Research article

Evidence for the involvement of G_{i2} in activation of extracellular signal-regulated kinases in hepatocytes

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Abstract

Background: Activation of the extracellular signal-regulated kinases ERK1 and ERK2 in hepatocytes by prostaglandin $(PG)F_{2\alpha}$ was recently found to be inhibited by pertussis toxin (PTX) suggesting a role for G_i proteins.

Results: Targeting the $Gi_{2\alpha}$ expression by a specific ribozyme inhibited the $PGF_{2\alpha}$-induced ERK1/2 activation in hepatocytes. On the other hand a non-cleaving form of the $Gi_{2\alpha}$ ribozyme did not significantly decrease the ERK1/2 activation. In ribozyme-treated cells the $Gi_{2\alpha}$ protein level was reduced, while the $G_{q\alpha}$ level was not affected thus confirming the specificity of the ribozyme.

Conclusion: The present data suggest an important role of G_{i2} in $PGF_{2\alpha}$-induced ERK1/2 signaling in hepatocytes.

Introduction

The extracellular regulated kinases ERK1 ($p44^{mapk}$) and ERK2 ($p42^{mapk}$) are believed to be implicated in regulation of cell growth and differentiation [1,2]. They are activated in response to stimulation both of heptahelical G protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). Epidermal growth factor (EGF), hepatocyte growth factor (HGF), $PGF_{2\alpha}$, norepinephrine, and several other agents activate ERK1/2 in hepatocytes [3,4,5,6]. Furthermore, it was observed in these cells that pretreatment with pertussis toxin (PTX) decreased activation of ERK1/2 in response to various agents acting on RTKs or GPCRs [6,7,8,9]. The data suggest an involvement of G_i protein(s) in the mechanisms of ERK1/2 activation in hepatocytes. However, it is not known which G_i protein(s) that mediate this effect. To approach this issue we have targeted the α subunit of G_{i2} by a catalytic RNA (ribozyme) [10,11]. The effect of the ribozyme on $PGF_{2\alpha}$-induced ERK1/2 activation, which is strongly sensitive to PTX, was subsequently assessed.

Results

Inhibition of ERK activation by pertussis toxin

Pretreatment of hepatocytes with pertussis toxin [12] was reported to decrease ERK1/2 activation by agents acting both on heptahelical G protein coupled receptors as well as receptor tyrosine kinases [6,7,8]. These observations are summarized in Fig. 1. In addition these data show the persistence with time of the marked inhibitory effect of PTX on ERK1/2 activation induced by $PGF_{2\alpha}$. The EGF- and HGF-induced responses are on the other hand only partially decreased. These findings suggest
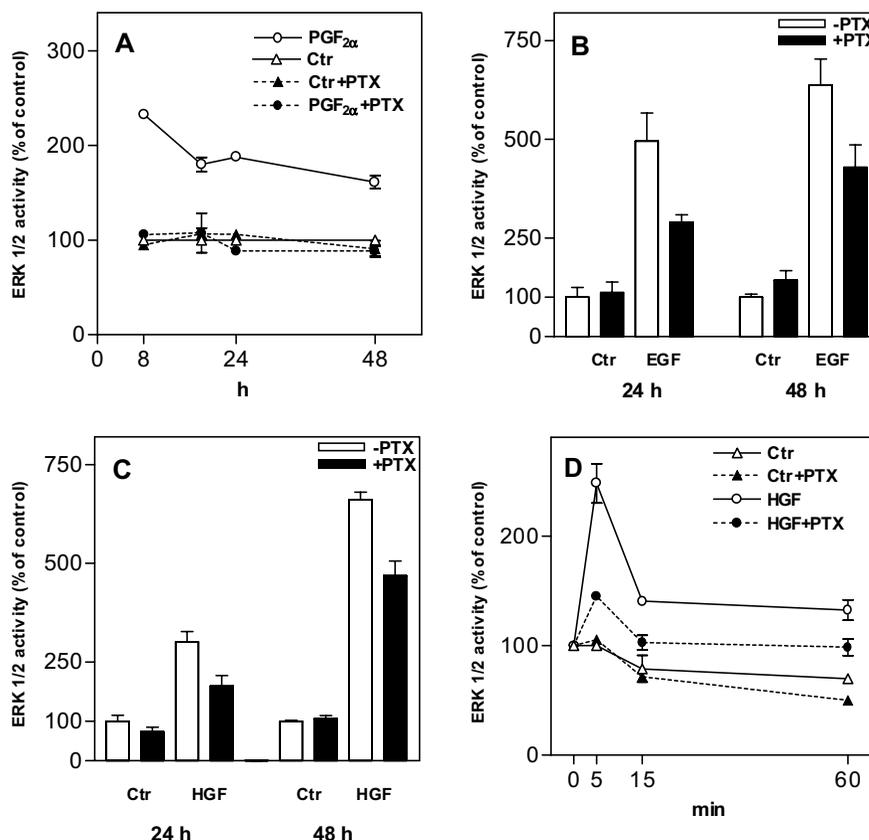
Figure 1
Effect of pertussis toxin (PTX) on ERK1/2 activation. PTX was added to cultured hepatocytes at 3 h after the time of seeding. A: ERK1/2 activation in the absence or presence of PTX (400 ng/ml) in response to stimulation with $PGF_{2\alpha}$ (10 μM) for 5 min in the period from 8 h to 48 h. B, C: Effect of PTX (300 ng/ml) on ERK1/2 activation in response to stimulation with EGF (10 nM) (B) or HGF (100 ng/ml) (C) for 5 min at 24 and 48 h. D: Time-course for HGF- (100 ng/ml) induced ERK1/2 activation in the absence or presence of PTX (300 ng/ml). Results in A-D represent means ± S.E.M. from three experiments.

that ERK1/2 activation in hepatocytes involve G_i protein(s).

Ribozyme targeting the $G_{i2\alpha}$

Although the data shown in Fig. 1 suggest that the activation of ERK1/2 implicated G_i, they do not determine the subtype of G_i involved in this process. Accordingly, we have evaluated the effects of a ribozyme specific for $G_{i2\alpha}$ upon ERK1/2 activation. The choice of target was based on the knowledge of G_{i2} as a major member of the G_i family in hepatocytes, which is also represented by G_{i3} in these cells [9,13,14], and furthermore on the α subunit as the unifying part of the heterotrimer which additionally comprises βγ variants of hitherto unknown subtype compositions and G protein specificity.

Ribozymes are RNA molecules that specifically cleave mRNAs [10,11]. These molecules have been shown to inhibit gene expression in various cell types [15,16]. To in-

crease the ribozyme stabililty, all hydroxyl pyrimidines were replaced by their 2'-amino analogs. This type of modification was shown to enhance the ribozyme stability without affecting its cleavage activity [15,17]. Fig. 2A shows the cleavage of the RNA substrate by the ribozyme.

Several approaches have been explored in order to introduce genes into hepatocytes [18,19]. As a first step, we have examined the usefulness of the cationic lipid-mediated ribozyme delivery into hepatocytes. In this respect, the hepatocytes were transfected with a 5'-carboxyfluorescein-conjugated ribozyme and analysed by fluorescence (Fig. 2B). As shown, most cells had taken the ribozyme molecules. Furthermore, no significant cytotoxic effect was observed at the concentration used. Thus DOTAP may represent a versatile transfection reagent for primary hepatocytes.
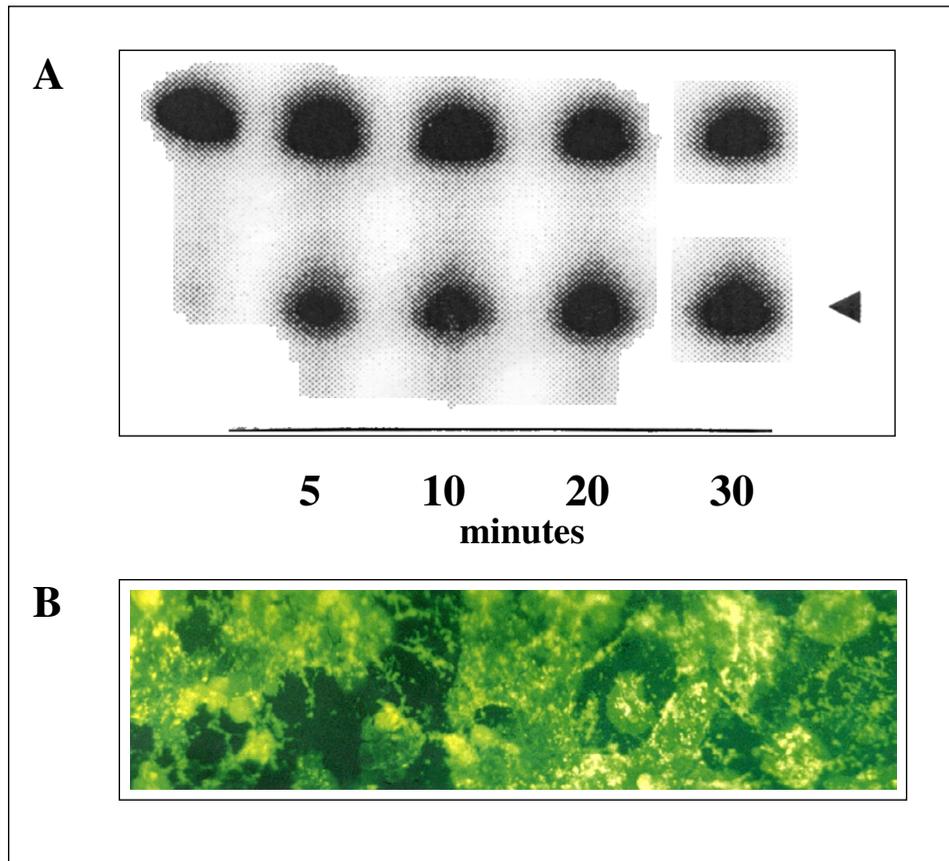
Figure 2
$G_{i2\alpha}$ ribozyme. A: Ribozyme *in vitro* cleavage activity. A PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) printout of a 15 % polyacrylamide gel with 7 M urea. The 5'-end-labelled RNA substrate (200 nM) was incubated with the ribozyme (75 nM) in reaction buffer as described in Materials and Methods. 10 μl samples were taken at the indicated time, added to 5 μl quenching solution and then analysed by electrophoresis. The arrow indicates cleavage products. B: Ribozyme uptake in cultured hepatocytes. Hepatocytes were transfected with 5'-FITC-labelled ribozyme for 30 h. Following washing with phosphate-buffered saline (PBS), cells were analysed by fluorescence microscopy.

Inhibition of $G_{i2\alpha}$ expression and ERK1/2 activation by ribozyme treatment

Having demonstrated a cellular uptake of ribozymes into cultured hepatocytes when DOTAP was used as delivery agent, in the next set of experiments we examined the effects of the $G_{i2\alpha}$ ribozyme on $G_{i2\alpha}$ protein levels as well as on the total ERK1/2 expression and activation. DO-TAP formulated test molecules were added to the hepatocyte cultures at 4–5 hours after the time of seeding. After 30–45 hours transfection time the expression of $G_{i2\alpha}$ protein was decreased in ribozyme-treated cells, while no significant effect was seen with its non-cleaving form (Fig. 3A). The expression of $G_{q\alpha}$ (Fig. 3B) or $G_{s\alpha}$ (not shown) was not affected by the ribozyme treatment

thus confirming the specificity of the ribozyme effects upon $G_{i2\alpha}$ gene expression. To investigate the functional roles of G_{i2} on ERK1/2 activation, we examined the phosphorylation of ERK1/2 in ribozyme-treated cells following $PGF_{2\alpha}$ stimulation (Fig. 3C, D). The basal level of ERK1/2 phosphorylation (i.e. in the absence $PGF_{2\alpha}$ stimulation) was not reduced following ribozyme treatment (Fig. 3C). However, the $PGF_{2\alpha}$-induced phosphorylation of ERK1/2 was decreased (Fig. 3C and 3D, lower panel). In contrast, the total ERK1/2 protein level was not affected by the treatment (Fig. 3D, upper panel). To further confirm the decrease in ERK1/2 phosphorylation, we also assessed their activity in response to $PGF_{2\alpha}$ stimulation (Fig. 4). A marked inhibitory effect was
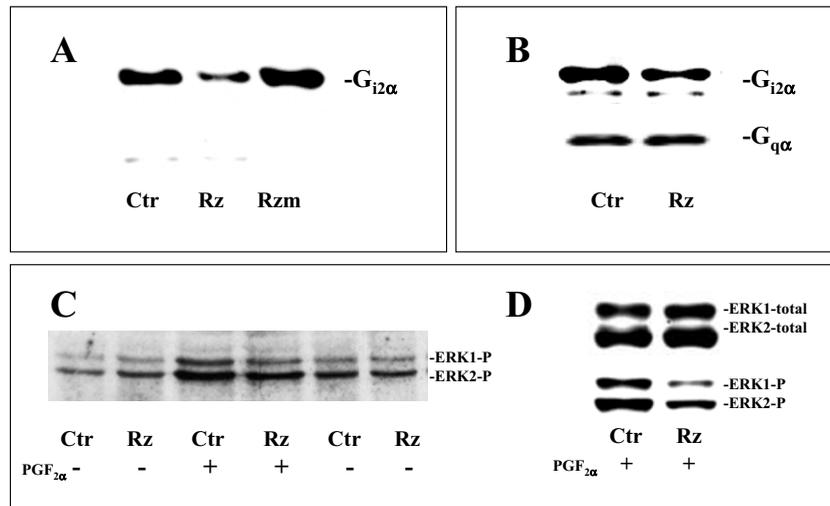
Figure 3
Western analysis of the effects of ribozyme on $G_{i2\alpha}$ and ERK1/2. $G_{i2\alpha}$ ribozyme (Rz) or non-cleaving $G_{i2\alpha}$ ribozyme (Rzm) complexed with DOTAP giving final ribozyme concentrations of 2.5 µM, or only DOTAP (Ctr) were added to hepatocyte cultures at 4–5 hours after the time of seeding. A: Expression of $G_{i2\alpha}$ protein was assessed after 45 h of ribozyme treatment using antibody (from Calbiochem) directed against C-terminal end of $G_{i1/i2\alpha}$. B: Expression of $G_{i2\alpha}$ and $G_{q\alpha}$ protein levels in the same samples subsequent to 30 h of ribozyme treatment using antibodies (from NEN™ Life Science Products) against C-terminal sequences of $G_{i1/i2\alpha}$ or $G_{q\alpha}$, respectively. The polyclonal antibodies used to assess $G_{i2\alpha}$ recognize both the α subunit of G_{i2} and G_{i1}. As shown previously hepatocytes do not express $G_{i1\alpha}$ [19], so the reactivity with these antibodies reflects only the $G_{i2\alpha}$ levels. C, D: After 45 h of ribozyme treatment cells were stimulated with or without $PGF_{2\alpha}$ (10 µM) for 5 min before they were harvested. Immunoblot using antibody against dually phosphorylated ERK1/2 (i.e. ERK1/2-P) (C) is depicted. In Fig. D is developed images from the same immunoblot using antibody detecting total amount of ERK1/2 (i.e. both phosphorylated and unphosphorylated forms) (upper panel) and antibody against dually phosphorylated fractions of ERK1/2 (lower panel).

found in ribozyme-treated cells. The activation of ERK1/2 by EGF was, however, reduced to a lesser extent. These results also show the absence of inhibition by the non-cleaving form of the $G_{i2\alpha}$ ribozyme.

Discussion

In the current study we have investigated the role of $G_{i2\alpha}$ upon $PGF_{2\alpha}$ -induced ERK1/2 activation in hepatocytes. The data obtained with the ribozyme suggest that G_{i2} is an important factor in ERK1/2 activation.

G_i proteins are believed to be involved in regulation of cell growth [20], and a role in activation of ERK1/2 is reported in different cells [21,22,23]. In hepatocytes it has

been observed that PTX inhibited activation of ERK1/2 both by agents acting on G protein coupled receptors including vasopressin, angiotensin II, norepinephrine and $PGF_{2\alpha}$ as well as by agents that bind to receptor tyrosine kinases like EGF and HGF [6,7,8], also suggesting roles of G_i. In the present study we explored closer the G_i dependency of $PGF_{2\alpha}$ -induced ERK1/2 activation, which is strongly PTX-sensitive. We used a ribozyme approach [10,11], which has been examined in a variety of experimental models to suppress gene expression [15,24], including in human hepatocytes and hepatoma cells [25,26,27]. Notably, ribozymes were recently reported to effectively suppress the expression of the $\gamma 7$ subunit of heterotrimeric G proteins in HEK 293 cells [28,29].
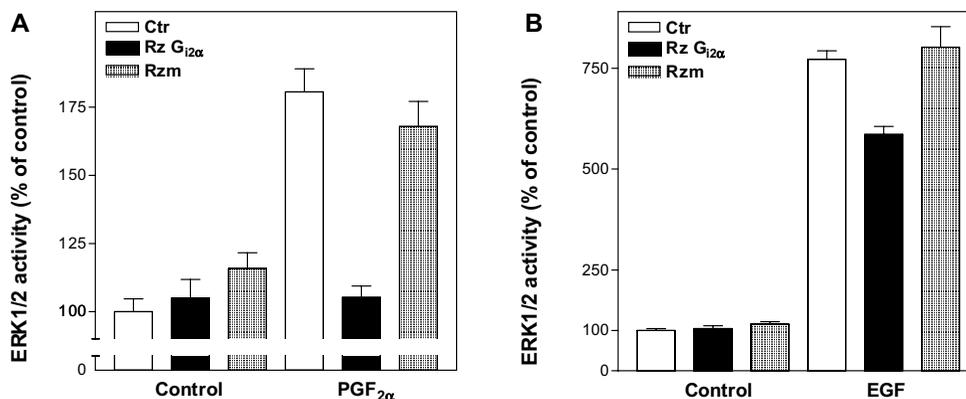
Figure 4
Effect of $G_{i2\alpha}$ ribozyme on ERK1/2 activation. $G_{i2\alpha}$ ribozyme (Rz) or non-cleaving $G_{i2\alpha}$ ribozyme (Rzm) complexed with DOTAP giving final ribozyme concentrations of 2.5 μM, or DOTAP without ribozyme, were added to hepatocyte cultures at 4–5 hours after the time of seeding. After 30 h of treatment the cell cultures were stimulated with $PGF_{2\alpha}$ (10 μM) or EGF (10 nM) for 5 min before they were harvested and ERK1/2 activity assessed. Results are expressed as percent of untreated control and represent mean ± S.E.M. from three experiments.

Pretreatment of the hepatocytes with the $G_{i2\alpha}$ ribozyme resulted in a marked inhibition of the $PGF_{2\alpha}$ -induced ERK1/2 activation. The findings might be explained by the ability of $PGF_{2\alpha}$ to act through receptors that couple directly to G_i in these cells [30]. In addition, the $G_{i2\alpha}$ ribozyme resulted in a partial decrease of the EGF-induced ERK1/2 activation, in accordance with a role of G_i. However, the explanation for an involvement of G_i in this pathway is not known. Observations in different cell types have indicated that G_i might play a role in EGF-induced cell signaling [31], and in hepatocytes a direct coupling of the EGF receptor to G_i has been proposed [32]. One explanation is that receptor-independent functions of G_i may be involved in signaling from receptor tyrosine kinases as well as from seven transmembrane receptors [9,33,34]. Since cyclic AMP was found to exert a negative control of ERK1/2 activation in hepatocytes [35], it can be speculated that the decreases in ERK1/2 responses observed subsequent to inhibition of G_i function might be caused by an elevation of intracellular levels of cyclic AMP. However, in experiments using pertussis toxin there were no detectable alterations of the cyclic AMP level under basal conditions (data not shown). Furthermore, as shown in the present study ribozyme treatment of the hepatocytes did not decrease the basal ERK1/2 ac-

tivation thus suggesting no significant alteration of cyclic AMP level under these experimental conditions.

The present data show a close correlation between the inhibitory effect on ERK1/2 activation produced by the $G_{i2\alpha}$ ribozyme compared to the effects obtained with PTX. This suggests that the PTX effects that have been observed, at least on ERK1/2 activation in response to $PGF_{2\alpha}$ as well as EGF stimulation, reflect G_{i2}-mediated mechanisms. However, the present data do not rule out a possible contribution of G_{i3} in ERK1/2 activation. In this regard, it is of note that observations in endothelial cells have suggested a role for G_{i2} proteins, but not G_{i3}, in ERK1/2 activation [36]. Further studies involving specific inhibition of G_{i3} proteins will be needed to clarify this issue in the hepatocyte model.

Previous findings have demonstrated that G_i dependent activations of ERK1/2 are mediated through βγ subunits. These results were derived from studies where ERK activation was elicited by overexpression of βγ [37,38], or antagonized by the βγ-inhibitory peptide β1ct (C-terminal fragment of the β-adrenergic receptor kinase-1) [39]. Although a possible role for βγ in ERK1/2 activation can not be ruled out, the present data might be compatible with a role for the α i2 subunit. This interpretation agrees

with observations reported by Hedin et al. [40] who found that in Jurkat T lymphocytes the δ-opioid activation of ERK1/2 was PTX-sensitive, but unaffected by β1ct treatment, suggesting an involvement of α_i. However, it should be noted that a strategy based on targeting a particular subunit of a G protein might affect the overall function of the heterotrimer.

Conclusion
The present study gives further support to a role of G_i proteins in ERK1/2 activation in hepatocytes and suggests a role of G_{i2}. On the other hand, the data can not exclude a possible involvement of G_{i3} in the mechanisms of ERK1/2 activation in these cells or define the precise contribution of the G protein subunit α_{i2}. The observation that primary hepatocytes are efficiently transfected with ribozymes may facilitate studies of cell signaling in this model system which represents features of normal cells. Thus, it will be interesting to explore the roles of different heterotrimeric G proteins and their subunits in activation of ERK1/2 as well as other mitogen-activated protein kinases by the nucleic acid enzyme strategy.

Materials and Methods
Materials
Dulbecco's modified Eagle's medium, Waymouth's medium MAB 87/3, penicillin and streptomycin were from Gibco, Grand Island, NY, U.S.A. Adenosine 5'-triphosphate, collagen, collagenase, phenylmethylsulfonyl fluoride, benzamidine, leupeptin, pepstatin A, myelin basic protein (MBP), epidermal growth factor, prostaglandin $F_{2\alpha}$, insulin, pertussis toxin, and 2-mercaptoethanol were from Sigma, St. Louis, MO, USA. Hepatocyte growth factor (human) was a gift from Magne Børset, NTNU, Trondheim, Norway. Sodium(meta)vanadate was from Fluka Chemie AG, Buchs, Switzerland. Phenyl Sepharose CL-4B was from Pharmacia Biotech., Uppsala, Sweden. Dexamethasone was from Norwegian Medicinal Depot, Oslo, Norway. DOTAP was from Boehringer Mannheim, Mannheim, Germany. T7 RNA polymerase and T4 polynucleotide kinase were from Promega Corporation, Madison, WI, USA. [γ-^{32}P] Adenosine 5'-triphosphate (3000 Ci/mol) was from Amersham International, Buckinghamshire, England.

Isolation and culture of hepatocytes
Male Wistar rats (170–220 g) fed ad libitum were used. Parenchymal liver cells were isolated by in vitro collagenase perfusion and low-speed centrifugation [41] with modifications [42]. Cell viability, measured as the ability to exclude trypan blue, was at least 95 %. The cells were suspended in culture medium and plated in Costar wells at 20.000 cells/cm^2. The culture medium (0.2 ml/cm^2) was a 1:1 mixture of Dulbecco's modified Eagle's medium and Waymouth's medium MAB 87/3 containing 16.8 mM glucose, supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), dexamethasone (25 nM) and insulin (100 nM). The cultures were gassed with 95 % air 5% CO_2 and kept at 37°C.

Measurement of ERK activity
The measurement of ERK1/2 activity was performed as previously described [5,6]. In brief, the hepatocyte cultures were exposed to agonists for 5 minutes before rinsing the cells and scraping them into a buffer containing 10 % ethylene glycol. The lysate was centrifugated (15,800 × g) for 10 minutes and the supernatant mixed with phenyl-Sepharose, which was washed twice in a 10 %, twice in a 35 % ethylene glycol buffer, before finally eluting ERK1/2 with 60 % ethylene glycol buffer [43]. The eluate was assayed for ERK1/2 activity, using MBP as substrate, in the presence of an inhibitor of protein kinase A (Sigma P-0300). The reaction mixture was spotted onto P81 paper (Whatman, Maidstone, UK), which was washed, dried and counted in a liquid scintillation counter. Protein content was determined with the BCA Protein Assay (Pierce, Rockford, IL, U.S.A.).

Immunoblotting
Aliquots with 20 µg cell protein (total cell lysate prepared in Laemmli buffer) were electrophoresed on 10 % polyacrylamide gels (acrylamide:N'N'-bis-methylene acrylamide 30:0.8) followed by protein electrotransfer to nitrocellulose membranes and immunoblotting with a polyclonal MAP kinase antibody against the dually threonine- and tyrosine phosphorylated forms of ERK1 (p44^{mapk}) and ERK2 (p42^{mapk}) or an antibody detecting both the phosphorylated and unphosphorylated forms (Promega Corporation, Madison, WI, USA). Antibodies against α-subunits of $G_{i1/i2}$ were from Calbiochem (La Jolla, CA, USA) and NEN™ Life Science Products (Boston, MA, USA). Antibody against the α-subunit of G_q was from NEN™ Life Science Products (Boston, MA, USA). Immunoreactive bands were visualised with ECL Western blotting detection reagents (Amersham International).

In vitro RNA synthesis
A 2'-amino pyrimidine modified hammerhead ribozyme having GUC as cleavage triplet corresponding to the nucleotide number 481 within the rat $G_{i2\alpha}$ mRNA [44] was synthesized by in vitro transcription using a short DNA template for the T7 RNA polymerase as previously described [15,45]. Subsequent to transcription ribozymes were PAGE gel-purified, ethanol-precipitated and then dissolved in water. The concentration was determined by assessment of absorbency at 260 nm. A non-cleaving form of the ribozyme was made by deleting the G12 from the catalytic core as indicated by lower case letter. The ribozyme short target was synthesized by in vitro tran-

scription of a synthetic DNA template with the T7 RNA polymerase. Subsequent to transcription, the gel-purified RNA was dephosphorylated by alkaline phosphate and then 5'-end labelled using T4 polynucleotide kinase and [γ-^{32} P]ATP. The ribozyme sequence is: 5'GGCAG-CACAGCU GAUGAGUCCGUGAGGACgAAACAGUGCGAACAGC3'. The sequence of the targeted site is: 5'GCUGUUCGCACUGUCCUGUGCUGCC3'. The cleavage site within the targeted sequences should be underlined.

In vitro cleavage activity of the $G_{i2\alpha}$ ribozyme
Cleavage reactions were performed at 37°C in a buffer containing 50 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$. Cleavage products were separated by electrophoresis on a 15 % polyacrylamide gel containing 7 M urea.

Transfection experiments
Cells were transfected with DOTAP- (25 μg/ml) formulated ribozyme 4–5 h after the time of plating. Only single transfections were used giving a ribozyme concentration in the culture medium of 2.5 μM. After 30–45 hours transfection time, immunoblotting experiments and assessment of ERK1/2 activity were performed.

List of abbreviations
Cyclic AMP, Adenosine 3',5'-cyclic monophosphate, DO-TAP, N- [1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium-methylsulphate; EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated kinase 1 and 2; G protein, guanine nucleotide binding (regulatory) protein; GPCR, G protein-coupled receptor; HGF, hepatocyte growth factor; MBP, myelin basic protein; PTX, pertussis toxin; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; RTK, receptor tyrosine kinase; Rz, ribozyme; Rzm, mutant ribozyme.

Acknowledgements

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