Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 35, Issue of August 27, pp. 24449–24452, 1999

GRFβ, a Novel Regulator of Calcium Signaling, Is Expressed in Pancreatic Beta Cells and Brain*

(Received for publication, June 15, 1999)

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By screening for genes expressed differentially in pancreatic beta cells, we have isolated a cDNA encoding GRFβ, a novel 178-amino acid protein whose N terminus is identical to that of GRF1, a calcium-dependent guanine nucleotide exchange factor, and whose C terminus is unrelated to known proteins. We show that both GRF1 and GRFβ are expressed selectively in beta cell lines, pancreatic islet cells and brain. Treatment of beta cell lines (βTC1 and HIT) with calcium ionophore led to a significant elevation in activity of the Ras signal transduction pathway, as determined by phosphorylation of extracellular signal-related kinase (ERK). Transfection of beta cells with a plasmid encoding a dominant negative variant of GRF1 led to 70% reduction in ERK phosphorylation, consistent with a role for GRF1 in calcium-dependent Ras signaling in these cells. To examine the possible function of GRFβ, cultured cells were transfected with a GRFβ expression vector. This led to a significant reduction in both GRF1-dependent ERK phosphorylation and AP1-dependent reporter gene activity. The results suggest that GRF1 plays a role in mediating calcium-dependent signal transduction in beta cells and that GRFβ represents a novel dominant negative modulator of Ras signaling.

Control of carbohydrate homeostasis is achieved largely through regulated secretion of insulin by pancreatic endocrine beta cells. The beta cell functions as a glucose sensor through specialized enzymatic machinery, including a high K_m glucose transporter (Glut2) and a high K_m glucokinase (reviewed in Refs. 1 and 2). Elevated blood glucose levels lead to increased intracellular ATP/ADP ratios, causing closure of ATP-dependent potassium channels, membrane depolarization, and a resultant increase in intracellular Ca^{2+} levels. This, in turn, is believed to cause increased insulin biosynthesis and secretion through mechanisms that are poorly defined. Elevated glucose concentrations can activate MAP[1] kinase pathways in cultured beta cells (3–5), accompanied by increased insulin secretion. However, specific inhibitors of MAP kinase showed little effect on insulin secretion (4–6). Since exposure of beta cells to glucose leads to nuclear translocation of ERK (4, 5), it is possible that one of the major effects of MAPK activation is modulation of transcription factor activity (7, 8), leading to changes in insulin biosynthetic rate. The precise actions of Ca^{2+} and the functional significance of elevated MAP kinase activity in beta cells remain to be established.

In order to better understand the molecular basis for cell-specific functions of beta cells, we have used representational difference analysis (9, 10) to identify genes expressed differentially between alpha and beta cells; this led to isolation of 26 cDNA clones (11, 12); using one of these, we have now isolated a clone encoding the novel protein GRFβ, which shares an identical region with GRF1, a calcium-dependent guanine exchange factor originally identified in brain cells (13, 14). In this report, we document the sequence of GRFβ, and demonstrate that both GRFβ and GRF1 are expressed preferentially in beta cells and brain. In addition we show that GRF1 plays a role in calcium signaling in beta cells and that GRFβ represents a naturally occurring, dominant negative form of GRF1.

EXPERIMENTAL PROCEDURES

Library Screening—A βTC1 cDNA library constructed in the vector λGT11 (15) was screened using a labeled DNA probe of clone 41 (12) generated by random priming. Inserts of positive clones were excised and subcloned to the vector pcDNA3. Sequencing was performed using an automated Applied Biosystems DNA sequencer.

Cell Lines—HIT T15 (16) (hamster insulinoma), NIH3T3, and 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). βTC1 cells (17) (mouse beta cell line) were grown in Dulbecco's modified Eagle's medium supplemented with 15% FCS and 2.5% horse serum.

Plasmids and Transfections—For reporter assay, chloramphenicol acetyltransferase (CAT) reporter plasmid containing nine copies of the AP1 binding sequence derived from the SV40 enhancer (4 μg), GRFβ expression vector (5 μg), and RSV-luciferase internal control plasmid (2 μg) were transfected using the calcium phosphate procedure (18). Glycerol shock (10%) was performed after 5 h. Cells were harvested 40 h later and extracts prepared for assay of luciferase (Promega luciferase reagent) and CAT activity. For phosphorylation analysis, HA-tagged ERK1 expression plasmid (4 μg) was transfected into HIT cells together with 12 μg of GRF1ΔC, encoding a dominant negative GRF1, prepared from a GRF1 expression vector by *Eco*RI digestion to remove the catalytic domain (19). Transfections into 293T cells were performed in six-well plates with 0.25 μg of HA-ERK plasmid, 0.25 μg of GRF1 expression vector, where indicated, and 3.5 μg of GRFβ expression vector or pcDNA3 parental vector alone.

RNA Preparation and RT-PCR Analysis—RNA was prepared using the TRI reagent kit (Molecular Research Center, Inc.) followed by DNase I treatment for 20 min at 37 °C. Mouse islets were prepared by collagenase digestion followed by Histopaque density gradient centrifugation (20). Islet RNA was prepared by the guanidinium thiocyanate procedure (21). RT-PCR was performed using the Access RT-PCR System (Promega) with 0.5 μg of total RNA.

The primers used to detect GRFβ were TCTAGCATCATGCAGAAAGCC (top strand primer corresponding to nt 130–150 of GRFβ) and GGGAGGAGAACCATAGATGG (bottom strand primer corresponding to nt 680–699). The predicted amplified fragment is 570 bp. The primers used to detect GRF1 were TCTAGCATCATGCAGAAAGCC (top strand primer, same as used for GRFβ, corresponding to nt 216–236 of

* This work was supported by a grant (to M. D. W.) from the Juvenile Diabetes Foundation International. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; FCS, fetal calf serum; CAT, chloramphenicol acetyltransferase; HA, hemagglutinin; ERK, extracellular signal-related kinase; RT-PCR, reverse transcriptase-polymerase chain reaction; nt, nucleotide(s); bp, base pair(s); PIPES, 1,4-piperazinediethanesulfonic acid; PH, pleckstrin homology; GEF, guanine nucleotide exchange factor.

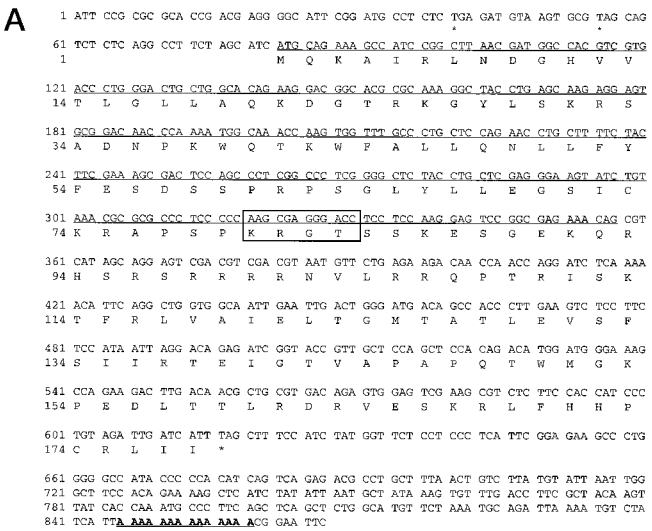

FIG. 1. *A*, DNA sequence and deduced amino acid sequence of GRFβ (GenBank™ accession number AF169826). The protein segment identical to mouse GRF1 ($CDC25^{Mm1}$) is *underlined*. An oligo(A) sequence (presumptive 3′ terminus) is shown in *bold*. A putative PKA site is *boxed*. * indicates stop codon. The sequence of GRFβ has been scanned against the data base at protein and DNA levels. Significant relatedness was observed only against GRF1 (GenBank™ accession number L20899), GRF2 (GenBank™ accession number U67326), and its species homologs. *B*, schematic map comparing GRFβ and GRF1. The region of identity between GRF1 and GRFβ is indicated by *vertical bars*. *PH*, pleckstrin homology domain. *PH1* and *PH2*, pleckstrin homology domains of GRF1. *C*, alignment of GRFβ (nt 301–375), GRF1 (nt 445–519; GenBank™ accession number L20899), and the genomic clone corresponding to GRF1/GRFβ (nt 901–975; GenBank™ accession number U55232). Also shown is the splice donor consensus sequence (23). The sequence GT, corresponding to the invariant nucleotides found at the 5′-end of introns, is shown in *bold letters*.

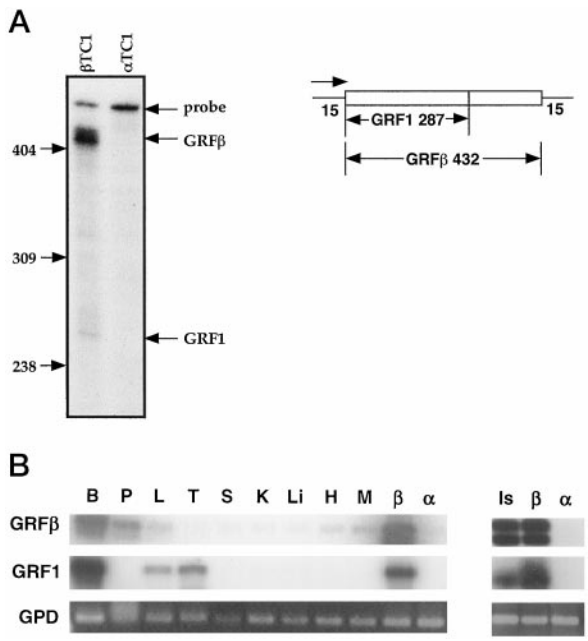

FIG. 2. *A*, RNase protection assay. Antisense probe was mixed with 20 μg of total RNA from βTC1 and αTC1. Following RNase digestion, the products were resolved on sequencing gel and exposed to x-ray film. The scheme indicates the expected sizes of the intact antisense probe (462 nt) and the predicted protected bands corresponding to GRFβ RNA (432 nt) and GRF1 (287 nt). The *arrows* indicate the migration of labeled DNA size markers. *B*, RT-PCR analysis of RNA extracted from the following mouse tissues: brain (*B*), pancreas (*P*), lung (*L*), testis (*T*), spleen (*S*), kidney (*K*), liver (*Li*), heart (*H*), muscle (*M*), βTC1 (β), αTC1 (α), and islets (*Is*). 0.5 μg total RNA (left panel) or 5 ng (*right panel*) was subjected to RT-PCR followed by hybridization with the indicated probes. The *lower panel* shows the ethidium bromide staining of PCR reactions performed with glyceraldehyde-3-phosphate dehydrogenase (*GPD*) primers, as a control for the efficiency of the RT-PCR reaction.

mouse GRF1) and GCGCAGGAAGTTGTTGACAAGG (bottom strand primer corresponding to nt 1014–1035). The predicted amplified fragment is 820 bp. The hybridization probe used for GRFβ was a restriction fragment corresponding to the GRFβ-specific region (nt 502–599). The hybridization probe used for GRF1 was a restriction fragment corresponding to nt 216–478.

One-fifth of the reaction was analyzed by Southern blot as described previously (12).

RNase Protection Assay—DNase I-treated RNA samples (20 μg) were mixed with a ^{32}P-labeled antisense probe produced by *in vitro* transcription reaction using T3 RNA polymerase. Samples were processed as described (21) except that RNase digestion was performed using 6 units of RNase ONE (Promega) in a total volume of 300 μl.

Immunoblot Analysis—Cells extracts (21) were resolved on 12% SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis using the following antibodies: rabbit anti-ERK (1:30,000), mouse antiphosphorylated ERK (1:30,000), and mouse anti-HA epitope (1:500). Quantitation was performed on autoradiograms in which signals were within the linear response range using a Bio-Rad model GS-690 densitometer and Multi-Analyst software. Relative ERK phosphorylation for each sample was determined from the ratio of absorbance using anti-P ERK to that obtained using anti-HA or anti-ERK. Statistical significance was evaluated using two-tailed Student's *t* test.

RESULTS

Cloning of GRFβ—Using clone 41, one of the products of the representational difference analysis (12), we screened a βTC1 cDNA library and obtained a 859-bp cDNA with a complete open reading frame of 178 amino acids (Fig. 1*A*). A second cDNA clone isolated in the screen was identical in sequence except that it was truncated at the 5′-end. Data base comparisons using the 859-bp cDNA showed that its 5′ portion is identical at the DNA level to the Ras guanine exchange factor, GRF1 ($CDC25^{Mm1}$) (Fig. 1*B*) (13, 14, 22). We term the encoded 178-amino acid protein GRFβ. Its N-terminal 92 amino acids are identical to the N terminus of mouse GRF1 (Fig. 1*B*), whereas the C-terminal 86 amino acids show no significant resemblance to known proteins. Thus GRFβ lacks all the characteristic motifs of GRF1, except for the N-terminal pleckstrin homology (PH) domain (Fig. 1*B*), half of which is identical to the corresponding portion of GRF1 and half of which derives from the novel C-terminal portion. The complete identity between 5′ regions of GRFβ and GRF1 at the DNA level suggests that these represent alternate splice products of a single gene. Consistent with this is the fact that both GRF1 and GRFβ sequences match perfectly the sequence of a mouse genomic clone U55232 up to a position where all three sequences diverge completely (Fig. 1*C*). The sequence of the genomic clone at this location matches exactly the splice donor consensus sequence (23) (Fig. 1*C*). To characterize GRF1 and GRFβ transcripts, we performed RNase protection assay, using a 462-nt antisense probe derived from the GRFβ DNA sequence, which

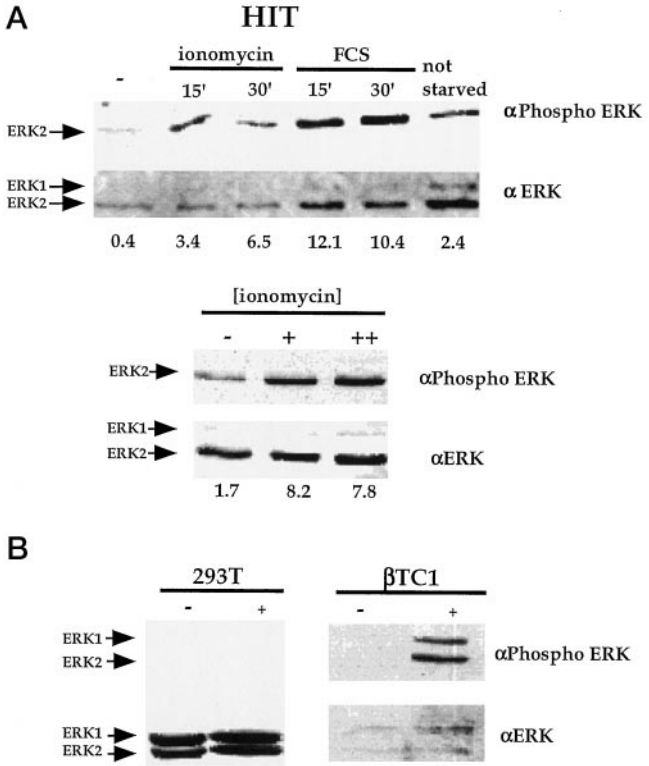

FIG. 3. **ERK activation in different cell lines.** Protein extracts (10 μg) were subjected to immunoblot using antibodies recognizing the phosphorylated form of ERK (*αPhospho ERK*) and antibodies recognizing both phosphorylated and nonphosphorylated ERK (*αERK*). *A*, hamster beta cells (*HIT*) were incubated overnight in medium containing 0.1% FCS and then incubated with ionomycin (5 μM) or FCS (10%) for the indicated time (*upper panels*) or with A23187 for 20 min (+ = 4 μM, ++ = 8 μM) (*lower panels*). Numbers shown are the ratios between the signals obtained with αPhospho ERK and αERK as measured by densitometer. *B*, 293T cells and βTC1 cells were incubated overnight in medium containing 0.1% FCS and then incubated for 5 min in the absence or presence of 5 μM ionomycin.

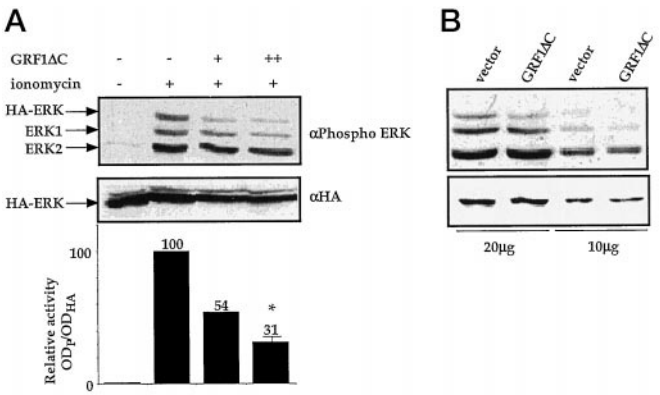

FIG. 4. **Inhibition of calcium activation by GRF1ΔC, the dominant negative form of GRF1.** *A*, HIT cells were transfected with HA-ERK expression vector, together with 6 μg (+) or 12 μg (++) of expression vector encoding a truncated form of GRF1 lacking the catalytic domain (GRF1ΔC) or control vector. Cells were incubated overnight in medium containing 0.1% FCS and for a further 5 min in the presence or absence of 5 μM ionomycin. Cell extracts were subjected to immunoblot using αPhospho ERK and αHA and signals were measured by a densitometer. The histogram shows mean values for normalized HA-ERK phosphorylation, determined as described under "Experimental Procedures." *, mean ± S.E. ($n = 5$); $p < 0.05$. *B*, HIT cells were transfected with HA-ERK, in the presence of expression vector encoding GRF1ΔC or control vector. Cells were incubated overnight in medium containing 0.1% FCS and for a further 5 min in the presence of 5 μM ionomycin. Cell extracts (20 μg or 10 μg of protein) were subjected to immunoblot as described for *A*.

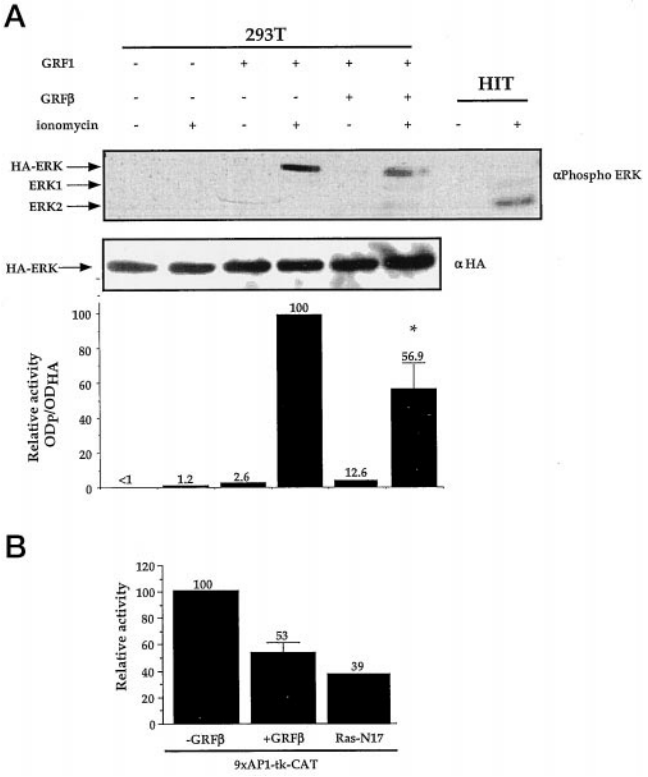

FIG. 5. **Effect of GRFβ on Ras signaling.** *A*, 293T cells were transfected with a plasmid encoding HA-ERK and the indicated expression vector (+) or an empty vector (−). Cells were then incubated in the absence or presence of ionomycin, collected, and subjected to immunoblot with the indicated antibodies. The same amount of extract from HIT cells, treated with the same ionomycin concentration, was loaded on the gel as a control. The histogram shows mean values for normalized HA-ERK phosphorylation, determined as described under "Experimental Procedures." *, mean ± S.E. ($n = 4$); $p < 0.05$. *B*, NIH3T3 cells were transfected with a CAT reporter plasmid containing nine copies of the AP1 binding site together with expression vectors encoding GRFβ or Ras (N17), a dominant negative mutated Ras, and RSV-luciferase as an internal control of transfection efficiency. Results are normalized CAT activity (mean ± S.E. ($n = 3$)) relative to cells transfected with the CAT reporter alone.

contained a 287-nt identity with GRF1 (Fig. 2*A*). A distinct band, corresponding in size to the expected 432-nt GRFβ protected fragment was obtained using βTC1 RNA, but not with αTC1 RNA (Fig. 2*A*). A faster migrating band corresponding to the expected 287-nt GRF1 protected fragment was also obtained with βTC1 but not with αTC1 RNA (Fig. 2*A*).

To study the tissue distribution of GRFβ, we performed RT-PCR analysis with RNA from different tissues using primers specific for each splice variant (Fig. 2*B*). Using the GRFβ primers two bands were observed. By isolating and sequencing these bands separately we showed that the upper band represents GRFβ and the lower band a truncated version lacking nucleotides 355–507. The highest levels of GRFβ mRNA were seen in βTC1 cells and in the brain, while GRF1 was expressed at highest levels in the brain and to a lesser extent in βTC1 cells. Both transcripts appeared in substantial amounts in mouse islets, at similar levels as in βTC1. Taken together, the RNase protection and the RT-PCR analyses indicate relatively high levels of GRFβ and GRF1 in the brain and beta cells. However, while GRF1 was expressed at higher levels in the brain than in beta cells, GRFβ showed the opposite pattern: higher expression in beta cells than in the brain.

GRF1 Involvement in ERK Activation by Calcium in Beta Cells—To examine the possible role of GRF1 in beta cells, we treated HIT cells with calcium ionophores. We observed strong

activation of the Ras pathway, as measured by the levels of activated ERK1/2, almost to the level of the activation obtained by 10% FCS (Fig. 3*A*). Activation was also observed following incubation with ionophore A23187 (Fig. 3*B*), KCl (50 mM for 10 min), and glucose (15 mM for 10 min) (data not shown). Furthermore, ionomycin led to activation of ERK also in βTC1 cells (Fig. 3*B*), but not in the cell line 293T, which lacks endogenous GRF1 (14) (Fig. 3*B*).

To determine the role of GRF1 in the calcium-dependent activation, we constructed a plasmid encoding a truncated GRF1 protein, GRF1ΔC, lacking its catalytic domain, which is expected to display dominant negative activity (19). When HIT cells were transfected with GRF1ΔC, ionomycin-dependent phosphorylation of co-transfected epitope-tagged HA-ERK was reduced by approximately 70% (Fig. 4, *A* and *B*).

GRFβ Is an Inhibitor of the Ras Signaling Pathway—We tested whether GRFβ (which contains the N-terminal 92 amino acids of GRF1) can affect the Ras/MAPK pathway. In 293T cells co-transfected with GRF1 and GRFβ, the extent of ionomycin-dependent ERK activation was significantly lower than in cells transfected with GRF1 alone (Fig. 5*A*). Co-transfection of serum-treated NIH3T3 cells with an AP1 reporter plasmid and an expression vector encoding the intact GRFβ protein produced a 2-fold lower CAT activity compared with co-transfection with control vector, almost to the inhibition level obtained with the dominant negative Ras variant N17 (Fig. 5*B*) (24). Thus GRFβ behaves as an inhibitor of both GRF1-mediated Ras/ERK signal transduction and of serum-dependent MAPK signaling pathways leading to AP1 activation.

DISCUSSION

Proteins of the Ras family play a crucial role in cell proliferation, differentiation, and tumorigenesis (25). Their activity depends on the presence of guanine exchange factors (GEFs), which are essential to promote dissociation of GDP-bound Ras to facilitate binding of GTP. The Sos-related GEF proteins are distributed broadly in many cell types and typically activate Ras following activation of growth factor receptor and a membrane recruitment mechanism mediated by the adapter protein Grb2 (26). The GRF-related GEFs, on the other hand, are less widely distributed; GRF1 is present in neuronal cells (14) and additional tissues and tumor cells (27); it activates Ras in a calcium-dependent fashion (14, 28). Calcium-dependent activation is mediated through calmodulin binding to an N-terminal IQ domain; however the precise mechanism leading to calcium-dependent activation remains to be determined and involves also pleckstrin and coiled-coil domains of the protein (28). A number of naturally occurring splice variants have been identified previously (29); these apparently represent N-terminal truncations rather than C-terminal truncations. The physiological significance of these truncations has not been addressed.

In this study, we have demonstrated for the first time that GRF1 is expressed in pancreatic beta cells. The inhibitory effect of a truncated dominant negative form of GRF1 suggests that GRF1 is essential for activation of calcium-dependent MAP kinase activity in the beta cell. We have further identified GRFβ, a novel 178-amino acid protein comprising the N-terminal 92 amino acids of GRF1, including a portion of the PH1 domain of GRF1 and 86 unrelated amino acids. GRFβ RNA was detected in beta cells and brain. We provide evidence that GRFβ represents a naturally occurring dominant negative form of GRF1, which may play a role in regulating the normal function of GRF1. The mechanism whereby GRFβ inhibits signaling is not clear. On the one hand, the N terminus of GRF1 has been shown in *in vitro* studies to inhibit the catalytic activity of GRF1 (30), perhaps through intra-molecular rearrangement. On the other hand, *in vivo* studies clearly show that deletion of the N-terminal PH1 domain leads to both intracellular redistribution of the protein and loss of calcium responsiveness (28). Probably the PH1 domain has multiple functions, including a role in proper intracellular localization.

The physiological role of GRF1 is not yet established. Mice lacking the GRF1 gene display impaired memory consolidation and slow growth (31, 32). The presence of GRF1 in beta cells and the ability of a dominant negative form to inhibit calcium-dependent MAPK activity suggest a key role for GRF1 in beta cell calcium signaling. GRFβ may function *in vivo* to modulate GRF1-dependent Ras activation. It remains to be established how GRF1 and GRFβ are regulated to activate MAPK activity and how MAPK activity influences beta cell function.

Acknowledgments—We thank C. Ezerzer, V. Ablamunits, and S. Weiss for advice and assistance; Dr. L. Feig for kindly providing the GRF1 expression plasmid; Dr. S. Michaeli for advice; and Dr. S. Lev for helpful comments on the manuscript.

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