The COOH-terminus of parathyroid hormone-related protein (PTHrP) interacts with β-arrestin 1B[1]

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Abstract Parathyroid hormone-related protein (PTHrP) has a diverse range of proposed biological activities participating in both extracellular and intracellular signaling. In order to identify candidate protein effectors, yeast two-hybrid screens were conducted using mature human PTHrP (residues 1–141) and the COOH-terminus (residues 107–141). Both PTHrP baits interacted with a β-arrestin 1B fragment, an important component of G-protein-coupled receptor desensitization and MAPK signaling. Co-immunoprecipitation, in vitro binding assays and colocalization experiments confirmed this interaction in human cells and this required residues 122–141 of PTHrP. These findings suggest that β-arrestin 1 acts as an effector for a novel function of PTHrP in cytoplasm.

Key words: β-Arrestin; Parathyroid hormone-related protein; Endosome; Yeast two-hybrid

1. Introduction

Parathyroid hormone-related protein (PTHrP) is a humoral factor secreted from tumors that mimics the actions of parathyroid hormone (PTH) on bone and kidney and is the major cause of hypercalcaemia in patients with cancer [1]. PTHrP is a key regulator of skeletal development via its interaction with the PTH1 receptor to influence chondrocyte proliferation, differentiation and apoptosis [2–5]. It also acts as a poly-functional hormone in several systems in a paracrine/autocrine manner via PTH-independent mechanisms to influence embryonic development, cell growth and differentiation [6].

In addition to its extracellular actions, it is evident that intracellular forms of PTHrP exist which can act via intracrine pathways. PTHrP forms lacking the pre-pro secretory signal have been demonstrated to influence mechanisms of proliferation, differentiation and apoptosis, effects that are dependent on its translocation between the cytoplasm and nucleus [7,8]. Nuclear/nucleolar targeting of PTHrP is required for enhancing survival of chondrocytes in culture under conditions that promote apoptosis [7] and also in the breast cancer cell line MCF-7 [9]. However, in other cell lines such as intestinal cell line, IEC-6, it has the opposing action [10]. In vascular smooth muscle cells, the nuclear localization of PTHrP increases cell proliferation, an opposing effect to extracellular PTHrP on vascular smooth muscle cells to inhibit cell proliferation [11]. The translocation of PTHrP into the nucleus is dependent on the preservation of the nuclear localization sequence (NLS) [7] and occurs via its interaction with importin-β to mediate nuclear transport of PTHrP in an active GTP-dependent manner [12]. This translocation is cell cycle regulated and PTHrP is retained in the cytoplasm when phosphorylated [13,14], suggesting active cytoplasmic retention via phosphorylation by unknown proteins. Upon entering the nucleus, PTHrP can be translocated to the dense fibrillar components of the nucleoli [7] where it has been shown to bind RNA, possibly to mediate ribosome synthesis [15].

To further elucidate the function of intracellular PTHrP, we have used the yeast two-hybrid system to identify interacting molecules. Mature full-length PTHrP that interacts with the PTH1R and two other forms, PTHrP (38–94) and PTHrP (107–141), that exhibit PTH1R-independent functions were used. β-Arrestin 1B protein was identified and further confirmed as an interactor with COOH-terminal 19 residues of human PTHrP (122–141). This is the first study to report a protein interaction with the COOH-terminus of PTHrP and support a novel role for PTHrP in the cytoplasm that is manifested through β-arrestin 1-mediated pathways.

2. Materials and methods

2.1. Antibodies

Anti-hemagglutinin (HA) mouse monoclonal antibody (12CA5) was from Roche. Polyclonal antiserum (#R90) to human PTHrP (67–94) peptide was raised in-house in rabbits, and recognizes a protein doublet of approximately 46 kDa in immunoblots of cell lysates overexpressing GFP-PTHrP (1–141). Mouse monoclonal anti-FLAG antibody M2 was from Sigma Chemical Company (St. Louis, MO, USA) and polyclonal rabbit anti-glutathione-*S*-transferase (GST) antibody (sc-459) was from Santa Cruz Biotechnology. Anti-mouse IgG conjugated to Texas red (Molecular probes T-862) was used for immunofluorescence.

2.2. Expression plasmids

For yeast two-hybrid assays, human PTHrP (1–141), mid region (38–94), COOH-terminal region PTHrP (107–141) and PTHrP (122–141) were generated by polymerase chain reaction (PCR) using oligonucleotides obrf 15.171 and 15.172, obrf 15.174 and 15.175, obrf 15.173 and 15.172, and obrf 15.179 and obrf 15.172, respectively, as

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[1] Deposited on MINT (http://cbm.bio.uniroma2.it/mint/index).

Abbreviations: DNA-AD, DNA activation domain; DNA-BD, DNA binding domain; GST, glutathione-*S*-transferase; GPCR, G-protein-coupled receptor; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; NLS, nuclear localization sequence; PCR, polymerase chain reaction; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein

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indicated in Table 1, then ligated into the *Eco*RI and *Bam*HI sited of pGBT9 to generate in-frame fusion proteins to the GAL4 DNA binding domain (DNA-BD). For yeast two-hybrid screening, a bidirectional cDNA library in pGAD10 was used (Clontech). FLAG-tagged human β-arrestin 1B (1–361) was generated using β-arr-3 (sense) and β-arr-4 (antisense) oligonucleotides for cloning into the *Hin*dIII and *Xho*I sites of pCDNA3. For HA-tagged PTHrP, $_{(COOH)}$HA-tagged PTHrP (1–141), $_{(NH2)}$HA-PTHrP and $_{(COOH)}$HA-PTHrP (−36–141) were generated using obrf 15.184 and 15.185, obrf 15.182 and 15.183, and obrf 15.186 and 15.185, respectively (Table 1), then ligated into the *Hin*dIII and *Xba*I sites of pCDNA3. For bacterial expression, GST-PTHrP (38–141) (kindly provided by M. Lam) and GST-RANKL (kindly provided by F.P. Ross) were used. For localization studies, p-enhanced green fluorescent protein (pEGFP)-C′-PTHrP (1–141) [14] encoded an in-frame COOH fusion to the GFP tag. GFP-human β-arrestin 1A was kindly provided by P. Sexton.

2.3. Yeast two-hybrid screens

The yeast two-hybrid system [16] used in this study was the GAL4 Matchmaker 1.0 (Clontech). *Saccharomyces cerevisiae* strain PJ69-4A (kindly provided by M. Southey) was transformed with bait constructs, clones selected and these were subsequently transformed with the pGAD10 activation domain cDNA library [17]. Transformants were selected for by growth on media lacking leucine and tryptophan. After 4 days, yeast were replica plated onto media selecting for activation of the reporter genes, i.e. lacking adenine, histidine, tryptophan and leucine (WAdHL−). Interacting clones were isolated and retransformed to confirm interaction. Interacting clones were also transformed alone, and with the empty DNA-BD vector, and an unrelated fusion encoded by DNA-BD-pLAM5′ control vector, to test for autoactivation. Interaction was further assayed by the production of β-galactosidase to catalyze ONPG over time in the Y190 yeast strain.

2.4. Cell lysis and co-immunoprecipitation

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM)+10% fetal bovine serum (FBS), then transfected for 24–48 h with 2 μg of plasmid DNA per well of a six-well plate using FuGENE (Roche) at 90% confluency. Cells were harvested using 1 ml of PLC lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1×Protease inhibitors) and disrupted with a syringe. Lysates were mixed with 1 μg of anti-FLAG antibody and incubated for 1 h at 4°C, followed by addition of 50 μl of protein G-agarose beads (Amersham) and incubated for 2 h at 4°C. Immunoprecipitates were washed three times with IP wash buffer (20 mM HEPES pH 7.5, 10% glycerol, 0.1% Triton X-100, 150 mM NaCl), then analyzed by immunoblotting.

2.5. In vitro binding assays

Protein expression was carried out in XL-1 blue cells with 1 mM IPTG for 3.5 h at 37°C. GST fusion proteins were purified by harvesting 100 ml of cells and lysing by sonication in 5 ml PBS. Cleared lysates were bound to 50 μl glutathione Sepharose beads for 30 min at 4°C. Bound proteins were then normalized to 250 μg/ml using fresh beads. Mammalian cell lysates (∼1 mg) were incubated with 5 μg of affinity-purified GST fusion protein coupled to 40 μl of glutathione Sepharose, for 2 h at 4°C, then washed four times with 10×volume of lysis buffer and analyzed by immunoblotting.

2.6. Immunofluorescence

HEK293 cells were grown in six-well plates containing poly-L-lysine (Sigma)-coated coverslips, transfected with recombinant plasmids for 24 h, washed twice with PBS, fixed with 3.2% PFA and permeabilized with 0.5% Triton X-100. Transfected cells were stained with anti-HA antibody of anti-FLAG antibody. Cells were visualized at 1000× using a Leica microscope and images acquired using a Bio-Rad MCR 1024 laser confocal imaging system.

3. Results

3.1. Isolation of protein interactors with human PTHrP

To identify proteins that interact with human PTHrP, three independent screens of a human placental cDNA library fused to a GAL4 activation domain were conducted using the full-length mature PTHrP (1–141), a mid region (38–94) and the COOH-terminal region (107–141), each fused to a DNA-BD. Using the PTHrP (1–141) bait, 4.4 million clones were screened and four clones were found to specifically interact supporting growth on WAdHL media. 1.2 million clones were screened with the PTHrP (107–141) bait from which four clones were also isolated on their ability to interact. Clones were sequenced and found to be identical in both screens encoding residues 1–361 of β-arrestin 1B, lacking 49 COOH-terminal residues. The DNA activation domain (DNA-AD)-β-arrestin 1B fusion was retransformed with controls and each of the PTHrP baits, and was demonstrated to specifically interact with PTHrP (1–141) and PTHrP (107–141), but not PTHrP (38–94) in both auxotrophic and colorimetric reporter gene assays (Fig. 1A).

Since sequences of PTHrP interacting with β-arrestin 1B 1–361 were confined to the COOH-terminus, a bait encoding human PTHrP (122–141) was cotransformed with DNA-AD-β-arrestin 1B (1–361) into PJ69-4A yeast (Fig. 1B). Nutritional and *lacZ* reporter gene readouts demonstrated positive interaction between human PTHrP (122–141) and human β-arrestin 1B (1–361), further confirming interaction in the yeast two-hybrid system and indicated involvement of the extreme COOH-terminus of PTHrP.

3.2. PTHrP and β-arrestin 1B form a protein complex

To confirm interaction of β-arrestin 1B (1–361) with PTHrP, bacterially expressed GST-PTHrP (38–141) conju-

Table 1
List of oligonucleotides used for PCR cloning

Oligonucleotide	Orientation	Sequence
obrf 15.171	S	5′-CCG**GAATTC**GCTGTGTCTGAACAATCAGCTC-3′
obrf 15.172	A	5′-GACA**GGATCC**TGCAATCTGTC-3′
obrf 15.173	S	5′-CC**GGAATTC**ACTCGCTCTGCCTGGTTAGAC-3′
obrf 15.174	S	5′-CC**GGAATTC**GCTACCTCGGAGGTGTCCCCTAAC-3′
obrf 15.175	A	5′-GCC**GGATCC**TTGCGTTTTCAGGGCTTGCCTTTCTTTTTCTTCCCAG-3′
obrf 15.179	S	5′-GCG**GAATTC**GAAGGGGACCACCTG-3′
obrf 15.182	S	5′-GCGCCC**AAGCTT**GGGCCATGTACCCATACGACGTCCCAGACTACGCTGCTGTGTCTGAACATCAGCTCCT-3′
obrf 15.183	A	5′-GGCGCC**TCTAGA**GCCTCATGCCCTCCGTGAAT-3′
obrf 15.184	S	5′-GGCGCCC**AAGCTT**GGGCCATGGCTGTGTCTGAACATCAGCTC-3′
obrf 15.185	A	5′-CGCGGC**TCTAGA**GCTCAAGCGTAGTCTGGGACGTCGTATGGGTAATGCCTCCGTGAATCGAGCTCCAGCGACGTTGT-3′
obrf 15.186	S	5′-GGCGCCC**AAGCTT**GGATGCAGCGGAGACTGGTTCA-3′
β-arr-3	S	5′-CGCGCGG**AAGCTT**GGACCATGGGCGACAAAGGGACGCG-3′
β-arr-4	A	5′-CGCGCTCGAGTCACTTGTCATCGTCGTCCTTGTAGTCCTCGTTCTCTGGAACTTCCCGATGC-3′

Restriction enzyme sequences are in bold. S, sense primer; A, antisense primer.

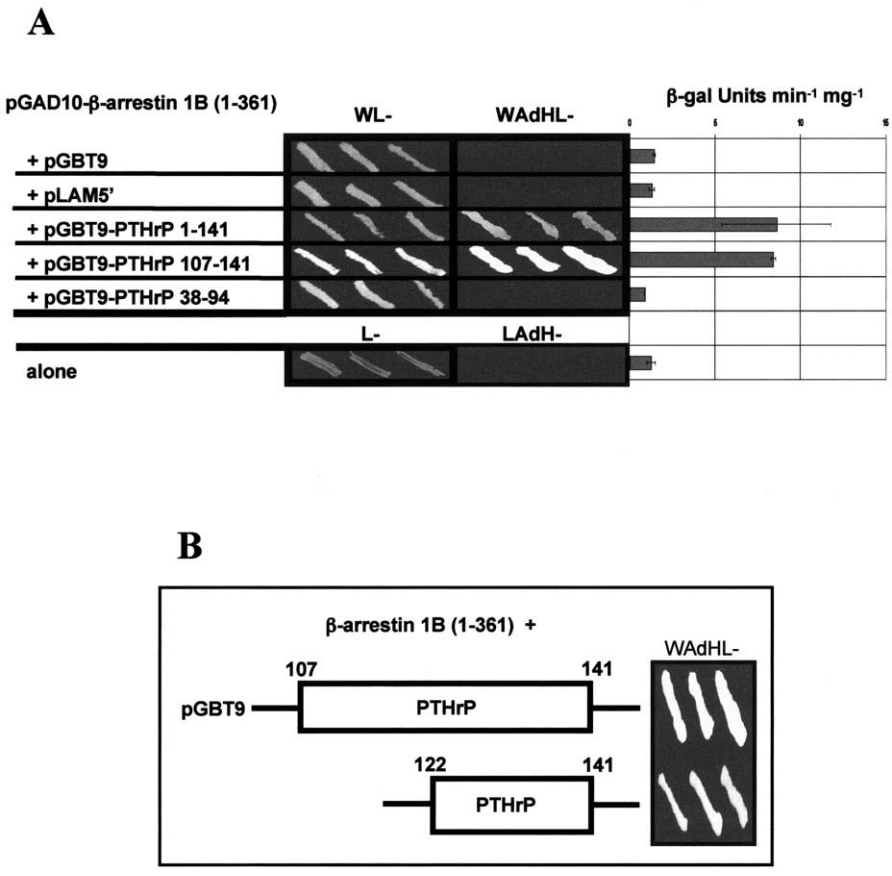

Fig. 1. A: Yeast cotransformed with pGAD10-β-arrestin 1B and a variety of baits. Activation of transcription of nutritional reporter genes was assayed by growth on media lacking adenine and histidine, as well as the plasmid markers leucine and tryptophan (WAdHL-). Activation of *lacZ* gene transcription was assessed by liquid β-galactosidase assays ± S.E.M. B: Interaction of human PTHrP COOH-terminal baits indicated with β-arrestin 1B (1–361) by growth on media lacking adenine and histidine (WAdHL−).

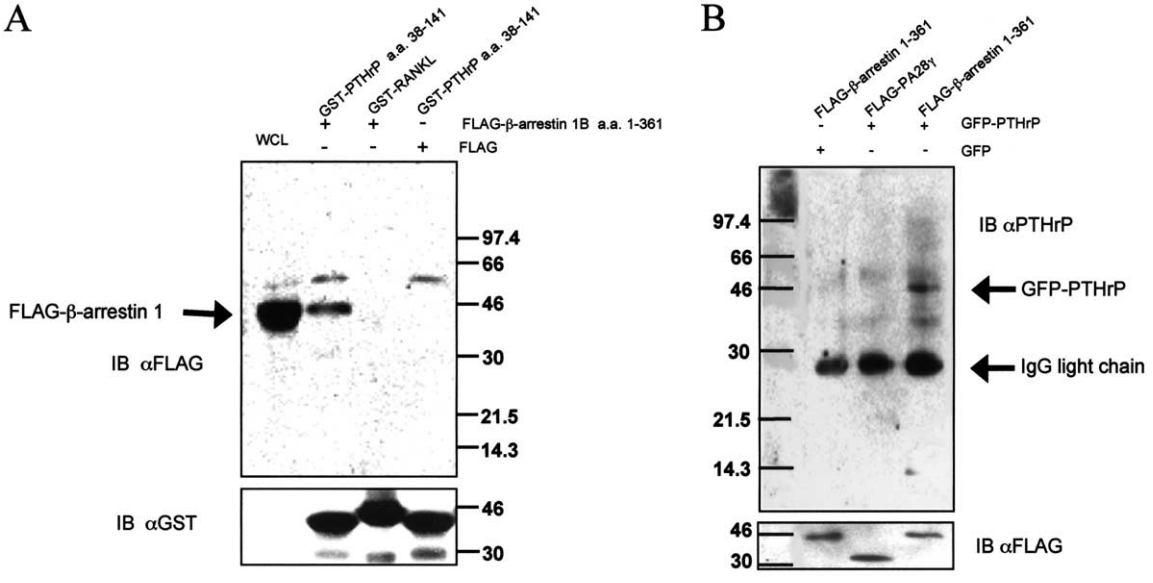

Fig. 2. Validation of binding of human PTHrP with β-arrestin 1. A: In vitro binding assays in which GST-PTHrP or negative control (GST-RANKL) was incubated with mammalian cell lysates expressing FLAG-β-arrestin 1B (1–361) or FLAG tag alone. Whole cell lysate (WCL) shows β-arrestin 1B (1–361) expression. B: Co-immunoprecipitation of GFP-PTHrP (1–141) with FLAG-β-arrestin 1B (1–361) but not with negative control FLAG-PA28γ. HEK293 lysates were immunoprecipitated with M2 αFLAG, and detected for the presence of GFP-PTHrP using R90 αPTHrP antibody.

gated to glutathione Sepharose was incubated with HEK293 lysates overexpressing FLAG-β-arrestin (1–361). FLAG-β-arrestin 1B (1–361) appears as a 45 kDa band in whole cell lysate. After stringent washing, the presence of FLAG-β-arrestin 1B (1–361) was detected on immunoblot when incubated with GSP-PTHrP and not with a control GST function (Fig. 2A). Importantly, this interaction was confirmed in human cells using co-immunoprecipitation. HEK293 cells were cotransfected with GFP or GFP-PTHrP (1–141) and FLAG-β-arrestin 1B (1–361). GFP-PTHrP co-immunoprecipitated with FLAG-β-arrestin 1B (1–361) as a 46 kDa protein (Fig. 2B). GFP-PTHrP did not co-immunoprecipitate with a negative control fusion, FLAG-PA28γ protein. Both of these binding assays confirm the yeast two-hybrid interaction and provide support for a novel function for PTHrP in vivo.

3.3. Subcellular colocalization of β-arrestin 1 and PTHrP

The subcellular localization of GFP-β-arrestin 1 was compared with that of three forms of HA-PTHrP. Mature PTHrP containing the secretory signal sequence, $_{(COOH)}$HA-PTHrP (−36–141) was co-overexpressed in the presence of GFP-β-arrestin 1 and demonstrated nuclear/nucleolar localization and co-localized with GFP-β-arrestin 1 around endosome-like structures located in the cytoplasm and around the nuclear periphery (Fig. 3A). The mature form of PTHrP (1–141) lacking the pre-pro region fused to HA tags at either the NH_2- (Fig. 3B) or the COOH-terminus (Fig. 3C) to exclude tag interference, demonstrated similar localization patterns, indicating that PTHrP does not have to be processed via the secretory pathway in order to interact with β-arrestin 1. Although PTHrP localized both to the nucleus and cytoplasm, the localization of GFP-β-arrestin 1 was coincident with some PTHrP localization in the cytoplasm and around the nucleus (Fig. 3).

4. Discussion

The yeast two-hybrid system has been employed using functional domains of human PTHrP in order to identify novel protein partners as targets for function. This study is the first to report the isolation of a protein interactor with the COOH-terminus of human PTHrP, a truncated form of β-arrestin 1B.

The PTHrP interaction with β-arrestin 1B (1–361) was confirmed by isolation of complexes by co-immunoprecipitation and in vitro assays. Further characterization of the PTHrP binding determinant revealed the interaction was dependent on residues within PTHrP (122–141). Subcellular localization studies demonstrated that PTHrP was observed on endosome-like vesicles when overexpressed with GFP-β-arrestin 1, a phenomenon analogous to that observed for ERK1 in which stoichiometrically equal expression of β-arrestin is required for ERK1′s endosome and nuclear localization. Both the pre-pro and mature forms of PTHrP colocalized with GFP-β-arrestin 1 on endosome-like vesicles, suggesting that the interaction occurs between intracellular forms of PTHrP, possibly initiated from internal ribosome entry sites to bypass the secretory pathway [18].

β-Arrestins are essential for the desensitization of ligand-stimulated G-protein-coupled receptors (GPCRs) in two steps: (1) by altering the affinity of the receptor for the G-protein and (2) by recruiting the clathrin adapter protein AP-2 and the NSF protein to signal clathrin-mediated endo-

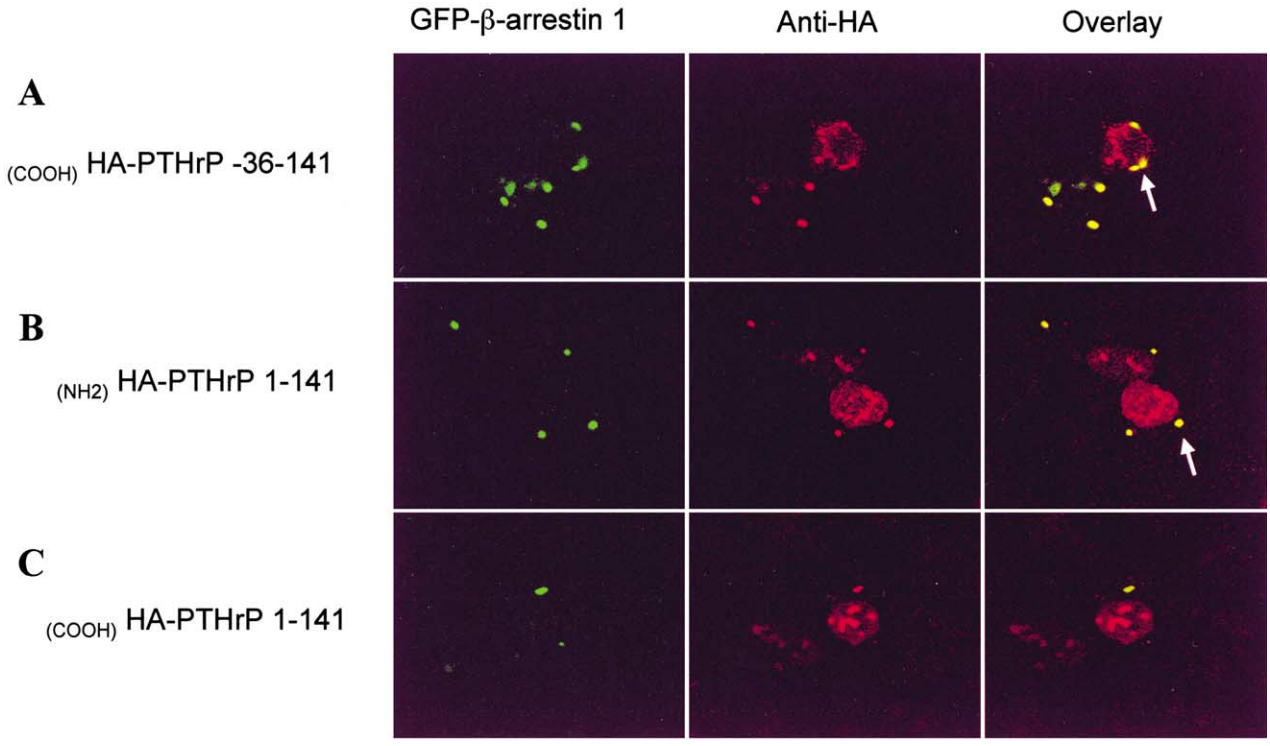

Fig. 3. Colocalization of HA-tagged PTHrP with GFP-β-arrestin 1. GFP-β-arrestin was cotransfected with either (A) COOH-terminally tagged HA-PTHrP (−36–141), (B) NH_2-tHA-tagged PTHrP (1–141), and (C) COOH-HA-tagged PTHrP (1–141). The localization was examined by direct fluorescence of GFP (left) and anti-HA mouse monoclonal with Texas red secondary antibody (center). Colocalization was examined by overlaying the two images (right). Colocalization around endosome-like structures is indicated by arrows.

cytosis of the activated receptor [19,20]. In addition, recent studies have demonstrated that receptor-bound β-arrestins act as scaffolds for members of the mitogen-activated protein kinase (MAPK) pathway such as c-src, raf, ASK1, ERK1/2 and JNK3 [21,22]. The association of these components with the scaffold prevents translocation of ERK1/2 to the nucleus, thereby attenuating its proliferative effects and promoting phosphorylation of its cytosolic substrates [23,24]. Thus the subcellular localization and specificity of ERK1/2 and its associated mitogenic potential are controlled by the formation of the β-arrestin scaffolding complex, most likely in response to GPCR activation. By analogy, the interaction of intracellular PTHrP with β-arrestin may allow cytoplasmic nuclear shuttling of PTHrP to be brought under control of the β-arrestin/MAPK pathway. In a manner similar to ERK1/2, the binding of PTHrP to the β-arrestin-MAPK scaffold may retain it in the cytosol, therefore preventing the proliferative effects associated with its uptake into the nucleus.

The association of PTHrP with β-arrestin 1 may bring it into contact with other components of the β-arrestin-dependent MAPK scaffold such as ras and c-src as a target for modifications such as phosphorylation, possibly as a means of retaining PTHrP in the cytoplasm. The influence of members of the MAPK pathway such as ras and c-src in modulating PTHrP expression has been previously demonstrated [25,26]. At the protein level, the presence of putative phosphorylation sites in the COOH-terminal regions of PTHrP may be potential targets of regulation by these β-arrestin interactors. Alternatively, PTHrP may interact with the β-arrestin 1 scaffold as a means of modulating GPCR signaling, perhaps to potentiate or antagonize the cellular response to other extracellular ligands. This novel interaction provides evidence for a cytoplasmic context for PTHrP function and this is manifested through a β-arrestin 1-mediated pathway.

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