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Expression of cAMP-Regulated Guanine Nucleotide Exchange Factors in Pancreatic β-Cells

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The insulinotropic hormone glucagon-like peptide-1 (GLP-1) binds to a Gs-coupled receptor on pancreatic β-cells and potentiates glucose-induced insulin secretion, insulin gene transcription, and β-cell growth. These stimulatory effects have been attributed to the elevation of intracellular cAMP levels, though it is now apparent that some stimulatory effects of GLP-1 occur independently of the cAMP-mediated activation of protein kinase A (PKA). The nature of this alternative, PKA-independent signaling pathway remains unknown. Here we present evidence for the expression of type 1 and type 2 cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs) in β-cells. GEFs are activated by their binding of cAMP. Because cAMP-GEFs activate Ras/MAPK proliferation signaling pathways, they may play an important role in PKA-independent, GLP-1-mediated, signaling pathways in the regulation of β-cell growth and differentiation.

Key Words: **cAMP-GEF; GLP-1; MAPK; IDX; PDX; Rap1A; β-cell.**

The GLP-1 receptor (GLP-1R) in pancreatic β-cells is coupled to Gs and stimulates cAMP production and activation of protein kinase A [PKA (1, 2)]. Activation of the GLP-1R results in the potentiation of glucose-dependent insulin secretion, activation of insulin gene transcription, and stimulation of β-cell growth (3–5). All of these effects are physiologically important in both normal, nondiabetic subjects and in the therapeutic efficacy of exogenous GLP-1 in type 2 diabetics (4, 6, 7). The effector sites for GLP-1-mediated signaling involved in the potentiation of insulin secretion include: (i) Enhanced closure of ATP-sensitive K^+ (K_{ATP}) channels (8); (ii) activation of nonselective cation channels (9, 10); (iii) potentiation of intracellular Ca^{2+} release (11–13); (iv) activation of L-type Ca^{2+} channels (14); and (v) effects on exocytosis (15). The closure of K_{ATP} channels by GLP-1 occurs by a cAMP-independent mechanism (16) that may involve regulation of the channels by the βγ subunits of Gs (17); a βγ binding site has been identified in SUR1 (18), a subunit of the β-cell K_{ATP} channel (19). Similarly, stimulation of the rat insulin gene promoter by GLP-1 is partially PKA-independent, an effect that may result from GLP-1-mediated stimulation of phosphatidylinositol 3-kinase (PI3K) or of mitogen-activated protein kinase (MAPK) signaling pathways (20). G-protein coupled receptors (GPCRs) can activate PI3K through the βγ dimer of heterotrimeric G-proteins (21) and this pathway may represent the mechanism by which GLP-1 activates PI3K and downstream effectors such as the transcription factor IDX-1 (PDX-1) to regulate insulin gene activity and also β-cell growth and differentiation (22). The MAPK signaling pathway also plays a role in the regulation of cell proliferation and also is activated by glucose and other secretagogues, including GLP-1, in β-cells (23, 24). GLP-1 and intracellular cAMP potentiate the glucose-induced activation of MAPK, but have no effect in the absence of glucose (23); however, cAMP alone stimulates MAPK-kinase [MEK-1, (23)]. Cyclic-AMP activates MAPK through a B-Raf and Rap1-dependent pathway initiated by PKA-dependent phosphorylation of Rap1 (25). An alternative signaling pathway for the activation of MAPK is formed by the cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs), cAMP-binding proteins that selectively activate Rap1A (26). The tissue distribution pattern of cAMP-GEFs indicates their expression in the pancreas (26) and therefore we reasoned that these proteins represent a potentially important pathway for the regulation of β-cell growth and differentiation by GLP-1. In view of this possibility, we examined β-cells for the expression cAMP-GEFs by PCR analysis. Here we re-

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TABLE 1

PCR Primer Pairs Used to Examine Expression of cAMP-GEFs in β-Cells

	PCR primer pairs	Sequence No.	
	cAMP-GEF1	U78167	Predicted size
1	5′ TAAGCCTGGGGTGGTGGTAG 3′	4–23	837
	5′ TAGTTCCCGTTTCACCGAGTTAGA 3′	818–840	
2	5′ GCCGCCCTCCTGCACCACTTC 3′	1376–1396	468
	5′ AGCTGCCATCACTTCCCTCACG 3′	1822–1843	
3	5′ CACAGCACCGCGCCTCTATCACC 3′	2651–2673	411
	5′ CCTCAATGGCCCCTTTTCCTT 3′	3041–3061	
	cAMP-GEF2	U78517	
1	5′ CAGAACGGTGCGCTACTACAGGA 3′	716–738	408
	5′ TGCCAACCGTGAAGAAGATGACTA 3′	1100–1123	
2	5′ GTGGGGACGTTTGAACTGATGAGC 3′	135–158	440
	5′ AGCCTGTACGCCTTGTGATTTCTG 3′	551–574	
3	5′ GCCAGAACGGTGCGCTACTACA 3′	714–735	595
	5′ ACCCTCCCCAGAACCCAGACC 3′	1288–1308	

port finding the expression of both cAMP-GEF1 and cAMP-GEF2 in rat islets and β-cell lines.

MATERIALS AND METHODS

PCR reactions were performed on cDNA generated in the presence or absence of reverse transcriptase using primers designed against the published sequences of cAMP-GEF types 1 and 2 as detailed in Table 1. PCR reactions for cAMP-GEF1 were performed with denaturation at 94°C for 30 s followed by 36 cycles for 30 s at 94°C, 30 s at 55°C, and 3 min at 72°C with a final extension for 10 min at 72°C. Cycling parameters for cAMP-GEF2 were 30 s at 95°C, 60 s at 57°C, and 90 s at 72°C for 25 cycles with a final extension time of 5 min. PCR products were then run on 1% Agar gels and bands excised and cloned into pCRII (Invitrogen, Carlsbad, CA). Vectors containing the inserts were transformed into competent cells and antibiotic-resistant colonies were selected. Mini-prep extracts (Qiaprep spin kit, Qiagen, Santa Clarita, CA) were sequenced and subject to Blast analysis against the GenBank database.

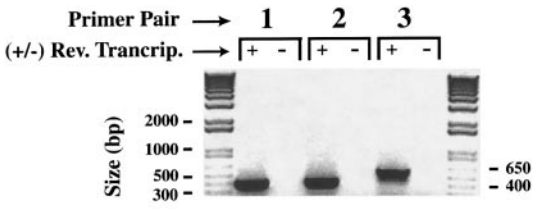
Western blots for Rap1A were performed using whole cell protein extracts in RIPA lysis buffer. Protein samples were run on SDS–PAGE gels and transferred to PVDF membranes. Membranes were blocked with 5% nonfat milk in TBS with 0.2% Tween 20 and probed with anti-Rap1A-C17 (Santa Cruz), diluted 1:200 in TBS, 0.2% Tween 20, and 2% nonfat milk for 1 h. The membrane was then washed (3×) with 80% TBS, 20% RIPA, 0.2% Tween 20, and then an HRP-coupled anti-goat secondary antibody (Santa Cruz; 1:20,000 in 50% TBS, 50% RIPA, and 0.2% Tween 20) was added for 40 min. The blot was then washed (as above) and developed by adding Luminol reagent (Santa Cruz) and exposing to film which was then scanned on a Molecular Dynamics densitometer.

RESULTS AND DISCUSSION

The three PCR primer pairs for cAMP-GEF1 all gave products of the predicted size from rat islet cDNA. Sequencing of these PCR products showed that they were identical to the GenBank sequence (U78167) for cAMP-GEF1 from rat brain (26). The product using primer pair #3 for cAMP-GEF1 (Table 1) with mouse-derived βTC6 cell cDNA was 95% identical to the rat cDNA sequence and the translated sequence contained an open reading frame matching 65/66 amino acids (A840G difference) at the 3′ end of the predicted full length rat protein sequence. These data suggest that the cAMP-GEF1 is expressed in rat islets and in β-cell lines, consistent with Northern blot data showing expression in the pancreas (26). No products of the appropriate size were obtained using hamster-derived HIT-T15 cell cDNA and products that were sequenced were unrelated to cAMP-GEF. The failure to generate PCR products from HIT cell cDNA probably reflects species differences between the rat-specific primers and hamster cAMP-GEF sequences.

The three primer pairs for cAMP-GEF2 also amplified bands of the predicted size (Fig. 1) from rat-derived INS-1 cell cDNA. The sequences of these products were identical to the rat brain sequence (U78517) recorded in GenBank (26) and these data indicate that β-cells express both type-1 and type-2 cAMP-GEFs.

The cAMP-GEFs specifically activate the Ras superfamily member Rap1A after cAMP binds to them (26). To further confirm that the cAMP-GEFs expressed in β-cells might play an important physiological role, we performed Western immunoblotting of various whole cell lysates with an antibody specific for Rap1A (Fig. 2). A Rap1A immunoreactive band was observed at about 21 kDa, the predicted size of the Rap1A protein. This immunoreactive band was observed in protein extracts from human islet, INS-1 cells, mouse-derived MIN6 cells and HIT-T15 cells. These observations confirm previous reports of the expression of Rap1 in mouse-derived βTC cells (27) and in human and rat islets,



Primer Pair	Sequence Amplified	Size (bp)
1	716 to 1123	408
2	135 to 574	440
3	714 to 1308	595

FIG. 1. Expression of cAMP-GEF2 in INS-1 cells. PCR reactions were performed using cDNA from INS-1 cells with three pairs of primers designed to amplify cAMP-GEF2 (Table 1). These three sets of primers each amplified specific bands of the predicted size based on the GenBank sequence U78517 for rat brain cAMP-GEF2. Sequencing of these bands revealed 100% identity with the published sequence indicating expression of cAMP-GEF2 in β-cells.

HIT-T15 and INS-1 cell lines (28) where it undergoes carboxyl methylation in response to nutrient stimulation and appears to play a role in glucose-stimulated insulin secretion.

Our results suggest that cAMP-GEFs act as downstream effectors of GLP-1-receptor signaling in β-cells (Fig. 3) and form a divergence point for cAMP-signaling through PKA or the cAMP-GEFs, both of these pathways may be important in the potentiation of glucose-induced insulin secretion by GLP-1. A potential mechanism for cAMP-GEFs in regulating insulin secretion is suggested by reports that Rap1A activates protein kinase C (PKC) (29) and thus the cAMP-GEF pathway may represent a convergence point for cross-talk between cAMP-mediated and PKC-signaling pathways. Additionally, Rap1A activates B-Raf leading to activation of MAPK (25). Activation of PKC by phorbol esters has also been reported to activate MAPK in β-cells (23) and thus GLP-1 may activate MAPK through cAMP-induced activation of cAMP-GEFs, Rap1A and then through two convergent pathways

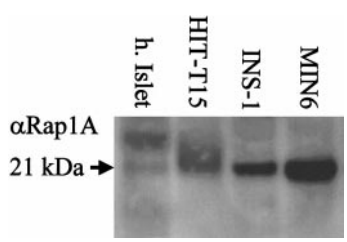


FIG. 2. Immunoblotting of β-cell protein lysates indicates expression of Rap1A. An antibody specific for human Rap1A was used to perform immunoblotting studies to identify the presence of Rap1A, the downstream effector of the cAMP-GEFs. Each lane was loaded with an equal amount of protein from various cell types as follows: lane 1, human islets; lane 2, HIT-T15; lane 3, INS-1; lane 4, MIN6. A strong band at about 21 kDa, the predicted migration size for Rap1A, can be seen in the three insulinoma cell lysates with a weaker band visible in human islet cell lysate.

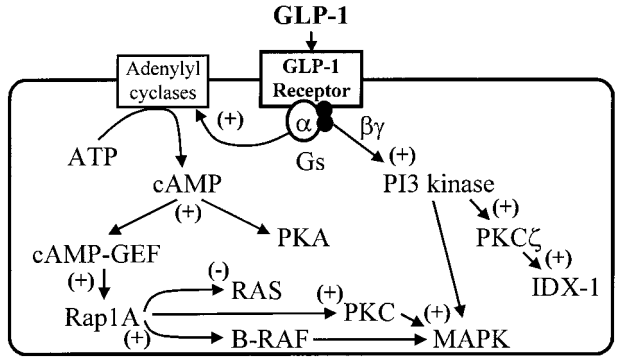


FIG. 3. A model for GLP-1-receptor signal transduction pathways in β-cells. Binding of GLP-1 to its receptor activates heterotrimeric Gs proteins, the Gsα subunit activates adenylyl cyclases to produce cAMP while the Gs$\beta\gamma$ dimer activates PI3K, which subsequently activates the homeodomain transcription factor IDX-1 (PDX-1) and possibly also activates MAPK. Cyclic AMP activates PKA and also binds to cAMP-GEFs which selectively activate Rap1A. Rap1A inhibits Ras and activates PKC and MAPK. The activation of both IDX-1 and MAPK by these signaling pathways may play an important role in mediating the growth factor-like effects of GLP-1.

downstream of Rap1A, PKC and B-Raf (Fig. 3). However, it should be noted that other reports failed to observe stimulation of MAPK by phorbol esters in β-cells (30) and this pathway therefore remains speculative. An alternative possibility is that PI3K has been shown to activate MAPK (31) and the GLP-1-induced activation of MAPK may, therefore, occur through Gs$\beta\gamma$-mediated activation of PI3K. Rap1A, the downstream effector of the cAMP-GEFs (26), is an inhibitor of Ras signaling. GLP-1 acting through cAMP and the cAMP-GEFs may thereby inhibit Ras. This circumstance would form a parallel pathway with the Ca^{2+}-dependent GRFβ, a dominant negative modulator of Ras signaling expressed in β-cells (32), likely to be activated by the elevation of intracellular Ca^{2+} observed in response to stimulation with GLP-1 and glucose (9, 10, 13).

In summary, β-cells express both type 1 and type 2 cAMP-GEFs. These two proteins may play an important role in mediating the β-cell growth promoting effects of GLP-1. We propose that these growth promoting effects of GLP-1 result from the activation of MAPK signaling in parallel with the activation of the transcription factor IDX-1. These two pathways may be activated by GLP-1R-induced activation of Gs through (i) Gsα stimulating the production of cAMP and the activation of cAMP-GEFs, Rap1A, PKC, and MAPK and (ii) Gs$\beta\gamma$ subunits activating PI3K, PKCζ, and IDX-1 (Fig. 3).

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