Gastrin Stimulates Tyrosine Phosphorylation of Insulin Receptor Substrate 1 and Its Association with Grb2 and the Phosphatidylinositol 3-Kinase*

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The growth-promoting effects of gastrin on normal and neoplastic gastrointestinal tissues have been shown to be mediated by the gastrin/CCKB receptor, which belongs to the family of G-protein-coupled receptors. However, the downstream signaling pathways activated by gastrin are not well characterized. In the present study, we demonstrate that gastrin stimulates tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1), the major cytoplasmic substrate of the insulin receptor. The gastrin-induced phosphorylation of IRS-1 was rapid and transient, occurring within 30 s of treatment and diminishing thereafter. IRS-1 binds several proteins containing SH2 domains through its multiple tyrosine phosphorylation sites. Following gastrin stimulation, we observed a time- and dose-dependent association of IRS-1 with the 85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-kinase). In addition, activation of PI3-kinase was detected in anti-IRS-1 immunoprecipitates from gastrin-treated cells, suggesting that tyrosine phosphorylation of IRS-1, which leads to the rapid recruitment of p85, might be one mechanism used by gastrin to activate PI3-kinase. We have previously reported that tyrosine phosphorylation of Shc and its association with the Grb2-Sos complex may contribute to the activation of the mitogen-activated protein kinase pathway by gastrin. We report here that Grb2 also interacts with tyrosine-phosphorylated IRS-1 in response to gastrin. Taken together, our results suggest that IRS-1 may serve as a converging target in the signaling pathways stimulated by receptors that belong to different families, such as the gastrin/CCKB G protein-coupled receptor and the insulin receptor.

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1 The abbreviations used are: G/CCK, gastrin/CCK; MAP, mitogen-activated protein; PI 3-kinase, phosphatidylinositol 3-kinase; SH, Src

homology; PtdIns, phosphatidylinositol; IGF-I, insulin-like growth factor I; IRS-1, insulin receptor substrate 1; INSERM, Institut National de la Santé et de la Recherche Médicale.
also been reported to activate PI 3-kinase in a number of cell systems (31–33); however, the mechanisms responsible for this activation are poorly understood. Since we recently reported that gastrin exerts growth-promoting effects on a tumor-derived pancreatic acinar cell line (AR4–2J) through the G/GCCK_{subunit} G protein-coupled receptor (7), we examined whether this peptide could regulate the activation of the PI 3-kinase in this cellular model. We also investigated the mechanisms responsible for gastrin-induced PI 3-kinase activity. We report here an activation of PI 3-kinase by gastrin receptors occupancy. In addition, we show that gastrin rapidly stimulates both tyrosine phosphorylation of IRS-1 and its association with PI 3-kinase, suggesting that IRS-1 may be an important signaling molecule involved in gastrin-induced PI 3-kinase activation.

**MATERIALS AND METHODS**

**Cell Culture—**AR4–2J cells, originally obtained by Jessop and Hay (34) from a rat exocrine pancreatic tumor (azaserine induced), were a gift from Dr. C. Logsdon (Department of Physiology, University of Michigan, Ann Arbor, MI). The cells, plated at 75,000 cells/ml, were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The medium was changed every 2 days.

**Immunoprecipitation—**AR4–2J cells growing in 100-mm culture dishes were serum starved in Dulbecco’s modified Eagle’s medium for 18 h before peptide addition. After stimulation, the cells were washed with ice-cold buffer A (50 mM Hepes, 150 mM NaCl, 10 mM EDTA, 10 mM Na_{subscript}3P_{subscript}O_{subscript}4, 100 mM NaF, 2 mM orthovanadate, pH 7.5) and homogenized in 500 μl of lysis buffer (buffer A containing 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 20 μg leupeptin, 100 IU/ml Trasylol) for 15 min at 4 °C. The solutes were clarified by centrifugation at 12,000 × g for 10 min at 4 °C and immunoprecipitated with the indicated antibodies preadsorbed on protein A- or protein G-Sepharose. Samples for immunoblotting were washed twice with 30 mM Hepes buffer, pH 7.5, containing 30 mM NaCl 0.1% Triton X-100, resuspended in SDS sample buffer, and boiled for 5 min.

**Western Blotting Analysis—**Whole cell lysates or immunoprecipitates, prepared as described above, were separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon, Millipore). Membranes were blocked with saline buffer (1 mM Tris, 14 mM NaCl, pH 7.4) containing 5% bovine serum albumin or nonfat dried milk and incubated overnight with the indicated antibodies. Membranes were washed three times with the buffer containing 0.5% bovine serum albumin, 0.05% dried milk and 0.5% Nonidet P-40 and incubated with 125I-protein A (500 000 cpm/ml) for 1 h at room temperature. Membranes were washed and autoradiographed.

**Phosphotyrosine Assay—**AR4–2J cells growing in 35-mm culture dishes were serum starved in Dulbecco’s modified Eagle’s medium for 18 h before hormone stimulation. After treatment of the cells, the proteins were solubilized and immunoprecipitated with the indicated antibodies as described above. The pellets were assayed for the PtdIns-3-kinase activity as described previously (30). The immunoprecipitates were washed twice with each of the three following buffers: (a) phosphate-buffered saline, pH 7.4, containing 1% Nonidet P-40; (b) 100 mM Tris, 0.5 mM LiCl, pH 7.4; and (c) 10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.4. The pellets were resuspended in 30 μl of 20 mM Hepes, 0.4 mM EDTA, and 0.4 mM Na_{subscript}3P_{subscript}O_{subscript}4. The substrate (PtdIns) was dried for 10 min, resuspended in 5 mM Hepes at 1 mg/ml, and sonicated for 15 min. The kinase reaction was started by addition of PtdIns at a final concentration of 0.2 μg/ml, 10 mM MgCl_2, and 50 μM PIP_2/ATP (10 μM/mml). After 15 min the reaction was stopped by addition of 13 μl of 4 M HCl, and the phosphoinositidal lipids were extracted with 130 μl of chloroform/methanol (1:1). The tubes were mixed by vortexing and centrifuged thereafter.

The phospholipids contained in the organic phase were recovered, dried, resuspended in 5 μl of chloroform, and separated by thin layer chromatography on Silica Gel 60A in chloroform/methanol/4.3 mM ammonia (90:70:20); the plate was analyzed by autoradiography.

**RESULTS**

PI 3-Kinase Is Activated in Gastrin-treated AR4–2J Cells—We first investigated the effect of gastrin on PI 3-kinase activity in AR2-J cells. Serum-starved cells were stimulated with 10 nM gastrin for varying lengths of time, and PI 3-kinase activity was measured after immunoprecipitation of the cell lysates with an antibody to the p85 regulatory subunit of PI 3-kinase. We observed a rapid and transient increase in PI 3-kinase activity similar to the p85 subunit of PI 3-kinase. We examined a rapid and transient increase in PI 3-kinase activity in response to gastrin (Fig. 1, A and B, lanes 1–4). The maximal activation obtained within 1 minute after peptide addition decreased toward the basal level at 3 min. Since insulin was known to activate PI 3-kinase in a number of cell systems (29), we used this hormone as a control in our experiments (Fig. 1, A and B, lanes 5 and 6). At 1 min, insulin (1 μM) induced an increase in PI 3-kinase activity similar to that observed with gastrin.

**Gastrin Induces Tyrosine Phosphorylation of IRS-1 and Its Association with p85 Subunit of PI 3-Kinase—**Next we examined whether the p85 subunit of PI 3-kinase could be phosphorylated on tyrosine residues in response to gastrin. Cells were treated for the indicated times with 10 nM gastrin. The cell extracts were immunoprecipitated with antibodies to the p85 subunit of PI 3-kinase, and the tyrosine-phosphorylated proteins were revealed by Western blotting with an antiphosphotyrosine antibody. We did not detect tyrosine phos-
phosphorylation of p85 in gastrin-treated cells (data not shown). However, Western blotting (Fig. 2) revealed a gastrin-dependent increase in the tyrosine phosphorylation of a 185-kDa protein that coprecipitated with PI 3-kinase antibodies. The phosphorylation induced by 10 nM gastrin was transient, with maximal stimulation detected at 30 s. This protein migrated with an apparent molecular weight appropriate for IRS-1 (Fig. 2A, lane 6), a protein known to associate with PI 3-kinase in response to insulin or IGF-I (29). To determine whether the 185-kDa protein that is tyrosine phosphorylated in response to gastrin is IRS-1, the cell lysates were immunoprecipitated with an antibody to IRS-1, and precipitates were analyzed by immunoblot with an antiphosphotyrosine antibody. As expected, phosphorylation of IRS-1 was observed in response to insulin, an effector known to induce tyrosine phosphorylation of IRS-1 in other cellular models (Fig. 3, A and B). IRS-1 was also phosphorylated on tyrosine residues after gastrin stimulation in a time- (Fig. 3, C and D) and dose-dependent manner (Fig. 4, A and B). The gastrin-induced phosphorylation was rapid and transient, occurring within 30 s of treatment and diminishing thereafter. The identity of this protein as phosphorylated IRS-1 was confirmed by its comigration with a protein immunoprecipitated with an antiphosphotyrosine antibody and recognized by immunoblotting with an antibody to IRS-1 (Fig. 4, C and D).

Immunoblot analysis demonstrated increased tyrosine phosphorylation of IRS-1 in gastrin-treated cells compared with control cells. We performed additional Western blotting experiments to confirm that gastrin could stimulate the association of tyrosine-phosphorylated IRS-1 with the p85 subunit of PI 3-kinase. Cells were incubated with 10 nM gastrin for the times indicated or for 30 s with increasing concentrations of peptide.

Solubilized proteins were subjected to immunoprecipitation with an anti-IRS-1 antibody, and precipitates were analyzed by immunoblot using an antibody to the 85-kDa subunit of PI 3-kinase. Following gastrin treatment of the cells, we observed an increase in the amount of p85 coprecipitated with the anti-IRS-1 antibody (Fig. 5). The time course and dose response for the association of IRS-1 with the p85 subunit of PI 3-kinase were consistent with those observed for IRS-1 phosphorylation induced by gastrin (Figs. 3 and 4).

Activation of PI 3-Kinase in Anti-IRS-1 Precipitates Occurs Following Gastrin Stimulation—Since PI 3-kinase activation by insulin has been shown to occur during its association with tyrosine-phosphorylated IRS-1 (29), we examined whether PI 3-kinase activity was detected in association with IRS-1 during gastrin stimulation of AR4–2J cells. PI 3-kinase assays were carried out on anti-IRS-1 immunoprecipitates. As shown in Fig. 6, A and B (lanes 1–3), an increase in PI 3-kinase activity was detected in the anti-IRS-1 precipitates after gastrin stimulation (10 nM, 30 s and 1 min). These results correlate with the gastrin-dependent increase in the amount of the 85-kDa subunit of PI 3-kinase, which coprecipitates with IRS-1 antibodies (Fig. 5). As expected, PI 3-kinase activity was also detected in association with IRS-1 after insulin stimulation (1 μM, 3 min; Fig. 6, A and B, lane 4). Similar results were obtained when PI 3-kinase activity was measured in antiphosphotyrosine immunoprecipitates (Fig. 6, C and D).

Gastrin-stimulated Recruitment of Grb2 by IRS-1—On insulin stimulation, tyrosine-phosphorylated IRS-1 has been shown to bind to several proteins containing SH2 domains, including PI 3-kinase as well as the adapter protein Grb2 (29). Following insulin stimulation (1 μM) of the AR4–2J cells, protein extracts were subjected to immunoprecipitation with an anti-Grb2 antibody, and an anti-IRS-1 antibody was used for Western blotting. As expected, Fig. 7, A and B, shows an increase in the...
amount of IRS-1 coprecipitated with anti-Grb2 antibodies in insulin-treated cells (lane 4) compared with control cells (lane 1), demonstrating that tyrosine-phosphorylated IRS-1 binds Grb2 on insulin stimulation in this experimental model. To determine whether phosphorylated IRS-1 associates with Grb2 following gastrin stimulation, immunoprecipitates from AR4–2J cell lysates, obtained with an anti-Grb2 antibody, were immunoblotted with either an antibody to IRS-1 (Fig. 7, A and B) or antiphosphotyrosine antibodies (Fig. 7, C and D). The amount of a 185-kDa tyrosine-phosphorylated protein was increased in the anti-Grb2 immunoprecipitates after gastrin

FIG. 4. Dose-dependent tyrosine phosphorylation of IRS-1 in response to gastrin. AR4–2J cells were incubated for 30 s with different concentrations of gastrin. Cellular proteins were immunoprecipitated (IP) with antibodies against IRS-1 (A) or with an antiphosphotyrosine (aPy) antibody (C). Immunoprecipitates were immunoblotted (IB) with either an antiphosphotyrosine antibody (A) or an antibody to IRS-1 (C). Arrow, migration of precipitated IRS-1. B and D, the autoradiograms were densitometrically analyzed, and the data were plotted as percentages of the control values. Data from three autoradiograms (from three separate experiments) are presented as means ± S.E. (bars).

FIG. 5. Gastrin-dependent association of the 85-kDa regulatory subunit of PI 3-kinase with IRS-1. AR4–2J cells were treated with 10 nM gastrin for varying lengths of time (A) or with increasing concentrations of gastrin for 30 s (C). Cell lysates were immunoprecipitated (IP) with an antibody to IRS-1, and the 85-kDa subunit of PI 3-kinase was revealed by Western blotting (IB) with an antibody to p85. Arrow, migration of the 85-kDa subunit of PI 3-kinase. B and D, the autoradiograms were densitometrically analyzed, and the data were plotted as percentages of the control values. Data from three autoradiograms (from three separate experiments) are presented as means ± S.E. (bars).

FIG. 6. Association of PI 3-kinase activity with IRS-1 in response to gastrin or insulin. AR4–2J cells were incubated for varying lengths of time in the absence or presence of gastrin (10 nM) or insulin (1 µM) as indicated. Cell lysates were immunoprecipitated (IP) with either an antibody against IRS-1 (A) or an antiphosphotyrosine (aPy) antibody (C). Immunoprecipitates were assayed for PI 3-kinase activity using PtdIns as substrate. The phospholipids were resolved on thin layer chromatography plates. Arrow, phosphorylated substrate PtdIns-P (PIP). B and D, the autoradiograms were densitometrically analyzed, and the data were plotted as percentages of the control values. Data from three autoradiograms (from three separate experiments) are presented as means ± S.E. (bars).

FIG. 7. Association of Grb2 with IRS-1 in insulin- or gastrin-treated AR4–2J cells. Cells were stimulated with gastrin (10 nM) or insulin (1 µM) for the indicated periods. Protein extracts were subjected to immunoprecipitation (IP) with an anti-Grb2 antibody, and the precipitates were analyzed by Western blot (IB) using either an anti-IRS-1 antibody (A) or an antiphosphotyrosine (aPy) antibody (C). Arrow, migration of coprecipitated IRS-1. B and D, the autoradiograms were densitometrically analyzed, and the data were plotted as percentages of the control values. Data from three autoradiograms (from three separate experiments) are presented as means ± S.E. (bars).
stimulation (Fig. 7, C and D). This phosphorylated protein comigrated with a protein that was immunoprecipitated with an anti-Grb2 antibody and identified as IRS-1 by immunoblotting using an anti-IRS-1 antibody (Fig. 7, A and B). Gastrin stimulated an increased association between phosphorylated IRS-1 and Grb2, with a time course identical to that observed for IRS-1 phosphorylation induced by gastrin. The association was maximal 30 s–1 min. after addition of 10 nM gastrin and decreased at 3 min (Fig. 7, C and D).

Activation of PI 3-kinase in Anti-Grb2 Immunoprecipitates Occurs in Response to Gastrin—Finally, we performed experiments in which immunoblotting with anti-p85 antibody (Fig. 8, A and B) and PI 3-kinase assays (Fig. 8, C and D) were performed on anti-Grb2 immunoprecipitates. A time-dependent increase in the level of the p85 subunit of PI 3-kinase coprecipitated with anti-Grb2 antibodies was detected by Western blotting after treatment of the cells with gastrin (10 nM; Fig. 8, A and B). Gastrin-induced coprecipitation of Grb2 with p85 was rapid and transient, occurring within 1 min of treatment and decreasing thereafter. This time course paralleled the increase in PI 3-kinase activity detected in the anti-Grb2 immunoprecipitates after gastrin stimulation (Fig. 8, C and D). In addition, increases in both p85 protein level and PI 3-kinase activity were also observed in immunoprecipitates from insulin-stimulated AR4–2J cells (Fig. 8, A–D, lane 5).

DISCUSSION

We have recently reported that gastrin stimulates the growth of the pancreatic carcinoma cell line AR4–2J through the G/CCKB G protein-coupled receptors (5, 7). Tyrosine phosphorylation is an important intracellular event that is implicated in the transmission of mitogenic signals induced by tyrosine kinase receptors, which bind cytosolic tyrosine kinases (growth hormone and cytokine receptors) as well as G protein-coupled receptors. Activation of insulin or IGF-I receptors that possess intrinsic tyrosine kinase activity regulates both metabolic and mitogenic events. One of the early steps in the insulin and IGF-I receptor signaling pathway is the tyrosine phosphorylation of IRS-1, an adaptor protein that links the receptor to downstream mediators (29). In response to insulin or IGF-I, IRS-1 is phosphorylated on multiple tyrosine residues recognized by the SH2 domains of specific proteins that activate different intracellular pathways. In the AR4–2J cells, G/CCKB receptors, which do not contain intrinsic tyrosine kinase activity, have been shown to mediate protein tyrosine phosphorylation (15). We undertook the present study to further characterize the signal transduction events induced by gastrin occupancy of the G/CCKB receptor. The work presented in this article is the first to demonstrate that gastrin, a G protein-coupled receptor agonist, rapidly and transiently stimulates tyrosine phosphorylation of IRS-1, the major cytoplasmic substrate of the insulin and IGF-I receptors. In addition, we showed that PI 3-kinase is associated with phosphorylated IRS-1 and activated following gastrin stimulation. Growth hormone and cytokine receptors have also been shown to mediate IRS-1 tyrosine phosphorylation and its association with PI 3-kinase (35–39). These receptors activate cytosolic tyrosine kinases of the Janus kinase family, which in turn associate and phosphorylate IRS-1. Tyrosine-phosphorylated IRS-1 subsequently associates PI 3-kinase via the SH2 domain of its regulatory subunit. More recently, IRS-1 has also been shown to be phosphorylated on tyrosine residues in response to angiotensin II, a ligand that binds specific seven-transmembrane domain receptors (40). These observations and our findings suggest that the tyrosine phosphorylation of IRS-1 that led to the rapid recruitment of the 85-kDa subunit of PI 3-kinase might represent a common mechanism for PI 3-kinase activation used by different families of receptors. Further studies are required to establish the mechanisms by which gastrin induces tyrosine phosphorylation of IRS-1. In particular, the tyrosine kinases stimulated by G protein-coupled receptor agonists that may be responsible for the phosphorylation of IRS-1 remained to be identified. Protein tyrosine kinases activated by this receptor family are potential candidates. Several published reports have shown that a number of G protein-linked receptors, including the G/CCKB receptors, mediate the autophosphorylation and activation of Src family protein tyrosine kinases (41–43). However, it remains to be answered whether these kinases can phosphorylate IRS-1.

We have recently demonstrated that gastrin stimulates MAP kinase activation in the AR4–2J cells. We have also characterized the molecular events, upstream of p21-Ras, that may link the MAP kinase pathway to G/CCKB receptors (15). Gastrin rapidly induces tyrosine phosphorylation of the adapter protein Shc, which subsequently interacts with the SH2 domain of Grb2, a second intermediate protein. Grb2 also possesses SH3 domains, which constitutively bind the prolin-rich motif of the p21-Ras activator termed Sos. The role of the activated p21-Ras is then to target the serine/threonine kinase c-Raf-1 to the plasma membrane, in which it can be activated by phosphorylation. Dual specific kinases (tyrosine/threonine kinases), termed MAP kinase kinases, are in turn activated by c-Raf-1 and directly phosphorylate the MAP kinases. In the present study, we demonstrate that Grb2 also interacts with tyrosine-phosphorylated IRS-1 in response to gastrin. Thus, the binding of IRS-1 to the Grb2-Sos complex, which is likely involved in insulin stimulation of MAP kinases (29), might be an alternative pathway used by gastrin to activate a Ras-dependent MAP kinase cascade in AR4–2J cells. A recent study has demonstrated that activation of the monocyte colony-stimulating factor receptor (a transmembrane protein tyrosine kinase) induces tyrosine phosphorylation of the 85-kDa subunit of PI 3-kinase and its direct association
with the Grb2-SOS complex via the SH2 domain of Grb2. This mechanism could contribute to the regulation of the Ras-signaling pathway in monocytes (44). Our results also demonstrate that activation of PI 3-kinase in anti-Grb2 immunoprecipitates occurs in response to gastrin. However, we did not detect tyrosine phosphorylation of p85 following gastrin stimulation. Since IRS-1 phosphorylated on tyrosine residues is able to directly interact with the SH2 domains of both p85 and Grb2, PI 3-kinase is likely coprecipitated with anti-Grb2 antibodies via IRS-1 in response to gastrin.

In summary, our results demonstrate for the first time that tyrosine phosphorylation of IRS-1 and its subsequent interaction with downstream signaling molecules such as Grb2 or PI 3-kinase can be induced by gastrin. This finding suggests that IRS-1 may serve as a converging target in the signaling pathway activated by gastrin. IRS-1 phosphorylation may contribute to the regulation of the Ras-signaling pathway via IRS-1 in response to gastrin.

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