies and can be inactivated by protein-tyrosine phosphatase 1B [7]. This pool of active Raf-1 in membranes from v-Ras-transformed NIH 3T3 cells can be inactivated by protein phosphatases stimulated by GTP [8]. Here we have studied whether transient expression of human somatostatin receptor 3 (hsstr$_3$) in v-Ras transformed NIH 3T3 cells can confer somatostatin-dependent activation of PTPase activity and inactivation of Raf-1.

MATERIALS AND METHODS

Materials

Somatostatin-14 and guanine nucleotides were purchased from Sigma–Aldrich and Boehringer-Mannheim respectively. NIH 3T3 cells transformed with Ha-Ras (G12V) [9] were a gift from L. Feig (Tufts University, Boston, MA, U.S.A). [^{125}I-Tyr^{11}]-somatostatin-14 was purchased from Amersham International.

Transfection and membrane preparation

v-Ras cells (at $\approx$60% confluency in 100 mm dishes) were transfected with 15 μg of total DNA of twice-CsCl-banded pCMV-hsstr$_3$ [10] or a control plasmid pGEM-3 (Promega) by the $CaPO_4$ precipitation method. Cells were used after 2 days of recovery in complete medium and 2 h of serum starvation. Membranes were prepared as described in [11], by flotation upon 39% (w/v) sucrose, washed, and resuspended to 1 mg/ml total protein in buffer A [25 mM Hepes (pH 7.6 at 4 °C), 8.6% (w/v) sucrose, 10 mM EDTA, 10 mM EGTA, 0.2% (v/v) 2-mercaptoethanol (2-ME)] containing the cocktail of proteinase inhibitors used in [11].

Superose 6 chromatography

Treated membranes were solubilized on ice by addition of Triton X-100 to 1% (v/v) final concentration with gentle trituration. Extracts were clarified by centrifugation (4 °C, 10 min) at $\approx$100000 ***g*** in an Airfuge (Beckman). Equal portions of supernatant (100–200 μl) were chromatographed (4 °C, 0.25 ml/min, 0.25 ml fractions) on a Superose 6 column (1 cm × 28 cm) (Pharmacia–LKB) equilibrated in buffer B

Abbreviations used: PTPase, protein tyrosine phosphatase; p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate; EGF, epidermal growth factor; sstrs, somatostatin receptors; hsstr$_3$, human somatostatin receptor 3; 2-ME, 2-mercaptoethanol; RCM, reduced, carboxymethylated and maleylated; SHPTP, Src homology domain 2-containing PTPase; PTx, pertussis toxin; MAPK, mitogen-activated protein kinase.

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Table 1 Somatostatin inactivates membrane-associated Raf-1 *in vivo*

Membranes were isolated (as in the Materials and methods, except buffers contained 1 μM microcystin-LR) from intact transfected v-Ras cells that had been treated with or without 1 μM somatatostatin for 10 min (30 ° C), and then assayed (20 μl) for membrane-associated Raf-1 activity (see the Materials and methods section). Results are averaged from two independent experiments; 100% = 6300 ± 250 c.p.m.

	Membrane-associated Raf-1 activity	
Transfection	Control	+ Somatostatin
pCMV-hsstr$_3$	100 ± 4.4	41.3 ± 3.2
pGEM-3	100 ± 1.7	100 ± 3.3

[25 mM Hepes (pH 7.4)/2 mM $MgCl_2$/0.1 mM EGTA/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 PMSF].

Assays

PTPase activity was measured by incubating (30 °C) portions of samples (0.5–5 μl) with 3 μl of reduced, carboxymethylated and maleylated (RCM) lysozyme containing phosphotyrosine [Tyr(P)] [12] [40 pmol of protein Tyr(^{32}P)] in buffer C [25 mM Hepes (pH 7.4)/0.1 mM EGTA/1 mM $MgCl_2$/0.2% (v/v) 2-ME/1 mg/ml BSA] (20 μl total assay volume). Reactions were terminated by sequential additions of 200 μl of cold 20% (w/v) trichloroacetic acid and 10 μl of 10 mg/ml BSA with mixing. Samples were centrifuged, and 210 μl of supernatants were counted for radioactivity in scintillant. Data were expressed as percentage release of total ^{32}P in the assay.

Column fractions were assayed for Raf-1 deactivating activity (see Figure 3 below), essentially as described [8], by incubation (30 °C, 15 min) of 2 μl of each fraction with 0.1 μg of partially purified FLAG-Raf-1, isolated from Sf9 cells co-expressing Ras and Src^{Y527F}, in buffer C containing 2.4 μM microcystin-LR; Raf-1 activity was then determined by MEK1 phosphorylation [8].

Raf-1 enzymic activity remaining in membranes after *in vivo* treatment of cells with somatostatin (Table 1) was measured by MEK-stimulated phosphorylation of kinase-defective $p42^{mapk}$ (K52R). Samples of isolated membranes (20 μl) were incubated (30 °C) with: (i) 45 μl of buffer [55 mM β-glycerol phosphate (pH 7.4)/40 mM $MgCl_2$/2.5 mM $MnCl_2$/0.25 mM vanadate/2.5 μM microcystin-LR/0.45 mM [γ-^{32}P]ATP (5 c.p.m./fmol)] for 5 min; (ii) 3 μl of 1 mg/ml $(His)_6$-MEK1 for 15 min; (iii) 20 μl of 0.1 mg/ml $p42^{mapk}$ (K52R) for 2 min. Reactions were quenched, and ^{32}P incorporation into K52R gel bands was determined by Čerenkov radiation.

Raf-1 deactivating activity in aliquots (20 μl) of membranes stimulated with somatostatin or guanine nucleotides *in vitro* (Table 2) was measured after dilution to 200 μl with cold, ultrapure water and pelleting the membranes. Membranes were resuspended in 50 μl of buffer A containing sucrose, proteinase inhibitors and 1 μM microcystin-LR, and incubated (10 min, 30 °C). Endogenous Raf-1 enzymic activity remaining after incubation was then measured by MEK-stimulated phosphorylation of kinase-defective $p42^{mapk}$ (K52R) (see above).

Analysis

Data shown are representative of three to four experiments unless otherwise indicated. Statistical comparisons were made using a paired Student's *t* test.

RESULTS AND DISCUSSION

NIH 3T3 fibroblasts do not appreciably express sstrs. Transfection of NIH 3T3 cells transformed with Ha-Ras (G12V) with a plasmid for cytomegalovirus promoter-driven expression of human sstr$_3$ [10] resulted in readily detectable, high-affinity binding for [^{125}I-Tyr^{11}]somatostatin in membranes (Figure 1). Membranes from cells transfected with an equal quantity of

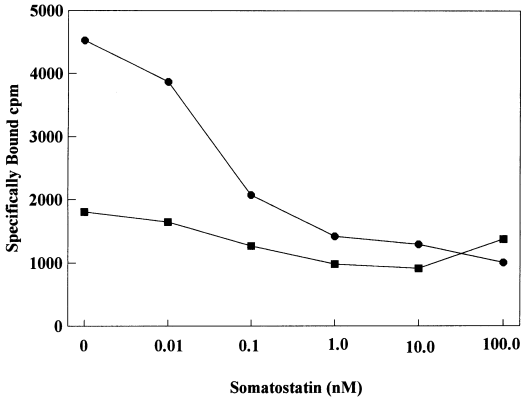

Figure 1 Somatostatin binding

Membranes were isolated as described in [13] from transfected Ha-Ras (G12V) NIH 3T3 cells, and equal portions (200 μg) were analysed for titration of specifically bound [^{125}I-Tyr^{11}]somatostatin-14 (ordinate) with somatostatin (abcissa), essentially as described [13]. ●, pCMV-hsstr$_3$; ■, control plasmid.

Table 2 Somatostatin stimulates PTPase and inactivates Raf-1 *in vitro*

Membranes (150 μl) from transfected cells were GDP-treated (as in Figure 2) and then incubated (50 μl per condition) *in vitro* with one of (final concns.): (i) 1 mM GDP, (ii) 0.1 mM p[NH]ppG or (iii) 0.1 mM p[NH]ppG plus 1 μM somatostatin on ice for 10 min prior to assay of 3 μl or 20 μl portions for PTPase or Raf-1 deactivating activities respectively (see the Materials and methods section). $n = 4$, 100% K52R phosphorylation = 6700 ± 300 c.p.m.); 100% ^{32}P release = pGEM-3, 10.6% ± 2.1, pCMV-hsstr$_3$, 15.3% ± 4.2 of total. *$P < 0.05$.

	PTPase		Raf-1 activity	
Treatment	pGEM-3	pCMV-hsstr$_3$	pGEM-3	pCMV-hsstr$_3$
GDP	100	100	100	100
p[NH]ppG	95.0 ± 5.6	102.0 ± 4.1	98.3 ± 11.8	94.3 ± 9.1
Somatostatin + p[NH]ppG	91.8 ± 4.0	184.8 ± 10.2*	92.0 ± 4.4	28.0 ± 13.1*

a control plasmid did not exhibit any appreciable binding (Figure 1).

Previous efforts to characterize the somatostatin-stimulated PTPase activity by column chromatography have utilized proteins solubilized from membranes isolated from intact cells or tissues stimulated with or without somatostatin [14]. Homogenization buffers contained phosphatase and proteinase inhibitors. This strategy is likely to be successful if the relevant PTPase is regulated by phosphorylation in a signalling pathway. However, we recently demonstrated that a PTPase present in membranes from v-Ras-transformed cells is an effector for PTx-sensitive G_α subunits [15]. The hypothesis that somatostatin liberates activated G_α subunits which regulate PTPase activity suggests an alternative strategy for recovering a somatostatin-stimulated increase in PTPase activity after chromatography.

We prepared membranes from v-Ras cells expressing $hsstr_3$ for *in vitro* experiments designed to foster somatostatin-stimulated activation of G-proteins by p[NH]ppG/Mg^{2+}. Membranes were first incubated with GDP (see the Materials and methods section) to ensure that G-proteins were in their basal state, and then treated for 10 min (30 °C) with either GDP (control), p[NH]ppG, or somatostatin plus p[NH]ppG. p[NH]ppG alone had no appreciable effect on PTPase activity in membranes under these conditions in comparison with GDP (results not shown). In contrast, stimulation of membranes with somatostatin plus p[NH]ppG increased [Tyr(^{32}P)]RCM-lysozyme phosphatase activity 1.5–2 fold, and this increase was blocked by PTx (results not shown).

The stimulated PTPase was characterized by Superose 6 chromatography (Figure 2) after solubilization of the membranes with 1% Triton X-100. Somatostatin plus p[NH]ppG robustly activated a PTPase, which was eluted as a distinct peak in the leading shoulder of a peak of PTPase activity that was not altered by somatostatin (Figure 1). The constitutive peak has been identified as PTP1B by Western blotting [15]. The apparent molecular mass of the stimulated PTPase in these experiments was 150–200 kDa, and may be affected by detergent binding.

Our success in recovering a large increase in somatostatin-stimulated PTPase activity is likely due to somatostatin-stimulated exchange of p[NH]ppG for GDP in G-proteins. p[NH]ppG is not hydrolysed by G_α subunits, resulting in persistent activation of G-proteins [16]. PTPase activity stimulated by somatostatin and p[NH]ppG/Mg^{+2} is co-eluted with G_α subunits following Superose 6 chromatography (results not shown). Recovery of an increase in PTPase activity after Superose 6 chromatography is suggestive of high-affinity binding of G_α-p[NH]ppG/Mg^{2+} to an effector. This complex appears to be more labile during ion-exchange chromatography. When Superose 6 fractions containing stimulated PTPase activity were pooled and subjected to MonoQ chromatography, approx. 80% of the activity was lost [15].

Activation of G-proteins by more prolonged incubation of membranes with p[NH]ppG also activated PTPase in comparison with incubation with GDP (Figure 3, bottom). Fractions containing p[NH]ppG-stimulated PTPase inactivated exogenous Raf-1, prepared from Sf9 cells co-expressing Ras and Src^{Y527F} (Figure 3, top). Raf-1 deactivating activity, like [Tyr(^{32}P)]RCM-lysozyme phosphatase activity, was robustly stimulated by p[NH]ppG versus GDP (Figure 3, top). The mobility of p[NH]ppG-stimulated PTPase during Superose 6 chromatography was nearly identical with that of somatostatin-stimulated PTPase (results not shown). Thus we conducted experiments to determine whether somatostatin could cause inactivation of the pool of constitutively active Raf-1 present in membranes from v-Ras-transformed NIH 3T3 cells.

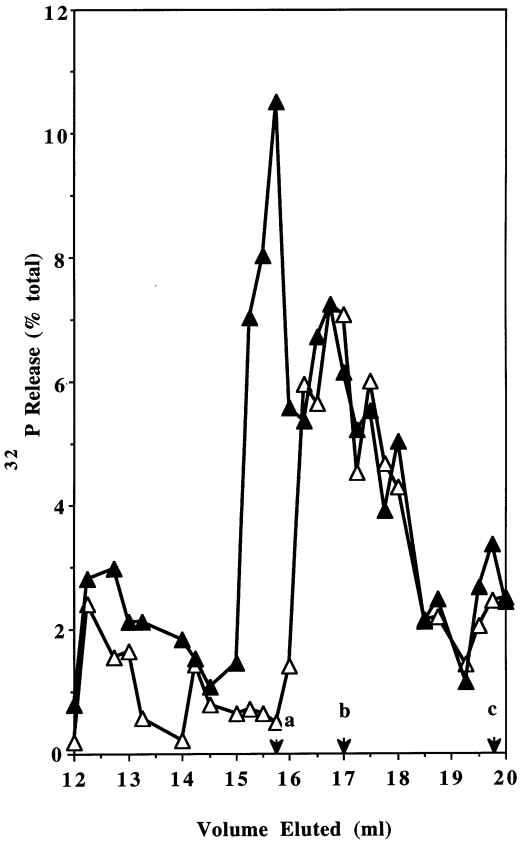

Figure 2 Preservation of somatostatin-stimulated PTPase activity after Superose 6 chromatography

Membranes (200 μg of total protein; 1 mg/ml) from pCMV-$hsstr_3$-transfected cells (see the Materials and methods section) were incubated on ice with 1 mM GDP (final concn.) for 5 min, and then for a further 15 min after adjustment to 2 mM Mg^{2+} (final concn. in excess of chelators). GDP-treated membranes were recovered by centrifugation (4 °C, 10 min, 100 000 ***g***) in an Airfuge (Beckman) and resuspended to 1 mg/ml in buffer A containing 1 μM microcystin-LR, proteinase inhibitors and 2 mM Mg^{2+} in excess of chelators. Equally divided portions were incubated (10 min, 30 °C) with 1 μM somatostatin plus 0.1 mM p[NH]ppG (▲) or 0.1 mM p[NH]ppG alone (△). Samples were solubilized, chromatographed, and 5 μl of the fractions assayed for PTPase activity (see the Materials and methods section). Arrows indicate elution of marker proteins: a, bovine γ-globulin; b, BSA; c, soybean trypsin inhibitor.

Transformed NIH 3T3 cells were transfected with pCMV-$hsstr_3$, or a control plasmid, and then briefly treated with or without somatostatin prior to isolation of membranes and assay (Table 1). Somatostatin caused a significant reduction in Raf-1 enzymic activity in membranes from cells transfected with pCMV-$hsstr_3$, but had no effect upon Raf-1 in membranes from cells transfected with a control plasmid (Table 1). To determine whether inactivation of this pool of Raf-1 correlated with activation of PTPase, membranes were isolated from pCMV-$hsstr_3$-transfected cells and assayed for the ability of somatostatin to cause activation of PTPase and inactivation of membrane-associated Raf-1. Somatostatin plus p[NH]ppG stimulated PTPase activity and reduced Raf-1 activity in membranes from pCMV-$hsstr_3$ transfected cells (Table 2). Inactivation of Raf-1 was blocked by inclusion of 0.1 mMvanadate, but not 2.4 μM microcystin-LR in the deactivation assay (results not shown). Neither p[NH]ppG nor GDP affected PTPase or Raf-1 activities under these conditions, and somatostatin had no effect on membranes from cells not expressing $hsstr_3$ (Table 2). Together,

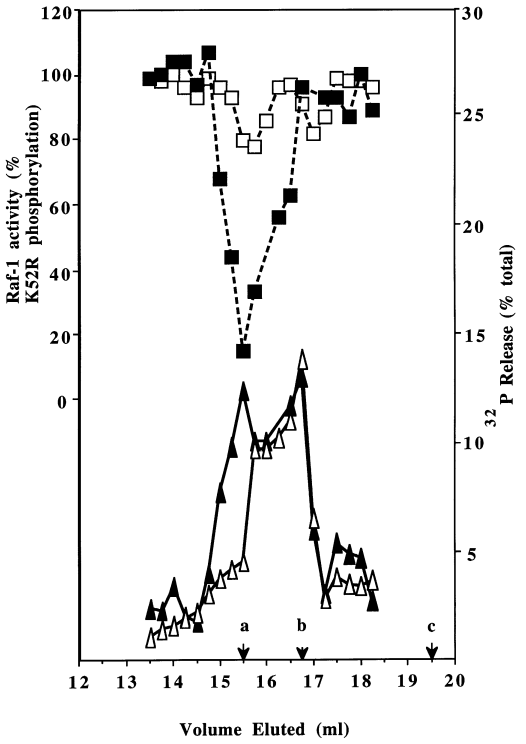

Figure 3 Inactivation of Raf-1 by p[NH]ppG-stimulated PTPase

Membranes (200 μg of total protein) isolated from v-Ras-transformed cells were incubated for 30 min on ice with 2 mM (final concn.) p[NH]ppG (■, ▲) or GDP (□, △) adjusted with a concentrated stock to 2 mM $MgCl_2$ in excess of chelators, solubilized by addition of Triton X-100 (1%, v/v, final concn.), and chromatographed (see the Materials and methods section). Fractions were assayed (see the Materials and methods section) for PTPase activity (right ordinate: ▲, △; 5 μl aliquots) or activity deactivating exogenous active Raf-1 (left ordinate: ■, □; 2 μl aliquots).

these data strongly suggest that activation of PTPase by somatostatin is causally related to inactivation of the membrane-associated form of Raf-1 in v-Ras cells. Our data also strongly support direct activation of PTPase activity by activated G-proteins, confirming and extending reports by the groups of Schally [2], Stork [3] and Sussini [14]. Clearly more vigorous investigation of PTPase regulation by G-proteins is warranted.

Mechanisms other than PTPase stimulation undoubtedly contribute to Raf-1 inactivation in other contexts. Ras inactivation, resulting from either decreased Ras guanine nucleotide exchange-factor activity or increased Ras GTPase activating protein activity, would be expected to play a major role in Raf-1 inactivation. Raf-1 is also regulated positively by phosphorylation on regulatory serine/threonine residues [11,17], and thus serine/threonine phosphatases may negatively regulate Raf-1.

Regulation of Raf-1 [and mitogen-activated protein kinase (MAPK)] by somatostatin is likely to be more complex than is apparent from the studies described. In a different context and temporal sequence, somatostatin has been reported to activate MAPK. Addition of somatostatin to serum-starved Chinese-hamster ovary cells, stably expressing rat $sstr_4$ activated both $p42^{mapk}$ and $p44^{mapk}$ [18]. Ras-dependent and PTx-sensitive activation of MAPK is also well established for a number of other G-protein-coupled receptors, e.g. the α_2-adrengergic receptor [19]. Activation of MAPK by G-protein-coupled receptors is also in many cases very transient. These data could be rationalized by a model in which MAPK activation by somatostatin is mediated by activation of Ras by $G_{\beta\delta}$ [20] and MAPK inactivation is initiated by $G_{\alpha i/o}$ via activation of a PTPase. Alternatively, PTPase(s) may also be required in steps leading to activation of the MAPK pathway. Several reports have implicated PTPases in both signal-generation and signal-termination steps for growth factors or cytokines [21,22].

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REFERENCES

1 Reubi, J. -C. and Laissue, J. A. (1995) Trends Pharmacol. Sci. **16**, 110–115
2 Liebow, C., Reilly, C., Serrano, M. and Schally, A. V. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 2003–2007.
3 Pan, G. P., Florio, T. and Stork, P. J. (1992) Science **256**, 1215–1217
4 Zeggari, M., Esteve, J. -P., Rauly, I., Cambillau, C., Mazarguil, H., Dufresne, M., Pradayrol, L., Chayvialle, J. -A., Vaysse and Sussini, C. (1994) Biochem. J. **303**, 441–448
5 Marshall, C. J. (1994) Curr. Opin. Genes Dev. **4**, 82–89
6 Traverse, S., Cohen, P., Paterson, H., Marshall, C. J., Rapp, U., and Grand, R. (1993) Oncogene **8**, 3175–3181
7 Jelinek, T., Dent, P., Sturgill, T. W. and Weber, M. J. (1996) Mol. Cell. Biol. **16**, 1027–1034
8 Dent, P., Jelinek, T., Morrison, D. K., Weber, M. J., and Sturgill, T. W. (1995) Science **268**, 1902–1906
9 Farnsworth, C. L., Marshall, M. S., Gibbs, J. B., Stacey, D. W., and Feig, L. A. (1991) Cell **64**, 625–633
10 Yasuda, K., Rens-Domiano, S., Breder, C. D., Law, S. F., Saper, C. B., Reisine, T. and Bell, G. I. (1992) J. Biol. Chem. **267**, 20422–20428
11 Dent, P., Reardon, D. B., Morrison, D. K., and Sturgill, T. W. (1995) Mol. Cell. Biol. **15**, 4125–4135
12 Brautigan, D. L. and Pinault, F. M. (1993) Mol. Cell. Biochem. **127/128**, 121–129
13 Rens-Domiano, S., Law, S. F., Yamada, Y., Seino, S., Bell, G. I. and Reisine, T. (1992) Mol. Pharmacol. **49**, 28–34
14 Colas, B., Cambillau, C., Buscail, L., Zeggari, M., Esteve, J. -P., Lautre, V., Thomas, F., Vaysse, N. and Sussini, C. (1992) Eur. J. Biochem. **207**, 1017–1024
15 Dent, P., Reardon, D. B., Wood, S. L., Lindorfer, M. A., Graber, S. G., Garrison, J. C., Brautigan, D. L. and Sturgill, T. W. (1996) J. Biol. Chem. **271**, 3119–3123
16 Neer, E. J. (1995) Cell **80**, 249–257
17 Yao, B., Zhang, Y., Delikat, S., Mathais, S., Basu, S. and Kolesnick, R. (1995) Nature (London) **376**, 307–310
18 Bito, H., Mori, M., Sakanka, C., Takano, T., Honda, Z., Gotoh, Y., Nishida, E. and Shimizu, T. (1994) J. Biol. Chem. **269**, 12722–12730
19 Alblas, J., van Corven, E. J., Hordjik, P. L., Milligan, G. and Moolenaar, W. H. (1993) J. Biol. Chem. **268**, 22235–22238
20 Crespo, P., Xu, N., Simonds, W. F. and Gutkind, J. S. (1994) Nature (London) **369**, 418–420
21 Yamauchi, K., Milarski., K. L., Saltiel, A. R. and Pessin, J. E. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 664–668
22 David, M., Chen, H. E., Goelz, S., Larner, A. L. and Neel, B. G. (1995) Mol. Cell. Biol. **15**, 7050–7058

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