Proline-rich Motifs in the Parathyroid Hormone (PTH)/PTH-related Protein Receptor C Terminus Mediate Scaffolding of c-Src with β-Arrestin2 for ERK1/2 Activation*

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Parathyroid hormone (PTH) stimulates ERK1/2 through both G-protein signaling and β-arrestin2-mediated internalization. β-Arrestin may serve as a scaffold for c-Src. However, the molecular mechanisms for ERK1/2 activation by PTH remain unclear. By using a targeted mutagenesis approach, we investigated the PTH/PTH-related protein receptor (PTH1R) structural determinants for ERK1/2 activation and transcriporntional activity in HEK-293 cells. First, ERK1/2 activation was inhibited by PTH1R mutations that specifically abrogated Gα-protein kinases C signaling with a decrease in cAMP-protein kinase A. Second, PTH1R C-terminal mutations and/or deletions that prevent interaction with β-arrestin inhibited ERK1/2 activation. Similar results were obtained in HEK-293 cells co-expressing wild-type PTH1R and a dominant-negative β-arrestin2. Third, the c-Src inhibitor PP2 and a kinase-dead c-SrcK295M mutant co-expressed with wild-type PTH1R both inhibited ERK1/2 activation. Furthermore, c-Src co-precipitated with both PTH1R and β-arrestin2 in response to PTH. Deleting the PTH1R-proximal C terminus abolished these interactions. However, the need for receptor interaction with β-arrestin to co-precipitate Src and activate ERK1/2 was obliterated by expressing a constitutively active c-SrcY527A mutant, suggesting direct binding of activated Src to PTH1R. Subsequently, we identified and mutated to alanine four proline-rich motifs in the PTH1R distal C terminus, which resulted in loss of both c-Src and arrestin co-precipitation and significantly decreased ERK1/2 activation. These data delineate the multiple PTH1R structural determinants for ERK1/2 activation and newly identify a unique mechanism involving proline-rich motifs in the receptor C terminus for reciprocal scaffolding of c-Src and β-arrestin2 with a class II G-protein-coupled receptor.

Mitogen-activated protein kinase (MAPK)2 activation is a central mechanism by which G-protein-coupled receptor (GPCR) agonists, including peptidic hormones, regulate crucial biological processes such as cell growth and differentiation (1). Recent studies on the β2-adrenergic receptor and the angiotensin type 1A receptor showed that ERK1/2 could be independently activated by the classical Gαi and Gαq signaling cascades and/or through receptor internalization with β-arrestins (2–4). β-Arrestin 1 and 2 are cytosolic proteins that interact with GPCRs that have been phosphorylated by a variety of kinases such as protein kinase A (PKA), protein kinase C (PKC), and G-protein-coupled receptor kinases (GRKs) (2, 5). In addition to uncoupling GPCRs from heterotrimeric G proteins and activating phosphodiesterase 4, thereby prompting cessation of cAMP signaling (6–9), arrestins may function as a scaffold for c-Src-mediated activation of MAPKs (10, 11). Alternatively, some GPCRs, such as the β2-adrenergic receptor, are capable of binding c-Src directly in the absence of β-arrestin (12). This scaffold is made possible through protein-protein interactions, including Src homology 3 (SH3) domains in c-Src and proline-rich motifs (PRX) in the β2-adrenergic receptor.

Parathyroid hormone (PTH) is the primary regulator of serum calcium homeostasis and plays a major role in bone metabolism (13). In osteoblasts and kidney cells, i.e. classical targets for PTH, PTH-induced ERK1/2 activation is correlated with cell proliferation, differentiation, and apoptosis (14–17). The PTH/PTHrP receptor (type 1 parathyroid hormone receptor, PTH1R) is a class II GPCR that transduces PTH (and PTHrP) signaling mainly through Gαq and Gα1 proteins (18, 19). The termination of receptor signaling involves receptor phosphorylation by PKC, GRKs, and to a lesser extent, PKA (20–22), followed by β-arrestin binding, desensitization, and internalization of ligand-receptor-arrestin complexes through clathrin-coated vesicles (7, 23, 24). Accordingly, PTH has been reported to activate ERK1/2 through arrestin-mediated internalization of PTH1R and subsequent transactivation of the epidermal growth factor receptor (EGFR) (25). Moreover, it was recently shown that Gβγ subunit interaction with the juxta-membrane region of the PTH1R cytoplasmic tail is implicated in MAPK signaling (26). However, most of the PTH1R structural determinants and related molecular events implicated in ERK1/2 activation remain to be elucidated. By using an extensive mutagenesis approach, we now identify a unique mechanism for ERK1/2 activation by PTH that engages scaffolding of type; dn, dominant negative; PKC, protein kinase C; PKA, protein kinase A; GRK, G-protein-coupled receptor kinase; SH3, Src homology 3; SRE, serum-responsive element; EGFR, epidermal growth factor receptor; FCS, fetal calf serum.
c-Src through both proline-rich motifs and β-arrestin-binding sites in the PTH1R C terminus. In turn, direct binding of c-Src to the receptor stabilizes its interaction with arrestins and may ensure a molecular switch from cAMP/IP_{3} intracellular signaling to MAPK signaling cascades.

**EXPERIMENTAL PROCEDURES**

*Adenylyl Cyclase Activity*—cAMP accumulation was determined in subconfluent cell cultures in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine as described previously (23). Briefly, cells were incubated for 2 h in medium containing [3H]adenine (2 μCi/ml), then preincubated for 15 min with 3-isobutyl-1-methylxanthine (1 mM), and subsequently exposed to PTH-(1–34) (100 nM) for the indicated time. In all cases, reactions were stopped with 1.2 M trichloroacetic acid, and cAMP was isolated by the two-column chromatographic method as described previously (23). Accumulation of [3H]adenine was then determined by a standard liquid scintillation technique. Results were expressed in percentage of WT PTH1R, and EC_{50} values were determined by performing PTH dose response for each mutant receptor (Fig. 1).

**Materials**—Bovine parathyroid hormone fragment 1–34 (PTH-(1–34)) and forskolin were purchased from Sigma. Dulbecco’s modified Eagle’s medium was from Invitrogen. c-Src inhibitor (PP2), PKA inhibitor (H89), and PKC inhibitor (Gö6983) were obtained from Calbiochem. Anti-phospho-p44/42 MAPK (pERK1/2) rabbit polyclonal antibody, anti-phosphotyrosine 416 c-Src, and anti-His tag (27E8) mouse monoclonal antibodies were from Cell Signaling (Billerica, MA). Mouse monoclonal anti-GFP antibody (JL-8) was from Santa Cruz Biotechnology (Nunningen, Switzerland). Mouse anti-GFP antibody (JL-8) from BD Biosciences. Protein A/G Plus immunoprecipitation reagent, anti-p44/p42 MAPK (ERK1/2), and anti-c-Src (Src-2) rabbit polyclonal antibodies were obtained from Santa Cruz Biotechnology (Nunningen, Switzerland).

**Cell Culture**—Human embryonic kidney cells (HEK-293) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 1% penicillin/streptomycin and 10% fetal calf serum (FCS) in a humidified 5% CO_{2} air incubator at 37 °C. Cells were plated at 9 × 10^{5} cells/100 mm culture flasks (Corning Glass) for MAPK assays and co-immunoprecipitation or at 10^{5} cells/well in 24-well dishes (Corning Glass) for luciferase assay, transfected as described previously (23), and then cultured in 1% FCS overnight before performing experiments.

**PathDetect Reporting System**—The SRE signal transduction pathways were assayed in vitro using the PathDetect SRE cis-reporting system according to the manufacturer’s instructions (Stratagene, La Jolla, CA). Briefly, 24-well tissue culture plates were seeded at a density of 10^{4} cells/well in 0.5 ml of complete Dulbecco’s modified Eagle’s medium and transfected using FuGENE® reagent (Roche Applied Science) 24 h after plating, with 400 ng of pSRE-reporter and various amounts of plasmid DNA to be tested. As needed, empty pcDNA3.1 vector was added to make the total transfected DNA amount equal in all wells. As an appropriate control for transfection efficiency, plasmid pRLTK coding for Renilla luciferase was also transfected using 10 ng/well. One night before PTH treatment, the medium was replaced by 1% FCS medium. The reporter luciferase activity was assayed by cell lysis and quantitation on a TD-20/20 luminometer (Turner BioSystems, Sunnyvale, CA) using the luciferase assay kit (Promega, Madison, WI). Results were expressed in PTH fold induction of luciferase activity.

**Plasmids Constructions**—Cloning, ligations, transformations in E. coli XL1 blue strain, and DNA mini preparations were carried out according to Sambrook et al. (27). Plasmid DNA was prepared using Qiagen kits (Basel, Switzerland). PCRs were performed using Expand High Fidelity PCR system (Roche Applied Science). The temperature cycling protocol was 94 °C for 1 min, 55 °C for 40 s, and 72 °C for 2 min for 30 cycles. The reactions were concluded by a 10-min elongation at 72 °C. Oligonucleotides were purchased from Microsynth AG (Balgach, Switzerland). Human PTH1R was cloned in pZeoSV2(+) (Invitrogen) as described in Bisello et al. (28). All subsequent mutants of the receptor were derived from this plasmid. Trimmed PTH1R receptor at aa 500 was first constructed using PCR amplification as described previously (7), and the appropriate mutations were thereafter added to obtain the β-arrestin-deficient PTH1R mutant (see below). The proximal C terminus-deficient PTH1R mutant (PCdel-PTH1R) holding a deletion of 28 amino acids between aa 472 and 500 has been created by PCR amplification of the DNA fragments located upstream and downstream of the sequence to be removed, using two sets of primers. H223R mutant was a generous gift of Dr. H. Juppner (Boston). Site-directed mutagenesis was performed using QuickChange™ site-directed mutagenesis kit (Stratagene, Basel, Switzerland) and the appropriate oligonucleotide primers and following the recommendations of the manufacturer. DNA sequence analysis of each construct confirmed the absence of PCR-introduced mutations. The β-arrestin-deficient PTH1R (ArD-PTH1R) results in the replacements of 7 proximal serine residues in the cytoplasmic tail of the truncated PTH1R at aa 500 with alanine (Ser-473, Ser-475, Ser-489, Ser-491, Ser-492, Ser-493, and Ser-495). The G_{q}α-deficient PTH1R mutant (G_{q}D-PTH1R) was obtained by site-directed mutagenesis and resulted in the change of EKKY motif into DSEL amino acid sequence within the second intracellular loop (29). The proline-rich motifs-deficient PTH1R mutant (PrD-PTH1R) resulted in the replacement of prolines (Pro-517, Pro-520, Pro-532, Pro-535, Pro-540, Pro-543, Pro-578, and Pro-581) by alanines in the four proline-rich motifs (PXXP) located in PTH1R C terminus. The resulting mutated proteins encoded by these plasmids are described in Fig. 1. All receptors used for immunoprecipitation were subcloned in the pcDNA3.1/V5-His-TOPO vector in-frame with V5 epitope (Invitrogen). The plasmid-encoding rat β-arrestin2-GFP was described by Ferrari and Bisello (23). The β-arrestin2 mini-gene (AA284–409), containing the major clathrin binding domain, was constructed by amplification of the 3′ end of the cDNA. The 5′ forward primer was designed, including a consensus Kozak sequence, an initial methionine ATG codon followed by the GCA glycine codon, and a 3′ reverse primer encoded the last 7 amino acids without a stop codon (30). PCR was performed on the plasmid encoding rat β-arrestins. The PCR fragments were cloned in the pcDNA3.1/V5-His-TOPO vector. We selected the clones in which the insert had the correct orientation, in-frame with the V5 epitope (Invitrogen).
c-Src, kinase-dead c-Src-K295M (SrkK\textsuperscript{−}) and constitutively active c-Src-Y527A (SrkK\textsuperscript{+}) (31) were the generous gifts of Dr. Marc Chanson (Geneva, Switzerland).

**Immunoprecipitation and Western Blot Analysis**—HEK-293 cells treated with PTH were rapidly frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until used for analysis. Cells were lysed at 4°C in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na\textsubscript{3}VO\textsubscript{4}, 0.01 μM calcyulin A, 0.1 μM microcystin LR, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS for 60 min. Lysates were then cleared by centrifugation at 12,000 \(\times\) g for 30 min, and a 75–μl sample was diluted with an equal volume of 2X reducing sample buffer containing 125 mM Tris buffer (pH 6.8), 4% SDS, 20% glycerol, 0.05% bromphenol blue, and 200 mM dithiothreitol. The mixture was then heated at 70°C for 30 min and subjected to gel electrophoresis on 6–15% gels. Following SDS-PAGE, proteins were transferred to Immobilon-P membranes and immunoblotted with either anti-pERK1/2 (1:10,000) or anti-ERK1/2 (1:20,000) antibodies. Detection was performed using peroxidase-coupled specific secondary antibodies, enhanced chemiluminescence reaction, and visualization by autoradiography (Amersham Biosciences). Evaluation of p-ERK1/2 quantification was made using the IQMac version 1.2 software. For immunoprecipitation analysis, equal aliquots of protein lysates were immunoprecipitated using 2 μg of specific antibody (anti-His6 or anti-GFP) and incubated overnight at 4°C in a rotating device. After 24 h, 20 μl of protein A/G Plus-agarose were added and incubated for 1 h at 4°C. Immunoprecipitates were then collected by centrifugation at 1200 \(\times\) g for 3 min, and pellets were washed four times with lysis buffer and resuspended in 50 μl of running buffer. Aliquots were then subjected to electrophoresis as described above, and membranes were immunoblotted with either anti-c-Src, anti-phosphotyrosine(416)-c-Src, anti-GFP or anti-His Tag antibodies. Immunoblots were probed with peroxidase-coupled specific secondary antibodies and visualized by enhanced chemiluminescence.

**Fluorescence Microscopy**—To perform real time fluorescence microscopy, HEK-293 cells were plated on 25-mm glass coverslips (1.5 \(\times\) 10\textsuperscript{5} cells/coverslip) and cultured for 48 h. After 24 h of culture, they were co-transfected with either PTH1R, PCdel-PTH1R, ArD-PTH1R or PrD-PTH1R and with β-arrestin2-GFP associated with or without dn β-arrestin2. 24 h after transfection, coverslips were washed twice with 1 ml of PBS, 0.1% bovine serum albumin, and the cell chamber was placed at 37°C in a temperature-controlled block for the duration of the experiment. Effects of PTH-(1–34) (100 nm) on β-arrestin2-GFP localization was evaluated by adding the agonist in the same buffer and by monitoring redistribution of the green fluorescent protein at 0.5, and 20 min. Coverslips were analyzed with a Zeiss Axiovert 200 microscope with a 100× oil immersion objective (Carl Zeiss, Göttingen, Germany).

**Statistics**—Data are presented as mean ± S.E. (n), where n indicates the number of independent experiments performed in triplicate. Statistical analyses were performed by two-factor analysis of variance using Statview (SAS Institute, Inc., Cary, NC).

**RESULTS**

**PTH-stimulated cAMP Signaling by WT and Mutant PTH1R**—To perform the structure-function analyses of PTH1R described in detail below, we generated a number of point mutations and/or deletions in the receptor second intracellular loop (IC2) and C terminus; H223R, Jansen mutant; GqD-PTH1R, Gq-deficient mutant; ArD-PTH1R, arrestin-deficient mutant; PCdel-PTH1R, proximal C-terminal deletion mutant; PrD-PTH1R, proline-deficient mutant.
bPTH-(1–34) at increasing concentrations. Maximal cAMP accumulation after 30 min was similar for all mutants (range, 88–110% of wild-type), and the EC_{50} for PTH-stimulated cAMP signaling was in the subnanomolar range for all mutants (Fig. 1C).

**Gq** Signaling-deficient **PTH1R** Mutants Abrogate ERK1/2 Activation—In HEK-293 cells expressing PTH1R, bPTH-(1–34) (100 nM) transiently activated ERK1/2 after 5 min, as evaluated by p42/44 phosphorylation (Fig. 2A). Prolonged exposure to PTH did not induce further activation of ERK1/2 (data not shown). In turn, the transcriptional activity of MAPK, as evaluated by an SRE-driven luciferase assay, was increased 7.9 ± 1.2-fold by PTH (mean ± S.E. from n = 9 independent experiments) (Fig. 2B).

It has been reported that ERK1/2 cannot be activated by a cAMP signaling-selective PTH1R ligand that also prevents arrestin binding and receptor internalization (25). To directly evaluate whether G_{q} mediated signaling is necessary for ERK1/2 activation, HEK-293 cells were transiently transfected with G_{q} signaling-deficient PTH1R mutants carrying either an EKKY to DSEL mutation in the second intracellular loop (G_{q}D-PTH1R) (Fig. 1) or the H223R mutation, i.e. a (cAMP) constitutively active mutant with deficient IP_{3} signaling (Jansen metaphyseal dysplasia mutant) (29, 32, 33). Both mutants displayed a significant reduction in PTH-stimulated ERK1/2 activation and related transcriptional activity (Fig. 2, A and B). This inhibition was mimicked by incubating HEK-293 cells expressing wild-type (WT) PTH1R with a specific PKC inhibitor (5 μM Go6983) (Fig. 2, A and C). In contrast, the PKA inhibitor H89 (10 μM) had no effect on ERK1/2 activation and luciferase activity in response to PTH, and conversely, ERK1/2 was unresponsive to direct stimulation of adenylate cyclase with forskolin (10 μM) (Fig. 2, A and C). These data indicate that PTH-stimulated ERK1/2 activation requires G_{q} binding at the PTH1R and IP_{3}/PKC signaling, whereas cAMP/PKA is not sufficient for this activation.

**PTH1R C-terminal Interaction with β-Arrestin and ERK1/2 Activation**—We reported previously that PTH-stimulated PKC activation plays an important role for β-arrestin recruitment to PTH1R and that this interaction is further stabilized by the PTH1R proximal C terminus (7, 23). In turn, deleting a 28-amino acid region of the proximal C terminus (PCdel-PTH1R) (Fig. 1) abolished β-arrestin recruitment to cell membrane and thus receptor internalization, as evaluated by fluorescence microscopy (Fig. 3A), as well as ERK1/2 activation and transcriptional activity in response to PTH (Fig. 3, B and C). Because the PTH1R proximal C terminus has been reported recently to also be a docking site for Gβγ subunits (26), we further generated a β-arrestin-deficient PTH1R mutant (ArD-PTH1R) by mutating proximal serine residues to alanine in addition to a C-terminal deletion at aa 500, i.e. distal to the Gβγ docking region (Fig. 1). Similar to the homologous PTH2R in which these mutations restrain β-arrestin binding while retaining its ability to activate PKC (28), ArD-PTH1R failed to recruit β-arrestin2 and thus to internalize itself in response to PTH (Fig. 3A). In turn, in HEK-293 cells expressing the ArD-PTH1R mutant, ERK1/2 activation and transcriptional activity in response to PTH were both significantly decreased (p-ERK1/2, −58 ± 6.6%, n = 3, p < 0.0001; luciferase activity, −48 ± 5.4%, n = 3, p < 0.0001) (Fig. 3, B and C).

To further delineate the role of β-arrestin in ERK1/2 activation, we used a dn β-arrestin2 mini-gene (aa 284–409) that contains the major binding domain to clathrin and thereby competes with endogenous β-arrestin2 for targeting the ligand-receptor complex to clathrin-coated vesicles (30). Although this dn β-arrestin2 did not interfere with β-arrestin recruitment to activated WT PTH1R without inducing its internalization (Fig. 3A), it significantly decreased ERK1/2 phosphorylation and luciferase reporter activity in response to PTH (p-ERK1/2, −46 ± 5.9%, n = 3, p < 0.0001; luciferase activity, −45 ± 4.8, n = 3, p < 0.0001) (Fig. 3, B and C).
of β-arrestin2 with PTH1R (Fig. 4B). In cells expressing the PCdel-PTH1R mutant that failed to recruit/bind arrestins (Fig. 3A), c-Src did not precipitate upon PTH stimulation. In contrast, a constitutively active c-Src-Y527A (SrcK+) mutant (31) was able to co-precipitate with PCdel-PTH1R, both before and after exposure to PTH, despite the absence of receptor-bound β-arrestin2 (Fig. 4B). This observation confirmed that, in addition to bind arrestins, the PTH1R proximal C terminus is involved in c-Src activation (26) and suggested the presence of PTH1R domain(s) (outside of the proximal C terminus) capable of interacting directly with activated c-Src.

**PTH1R Proline-rich Motifs Stabilize the Src-Arrestin Complex for ERK1/2 Activation**—Four proline-rich (PXXP) motifs were identified in the receptor C terminus distal to aa 515 and mutated to alanine (PXXP → AXAX) to generate a proline-deficient PTH1R mutant (PrD-PTH1R) (Fig. 1). Similar to the other mutant receptors used in this study, PrD-PTH1R had a cAMP response to PTH similar to WT (Fig. 1). PrD-PTH1R resulted in a complete loss of c-Src and, surprisingly, β-arrestin2 co-precipitation (Fig. 4B). In contrast, constitutively active c-Src (SrcK+) co-precipitated with proline-deficient PTH1R, both before and after PTH stimulation, and co-precipitation of arrestin with the receptor was also restored (Fig. 4B). Fluoroscopy of β-arrestin2-GFP showed that the initial phase of arrestins recruitment to

Taken together, these data indicate that PTH1R interaction with arrestins accounts for nearly half of ERK1/2 activation.

**Arrestins Mediate Src-dependent Activation of ERK1/2—**
Next, we investigated the role of c-Src in PTH-stimulated, arrestin-mediated activation of ERK1/2. The kinase-dead c-Src-K295M mutant (SrcK−) (31) inhibited PTH-stimulated ERK1/2 activation and significantly decreased its transcriptional activity (−43 ± 3.8%, n = 3, p < 0.001) (Fig. 4, A and C). Similar results were obtained with PP2, a specific c-Src tyrosine kinase inhibitor (−38 ± 4.4%, n = 4, p < 0.001) (Fig. 4C).

HEK-293 cells were then transfected with His6-tagged PTH1R, c-Src, and GFP-tagged β-arrestin2. PTH treatment resulted in the co-precipitation of total and activated c-Src and PrD-PTH1R in response to PTH was conserved, but β-arrestin2-GFP failed to re-translocate to cytoplasmic vesicles (Fig. 4D). These observations indicate that, in the absence of c-Src activation and/or binding to PTH1R proline motifs, β-arrestin2 is released early from PTH1R. It also suggests that activated c-Src and arrestin form a stable complex with PTH1R during endocytosis (Fig. 5).

The profile of ERK1/2 activation and transcriptional activity in cells expressing PrD-PTH1R paralleled the changes observed in the co-precipitation assay, with a significant decrease in PTH-stimulated ERK1/2 phosphorylation (−61 ± 5.4%, n = 4, p < 0.0001) (Fig. 4A) and a proportional decrease in luciferase-reporter activity (−52 ± 6.4%, n = 4, p < 0.001) (Fig. 5C).
**PTH1R Proline-rich Motifs and ERK1/2 Activation**

**A.**

|            | PTH1R + c-Src | PTH1R + c-SrcK
|------------|---------------|---------------|
| p-ERK1/2   |               |               |
| Time (min) | 0 5 15        | 0 5 15        |
| ERK1/2     |               |               |

**B.**

IP: 6His

|            | PTH1R + c-Src | PCDel-PTH1R + c-Src | PCDel-PTH1R + c-SrcK |
|------------|---------------|---------------------|----------------------|
| p-Tyr(416) c-Src |               |                     |                      |
| βarr2-GFP |               |                     |                      |
| 6-His     |               |                     |                      |

**C.**

Relative luciferase expression (fold change)

**D.**

|            | PTH1R | PTD-PTH1R |
|------------|-------|-----------|
| βarr2-GFP  |       |           |

**FIGURE 4. c-Src binding to arrestin and PTH1R proline-rich motifs for ERK1/2 activation.** HEK-293 cells expressing GFP-tagged β-arrestin2 were transiently co-transfected with His6-tagged wild-type PTH1R, proximal C-terminal deletion mutant (PCdel) and proline-deficient (PrD) receptors, and either wild-type c-Src, kinase-dead c-Src-K295M mutant (c-SrcK), or constitutively active c-Src-Y527A mutant (c-SrcY527A), as indicated. A, phosphorylated (upper panel) and total (lower panel) ERK1/2 (p42/44) in whole-cell lysates were determined by immunoblotting after stimulation with PTH-(1-34) (100 nM). B, upon immunoprecipitation (IP) of wild-type PTH1R by anti-His, antibody activated c-Src (p-Tyr(416)), total c-Src, and β-arrestin2-GFP were co-precipitated for 5 min after PTH stimulation using specific antibodies as indicated under “Experimental Procedures.” In cells expressing PCdel and PrD PTH1R mutants, PTH did not stimulate co-precipitation of c-Src nor β-arrestin2 (p-ar2) with the receptor. Constitutively active Src (c-SrcY527A) co-precipitated with PCdel mutant receptor before and after PTH stimulation in absence of receptor-bound arrestin. Constitutively active Src (c-SrcY527A) and β-arrestin2 co-precipitated with PrD mutant receptor before and after PTH stimulation. The lowest panel indicates expression of each receptor. C, inhibition of ERK1/2 transcriptional activity in HEK-293 cells expressing wild-type PTH1R with dominant-negative Src (c-SrcK) or the Src inhibitor PP2 (5 μM) and in cells expressing proline-deficient mutant receptors is shown 4 h after incubation with vehicle (Veh) or PTH-(1-34) (100 nM). *p < 0.001 compared with PTH-stimulated PTH1R. D, fluorescence microscopy of β-arrestin2-GFP shows its recruitment to cell membrane 5 min after PTH-(1-34) (100 nM) stimulation for both PTH1R and proline-deficient (PrD) mutant, and a marked decrease of arrestin trafficking 15 min after PTH stimulation in cells expressing proline-deficient (PrD) mutant compared with wild-type (PTH1R) receptors. For all experiments, similar results were at least reproduced three times.

**DISCUSSION**

It is now well recognized that GPCR-mediated signaling not only activates protein kinases such as PKA and PKC but also involves adaptor proteins that provide a molecular scaffold for MAPK activation (34–36). In turn, these signaling pathways ensure distinct functions of hormones and other GPCRs agonists, including the systemic control of homeostasis as well as the local regulation of cell growth, differentiation, and apoptosis (1, 37). As a paradigm, PTH-stimulated cAMP and IP3/Ca2+ signaling in bone and kidney is central to its hormonal effects on serum calcium homeostasis and bone turnover (13, 38), whereas MAPK signaling in osteoblasts may play an important role in bone formation (15–17, 39, 40). However, how a limited number of intracellular signaling pathways may mediate the specific activity of a host of different stimuli in a given cell type remains unclear. For instance, the GPCRs for prostaglandin E2, adrenergic agonists and PTH/PTHrP are all expressed on osteoblastic cell surfaces and signal primarily through Goα and cAMP, yet these stimuli have contrasting and sometimes opposite effects on bone formation (41–44). Hence, to ensure a specific cellular response to a given agonist, distinct receptor structures capable of targeting the signaling scaffolds to a determined receptor are required. By using a targeted mutagenesis approach, we have now identified a novel mechanism for copying ERK1/2 activation to the PTH1R. This mechanism involves cooperation between arrestin-binding sites and proline-rich motifs in the receptor C terminus, allowing for scaffolding of c-Src in response to PTH. In addition, we provide evidence that activated c-Src stabilizes arrestin binding to the receptor, a process known to prompt internalization of the ligand-receptor complex (7, 23).

Thus, the reciprocal scaffolding of c-Src and arrestins at PTH1R may concomitantly favor arrestin-mediated uncoupling of receptor from G proteins and localize MAPK activation complexes to distinct subcellular compartments.

ERK1/2 activation in response to PTH may be mediated through cAMP/PKA signaling, primarily in B-Raf expressing cells (16). However, using a PTHRp-derived analog (Bpa1-PTHRp(1-36)) that activates cAMP but not Ca2+/IP3, Syme et al. (25) failed to stimulate ERK1/2 activity in HEK-293 cells expressing PTH1R. We reported previously that this analog also fails to recruit arrestins and internalize PTH1R (45). Hence, to specifically evaluate the importance of Goα signaling on ERK1/2 activation by PTH, we used Goα-deficient PTH1R mutants, including the (cAMP) constitutively active H223R Jansen receptor mutant (32) that remains capable of recruiting arrestins and internalizing with PTH (23). Because these mutants completely failed to activate ERK1/2, the crucial role of PTH1R coupling to Goα for ERK1/2 activation is now clearly established. The downstream molecular events leading from Goα-PKC to ERK1/2 activation could be
PTH1R Proline-rich Motifs and ERK1/2 Activation

FIGURE 5. Integrated illustration of the multistep activation of ERKs through PTH1R. Step 1, Gq-mediated events. PTH binding to PTH1R triggers Gq, binding to PTH1R IC2 (EKKY motif), activating the phospholipase C/PKC signaling cascade. PKC- and GPCRs-related kinases (GRKs) phosphorylate serine residues in the receptor C-terminus (2, 5). PKC may directly activate RAF-1 and ERK1/2 subsequently (dotted arrow; adapted from Refs. 46 and 54). Step 2, β-arrestin binding and Src activation. The phosphorylated PTH1R recruits β-arrestins, prompting receptor uncoupling from Gq, whereas Gqα subunits bind the juxta-membrane region of the C-terminus, which in turn activates c-Src (adapted from Ref. 49). Activated c-Src (c-Src*) will interact through its SH3 domain with proline-rich (PXXP) motifs in PTH1R cytoplasmic tail. Step 3, Src-mediated activation of ERK1/2. Through its catalytic or kinase domain (SH1) (52), c-Src concomitantly binds to receptor-bound β-arrestin, ensuring reciprocal stabilization of the receptor-arrestin-Src complex. Arrestin-mediated internalization of the complex and Src-mediated activation of RAS in turn will lead to ERK1/2 activation. Further transactivation of EGFR-mediated ERK1/2 activation (25) is not shown here.

Both a direct activation of the classical Raf-1/MEK/ERK signaling pathway (46) and PKC-mediated phosphorylation of PTH1R. The latter indeed favors arrestin binding (7) and thereby arrestin-dependent activation of ERK1/2 (Fig. 5). In turn, by using PTH1R mutants that are incapable of recruiting arrestins (PCdel- and ArD-PTH1R) as well as a dn β-arrestin2 that competes with arrestin-dependent internalization, we confirm and extend the importance of this adaptor molecule for ERK1/2 activation through GPCRs (25, 47). Interestingly, Syme et al. (25) had also found that arrestin-mediated ERK1/2 activation implicated transactivation of the EGFR. By showing that c-Src co-precipitates with both PTH1R and arrestin, our data provide some evidence that ERK1/2 activation may also occur directly downstream of PTH1R. In this case, we propose that EGFR transactivation could represent a mechanism for secondary amplification of ERK1/2 signaling in response to PTH.

Studies on the angiotensin type 1A receptor have shown that G-protein (Gq)-mediated activation of ERKs is rapid, transient, and leads to nuclear translocation of the activated ERK1/2 to regulate transcription (3, 48). In contrast, β-arrestin-mediated activity was characterized by slower onset, retention of activated ERK1/2 in cytoplasmic endosomal vesicles, and a weak transcriptional regulation (3, 48). Gesty-Palmer et al. (47) recently reported a biphasic response of ERK1/2 to PTH, with an early activation phase implicating signal transduction by G proteins and a later phase mediated by arrestins. In our experimental conditions, we confirm the role of Gqα-mediated signaling in the early activation of ERK1/2 but also found arrestins to be implicated in signal transduction to ERK1/2 from an early time point. Indeed, the presence of dn β-arrestin2, as well as the expression of an arrestin-deficient PTH1R mutant, decreased ERK1/2 activation at 5 min (Fig. 3B). Our observation is consistent with the study by Syme et al. (25) who reported a marked decrease of PTH-stimulated ERK1/2 activation in the presence of dominant-negative dynamin, which prevents arrestinin-mediated PTH1R internalization. Moreover, our data indicate that the mechanisms for rapid phosphorylation of cytoplasmic ERK1/2 are indistinguishable from those leading to a MAPK transcriptional response in response to PTH. Contrasting results concerning the time course of ERK1/2 activation may be because of differences in the ligands used, for instance bovine PTH(1–34) (this study and see Ref. 25) versus human PTH(1–34) (47); in the cell-based assays, culture conditions, and other experimental procedures. In any case, by systematically performing both immunoblotting and transcriptional assays, we unequivocally demonstrate the implication of arrestins and scaffolded c-Src on ERK1/2 transcriptional activity.

Recently, a docking site for Gβγ subunits was identified in the juxta-membrane region of the PTH1R C terminus, and mutations that disrupted Gβγ interaction with the receptor prevented signaling via Gq, and blunted cAMP signaling (26). Interestingly, Gβγ subunits trigger phosphorylation of Shc through c-Src and may thereby link GPCR activation to RAS-MAPK signaling (49). Accordingly, we found that the proximal C-terminal deletion (PCdel-PTH1R) resulted in a more severe inhibition of ERK1/2 compared with arrestin-deficient receptor mutant (ArD-PTH1R), which includes a C-terminal deletion distal to the Gβγ docking site. Taken together, these data lead us to propose a sequence of events by which c-Src may be activated through Gβγ and then interact with proline-rich motifs in the receptor C terminus and eventually bind to arrestins to form a molecular scaffold at the PTH1R that triggers the MAPK signaling cascade (Fig. 5). Concerning the putative mechanisms by which active c-Src may stabilize arrestin binding to the receptor, it is known that SrcK− may directly activate GRKs (50). In turn GRKs are known to phosphorylate serine residues in the PTH1R proximal C terminus, prompting arrestin binding (24).

Noteworthy, ERK1/2 activation through PTH1R appears to recapitulate the molecular mechanisms of both β2-adrenergic receptor-mediated ERK1/2 activation (involving β-arrestin binding of c-Src) and β2-adrenergic receptor (binding c-Src through its proline-rich motifs) (12, 51). In the case of PTH1R, we postulate that c-Src interacts with the PTH1R proline-rich motifs through its SH3 domain, contributing to Src activation,
and with β-arrestin through its catalytic domain (SH1) (52). Other class I GPCRs such as the dopamine D3 and D4 receptors, muscarinic receptor M4, and β1-adrenergic receptors present proline-rich motifs that can serve as potential SH3 ligands (53). In contrast, we have not identified PXPP motifs in class II GPCRs beyond PTH1R itself. Hence, the molecular mechanisms for PTH1R-mediated ERK1/2 activation could be unique among class II GPCRs. The functional relevance of this particular mechanism of ERK1/2 activation by PTH, primarily on osteoblastic functions, remains to be investigated.

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**PTHR1 Proline-rich Motifs and ERK1/2 Activation**

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