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Gastrin induces tyrosine phosphorylation of Shc proteins and their association with the Grb2/Sos complex

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Abstract **Gastrin/CCK_B G protein-coupled receptors have been shown to mediate proliferative effects of their endogenous ligands. In the present study, we examined the signal transduction mechanisms linked to the G/CCK_B receptor occupancy. We report here that gastrin stimulates MAP kinase activation in a dose- and time-dependent manner, a pathway known to play a key role in cell proliferation. We also characterized the molecular events, upstream of p21-Ras, that may link the MAP kinase pathway to G/CCK_B receptors. Gastrin induced a rapid and transient increase in tyrosine phosphorylation of several proteins including the 2 isoforms (46 and 52 kDa) of the adaptor protein Shc. Phosphorylated Shc subsequently associated with a complex that includes Grb2 and the p21-Ras activator, Sos. Our results also indicate that Sos becomes phosphorylated in response to gastrin as shown by a reduction in electrophoretic mobility of the protein. Tyrosine phosphorylation of Shc and subsequent complex formation with Grb2 and Sos appear to be a common mechanism by which tyrosine kinase receptors and the G/CCK_B G protein-coupled receptor stimulate the Ras-dependent MAP kinase pathway.**

Key words: Gastrin; G protein-coupled receptor; MAP kinase; Shc; Grb2; Sos

1. Introduction

Gastrin, a regulatory peptide known to be a potent stimulant of gastric acid secretion [1], has also been shown to have growth-promoting effects on normal and neoplastic gastrointestinal tissues. Trophic effects of gastrin have been reported on normal digestive mucosa [2] as well as on colon carcinomas 'in vivo' [3,4]. Gastrin also stimulates the growth of colon, gastric and pancreatic cancer cell lines in culture [5–8] as well as the colony formation of small cell lung cancer cells [9]. The proliferative effect of gastrin have been shown to be mediated by the gastrin/CCK_B (G/CCK_B) receptor in different cellular models [8,9] including cells transfected with the human G/CCK_B receptor cDNA [10–12]. This receptor which has been recently cloned from different species by several laboratories [13–16], belongs to the family of G protein-coupled receptors which are known to be linked to the phospholipase C/protein kinase C (PLC/PKC) pathway. Gastrin-dependent activation of the G/ CCK_B receptors has been shown to induce phosphatidyl-inositol biphosphate (PIP2) hydrolysis by PLC that results in inositol triphosphate (IP3) and diacylglycerol (DAG) production leading to intracellular Ca^{2+} mobilization and stimulation of PKC [17,18]. Gastrin, like many ligands that bind G protein-coupled receptors, has also been shown to induce tyrosine kinase activity [19].

It is now well established that the MAP kinase (MAPK) pathway, stimulated by growth factor receptors with intrinsic tyrosine kinase activity or by growth hormone and cytokine receptors coupled to cytosolic protein tyrosine kinase, plays an important role in cell proliferation and differentiation [20]. P42-MAPK and p44-MAPK, also known as ERK-1 and ERK-2 (Extracellular Regulated kinase-1 and -2) are two members of the MAPK family that are activated by phosphorylation on both tyrosine and threonine residues [21–23]. Both isoforms regulate the activity of several proteins involved in cell proliferation by phosphorylation on serine/threonine residues [24].

The best characterized pathway coupled to tyrosine kinase receptors that leads to MAPK activation, involves the protooncogene product p21-Ras, a member of the small GTPases family [24]. P21-Ras is converted from its inactive GDP-bound state to its active GTP-bound state upon tyrosine kinase receptor stimulation. The role of the activated p21-Ras is then to target the serine/threonine kinase c-Raf-1 to the plasma membrane where it can be activated by phosphorylation. Dual specific kinases (tyrosine/threonine kinases) termed MEK (or MAPK kinase) are in turn activated by c-Raf-1 and directly phosphorylate MAPK. Recently, an alternative pathway that activates MEK in a Raf-independent manner, has been shown to regulate MAPK activation [25].

Recent studies have shown that G protein-coupled receptors also stimulate the activation of MAPK in a Raf-dependent or -independent manner [26]. However, the precise mechanisms of the different pathways used by these receptors to activate MAPK are still poorly understood. Several adaptor proteins upstream of p21-Ras have been recently identified. These proteins link the Ras/Raf pathway to tyrosine kinase receptors as well as receptors which activate cytosolic tyrosine kinase [27]. The three isoforms (46-, 52-, 66-kDa) of the adapter protein Shc (for *S*rc *h*omology 2/alpha-*c*ollagen-related) that bind to specific phosphotyrosine-containing sequences via its SH2 (for *S*rc *H*omology *2*) domains are phosphorylated on tyrosine residues upon stimulation of these receptors. Tyrosine-phosphorylated Shc subsequently interacts with the SH2 domains of a second intermediate protein of 25 kDa, Grb2 [28,29]. This second adapter protein also possess SH3 (for *S*rc *H*omology *3*) domains which in turn interact with the proline-rich motifs of the Ras-GTP/GDP exchange factor, termed Sos [30–35]. Several receptors (such as EGF-receptor) have also been shown to

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interact directly with the Grb2/Sos complex to activate the Ras pathway [31–35].

It has been shown that the cloned human G/CCK$_B$ receptor transfected in mouse NIH3T3 fibroblasts stimulated the MAPK pathway [11]. However, the upstream molecular events that link this receptor to MAPK activation still remain to be elucidated. We have previously shown that gastrin proliferation of a tumor-derived pancreatic acinar cell line (AR4-2J) through the G/CCK$_B$ receptor [8]. In the present study, we have analysed, in this cellular model, the possibility that gastrin might activate, upstream of p21-Ras, the complex Shc/Grb2/Sos that leads to MAPK activation.

2. Materials and methods

2.1. Cell culture

AR4-2J cells, originally obtained by Jessop and Hay [36] from a rat exocrine pancreatic tumor (azaserine induced), were a gift from Dr. C. Logsdon (Ann Arbor, MI). The cells, plated at 75,000 cells/ml, were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum. The medium was changed every 2 days.

2.2. Immunoprecipitation

AR4-2J cells growing in 100 mm culture dishes were serum starved in DMEM 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 $Na_4P_2O_7$, 100 mM NaF, 2 mM orthovanadate, pH 7.5) and homogenized in 500 μl lysis buffer (buffer A containing 1% Triton X-100, 0.5 mM phenylmethylsulfonylfluoride, 20 μM leupeptin, 100 IU/ml Trasylol) for 15 min at 4°C. The solubilizates 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 and 0.1% Triton X-100, resuspended in SDS sample buffer and boiled for 5 min.

2.3. Western Blotting Analysis

Whole cell lysates or immunoprecipitates, prepared as described above, were separated by SDS-PAGE. Proteins were transfered to PVDF membrane (Immobilon PVDF Millipore). Membranes were blocked with saline buffer (1 mM Tris, 14 mM NaCl, pH 7.4) containing 5% BSA or non-fat dried milk and incubated overnight with the indicated antibodies. Membranes were washed 3 times with saline buffer containing 0.5% BSA or non-fat dried milk and 1% Triton X-100 and incubated with [^{125}I]protein-A (500,000 cpm/ml) for 1 h at room temperature. Membranes were washed and autoradiographed.

2.4. Immune complex assays for MAPK activation

Cells growing in 60 mm culture dishes were serum starved in DMEM for 18 h before peptide addition. Cell lysates were immunoprecipitated as described above with anti p42-MAPK or p44-MAPK antibodies. Precipitated immune complexes were washed twice in lysis buffer and 3 times in 20 mM HEPES buffer, pH 7.5 containing 150 mM NaCl, 200 μM orthovanadate, 0.1% Triton X-100, 10% glycerol, 0.5 mM phenylmethylsulfonylfluoride, 20 μM leupeptin, 100 IU/ml Trasylol. The phosphorylation was performed for 30 min at room temperature with Myelin Basic Protein (MBP) at 0.2 mg/ml, 10 mM Mg Acetate and [γ-^{33}P]ATP (3000 Ci/mmole). The reaction was stopped by depositing the samples on P81 Whatmann chromatography paper immersed in 1% orthophosphoric acid. After 3 washes in 1% orthophosphoric acid, the radioactivity was counted.

2.5. Materials

Human gastrin$_{2\text{-}17ns}$ was purchased from Bachem (Switzerland). [^{125}I]Na (100 mCi/ml) was obtained from Amersham and [γ^{33}P]ATP (3000 Ci/ mmol) from Isotopchim. Myelin Basic Protein (MBP), Protein-A sepharose-CL4B, Protein-G sepharose-CL4B, orthovanadate, aprotinin, leupeptin, phenylmethylsulfonylfluoride and BSA 7030 were purchased from Sigma. Anti-phosphotyrosine and anti-Shc antibodies were from Upstate Biotechnology. Anti-p42-MAPK, anti-p44pMAPK, anti-Sos and anti-Grb2 antibodies were obtained from Santa Cruz.

3. Results

3.1. Tyrosine phosphorylation of cellular proteins in response to gastrin

Since tyrosine phosphorylation is believed to play a key role in mediating the proliferative effects of many growth factors, we examine the effect of gastrin on tyrosine phosphorylation of cellular proteins. Serum-starved cells were treated with gastrin and cell lysates were subjected to SDS-PAGE electrophoresis followed by immunoblot analysis using an anti-phosphotyrosine antibody (as described in section 2). As shown in Fig. 1, treatment of the cells with 10 nM gastrin induces a rapid and transient increase in tyrosine phosphorylation of six proteins with approximate molecular weights of 123-, 96-, 65-, 55-, 43- and 41-kDa.

For all the proteins, the stimulation reached a peak value 3 min after peptide addition before decreasing at 5 and 10 min.

3.2. MAPK activation in gastrin-treated AR4-2J cells

To examine whether G/CCK$_B$ receptors could mediate stimulation of the MAPK pathway in AR4-2J cells, we investigated the effect of gastrin on MAPK activity by performing immune complex assays for MAPK activation (as described in section 2). As shown in Fig. 2, gastrin stimulated the MAPK activity of p42-MAPK in a manner similar to that observed with the

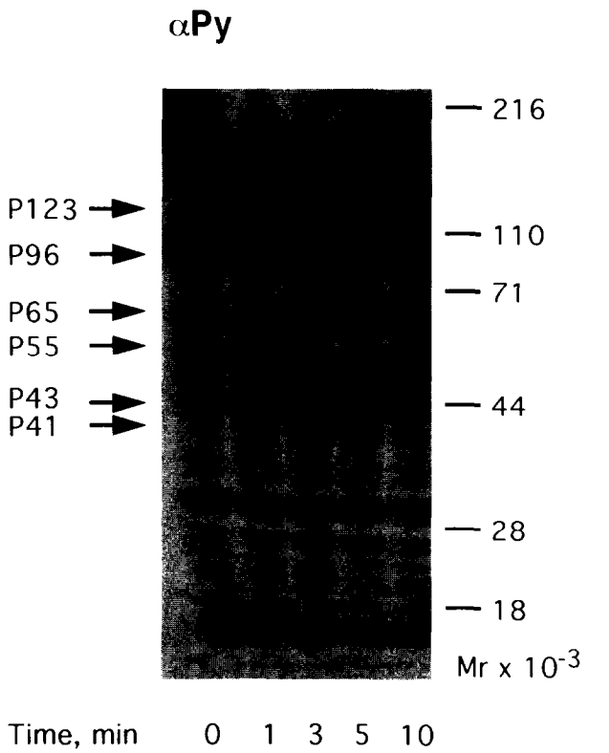

Fig. 1. Tyrosine phosphorylation of cellular proteins in response to gastrin. AR4-2J cells were stimulated with 10 nM gastrin for the times indicated. Whole-cell lysates were prepared as described in section 2 and immunoblotted with an anti-phosphotyrosine antibody. Molecular weight markers are indicated on the right. The arrows on the left indicate the migration (kDa) of proteins tyrosine-phosphorylated in response to gastrin.

tyrosine phosphorylation of cellular proteins. The stimulation was time- and dose-dependent and maximal phosphorylation of the MAPK substrate (MBP) by immunoprecipitated p42-MAPK was achieved 3–5 min after peptide addition (10 nM) before decreasing towards the basal level at 10 min. Similar results were obtained with p44-MAPK (data not shown).

3.3. *Tyrosine phosphorylation of Shc in gastrin-treated AR4-2J cells*

It has been shown that Shc proteins are tyrosine phosphorylated upon stimulation of tyrosine kinase receptors or receptors directly coupled to cytosolic tyrosine kinase. In order to determine whether gastrin could induce tyrosine phosphorylation of Shc via the G/CCK_B G protein-coupled receptor, cell lysates were immunoprecipitated with an anti-Shc antibody and precipitates were analysed by immunoblot with an anti- phosphotyrosine antibody. As shown in Fig. 3, the 46- and 52-kDa Shc isoforms were tyrosine phosphorylated upon gastrin stimulation in a time- (A) and dose-dependent manner (B). The increase in tyrosine phosphorylation of both isoforms was maximal 3–5 min after addition of 10 nM and decreased after 10 min.

3.4. *Association of Grb2 with tyrosine phosphorylated Shc in response to gastrin*

Since Grb2 has been shown to interact via its SH2 domains with tyrosine-phosphorylated Shc, we examined the association of Grb2 with Shc in gastrin-treated AR4-2J cells. Cells were stimulated with 10 nM gastrin for varying lengths of time and lysates immunoprecipitated with the anti-Grb2 antibody. Western blotting with an anti-phosphotyrosine antibody revealed an increased phosphorylation of two proteins coprecipitated with Grb2 that migrated with an apparent molecular weight identical to the Shc proteins (Fig. 4A), suggesting the association of both Shc isoforms with Grb2 in response to gastrin. The time-dependent stimulation was similar to that observed with the tyrosine phosphorylation of Shc isoforms. To confirm that gastrin could stimulate the association of Grb2 with tyrosine phosphorylated Shc, we performed additional experiments. Cell lysates were immunoprecipitated with an anti-Shc antibody and an anti-Grb2 antibody was used for Western blotting. As seen in Fig. 4B, a 23 kDa protein recognized by the anti Grb2 antibody in Western blots, was coprecipitated with the anti-Shc antibody in a gastrin-dependent manner. The time course and dose response of Grb2/Shc association are consistent with those observed for Shc phosphorylation and MAPK activation induced by gastrin.

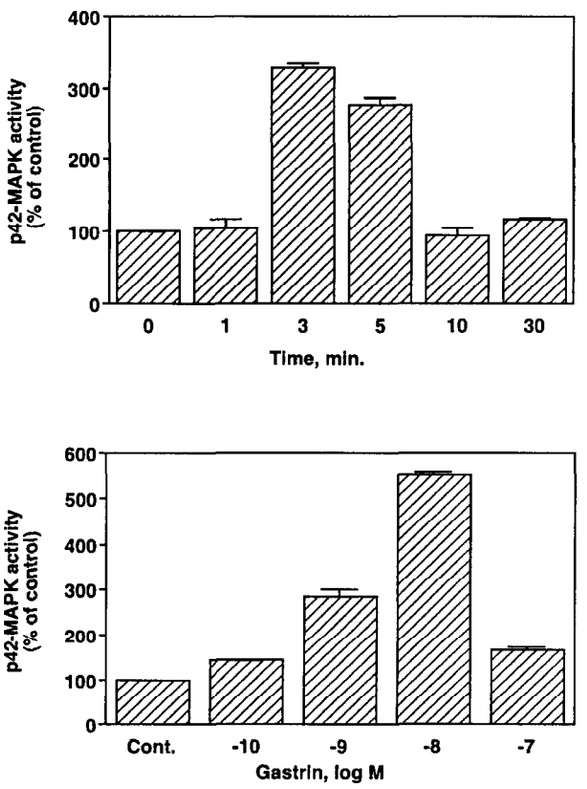

Fig. 2. Time- and dose-dependent activation of p42-MAPK by gastrin. Immune complex assays for MAP kinase activity were performed as described in section 2. Cells were treated (A) with 10 nM gastrin for the times indicated or (B) for 3 min with increasing concentrations of gastrin.

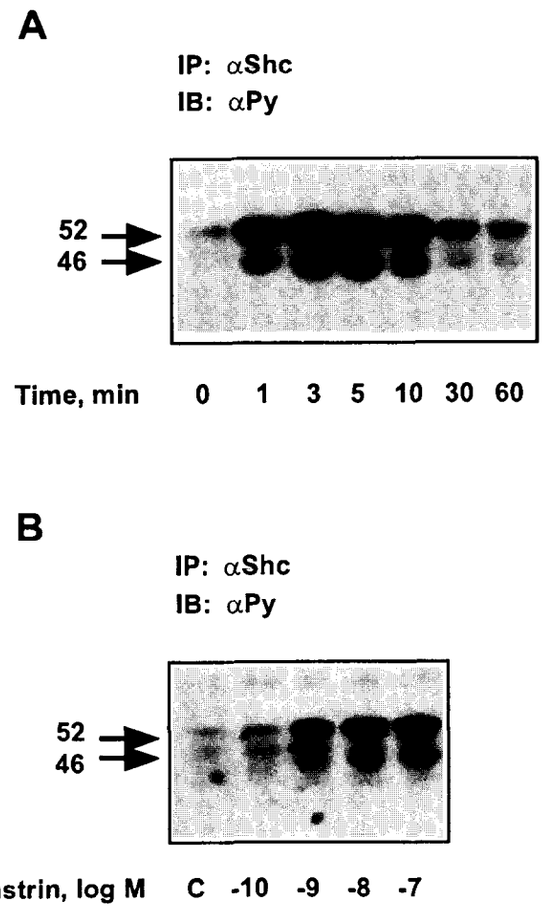

Fig.3. Tyrosine phosphorylation of Shc in gastrin-treated AR4-2J cells. Cells were incubated with (A) 10 nM gastrin for the times indicated or (B) with increasing concentrations of gastrin for 3 min. Cellular proteins were immunoprecipitated with an anti-Shc antibody and Western blotted with an anti-phosphotyrosine antibody. The arrows indicate the migration of precipitated 52- and 46-kDa Shc.

3.5. Association of Grb2 with Sos in gastrin-treated AR4-2J cells

It has been established that Sos, the guanyl-nucleotide exchange factor of Ras, can interact with the SH3 domains of Grb2. We therefore assessed the association of Grb2 with Sos in AR4-2J cells. Immunoprecipitation of cell extracts with an anti-Grb2 antibody revealed coprecipitation of a 170 kDa protein identified as Sos by immunoblotting using an anti-Sos antibody (Fig. 5A,B). After gastrin treatment (10 nM) from 1 to 30 min, an equivalent amount of Sos was coprecipitated with the anti Grb2 antibody indicating that gastrin did not affect the association of Grb2 with Sos in AR4-2J cells. However, 3 min of gastrin stimulation resulted in a reduction of the electrophoretic mobility of coimmunoprecipitated Sos, suggesting that gastrin induced Sos phosphorylation in a time- (A) and dose-dependent manner (B). The shift in Sos mobility was maximal between 3 and 5 min and decreased at 10 min but remained detectable for at least 30 min. Similar results were obtained with whole cell lysates analyzed by immunoblot with an anti-Sos antibody (Fig. 5C).

4. Discussion

Tyrosine phosphorylations are believed to play a key role in the regulation of cellular growth induced by multiple receptors, such as tyrosine kinase receptors, receptors coupled to cytosolic tyrosine kinase (growth hormone and cytokine receptors) as well as G protein-coupled receptors.

In the present study, we show that gastrin, which stimulates proliferation of a pancreatic tumor cell line (AR4-2J) [7,8], induces in this model tyrosine phosphorylation of several proteins including the 2 isoforms (46-, 52-kDa) of the adaptor protein, Shc. Tyrosine phosphorylation of Shc and subsequent complex formation with Grb2 and Sos appears to be the major pathway upstream of p21-Ras, that leads to Ras-dependent MAPK activation [37–40].

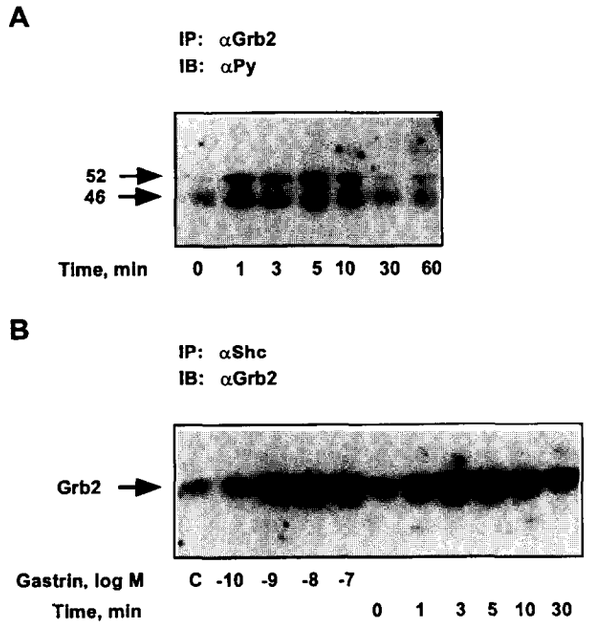

Fig. 4. Association of tyrosine-phosphorylated Shc with Grb2 upon gastrin stimulation. (A) AR4-2J cells were treated for the indicated times with 10 nM gastrin. Cell lysates were immunoprecipitated with anti-Grb2 antibody and immunoblotted with an anti-phosphotyrosine antibody. The migration of coprecipitated 52- and 46-kDa Shc is indicated on the left. (B) Cells were stimulated for 3 min with increasing concentrations of gastrin or with 10 nM gastrin for varying lengths of time. Cell extracts were immunoprecipitated with an anti-Shc antibody and Western blotted with an anti-Grb2 antibody. The migration of coprecipitated 23 kDa Grb2 is indicated on the left.

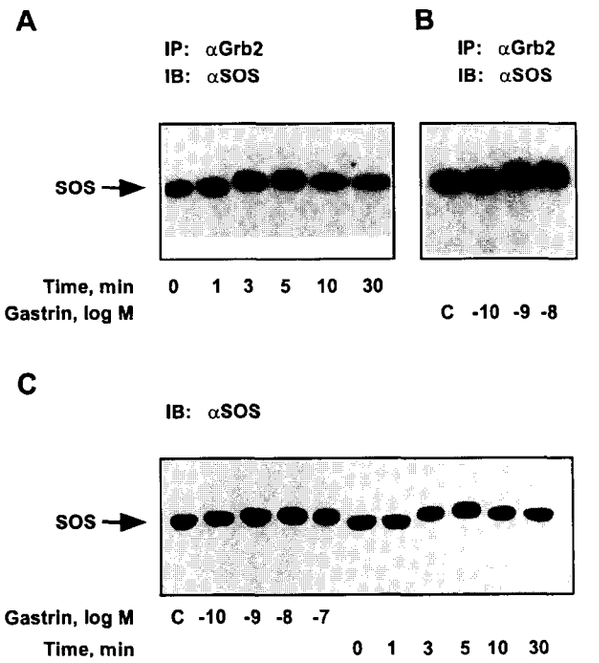

Fig. 5. Association of Grb2 with Sos and the increase in electrophoretic mobility of Sos in gastrin treated AR4-2J cells. Cells were incubated with (A) 10 nM gastrin for the times indicated or (B) for 3 min with increasing concentrations of gastrin. Cell lysates were immunoprecipitated with an anti-Grb2 antibody and the immunoprecipitates were Western blotted with an anti-Sos antibody. The migration of coprecipitated 170 kDa Sos is indicated on the left. (C) AR4-2J cells were treated for 3 min with increasing concentrations of gastrin or with 10 nM gastrin for varying lengths of time. Whole-cell lysates were immunoblotted with an anti-Sos antibody. The migration of 170 kDa Sos is indicated by the arrow.

This is the first report demonstrating that the G/CCK$_B$ G protein-coupled receptor mediates p46- and p52-Shc tyrosine phosphorylation and their association with the Grb2/Sos complex. Grb2 recruitment to phosphorylated Shc was observed within 3 min after gastrin stimulation, however, this increase in tyrosine phosphorylation of Shc did not result in a greater association between Grb2 and the Ras activator, Sos. Our data suggest that Grb2 and Sos exist in a constitutive complex in AR4-2J cells and gastrin does not affect the level of Grb2 complexed with Sos. These results are consistent with those observed in several studies with growth factors [30,33,34]. Regulation of Grb2/Sos association has been reported for EGF receptor stimulation in cells overexpressing Grb2 [41] as well as for the occupancy of the T-cell antigen receptor [42].

The results of our experiments also indicate that Sos becomes phosphorylated in response to gastrin as shown by a reduced electrophoretic mobility of the protein. The time course and dose response of Sos phosphorylation by gastrin paralleled tyrosine phosphorylation of Shc and its association with the

Grb2/Sos complex as well as MAPK activation. Like gastrin, several growth factors have been reported to induce the phosphorylation of Sos [32], however, the role of this phosphorylation remains to be elucidated.

Recently it has been reported that in fibroblasts inhibition of Sos phosphorylation did not influence the activation of p21-Ras nor the interaction between Sos and Grb2/tyrosine phosphorylated-Shc complex [43].

In summary, our results clearly demonstrate that phosphorylation of Shc and its association with the Grb2/Sos complex may be one mechanism, upstream of p21-Ras, by which the G/CCK$_B$ G protein-coupled receptor mediates MAPK activation.

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