Implication of G $\beta\gamma$ proteins and c-SRC tyrosine kinase in parathyroid hormone-induced signal transduction in rat enterocytes

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Abstract

Parathyroid hormone (PTH) interacts in target tissues with a G protein-coupled receptor (GPCR) localized in the plasma membrane. Although activation of GPCR can elicit rapid stimulation of cellular protein tyrosine phosphorylation, the mechanism by which G proteins activate protein-tyrosine kinases is not completely understood. In the present work, we demonstrate that PTH rapidly increases the activity of non-receptor tyrosine kinase c-Src in rat intestinal cells (enterocytes). The response is biphasic, the early phase is fast and transient, peaking at 30 s (+120%), while the second phase progressively increases up to 5 min (+220%). The hormone activates c-Src in intestinal cells through fast changes in tyrosine phosphorylation of the enzyme. The first event in the activation of c-Src is the dephosphorylation of Tyr527 (which happens after a few seconds of PTH treatment), followed by a second event of activation with phosphorylation at Tyr416 (+twofold, 5 min). Removal of external Ca²⁺

(EGTA, 0.5 mM) and chelation of intracellular Ca²⁺ with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetracetic acid acetoxymethyl ester (BAPTA) (5 μ M) suppressed Tyr527 dephosphorylation and Tyr416 phosphorylation, indicating that Ca²⁺ is an upstream activator of c-Src in enterocytes stimulated with PTH. The G protein subunits, G α s and G β , are associated with c-Src in basal conditions and this association increases two- to threefold in cells treated with PTH. Blocking of G β subunits by preincubation of cells with a G β antibody abolished hormone-dependent c-Src Tyr416 phosphorylation and ERK1/ERK2 activation. The results of this work indicate that PTH activates c-Src in intestinal cells through conformational changes via G proteins and calcium-dependent modulation of tyrosine phosphorylation of the enzyme, and that PTH receptor activation leads via G $\beta\gamma$ -c-Src to the phosphorylation of the MAP kinases, ERK1 and ERK2.

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Introduction

Parathyroid hormone (PTH) is an 84-amino-acid polypeptide hormone functioning as a major mediator of bone remodeling and as an essential regulator of calcium homeostasis (Rosenblatt *et al.* 1989). In rat intestinal cells (enterocytes), PTH initiates its effects by interacting with the heterotrimeric G protein-coupled receptor, PTHR1 (Gentili *et al.* 2003b). The transduction of PTH signal through the plasma membrane of rat enterocytes involves both a Gs-mediated stimulation of adenylyl cyclase with cAMP production and protein kinase (PK) A activation (Picotto *et al.* 1997), and a Gq-mediated activation of phospholipase (PL) C β , leading to generation of inositol 1,4,5 trisphosphate (IP₃) and diacylglycerol, followed by activation of PKC (Massheimer *et al.* 2000). PTH also increases intracellular Ca²⁺ levels in rat enterocytes by promoting an initial acute IP₃-mediated mobilization of Ca²⁺ from a thapsigargin-sensitive store, and a sustained phase due to Ca²⁺ influx through voltage-dependent Ca²⁺-channels (Gentili *et al.* 2003a). Activation of PTH

seven transmembrane receptor in enterocytes also leads to tyrosine phosphorylation of a number of intracellular proteins, the most prominent being PLC γ (Gentili *et al.* 2001a) and the mitogen-activated protein kinases, ERK1 and ERK2 (Gentili & de Boland 2000) which leads to an increase in DNA synthesis (Gentili *et al.* 2001b). Initial studies on the mechanisms underlying PTH activation of the enterocyte tyrosine phosphorylation revealed that the cytosolic tyrosine kinase c-Src plays a central role in these processes, demonstrating that pharmacological inhibition of c-Src abolishes PTH-dependent PLC γ phosphorylation, the ERK cascade activation (Gentili & de Boland 2000, Gentili *et al.* 2001a) and ERK-induced enterocyte proliferation (Gentili *et al.* 2001b). The c-Src tyrosine kinase has been shown to regulate a diverse number of cellular effects including stimulating and inhibiting cell growth (Roche *et al.* 1995, Broome & Hunter 1996), regulating cell adhesion (Parsons & Parsons 1997, Cary *et al.* 2002), and regulating apoptosis (Carragher *et al.* 2001).

The heterotrimeric guanine nucleotide binding (G) proteins control diverse biological processes by conveying

signals from cell-surface receptors to intracellular effectors. They are a family of proteins that transduce an extracellular signal to an intracellular response via a seven helical transmembrane receptor (G protein-coupled receptor, GPCR). Upon activation, the receptor facilitates the exchange of GDP for GTP in the G α subunit. G α is then thought to dissociate from the G $\beta\gamma$ heterodimer, allowing both complexes to individually activate a number of effectors (Neer 1995, Hamm 1998). Although function was originally ascribed to the GTP-bound α -subunit, it is now well established that the $\beta\gamma$ -dimer plays active roles in the signaling process through upstream recognition of receptors and downstream regulation of effectors (Clapham & Neer 1997). Molecular cloning has identified at least 5 β - and 12 γ -subunit genes in the mouse and human genomes. Structurally, γ -subunits are the most diverse, with four subgroups that show less than 50% identity to each other (Balcueva *et al.* 2000). Moreover, γ -subunits exhibit very different temporal (Morishita *et al.* 1999, Schuller *et al.* 2001) and spatial (Betty *et al.* 1998) patterns of expression. These characteristics suggest that γ -subunits have heterogeneous functions. Free G $\beta\gamma$ interacts with a large assortment of effector proteins, including phospholipases (Rhee & Bae 1997), adenylyl cyclases (Sunahara *et al.* 1996), ion channels (Schneider *et al.* 1997), and G protein-coupled receptor kinases (Pitcher *et al.* 1992). There are, however, G protein-coupled receptor responses, such as MAP kinase activation (Koch *et al.* 1994, Luttrell *et al.* 1996, 1997), receptor internalization (Liu *et al.* 1997, Lin *et al.* 1998), and organelle transport (Stow & Heimann 1998, Jamora *et al.* 1999) that are mediated through the G $\beta\gamma$ subunit but which have not definitively been linked to known G $\beta\gamma$ effectors. Although activation of G protein-coupled receptors can elicit rapid stimulation of cellular protein tyrosine phosphorylation, the mechanism by which G proteins activate protein-tyrosine kinases is not completely understood. In the present study, we identified c-Src tyrosine kinase as a direct effector of G proteins and characterized the G proteins subunits involved in the mechanism by which PTH regulates ERKs via c-Src tyrosine kinase.

Materials and Methods

Chemicals

Synthetic rat PTH(1–34), immobilon P (polyvinylidene difluoride, PVDF) membranes, enolase and protein A-Sepharose were from Sigma Chemical Co. (St Louis, MO, USA). Rabbit polyclonal anti-phosphotyrosine antibody, rabbit anti-phospho c-Src Y527 and Y416 antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-PLC γ , anti-P-ERK, anti-ERK and anti-c-Src antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). G protein

antibodies were generously provided by Dr Maria Julia Marinissen (NIH, MD, USA). Secondary antibody goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG and the Super Signal CL-HRP substrate system for enhanced chemiluminescence (ECL) were obtained from Amersham Corp. (Arlington Heights, IL, USA). [γ - 32 P]ATP (3000 Ci/mmol) was from New England Nuclear (Chicago, IL, USA). All other reagents were of analytical grade.

Animals

Three-month-old male Wistar rats were fed with standard rat food (1.2% Ca, 1.0% phosphorus), with water available *ad libitum*, and were maintained on a 12 h light–12 h darkness cycle. Animals were killed by cervical dislocation. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals (1996 (7th edn) Washington, DC: National Academy Press, aka National Research Council Guide).

Isolation of duodenal cells

Duodenal cells were isolated as described previously (Massheimer *et al.* 1994). The method employed yields preparations containing only highly absorptive epithelial cells that are devoid of cells from the upper villus or crypt (Weiser 1973). The duodenum was excised, washed and trimmed of adhering tissue. The intestine was slit lengthwise, cut into small segments (2 cm length) and placed into solution A: 96 mM NaCl, 1.5 mM KCl, 8 mM KH $_2$ PO $_4$, 5.6 mM Na $_2$ HPO $_4$, 27 mM Na citrate, pH 7.3, for 10 min at 37 °C. The solution was discarded and replaced with solution B (isolation medium): 154 mM NaCl, 10 mM NaH $_2$ PO $_4$, 1.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 5.6 mM glucose, pH 7.3, for 15 min at 37 °C with vigorous shaking. The cells were sedimented by centrifugation at 155 $\times g$ for 10 min, washed twice with 154 mM NaCl, 10 mM NaH $_2$ PO $_4$, 5.6 mM glucose, pH 7.4 and resuspended in the incubation medium (solution D): 154 mM NaCl, 5 mM KCl, 1 mM Na $_2$ HPO $_4$, 1 mM MgCl $_2$, 10 mM NaMOPS pH 7.4, 5.6 mM glucose, 0.5% BSA, 1 mM CaCl $_2$, 2.5 mM glutamine. All the above steps were performed under a 95% O $_2$: 5% CO $_2$ atmosphere using oxygenated solutions. The enterocytes were used between 20 and 60 min after their isolation. Cell viability was assessed by trypan blue exclusion in dispersed cell preparations; 85–90% of the cells were viable for at least 150 min.

In vitro treatments

Isolated duodenal cells were pre-equilibrated in solution D for 15 min and then exposed for short intervals (15 s–10 min) to PTH (10 $^{-8}$ M). After treatment, enterocytes were lysed in 50 mM Tris–HCl (pH 7.4), 150 mM

NaCl, 2 mM EGTA, 25 mM NaF, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml leupeptin, 2 μ g/ml aprotinin, 0.25% sodium deoxycholate and 1% NP40. Insoluble material was pelleted in a microcentrifuge at 14 000 r.p.m. for 10 min. The protein content of the clear lysates was determined according to the method of Bradford (1976).

Immunoprecipitation

Lysate aliquots (500–700 μ g protein) were incubated overnight at 4 °C with anti-phosphotyrosine antibody, followed by precipitation of the complexes with protein A conjugated with Sepharose. The immune complexes were washed three times with cold immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 0.2 mM sodium orthovanadate, 1% Triton X-100 and 1% NP40), two times with PBS and then subjected to Western blot analysis.

Co-immunoprecipitation

Co-immunoprecipitation assays were performed under native conditions in order to preserve protein-protein associations. After hormone treatment, cells were lysed (15 min at 4 °C) in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 3 mM KCl, 0.5 mM EDTA, 0.2 mM Na₃VO₄ (OV), 1 mM NaF, 1 mM PMSF, 6 μ g/ml leupeptin, 8 μ g/ml aprotinin, and 1% Tween-20. Lysates were clarified by centrifugation (14 000 $\times$ g, 10 min) and immunoprecipitation of the supernatants was performed with anti-G α s, anti-G β or anti-c-Src antibodies, the precipitated immunocomplexes were washed and processed as described above. To confirm co-immunoprecipitation of both proteins, immunoprecipitation and immunoblotting were performed with the same antibodies used in reverse order.

SDS-PAGE and immunoblotting

Immunoprecipitated proteins (or lysate proteins) dissolved in Laemmli sample buffer were separated on SDS-polyacrylamide (10%) gels (Laemmli 1970) and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 2 h at room temperature in TBST (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Tween 20 containing 1% dry milk). Anti-phospho c-Src (Tyr527), anti-phospho c-Src (Tyr416), anti-c-Src, anti-phospho ERKs (p42 and p44 isoforms), anti-G α s, or anti-G β antibodies were allowed to react with the membrane overnight at 4 °C. Next, the membranes were washed three times in TBST, incubated with a 1:10 000 dilution of peroxidase-conjugated anti-rabbit secondary antibody for 1 h at room temperature and washed three additional times with TBST. The membranes were then visualized using an enhanced

chemiluminescent technique, according to the manufacturer's instructions. Images were obtained with a model GS-700 Imaging Densitometer from Bio-Rad (Hercules, CA, USA) by scanning at 600 dpi and printing at the same resolution. Bands were quantified using the Molecular Analyst program (Bio-Rad).

Measurement of c-Src kinase activity

Cell lysates (700 μ g protein) were prepared followed by immunoprecipitation of c-Src as described above. After three washes with immunoprecipitation buffer and two washes with kinase buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 1 mM DTT, 0.1 mM sodium orthovanadate), the immune complexes were incubated at 30 °C for 10 min in kinase buffer (30 μ l/sample) containing enolase as an exogenous substrate for Src (2.5 μ g/assay), 50 μ M ATP and [γ -³²P]ATP (2 μ Ci/assay). To terminate the reaction, the phosphorylated product was separated from free isotope on ion-exchange phosphocellulose filters (Whatman P-81). Papers were immersed immediately onto ice-cold 75 mM H₃PO₄, washed (1 $\times$ 5 min, 3 $\times$ 20 min) and counted in a scintillation counter.

Measurement of adenylyl cyclase activity

Adenylyl cyclase activity was determined by measuring the cAMP generated after hormonal treatment of microsomal membranes (Farndale *et al.* 1994). Microsomal membranes were isolated by centrifugation at 100 000 $\times$ g (60 min). Microsomal protein (75 μ g) was incubated in 500 μ M ATP, 10 mM MgCl₂, 10 mM phosphocreatine, 50 U/ml creatine kinase, 100 μ M isobutylxanthine, 1 mM DTT, 10 mM Tris-HCl, pH 7.4, for 3 min at 30 °C. Treatment was stopped by the addition of perchloric acid (6%) followed by centrifugation. Cyclic AMP was measured in the supernatant by a protein binding assay (Tovey *et al.* 1974) using a commercial kit.

Statistical evaluation

Statistical significance of the data was evaluated using Student's *t*-test (Snedecor & Cochran 1967) and probability values below 0.05 (*P* < 0.05) were considered significant. Results are expressed as means $\pm$ standard deviation (s.d.) from the indicated set of experiments.

Results

To evaluate whether the tyrosine kinase c-Src is a downstream effector of G proteins in the PTH signaling mechanism in rat intestinal cells, we first investigated the effect of the hormone on c-Src kinase activity. To that end, the enzyme from lysates of rat enterocytes exposed for different times to PTH (10⁻⁸ M) was immunoprecipitated with a highly specific anti-c-Src monoclonal

Figure 1 Time-course of PTH stimulation of c-Src kinase activity. Rat enterocytes were incubated in the presence of PTH(1–34) (10^{-8} M) for the indicated times. Immunoprecipitation of c-Src and assay of c-Src tyrosine kinase activity using [γ - 32 P]ATP and enolase as exogenous substrate were carried out as detailed in Materials and Methods. Results are the average of three independent experiments performed in triplicate $\pm$ s.d. * $P < 0.05$. Prot., protein.

antibody and then [γ - 32 P]ATP and enolase, acting as exogenous c-Src substrate, were added. As shown in Fig. 1, PTH caused a time-dependent increase in Src kinase activity in rat enterocytes. Phosphorylation of the c-Src substrate is biphasic, with an early phase peaking at 30 s (+120%) and a second phase gradually increasing up to 5 min of treatment with the hormone (+220%). No time-dependent changes in c-Src activity were observed under basal conditions (data not shown). We then investigated PTH-induced changes in tyrosine phosphorylation of c-Src. Enterocytes were treated with PTH (10^{-8} M, 30 s–5 min) and cell lysates were immunoprecipitated with anti-P tyrosine antibody, followed by Western blotting with a specific anti-cSrc antibody. As shown in Fig. 2, PTH increased the level of tyrosine phosphorylation of c-Src with a kinetics roughly similar to that found for the increase in kinase activity. Then we monitored the phosphorylation state of Tyr527 and Tyr416 of c-Src. To that end, enterocytes were exposed to 10^{-8} M PTH (15 s–10 min), followed by Western blot analysis of cell lysates with anti-c-Src-phospho Tyr527 and anti-c-Src-phospho Tyr416. Total c-Src was measured in the same immunoblot by stripping the membrane and reincubating with anti-c-Src antibody. As shown in Fig. 3A, PTH transiently induces the dephosphorylation of Tyr527 at 15 s (– twofold), and increases the phosphorylation on Tyr416 of c-Src, with maximal effects at 5 min (+threefold) (Fig. 3B).

The relationship of the rise in intracellular Ca^{2+} by PTH leading to Src activation is not defined. We have previously found that PTH increases cytosolic Ca^{2+} levels [Ca^{2+}]_i in rat enterocytes, which involves Ca^{2+} mobilization from endogenous stores followed by cation influx from the extracellular milieu (Picotto *et al.* 1997, Gentili *et al.* 2003a). To evaluate the contributions of intracellular and extracellular Ca^{2+} pools, PTH-dependent changes in

Figure 2 PTH stimulates c-Src tyrosine phosphorylation. Enterocytes were exposed to 10^{-8} M PTH(1–34) for the indicated times. Cell lysates were immunoprecipitated with anti-P tyrosine antibody, and the c-Src tyrosine phosphorylation state was assayed by immunoblotting with anti-c-Src antibody. A representative immunoblot and densitometric analysis of changes in c-Src tyrosine phosphorylation from three immunoblots are shown. * $P < 0.05$, ** $P < 0.025$. Blotted membranes shown were stained with Coomassie brilliant blue in order to evaluate the equivalence of protein content among the different experimental conditions.

the tyrosine phosphorylation/dephosphorylation of c-Src were examined in a Ca^{2+} -free medium (EGTA, 0.5 mM), and after chelating intracellular Ca^{2+} with 1,2-bis(2-amino-phenoxy)ethane- N,N,N',N' -tetracetic acid tetrakis (BAPTA-AM) (5 μM). As shown in Fig. 4A, BAPTA-AM fully suppressed PTH-dependent c-Src Tyr416 phosphorylation. In the presence of EGTA, the phosphorylation of this residue is also abolished, except at 1 min, the latter suggesting that the PTH-stimulated release of calcium from intracellular stores is responsible for the early effect of PTH on c-Src activation. Chelation of extracellular or intracellular Ca^{2+} also inhibited hormone-dependent rapid dephosphorylation of c-Src Tyr527 (Fig. 4B). These results point to a role for Ca^{2+} as an upstream activator of c-Src in rat enterocytes exposed to PTH.

To further explore PTH-signal transduction in enterocytes, we initially determined whether G proteins are upstream mediators of PTH-induced c-Src activation. To investigate whether there is a direct interaction between c-Src and G proteins, we performed co-immunoprecipitation studies with *G α* s and *G β* subunits in PTH-stimulated enterocytes. As shown in Fig. 5, association of both subunits to c-Src was clearly detectable under basal conditions and stimulation with PTH significantly increased the formation of the complex. The hormone induces the greatest association of the kinase with *G α* s and *G β* at 30 and 15 s respectively. Similar results were obtained when the antibodies were used in reverse order

Figure 3 PTH induces c-Src Tyr527 dephosphorylation and Tyr416 autophosphorylation. Enterocytes were exposed to 10^{-8} M PTH(1–34) for the indicated times. The c-Src tyrosine phosphorylation state was assayed in cell lysates by immunoblotting with anti-c-Src-phospho (P)-Tyr527 (A) and anti-c-Src-P-Tyr416 (B) antibodies as described in Materials and Methods. A representative immunoblot and densitometric analysis of changes in c-Src tyrosine phosphorylation from three immunoblots are shown; * $P < 0.05$, ** $P < 0.025$, *** $P < 0.01$. Blotted membranes shown were re-probed with anti-c-Src antibody in order to evaluate the equivalence of c-Src kinase content among the different experimental conditions.

(not shown). These results suggest that G proteins may be required for PTH-induced c-Src activation. It is likely that c-Src association with G protein subunits changes the enzyme conformation leading to increased accessibility of the active site to substrates. It has been reported that direct G protein regulation of c-Src does not involve the dephosphorylation of Tyr527 (Ma *et al.* 2000). Therefore, in an attempt to further evaluate whether PTH-induced G

protein-Src association stimulates the PTH-mediated increase in c-Src Tyr416 autophosphorylation, enterocyte homogenates were preincubated on ice for 10 min in the presence of anti- $G\beta$ or anti- $G\alpha$ s antibodies, followed by exposure to 10^{-8} M PTH(1–34) for 5 min. Proteins were resolved by electrophoresis, electroblotted into PVDF membranes and incubated with anti-c-Src-phospho Tyr416. In order to evaluate the equivalence of c-Src

Figure 4 Ca^{2+} dependence of PTH-induced c-Src Tyr416 phosphorylation and Tyr527 dephosphorylation. Enterocytes were exposed to 10^{-8} M PTH(1–34) for the indicated times in the presence or absence of EGTA (0.5 mM) or BAPTA-AM (5 μ M). Cell lysates were obtained and comparable aliquots of lysate proteins were separated by SDS-PAGE followed by Western blotting with anti-c-Src-phospho-Tyr416 (A) or anti-c-Src-phospho-Tyr527 (B) antibodies. Blotted membranes shown were re-probed with anti-c-Src antibody in order to evaluate the equivalence of c-Src kinase content among the different experimental conditions. Representative immunoblots from 3 independent experiments are shown.

Figure 5 Interaction of Gas and Gβ with c-Src in enterocytes stimulated with PTH. Enterocytes were exposed to 10^{-8} M PTH(1–34) for the indicated times. Cell lysates were obtained, c-Src was immunoprecipitated (IP) with anti-c-Src monoclonal antibody under native conditions, resolved onto 10% SDS-PAGE gels and then immunoblotted (IB) with anti-Gas or anti-Gβ antibodies as described in Materials and Methods. Representative immunoblots from 3 independent experiments are given.

kinase content among the different experimental conditions, blotted membranes were re-probed with anti-Src antibody. When enterocyte homogenates were preincubated with anti-Gβ antibody followed by a brief exposure to PTH, the effect of the hormone on c-Src Tyr416 phosphorylation was abolished (Fig. 6). Incubation with anti-Gas did not alter the PTH-dependent increase in c-Src Tyr416 phosphorylation. In rat enterocytes, we have previously reported that PTH activates the MAP kinases, ERK1 and ERK2, by a mechanism dependent on c-Src, the adenylyl cyclase pathway and Ca^{2+} (Gentili & de Boland 2000, Gentili *et al.* 2001b). To examine whether Gβγ and Gas subunits could mediate PTH-dependent activation of ERKs, we determined whether the hormone effect is sensitive to antibody blockade of these G protein subunits. To that end, enterocyte homogenates were preincubated in the presence or absence of anti-Gβ or anti-Gas antibodies followed by a brief exposure to 10^{-8} M PTH(1–34). Then, cell lysates were probed with an anti-phospho ERKs antibody, which recognizes both the 42 and 44 kDa species. As shown in Fig. 7, and in agreement with our previous results (Gentili & de Boland 2000, Gentili *et al.* 2001b), PTH induces a marked increase in the phosphorylation of both isoforms. Hormone-dependent ERK1 and ERK2 tyrosine phosphorylation was sensitive to antibody treatment of Gβ subunits, suggesting that c-Src via the dimer Gβγ activates the mitogen-activated protein kinases in cells stimulated with PTH. Antibody blockade of Gas had no effect on PTH-dependent increase in ERK1 and ERK2 phosphorylation. The efficacy of the antibody against Gas was tested by evaluating its effects on adenylyl cyclase activity. As shown in Table 1, incubation of microsomal membranes with PTH (10^{-8} M) induced a 200% increase in adenylyl cyclase activity, an effect that was not seen in membranes preincubated with anti-Gas.

Figure 6 Inhibition of PTH-dependent c-Src Tyr416 autophosphorylation by enterocyte treatment with anti-Gβγ antibody. Enterocytes isolated from 3-month-old rats were homogenized and preincubated on ice for 10 min in the presence of anti-Gβγ or anti-Gas (1:250) antibodies followed by exposure to 10^{-8} M PTH(1–34) for 5 min. Proteins were resolved by electrophoresis, electroblotted into PVDF membranes and incubated with anti-c-Src-phospho-Tyr416 antibody as indicated in Materials and Methods. Blotted membranes shown were re-probed with anti-c-Src antibody in order to evaluate the equivalence of c-Src kinase content among the different experimental conditions. Representative immunoblot and quantification by scanning volumetric densitometry of blots from 3 independent experiments are shown; averages $\pm$ S.D. are given. * $P < 0.05$.

Discussion

c-Src kinase activity could be modulated by tyrosine phosphorylation and conformational changes that affect the intramolecular interactions. The kinase activity of c-Src is maintained at a low basal level by two intramolecular interactions, one is between the SH3 domain and the linker (between the SH2 domain and the kinase domain) and the other is between the SH2 domain and the phosphorylated tyrosine residue 527 (Tyr527) in the carboxyl-terminal tail (Xu *et al.* 1997). The tyrosine kinase Csk phosphorylates Tyr527, repressing the kinase activity of c-Src to generate a downregulated state (Brown & Cooper 1996). In addition, autophosphorylation of Tyr416 at the activation loop is a critical step leading to full activation of c-Src tyrosine kinase activity. In the active state, the activation loop swings away from the entrance of the catalytic cleft, allowing access of the substrate to the active site. Phosphorylation of Tyr416 has been proposed to stabilize this extended conformation and activate kinase activity (Xu *et al.* 1999).

Figure 7 Gβγ subunits mediate the early phosphorylation of ERK1/2 in enterocytes stimulated with PTH. Enterocytes isolated from 3-month-old rats were homogenized and preincubated on ice for 10 min in the presence of anti-Gβγ or anti-Gαs (1:250) antibodies followed by exposure to 10^{-8} M PTH(1–34) for 1 min. Proteins were resolved by electrophoresis, electroblotted into PVDF membranes and incubated with anti-phospho ERK 1/2 antibody as indicated in Materials and Methods. Blotted membranes shown were re-probed with anti-ERK antibody in order to evaluate the equivalence of ERK kinase content among the different experimental conditions. A representative Western blot and quantification by scanning volumetric densitometry of blots from 3 independent experiments are shown; averages $\pm$ S.D. are given. * $P < 0.025$.

In the present work, we demonstrate, for the first time, that PTH rapidly increases, in a biphasic manner, the activity of non-receptor tyrosine kinase c-Src in rat intestinal cells. The biphasic nature of c-Src activity has also been observed in rat colonocytes stimulated with the steroid hormone $1\alpha,25(\text{OH})_2$ -vitamin D_3 (Khare *et al.* 1997). The hormone activates c-Src in intestinal cells through fast changes in tyrosine phosphorylation of the enzyme. The first event in the activation of c-Src is the dephosphorylation of Tyr527 (which happens after a few seconds of PTH treatment), which rapidly undergoes re-phosphorylation most likely catalyzed by Csk as discussed above. This is followed by a second event of activation related to the phosphorylation at Tyr416. In line with these observations, there is evidence demonstrating that PTH activates c-Src in osteoblastic cells through changes in tyrosine phosphorylation (Izbicka *et al.* 1994). Our results show that removal of external Ca^{2+} and

Table 1 Effect of anti-Gαs antibody on PTH-dependent adenylyl cyclase activity. Data are presented in pmol cAMP/mg protein/min and in percentage of the stimulation in respect to control values (in parentheses). Results are the mean $\pm$ S.D. of three independent experiments performed in quadruplicate

	cAMP levels pmol/mg protein/mg)
Control	2.90 $\pm$ 0.52
PTH	9.05 $\pm$ 0.81 (+212%)*
Anti Gαs	3.02 $\pm$ 0.40
PTH+anti Gαs	3.14 $\pm$ 0.67 (+8%)

Enterocyte microsomal membranes isolated from 3 month-old rats, were homogenized and preincubated on ice for 10 min in the presence or absence of anti-Gαs (1:250) antibody followed by exposure to 10^{-8} M PTH(1–34) for 2 min. Adenylyl cyclase activity was determined as described under Materials and Methods.

* $P < 0.005$ with respect to control.

chelation of intracellular Ca^{2+} suppressed Tyr527 dephosphorylation and Tyr416 phosphorylation, indicating that Ca^{2+} is an upstream activator of c-Src in enterocytes stimulated with PTH. Extracellular Ca^{2+} has been shown to be required for the activation of the Src kinase pathways in pancreatic acinar cells (Tsunoda *et al.* 1996). Furthermore, Src family kinases have been implicated in the control of receptor-operated Ca^{2+} influx in various cell types (Niklinska *et al.* 1992, Lee *et al.* 1993, Bonaccorsi *et al.* 1995, Fleming *et al.* 1995). Of interest, in colonic smooth muscle cells, the biphasic activation of ceramide-induced Src kinase activity was shown to be dependent on extracellular Ca^{2+} in the second, sustained phase of activation (Ibitayo *et al.* 1998).

c-Src family tyrosine kinases emerged to play a role in heterotrimeric G protein signaling. There is evidence suggesting the importance of c-Src in GPCR-signal transduction pathways, but the biochemical mechanisms used by G proteins to activate c-Src remains largely elusive. Recent studies demonstrated that Gαs and Gαi directly stimulate the kinase activity of the downregulated c-Src and found that activated G proteins interact with the kinase domain of c-Src (Ma *et al.* 2000). Our results indicate that the G protein subunits, Gαs and Gβ, are associated with c-Src in basal conditions and this association increases two- to threefold in rat intestinal cells treated with PTH. Blocking of Gβ subunits by preincubation of cells with a Gβ antibody abolished hormone-dependent c-Src Tyr416 phosphorylation, indicating that c-Src is a downstream effector of Gβγ complexes. In line with this observation, it has been reported that Gβγ subunits, when overexpressed in COS-7 cells, increased (by $\sim$ twofold) c-Src Tyr416-autophosphorylation (Luttrell *et al.* 1996). Moreover, in other G protein signaling systems, Gβγ plays significant roles in downstream effector activation (Clapham & Neer 1997).

Figure 8 A model for the stimulation of the ERK pathways upon binding of PTH to its G protein-coupled seven transmembrane receptor in rat enterocytes. PTH, upon binding to its seven transmembrane receptor, activates the receptor-coupled Gβγ protein subunits that associate and activate c-Src. Then, c-Src kinase creates SH2 domain binding motifs and an Shc-, Grb2-, and Sos-containing complex is formed to activate Ras and, in turn, Raf-1. The increase in Raf-1 activity is subsequently transduced through the phosphorylation of the MEK-ERK module. PKA positively regulates the ERK pathway, but whether it does so through Raf-1 activation or inhibition of MAP kinase phosphatases (MKPs) is not known at present. Y, tyrosine; T, threonine.

There is increasing evidence suggesting that Src tyrosine kinases have a pivotal role in the regulation of various cellular processes (Erpel & Courtneidge 1995). Members of this family are thought to be involved in signal transduction mechanisms linked to the mitogen-activated protein kinases (ERK1 and ERK2) cascade underlying the regulation of cell proliferation and differentiation by agonists of receptor tyrosine kinases or heterotrimeric G protein-coupled receptors (Marshall 1995). While the tyrosine kinase growth factor receptors transmit signals to ERKs in a well defined multistep process, the stimulation of ERK activity by G protein-coupled receptors may be mediated by different classes of G proteins, including Gs, Gi, Gαq/11, βγ complexes, or Gi/o (Liebmann 2001, Marinissen & Gutkind 2001). We found that blockade of Gβ subunits by preincubation of cells with a Gβ antibody abolished PTH-dependent ERK1/ERK2 activation in rat intestinal cells. In agreement with these observations, it was reported (Luttrell *et al.* 1996) that Gβγ subunit-mediated formation of Shc-c-Src complexes and c-Src kinase activation are early events in Ras-dependent activation of MAP kinase via pertussis toxin-sensitive G protein-coupled receptors. Nishida *et al.* (2000) reported that inhibition of the βγ subunit attenuates hydrogen

peroxide (H₂O₂)-induced ERK activation via c-Src in rat neonatal cardiomyocytes. c-Src can also activate Ras through the transactivation of the epidermal growth factor receptor and related receptors following stimulation of GPCRs coupled to either Gi or Gq (Della Rocca *et al.* 1997, Andreev *et al.* 2001, Shah & Catt 2002).

In summary, the results of this work show that PTH activates c-Src in intestinal cells through conformational changes via G proteins and a calcium-dependent modulation of tyrosine phosphorylation of the enzyme, and that PTH receptor activation leads via Gβγ-c-Src to the phosphorylation of the MAP kinases, ERK1 and ERK2 (Fig. 8).

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