Glucose-dependent Insulinotropic Polypeptide Activates the Raf-Mek1/2-ERK1/2 Module via a Cyclic AMP/cAMP-dependent Protein Kinase/Rap1-mediated Pathway*

Received for publication, May 22, 2002, and in revised form, July 1, 2002 Published, JBC Papers in Press, July 22, 2002, DOI 10.1074/jbc.M205055200

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The gastrointestinal hormone, glucose-dependent insulinotropic polypeptide (GIP), 1 is one of the most important regulators of insulin secretion following ingestion of a meal. GIP stimulates insulin secretion from the pancreatic β-cell via its G protein-coupled receptor activation of adenylyl cyclase and other signal transduction pathways, but there is little known regarding subsequent protein kinase pathways that are activated. A screening technique was used to determine the relative abundance of 75 protein kinases in CHO-K1 cells expressing the GIP receptor and in two pancreatic β-cell lines (βTC-3 and INS-1 (832/13) cells). This information was used to identify kinases that are potentially regulated following GIP stimulation, with a focus on GIP regulation of the ERK1/2 MAPK pathway. In CHO-K1 cells, GIP induced phosphorylation of Raf-1 (Ser-259), Mek1/2 (Ser-217/Ser-221), ERK1/2 (Thr-202 and Tyr-204), and p90 RSk (Ser-380) in a concentration-dependent manner. Activation of ERK1/2 was maximal at 4 min and was cAMP-dependent protein kinase-dependent and protein kinase C-independent. Studies using a β-cell line (INS-1 clone 832/13) corroborated these findings, and it was also demonstrated that the ERK1/2 module could be activated by GIP in the absence of glucose. Finally, we have shown that GIP regulation of the ERK1/2 module is via Rap1 but does not involve Gβγ subunits nor Src tyrosine kinase, and we propose that cAMP-based regulation occurs via B-Raf in both CHO-K1 and β-cells. These results establish the importance of GIP in the cellular regulation of the ERK1/2 module and identify a role for cAMP in coupling its G protein-coupled receptors to ERK1/2 activity in pancreatic β-cells.

The incretin hormone, glucose-dependent insulinotropic polypeptide (GIP), 1 is a potent stimulator of insulin secretion and an essential component of the enteroinsular axis in mammals (1–3). GIP transduces its biological effects by interacting with its G protein-coupled receptor (GPRC), a member of the class II GPRC superfamily that includes receptors for glucagon, glucagon-like peptide-1 (GLP-1), secretin, pituitary adenyl cyclase-activating protein (PACAP), and vasoactive intestinal polypeptide (4–7). All of these peptide hormone receptors are coupled to the production of cyclic AMP (cAMP) and have been shown to activate the mitogen-activated protein kinases (MAPK) ERK1 and -2 (extracellular signal-regulated kinase) (8–12). GIP receptor activation has been shown to induce MAPK activity and was only shown recently (13, 14) to activate ERK1/2 in a β-cell model (INS-1). However, elucidation of the mode of coupling between class II receptors and MAPK activation has been largely unexplored, and no distinct biological function has been definitively linked to its activation in β-cells.

At least five distinct mammalian MAPK signaling modules have been defined and include ERK1/2, p38 MAPK, Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), ERK3, and ERK5 (BMK1; big MAPK) (15–18). These cytoplasmic protein-serine/threonine kinases are cellular regulators of numerous processes including gene transcription, differentiation, and proliferation (18). The core module of the MAPK pathways consists of three kinases; the kinases directly upstream of the MAPK are members of the Mek family, and these are activated by the Mek kinases (Mekk). Originally, the ERK1/2 module was shown to be coupled to growth factor activation of receptor-tyrosine kinases (RTKs). However, coupling of GPRCs to this MAPK cascade has now been demonstrated for Gs, Gi, Gα11, and Gβγ classes of G proteins (19, 20). The class II Gα-coupled receptors for PACAP and vasoactive intestinal polypeptide have been shown to regulate ERK1/2 activity via the cAMP-dependent protein kinase A (PKA) pathway (20). At the time they were conducted, however, these studies could not fully explain the interaction of this pathway with the ERK1/2 module.

Signals conveyed through Gαs-coupled GPRCs are extremely diverse. Although these receptors generally influence cellular events through their regulation of adenylyl cyclase, it is now evident that other proximal signals can mediate regulation of the ERK1/2 module. Depending on the cell type, such receptors change the cellular signal-regulated kinase kinase; p38, p38 MAPK; Mos, v-Mos Moloney murine sarcoma viral oncogene homolog 1; Mst1, mammalian sterile 20-like 1; PAK, p21 activated kinase; Raf1, oncogene Raf1; RafB, v-Raf murine sarcoma viral oncogene homolog B1; RSK, 90-kDa ribosomal S6 kinase; Src, oncogene Src; ZAP70, c-chain-associated protein kinase; GPCR, its G protein-coupled receptor; MOPS, 4-morpholinepropanesulfonic acid; ANOVA, analysis of variance; CHO, Chinese hamster ovary; AA, arachidonic acid; PMA, phorbol myristic acid.

* This work was supported in part by the Canadian Institutes of Health Grant MOP-13192 (to R. A. P. and C. H. S. M.). 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.

‡ Supported by the Canadian Institutes of Health and the Michael Smith Foundation.

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1 The abbreviations used are: GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; PACAP, pituitary adenylyl cyclase-activating protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK/SAPK, Jun N-terminal kinase/stress-activated protein kinase; RTK, receptor tyrosine kinase; PKA, protein kinase A; PKC, protein kinase C; Cot, Tpl-2, cancer Oska thyroid oncogene; Mek, mitogen-activated protein kinase/extra-
GIP Activates the ERK1/2 MAPK Module via cAMP PKA/Rap1

have been shown to activate MAPK via PKA, protein kinase C (PKC), phosphatidylinositol 3-kinase, and/or RTKs (20, 21). Furthermore, the prototypical GPCR, the β2-adrenergic receptor, has even been reported to undergo a switch from Goα to Goγ, with subsequent activation of ERK1/2 regulated by Gβγ subunits (22, 23). Finally, Goα has been shown to interact directly with, and activate, Src (24), and this protein-tyrosine kinase can regulate the ERK1/2 module due to its ability to transactivate RTKs and influence the activity of the small G protein Ras (via Grb2 and Sos) (23).

The cascades involving cAMP and MAPK are highly conserved, stringently regulated, and versatile signal transduction pathways essential to numerous physiological functions. The paradigm of the cAMP and PKA pathway now includes additional cAMP effectors such as CAMP GTPase exchange factors, and the small GTPases Rap1 and Rap2, thus identifying the mechanism by which GIP-stimulated cAMP production positively regulates ERK1/2.

For the first time, GIP is shown to influence the activity of all four MAPK cascades, it is evident that four modules (Raf1/B, MEKk, and Rap1) are expressed in the cell of neuronal cells, and the commonly used HEK and CHO-K1 cell lines (30). These effectors form the basis for cAMP interaction with the MAPK module. Initial studies on the interaction of these two pathways documented an inhibitory effect of cAMP on ERK1/2 module activation by growth factors (30), but cAMP has since been shown to stimulate ERK1/2 activity in several cell types including 3T3-F442A adipocytes, ovarian granulosa cells, pituitary and neuronal cells, and the commonly used HEK and CHO-K1 cell lines (30). This ability of cAMP to activate ERK1/2 depends on which isoform of Raf (the Mekk) is expressed in the cell of interest (31). In this manner, cAMP has been shown to regulate either transient or sustained activation of ERK1/2 in the neuronal PC12 cell line (32).

In the present study, we sought to determine the mechanism by which GIP activates ERK1/2. In doing so, we have characterized a GIP receptor-transfected CHO-K1 (rGIP-15) cell line and two β-cell lines by screening for the relative expression of 75 different protein kinases. From this, we were able to conclude that at least four MAPK modules are potentially regulated by GIP and that the pancreatic β-cell lines, βTC-3 and INS-1 (832/13) cells, express 70 and 60% of the protein kinases examined, respectively, at similar levels to the rGIP-15 cells. For the first time, GIP is shown to influence the activity of all known isoforms of the ERK1/2 module, including Raf-1, Mek1/2, ERK1/2, and p90 RSK in CHO-K1 cells and in the INS-1 (clone 832/13) β-cell line. This regulation is shown to occur via PKA and Rap1, thus identifying the mechanism by which GIP-stimulated cAMP production positively regulates ERK1/2.

Experimental Procedures

Tissue Culture, Cell Transfections, and Materials—Chinese hamster ovary cells (CHO-K1), cultured in Dulbecco’s modified Eagle’s medium/ Ham’s F-12 (Invitrogen) supplemented with 10% newborn calf serum (Cansera, Rexdale, Ontario, Canada), were stably transfected with the wild type rat GIP receptor as described previously (5). The CHO-K1 cell line obtained by pooling clones was termed rGIP-15 and has been shown previously (33) to express receptors at similar levels to high level expressed GIP, and that the pancreatic β-cell lines, βTC-3 and INS-1 (832/13) cells, express 70 and 60% of the protein kinases examined, respectively, at similar levels to the rGIP-15 cells. For the first time, GIP is shown to influence the activity of all known isoforms of the ERK1/2 module, including Raf-1, Mek1/2, ERK1/2, and p90 RSK in CHO-K1 cells and in the INS-1 (clone 832/13) β-cell line. This regulation is shown to occur via PKA and Rap1, thus identifying the mechanism by which GIP-stimulated cAMP production positively regulates ERK1/2.

Quantification of the immunoreactive bands on the Kinetwork blots (trace quantity) with ECL detection was performed with a Bio-Rad FluoroS Max Imager and Bio-Rad Quantity One software. This led to follow up studies in which the phosphorylation states of Raf-1, Mek1/2, p90 RSK, and Elk-1 were assessed. Phospho-Thr-202, Tyr-204-ERK1 (clone 832/13)) was purchased from Santa Cruz Biotechnologies, and phospho-Mek1/2 was purchased from Cell Signaling Technology. Phospho-Thr-202, Tyr-204-ERK1 (clone 832/13)) was purchased from Santa Cruz Biotechnologies, and phospho-Mek1/2 was purchased from Cell Signaling Technology. Phospho-Thr-202, Tyr-204-ERK1 and phospho-Mek1/2 were obtained from Cell Signaling Technology (New England Biolabs). Total ERK1/2 was assessed using a C-terminal targeted antibody from Santa Cruz Biotechnologies. Briefly, cells were harvested and plated into 6-well plates 2 days prior to overnight serum starvation, and subsequent stimulation was performed on day 3. Cells were harvested for 1 h at 37 °C in modified Krebs-Ringer solution prior to the addition of agonists or pharmacological inhibitors. Following the elapsed stimulation period, cells were washed once with ice-cold Krebs-Ringer and lysed on ice. Immunoblotting, protein quantification, and Western blotting were performed as described previously (33) to express receptors at similar levels to high level expressed GIP, and that the pancreatic β-cell lines, βTC-3 and INS-1 (832/13) cells, express 70 and 60% of the protein kinases examined, respectively, at similar levels to the rGIP-15 cells. For the first time, GIP is shown to influence the activity of all known isoforms of the ERK1/2 module, including Raf-1, Mek1/2, ERK1/2, and p90 RSK in CHO-K1 cells and in the INS-1 (clone 832/13) β-cell line. This regulation is shown to occur via PKA and Rap1, thus identifying the mechanism by which GIP-stimulated cAMP production positively regulates ERK1/2.

In the present study, we sought to determine the mechanism by which GIP activates ERK1/2. In doing so, we have characterized a GIP receptor-transfected CHO-K1 (rGIP-15) cell line and two β-cell lines by screening for the relative expression of 75 different protein kinases. From this, we were able to conclude that at least four MAPK modules are potentially regulated by GIP and that the pancreatic β-cell lines, βTC-3 and INS-1 (832/13) cells, express 70 and 60% of the protein kinases examined, respectively, at similar levels to the rGIP-15 cells. For the first time, GIP is shown to influence the activity of all known isoforms of the ERK1/2 module, including Raf-1, Mek1/2, ERK1/2, and p90 RSK in CHO-K1 cells and in the INS-1 (clone 832/13) β-cell line. This regulation is shown to occur via PKA and Rap1, thus identifying the mechanism by which GIP-stimulated cAMP production positively regulates ERK1/2.

Expression Profiling of Protein Kinases—The expression levels of protein kinases are cell-specific. Our approach to delineating the signaling pathways responding to GIP receptor activation included an initial assessment of the various protein kinases expressed (Table I–III), with a comparison between a heterologous GIP receptor expression system (rGIP-15) and the two β-cell lines (βTC-3 and INS-1 (832/13)). With respect to MAPK cascades, it is evident that four modules (Raf1/B}
Proteins were extracted in ice-cold lysis buffer, followed by sonication prior to centrifugation. Protein was quantified using the BCA assay, and 20 µg of protein were loaded/well for Western blotting. Quantitative assessment (trace quantities) was based on band densities (n = 3). The abbreviations used are as follows: β2M, bone marrow x-linked protein; βtk, Bruton tyrosine kinase; CaMK, Ca-modulin-dependent kinase; Cdk, cyclin-dependent kinase; CK, casecin kinase; COT,Tpl-2, cancer Osaka thymic oncogene; CSK, C-term Ssrc kinase; DAPK, death-associated protein kinase; DNAPK, DNA-activated protein kinase; ERK, extracellular regulated protein kinase; FAK, focal adhesion kinase; Fyn, Fyn oncogene related to SRC; GCK, general center kinase; GGRK, G-protein-coupled receptor kinase; GSK3, glycogen synthase kinase 3; HPK, hematopoietic progenitor kinase; IKK, inhibitor NF-κB kinase; JAK, Janus kinase; Ksc, kinase suppressor of Ras 1; Lck, lymphocyte specific protein-tyrosine kinase; Lyn, oncogene Lyn, Mek, MAPK kinase; Mek1/2, MAPK kinase; Mnk, MAPK-interacting kinase; Mos, mos Moloney murine sarcoma viral oncogene homolog 1; Mst1, mammaliansteric 20-like 1; Nek, NIMA-like mitotiskin-related kinase 2; PKA, p38 MAPK; PAK, p21-activated kinase; PDK, 3-phosphoinositide-dependent kinase; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PKR, dsRNA-dependent protein kinase; Pyk, protein-tyrosine kinase; Raf1, oncogene Raf1; RafB, v-Raf murine sarcoma viral oncogene homolog B1; ROK, RhoA kinase; RSK, 90-kDa ribosomal S6 kinase; S6K, ribosomal S6 kinase; Src, oncogenic Src; Syk, spleen tyrosine kinase; Yes, Yamaguchi cell kinase; 3). The expression profiles of rGIP-15, βTC-3, and INS-1 (83/13) ranked as highly expressed.

| CHO cells with GIPR | Trace quantity | βTC-3 cells | Trace quantity | INS-1 | Trace quantity |
|---------------------|----------------|-------------|----------------|-------|--------------|
| BMX                 | 5219 ± 584     | 3825 ± 468  | 5374 ± 1252    |       |              |
| CaMK4               | 4965 ± 380     | 5949 ± 194  | 13169 ± 957    |       |              |
| Cdk 5               | 4210 ± 819     | 7357 ± 833  | 9882 ± 1306    |       |              |
| Cdk 6               | 6781 ± 1875    | 9520 ± 556  | 4946 ± 544     |       |              |
| Cdk 7               | 4588 ± 805     | 6243 ± 2437 | 22371 ± 2553   |       |              |
| CK 2 α              | 8829 ± 1878    | 19357 ± 3056| 17283 ± 2564   |       |              |
| CK 2 α’             | 11170 ± 3900   | 42835 ± 4096| 8011 ± 861     |       |              |
| CK 2 α”             | 6170 ± 1460    | 17907 ± 1176| 30573 ± 4908   |       |              |
| ERK 1               | 26050 ± 3058   | 6205 ± 1446 | 10992 ± 1206   |       |              |
| ERK 2               | 12901 ± 3408   | 6882 ± 1163 | 7643 ± 819     |       |              |
| ERK 3               | 6679 ± 1276    | 13105 ± 2244| 5281 ± 609     |       |              |
| FAK                 | 13642 ± 4532   | 11655 ± 2294| 7400 ± 1456    |       |              |
| Fyn                 | 11057 ± 1948   | 21203 ± 3624| 4743 ± 348     |       |              |
| GRK 2               | 9585 ± 2229    | 9405 ± 1074 | 6741 ± 506     |       |              |
| GRK 3 α             | 10560 ± 3460   | 5925 ± 835  | 4092 ± 346     |       |              |
| GRK 3 β             | 6637 ± 2365    | 9751 ± 326  | 24364 ± 1721   |       |              |
| Hpk 1               | 8122 ± 2659    | 7035 ± 1485 | 11847 ± 2466   |       |              |
| JAK 1               | 5444 ± 2335    | 19320 ± 3621| 21497 ± 3615   |       |              |
| JAK 2               | 4364 ± 913     | 19196 ± 3587| 6436 ± 1599    |       |              |
| Mek 2               | 8117 ± 1373    | 11776 ± 1641| 4573 ± 1332    |       |              |
| Mek 4               | 4219 ± 921     | 14718 ± 5307| 12211 ± 2241   |       |              |
| P38 α               | 26243 ± 1831   | 6343 ± 1295 | 6954 ± 1383    |       |              |
| p70 S6K             | 10663 ± 1834   | 3824 ± 1295 | 15117 ± 3993   |       |              |
| PKA α               | 7912 ± 2854    | 12306 ± 1083| 8782 ± 1791    |       |              |
| PKB α               | 8831 ± 907     | 18832 ± 2631| 17931 ± 4748   |       |              |
| PKC μ               | 11420 ± 1384   | 4987 ± 972  | 19662 ± 2319   |       |              |
| PKC ζ               | 37137 ± 3665   | 11153 ± 243 | 15117 ± 3993   |       |              |
| Raf B               | 4982 ± 476     | 34330 ± 3633| 30573 ± 4908   |       |              |
| Raf 1               | 16495 ± 4985   | 29021 ± 4795| 19662 ± 2319   |       |              |
| Raf 1’              | 12123 ± 3646   | 8920 ± 749  | 81092 ± 1206   |       |              |
| ZIPK                | 7087 ± 1977    | 10445 ± 501 | 3925 ± 6400    |       |              |

Mek1/2 → ERK1/2, Cot/Tpl-2 → Mek4/7 → SAPK, PAKa → Mek6 → p38α, and ERK3) were all present in rGIP-15, βTC-3, and INS-1 cells (Tables I–III; ERK5 was not blotted for). Furthermore, a pathway regulating cell survival (3-phosphoinositide-dependent kinase 1 → protein kinase B → glyocgen synthase kinase 3), the multiple isoforms of PKC (α, β, δ, ε, γ, λ, μ, θ, and ι), and the ubiquitous cyclin-dependent kinases (kinases 1, 2, 4–7, and 9) regulating cell cycle progression are also potential regulatory effector molecules for GIP in the cell lines (Tables I–III). On comparing the kinase expression profiles of rGIP-15, βTC-3, and INS-1 cells, there were few major differences in expression levels of the ubiquitous kinases of the MAPK modules mentioned above. Differences were confined mainly to the relative expression level of the upstream Meks (Mek 1, 2, 4, 6, and 7; Mek3 was not blotted), the Meks (Mos, Cot, germinal center kinase, hematopoietic progenitor kinase, Mst1), and the numerous PKC isoforms (Table I–III). The most reassuring finding was that 50–70% of the protein kinases probed for were expressed at similar levels in the three cell lines (48% across all three; 59% for rGIP-15 versus INS-1; 61% for βTC-3 versus INS-1; and 73% for rGIP-15 versus βTC-3).
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Expression levels of protein kinases in rGIP-15, βT3-3, and INS-1 (832/13) ranked as minimally expressed

See Table I for abbreviations.

| Protein kinases not detected | CHO cells with βGIP | βT3-3 cells | INS |
|------------------------------|-------------------|-------------|-----|
| Btk                          | Btk               | BMX         | BMX|
| CaMK1                        | Btk               | CaMKK       | CaMKK|
| CK 1 ε                       | CaMKK             | CaMKK       | CaMKK|
| DAPK                         | Cdk 1, 2          | Cdk 1       | Cdk 1|
| DNAK                         | CSK               | CSK         | CSK|
| ERK 6                        | DAPK              | DAPK        | DAPK|
| KSR 1                        | DNAK              | DNAK        | DNAK|
| Lck                          | Cot (Tpl-2)       | DNAPK       | DNAPK|
| Lyn                          | ERK 3             | Fyn         | Fyn|
| Mek 6                        | GCK               | GCK         | GCK|
| MST 1                        | KSR 1             | Hpk         | Hpk|
| Mek 2                        | Lck               | JAK 1, 2    | JAK 1, 2|
| Pin 1                        | Lck               | Lck         | Lck|
| PKC β, γ, θ                  | Mek 1, 7          | Mek 7       | Mek 7|
| PKG                          | Mos               | Mink 2      | Mink 2|
| PKR                          | MST 1             | MST         | MST|
| Pyk 2                        | PKC γ, θ          | Nek 2       | Nek 2|
| RSK 2                        | Pim 1             | Pim 1       | Pim 1|
| Srr                          | Pyk 2             | PKC γ, λ, θ| PKC γ, λ, θ|
| Syk                          | Srr               | PKG         | PKG|
| Yes 1                        | Yes 1             | PKR         | PKR|
| ZAP70                        | ZAP70             | ROK α       | ROK α|
|                               |                   | RSK 2       | RSK 2|
|                               |                   | Srr         | Srr|
|                               |                   | Syk         | Syk|
|                               |                   | Yes 1       | Yes 1|

Table II

Expression levels of protein kinases in rGIP-15, βT3-3, and INS-1 (832/13) ranked as minimally expressed

See Table I for abbreviations.

| CaMKK | CaMK 1 | CaMK1 | 1390 ± 125 | CaMK1 | 636 ± 90 |
|-------|--------|-------|------------|-------|---------|
| Cdk1  | 1682 ± 117 | Cdk 1 | 2521 ± 62  | Cdk4 | 983 ± 150 |
| Cdk 2 | 2581 ± 683 | Cdk 4 | 4029 ± 207 | Cdk6 | 1078 ± 253 |
| Cdk 8 | 2000 ± 158 | Cdk 6 | 1982 ± 947 | Cdk9 | 1015 ± 194 |
| CK 1 δ | 2145 ± 165 | Cdk 9 | 1898 ± 314 | CK 1 δ | 469 ± 110 |
| Cot (Tpl-2) | 1929 ± 640 | CK 2 α | 3263 ± 578 | GSK 3 β | 2146 ± 373 |
| CSK | 2784 ± 562 | Fyn | 3444 ± 762 | RSR 1 | 692 ± 84 |
| GCK | 4019 ± 881 | IKK α | 1884 ± 262 | Lyn | 758 ± 275 |
| IKK α | 2265 ± 797 | Mek 6 | 2539 ± 615 | Mek 1 | 2052 ± 313 |
| Mek 1 | 2606 ± 111 | Mek 2 | 1400 ± 194 | Mek 6 | 3245 ± 332 |
| Mek 7 | 3756 ± 269 | Neck 2 | 2917 ± 450 | Pak α | 2851 ± 112 |
| Mnk 2 | 1726 ± 56 | PDK 1 | 3086 ± 1596 | p70 S6K | 2105 ± 159 |
| Mos | 1357 ± 291 | Pka | 3273 ± 1015 | PDK1 | 1121 ± 69 |
| PDK 1 | 2514 ± 86 | PKC α | 1895 ± 1004 | PKA | 3107 ± 803 |
| PKA | 450 ± 50 | PKG | 1778 ± 149 | PKC α | 3497 ± 1304 |
| PKC α | 1831 ± 170 | ROK α | 3751 ± 775 | PKC α | 3648 ± 1135 |
| PKC δ | 1822 ± 348 | RSK 2 | 2200 ± 1200 | Pyk 2 | 2042 ± 535 |
| PKC ε | 1646 ± 732 | SAPK (JNK) | 2855 ± 231 | SAPK (JNK) | 1666 ± 106 |
| PKC λ | 3282 ± 152 | | | | |
| ROK α | 2426 ± 959 | | | | |
| SAPK (JNK) | 2458 ± 595 | | | | |

Table III

Expression levels of protein kinases in rGIP-15, βT3-3, and INS-1 (832/13) ranked as not expressed

See Table I for abbreviations.

(11 mM) in these cells. Glucose caused a distinct leftward shift in the concentration-response profile of GIP-mediated ERK1/2 phosphorylation, without enhancing the fold maximal phosphorylation attained (Fig. 2, B and C). Supportive studies with a third β-cell line, Brin-D11, also mimic these actions of GIP (data not shown).

GIP signals via cAMP and arachidonic acid (AA) in rGIP-15 cells and β-cell lines (5, 38). To identify the proximal regulator of the ERK module, we tested the ability of these two second messengers to modulate ERK1/2 phosphorylation, while using the phorbol ester, phorbol myristic acid (PMA), as a positive control for ERK1/2 activation (Fig. 3). Both forskolin and AA increased ERK1/2 activity in rGIP-15 cells. However, only forskolin was active in INS-1 cells. cAMP also activated ERK1/2 in βT3-3 cells (data not shown), implying that cAMP is a positive regulator of ERK1/2 activity in β-cells.

GIP Regulates the ERK1/2 Module Upstream of Raf-1 via PKA—GIP has been shown previously (13, 14) to activate ERK1/2, but the other kinases in this module were not examined, and no attempt was made to link ERK1/2 activation to GPCR effector coupling. Fig. 4A illustrates the concentration-dependent actions of GIP on phosphorylation of Ser-259 of Raf-1, Ser-217/221 of Mek1/2, and Ser-380 of p90 RSK, after a 5-min stimulation in rGIP-15 cells. There is a high correlation in responses among these three kinases, but we found no evidence for Elk-1 (Ser-383) phosphorylation after 5 min (n = 3). Evidence supporting a role for Raf-1 and p90 RSK in responses to GIP in the β-cell (INS-1 clone 832/13) is shown in Fig. 4B, under 0 mM glucose conditions. Under these conditions the state of Ser-217/221 phosphorylation of Mek1/2 was already high, and further enhancement of activity by GIP could not be detected. However, the Mek1/2-specific inhibitors, PD98059 and U0126, both completely abolished forskolin, PMA, and GIP-mediated ERK1/2 phosphorylation, supporting a role for Mek1/2 in the cascade (Fig. 4C). Thus, GIP is able to regulate the Raf1 → Mek1/2 → ERK1/2 → RSK module in both CHO-K1 and INS-1 β-cells.

The general PKC and PKA inhibitors, GF109203x (Bis) and H89, respectively, were used to evaluate the role of these protein-serine/threonine kinases in mediating GIP actions on the ERK module (Figs. 5 and 6). These studies were conducted in rGIP-15 cells using forskolin, PMA, and AA as agonists representing three potential activation pathways (PKA, PKC, and AA, respectively). PKC inhibition (2 μM GF109203x) reduced basal ERK1/2 phosphorylation as well as significantly abrogating forskolin and PMA-induced ERK1/2 phosphorylation (p < 0.05, n = 4). The specificity of GF109203x for PKC is demonstrated by the complete ablation of PMA effects. Fig. 5 clearly demonstrates that GIP-mediated activation of ERK1/2 can be PKC-independent.

In contrast, PKA inhibition suppressed basal, forskolin-, AA-, and GIP-stimulated ERK1/2 phosphorylation, while not altering PMA effects (Fig. 6, A and B). We conclude that GIP regulates ERK1/2 activity through activation of PKA. To determine the step at which PKA exerts its regulation of the 3-tiered ERK1/2 module, the effect of H89 on agonist-induced changes...
in phospho-Raf-1 and phospho-Mek1/2 was studied (Fig. 6). It is evident that PKA regulation occurred upstream of Raf-1, as H89 was able to block the phosphorylation of Ser-259 of Raf-1 and Ser-217/221 of Mek1/2. Thus, GIP seemingly inhibits Raf-1 activity via increased phosphorylation of Ser-259 (39), while positively regulating Mek1/2, ERK1/2, and p90 RSK.

It should also be noted that PMA reduced the phosphorylation of Raf-1, while activating both Mek1/2 and ERK1/2 (Fig. 6). Furthermore, AA seemingly reduced Raf-1 phosphorylation while bypassing Mek1/2 and activating ERK1/2. These findings illustrate that PKC mediates a reduction in Raf-1 Ser-259 phosphorylation, while enhancing downstream Mek1/2 and ERK1/2 activity.

**GIP Activates ERK via Rap1 Independently of Gβy and Src—** The above data reveal a positive effect of GIP on the Mek1/2 → ERK1/2 → RSK module, in contrast to its inhibitory influence on Raf-1 activity through promotion of Ser-259 phosphorylation (40–42). It was hypothesized that another kinase must be activated by GIP in order to phosphorylate Ser-259 of Raf-1. We therefore studied whether GIP activates ERK1/2 via Rap1 independently of Gβy and Src

**Fig. 1.** GIP stimulates ERK1/2 phosphorylation in a transient (A) and concentration-dependent (B) manner in rGIP-15 cells. Cells were stimulated in Krebs-Ringer buffer for 5 min (A) or indicated times. B, stimulation was stopped by addition of ice-cold lysis buffer followed by sonication prior to centrifugation. Protein was quantified using the BCA assay, and 50 μg of protein were loaded per lane for Western blotting. Membranes were blotted with phospho-Thr-202, Tyr-204-ERK (p-ERK) antibody, prior to densitometric analysis. Data represent mean ± S.E. (A, n = 4–5; B, n = 4), where * indicates p < 0.05 compared with respective controls (ANOVA, with Dunnett’s multiple comparison test).

**Fig. 2.** GIP stimulates ERK1/2 phosphorylation in the presence of glucose in βTC-3 cells (A), and in both the absence (B) and presence (C) of glucose in INS-1 (832/13) cells. Cells were stimulated in Krebs-Ringer buffer for 5 min containing 11 mM glucose (Gluc) (A and C) or 0 mM (B). Fifty-μg protein samples were separated by SDS-PAGE, and membranes were blotted with phospho-Thr-202, Tyr-204-ERK (p-ERK) antibody, prior to densitometric analysis. Data represent mean ± S.E. (A, n = 3; B, n = 4; C, n = 4). * indicates p < 0.05 compared with basal controls; # indicates p < 0.05 compared with glucose control (ANOVA, with Newman-Keuls post hoc).
GIP Activates the ERK1/2 MAPK Module via cAMP/PKA/Rap1

Several members of the glucagon/PACAP superfamily of peptide hormones have been identified as regulators of MAPK modules. PACAP, glucagon, and the incretin GLP-1 have been shown to positively regulate ERK1/2 in neuronal PC12 cells (8), HEK cells (9), and in β and non-β cells, respectively (11, 45). Both PACAP- and glucagon-mediated cAMP elevations were found to regulate these protein-serine/threonine kinases. Until recently, however, the role of cAMP in ERK1/2 regulation was controversial with some studies demonstrating positive effects and others negative effects. A recent study (9) on the glucagon receptor provided the first insight into how this superfamily of peptide hormones may regulate ERK1/2 activity in a cAMP-PKA-dependent manner. In the present study, we demonstrate that GIP can activate the ERK1/2 module via proximal cAMP-PKA-Rap1 activation, and we propose a role for B-Raf in the positive regulation of this module.

In attempting to delineate GIP receptor signaling pathways in CHO and β-cells, we have begun to map the expression of a wider range of protein kinases (Tables I–III). Although various protein kinases have been identified in pancreatic β-cells, few have been identified in a single cell simultaneously (46). We confirmed the presence of four intact MAPK modules (Raf1/B)–subunits (22), we investigated whether a similar pathway was involved in regulating downstream ERK1/2 signaling. An intervening peptide (C terminus of β-adrenergic receptor kinase) that we previously employed to investigate GIP signaling (38) was expressed in increasing amounts in rGIP-15 cells, but it was unable to reverse 1 nM GIP-mediated activation of ERK1/2 phosphorylation (Fig. 8), thus providing evidence against a role for Gβγ signaling in ERK1/2 regulation. Recent findings have implicated Gαs in the activation of the tyrosine kinase Src (24) and Src in the regulation of ERK1/2 (23). We found no evidence for Src involvement in the GIP activation of ERK1/2 using dominant negative and constitutively active constructs (Src Y527F and Src RF; n = 3, data not shown) (44). This is consistent with the absence of detectable Src from our protein kinase profile data (Table III).

**DISCUSSION**

In most cell types, cAMP inhibits cell growth, and initial evidence detailing the molecular basis for this effect focused on cAMP inhibition of ERK1/2 activity. This was mapped to occur at the level of Raf-1, via PKA-mediated phosphorylation of Ser-43 (47), Ser-259 (48), and Ser-621 (49), resulting in enzyme inhibition. However, recent studies (30) have now highlighted a role for cAMP in positively regulating ERK1/2 activity in pituitary, ovarian, and neuronal cells. The basis for this interaction has been most extensively studied in the rat pheochromocytoma cell line, PC12 cells. These studies have culminated in a model where cAMP activates ERK1/2 when B-Raf is expressed in cells (30). Unlike Raf-1, which undergoes complex protein interactions to regulate its activity, B-Raf is mainly regulated by small GTPase proteins (40). The key regulator is the small G protein, Rap1, which is directly activated by elevated cAMP.
levels via PKA (27, 30). Recent work (29) has demonstrated that Rap1 can be activated by both PKA-dependent and -independent mechanisms. Furthermore, data indicate that the amount of 14-3-3-binding protein associated with B-Raf may also explain the tissue-specific effects of cAMP on B-Raf and ERK1/2 activity (50). The presence of B-Raf in our cell model

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**Fig. 4.** GIP regulates the ERK1/2 module in CHO (A) and INS-1 (832/13; B and C) cells. rGIP-15 (A) or INS-1 (B and C) cells were stimulated in Krebs-Ringer buffer for 5 min containing 0 mM glucose. The Mek1/2 inhibitors, PD98059 (100 μM) or UO126 (10 μM), were added 15 min prior to and during stimulations (C). Fifty-μg protein samples were separated by SDS-PAGE, and membranes were blotted with antibodies against phospho-Thr-202, Tyr-204-ERK (p-ERK), p-Raf (Ser-259), p-MEK (Ser-217, Ser-221), p-p90RSK (Ser-380), and p-Elk-1 (Ser-383). p-Elk-1 was not detected at 5 min. Data represent mean ± S.E. (A, n = 3; B, n = 3–4; C, blots representative of 3 and 2 independent experiments with similar results), where * indicates p < 0.05 compared with respective controls (ANOVA, with Dunnett’s multiple comparison test).
supports the notion that cAMP-mediated signaling via GIP can positively influence ERK1/2 activity.

We propose, therefore, that GIP stimulates ERK1/2 by influencing B-Raf activity in pancreatic β-cells. In support of this hypothesis, other GPCRs have also been shown to regulate ERK1/2 activity via B-Raf and Rap1. For example, the adenosine receptor (A2A), the prototypical β2-adrenergic receptor, and the M1 muscarinic receptor have all been found to stimulate ERK1/2 via Rap1 and B-Raf in CHO, HEK, and PC12 cells, respectively (51–53). These cells all express the Raf isoform B-Raf and are therefore able to activate ERK1/2 via cAMP signaling. Since Rap1 is able to increase GIP-mediated ERK1/2 activity, it is likely that PKA-dependent activation of Rap1 is able to regulate B-Raf and thereby activate Mek1/2 → ERK1/2 → p90RSK.

Neuronal and endocrine cells share many similar phenotypic features. Therefore, it may be expected that cAMP can positively influence ERK1/2 activity and cellular fate in both cell types. cAMP regulation of ERK1/2 activity in CHO cells has been controversial, with studies showing both activating and inhibitory affects (51, 54, 55). A previous report (13) investigated GIP signaling demonstrated an inhibitory effect of cAMP on ERK1/2 activity in CHO cells. In the present study, however, we report that cAMP is able to influence positively the ERK1/2 module in both CHO (rGIP-15) and β-cells (INS-1, βTC-3, and BrinD11 cells). This similarity in cAMP actions in

![Fig. 5. GIP regulation of ERK1/2 is PKC-independent in rGIP-15 cells.](image)

![Fig. 6. GIP regulation of ERK1/2 (A and B), Raf-1 (B), and Mek1/2 (B) is PKA-dependent in rGIP-15 cells.](image)
these cell lines, together with the similar expression level of protein kinases profiled in our cell model versus \( \beta \)-cell lines, supports our model system as a means for elucidating GIP receptor signaling.

It may seem counterintuitive for GIP to inhibit Raf1 activity, while positively regulating downstream ERK1/2 via Rap1 activation (Figs. 5 and 7). However, similar results have been found for neuronal growth factor and \( \beta \)-adrenergic receptor signaling (32, 52). The balance between Raf-1/B-Raf activation or inhibition represents a mechanism by which downstream kinases of ERK1/2 activity may be regulated, thereby affording different physiological processes. For example, both neuronal growth factor and the \( \beta \)-adrenergic receptor kinase (\( \beta ARKct \)) and the vector (pRK5) were provided by Dr. R. J. Lefkowitz (Duke University). Proteins were separated by SDS-PAGE, and membranes were blotted with Ras and ERK1/2 rapidly and transiently via Raf-1 (32, 52). However, sustained (prolonged) activation of the ERK1/2 module occurs through B-Raf in these systems. This attractive model may explain the molecular basis responsible for regulating differentiation (sustained ERK1/2 activation) versus proliferation (transient ERK1/2 activation) in PC12 cells. Although we provide evidence for extremely rapid kinetics of ERK1/2 activation, there may be prolonged effects mediated by GIP via Raf-1 in \( \beta \)-cells, which still need to be elucidated. Whereas GIP is able to regulate \( \beta \)-cell proliferation (14, 56), the second incretin hormone, GLP-1, has been identified recently as a growth factor and independently of G\( \gamma \)-subunits (5). GIP-15 cells were transfected with vectors \( \pm \) constructs 2 days prior to experiments using Lipofect2000 (Invitrogen). Vector (pCGN) and wild type Rap1b were a kind gift from Dr. D. Altschuler (University of Pittsburgh), and the C terminus of \( \beta \)-adrenergic receptor kinase (\( \beta ARKct \)) and the vector (pRK5) were provided by Dr. R. J. Lefkowitz (Duke University). Proteins were separated by SDS-PAGE, and membranes were blotted with phospho-Thr-202, Tyr-204-ERK (\( p \)-ERK), and a C-terminal targeted ERK1/2 antibody from Santa Cruz Biotechnology to assess total ERK1/2. Blots are representative of two (A) or three (B) independent experiments with similar results.

Acknowledgments—We are grateful for the technical assistance of Cuilan Nian. We also thank Dr. D. Altschuler (University of Pittsburgh), Dr. R. J. Lefkowitz (Duke University), and Dr. J. Brugge (Harvard University) for kindly providing reagents, and Dr. C. B. Newgard (University of Texas Southwestern Medical Center) for the INS-1 cells (clone 832/13).

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