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Dephosphorylation is the mechanism of fibroblast growth factor inhibition of guanylyl cyclase-B

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ABSTRACT

Activating mutations in fibroblast growth factor receptor 3 (FGFR3) and inactivating mutations of guanylyl cyclase-B (GC-B, also called NPRB or NPR2) cause dwarfism. FGF exposure inhibits GC-B activity in a chondrocyte cell line, but the mechanism of the inactivation is not known. Here, we report that FGF exposure causes dephosphorylation of GC-B in rat chondrosarcoma cells, which correlates with a rapid, potent and reversible inhibition of C-type natriuretic peptide-dependent activation of GC-B. Cells expressing a phosphomimetic mutant of GC-B that cannot be inactivated by dephosphorylation because it contains glutamate substitutions for all known phosphorylation sites showed no decrease in GC-B activity in response to FGF. We conclude that FGF rapidly inactivates GC-B by a reversible dephosphorylation mechanism, which may contribute to the signaling network by which activated FGFR3 causes dwarfism.

1. Introduction

C-type natriuretic peptide (CNP) is a paracrine factor that stimulates the growth of long bones and vertebrae, promotes axonal bifurcation in the spinal cord, and prevents resumption of meiosis in the ovarian follicle [1–3]. These physiologic functions of CNP are mediated by guanylyl cyclase-B (GC-B), which elevates intracellular cGMP in response to CNP binding. Female mice lacking GC-B are infertile, and mice of both sexes lacking functional CNP or functional GC-B exhibit disproportionate dwarfism caused by reduced chondrocyte proliferation and hypertrophy [4,5]. In humans, genetic mutations that inactivate both alleles encoding GC-B cause acromesomelic dysplasia, type Maroteaux (AMDM) dwarfism [6]. Conversely, mutations that increase CNP expression [7,8] or mutations that activate a single GC-B allele in the absence of CNP cause skeletal overgrowth [9–11]. CNP levels in plasma are also predictive of longitudinal bone growth [12,13].

GC-B is a single membrane-spanning enzyme that exists as a higher ordered oligomer, possibly a dimer, that catalyzes the synthesis of cGMP from GTP in response to CNP binding [14]. The extracellular domain of GC-B is glycosylated and terminal N-linked glycosylation is required for the formation of an active GC catalytic domain [15,16]. AMDM dwarfism-causing missense mutations are most often associated with receptors lacking terminal N-linked glycosylation that markedly reduces or abolishes the ability of GC-B to form an active catalytic domain [16]. The intracellular portion of GC-B consists of a kinase homology domain that contains six chemically identified serine/threonine phosphorylation sites and one putative, functionally identified serine phosphorylation site [17–19], a short coiled-coiled dimerization region, and a carboxyl-terminal GC domain [14,20].

Previous studies have established that phosphorylation of GC-B is required for activation of GC-B. Conversion of all six chemically determined serine and threonine phosphorylation sites in GC-B to alanines produced a properly folded enzyme that retained GC activity under synthetic detergent activation conditions but had only 6% of the CNP-dependent activity observed with the phosphorylated wild type enzyme [17]. Conversely, mutating all chemically identified phosphorylation sites and one putative functionally identified phosphorylation site to glutamate to mimic the negative charge of phosphate produced an enzyme called GC-B-7E that is activated by CNP like the phosphorylated

Abbreviations: cGMP, cyclic guanosine monophosphate; GC, guanylyl cyclase; NP, natriuretic peptide; PBS, phosphate buffered saline; WT, wild type

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WT enzyme [19].

Early studies showed that activation of several G protein coupled receptors inactivates GC-B, through a process involving dephosphorylation [21,22]. Recently, luteinizing hormone (LH) was shown to stimulate GC-B dephosphorylation and inactivation in ovarian follicles by a process that requires a PPP family serine and threonine protein phosphatase [23]. Importantly, a knock-in mouse (GC-$B^{7E/7E}$) expressing GC-B-7E at the normal GC-B genetic locus was immune to LH-dependent GC-B inactivation and displayed a 5-h delay in the resumption of meiosis in the oocyte [24]. These findings indicate that hormones that activate G protein coupled receptors inactivate GC-B by dephosphorylation.

The present paper investigates the possibility that not only GPCR signaling, but also growth factor receptor signaling could inactivate GC-B by dephosphorylation. Multiple mechanisms could contribute to FGFR3 regulation of long bone growth [25], including FGF inhibition of GC-B [26]. However, although FGF2 exposure was shown to inactivate GC-B in the ATDC5 chondrocyte cell line [26] and in BALB/3T3 fibroblasts [27], the molecular basis for the inactivation was not determined. Here, we used multiple approaches to examine the molecular mechanism of FGF2-dependent GC-B inactivation in rat chondrosarcoma (RCS) cells, a highly physiologic chondrocyte cell line [28].

2. Material and methods

2.1. Materials

^{125}I cGMP radioimmunoassay kits were from PerkinElmer Life Sciences (Waltham, MA). CNP and heparin were from Sigma-Aldrich (St. Louis, MO), and FGF2 was from R & D Systems (Minneapolis, MN). IPA300 Protein A-conjugated resin was from Repligen (Waltham, MA, USA).

2.2. Cell culture

RCS cells are derived from a Swarm rat chondrosarcoma and express FGFR2 and FGFR3, but the mRNA for FGFR3 is at least seven-fold higher than the mRNAs of the other FGF receptor [28–33]. The RCS cells were a gift from Professor Benoit de Crombrugghe (MD Anderson Cancer Center, Houston, TX) and were maintained in DMEM with 1% penicillin/streptomycin and 10% fetal bovine serum. Except as indicated, FGF2 was used at a concentration 100 ng/m in the presence of 1 μg/ml heparin. Control cells were treated with heparin alone.

2.3. Construction of adenovirus-based vectors

The replication-deficient CMV promoter-driven rat GC-B-expressing vectors (RGD-CMV-GC-B-7E and RGD-CMV-GC-B-WT) were constructed through homologous recombination with the RGD fiber-modified Ad backbone plasmid (RGD-Ad-Easy). All vectors are identical and contain the CMV promoter-driven GC-B transgene cassette inserted in place of the deleted E1 region of a common Ad vector backbone. First, full-length GC-B-WT or GC-B-7E sequences derived from pRK5-GC-B were cloned into pShuttle-CMV plasmid. The resultant plasmids were linearized with *Pme* I digestion and subsequently co-transformed into *E. coli* BJ5183 with the RGD fiber-modified Ad backbone plasmid (RGD-Ad-Easy). After selection of recombinants, the recombinant DNA was linearized with *Pac* I digestion and transfected into 911 cells to generate viral vectors. The virus was propagated in 911 cells, dialyzed in phosphate-buffered saline (PBS) with 10% glycerol, and stored at − 80 °C. Titering was performed with a plaque-forming assay using 911 cells and optical density-based measurement.

2.4. Adenoviral transduction

A 10 cm dish of RCS cells at 50% confluency was transduced with either RGD-CMV-GC-B-7E or RGD-CMV-GC-B-WT using a multiplicity of infection of 100. Cells were incubated overnight, followed by a change in medium. GC activity was assayed in membranes from serum-starved cells harvested two days after viral transduction.

2.5. GC assays

Crude membranes were prepared in phosphatase inhibitor buffer as previously described [34]. Assays were performed at 37 °C for the indicated times in a cocktail containing 25 mM HEPES pH 7.4, 50 mM NaCl, 0.1% BSA, 0.5 mM isobutylmethylxanthine, 1 mM EDTA, 5 mM $MgCl_2$, 0.5 μM microcystin, and 1 × Roche Complete protease inhibitor cocktail. Unless indicated, the mixture also included 1 mM ATP and 1 mM GTP. If not indicated otherwise, CNP concentrations were 1 μM. Assays with 1% Triton X-100 and 5 mM $MnCl_2$ substituted for $MgCl_2$ were used to determine the total amount of GC-B in the membranes, since phosphorylation does not affect GC activity measured in detergent. Reactions were initiated by adding 80 μl of the mixture to 20 μl of crude membranes containing 5–15 μg of crude membrane protein. Reactions were stopped with 0.4 ml of ice-cold 50 mM sodium acetate buffer containing 5 mM EDTA. Cyclic GMP concentrations were determined by radioimmunoassay as described [35].

2.6. Immunoprecipitations and ProQ or SYPRO ruby staining

RCS cells were lysed for 30 min at 4 °C on a rotator in RIPA buffer containing: 50 mM HEPES pH 7.4, 50 mM NaF, 2 mM EDTA, 0.5% deoxycholate, 0.1% SDS, 1% IGEPAL CA-630, 100 mM NaCl, 10 mM NaH_2PO_4, 1 × Roche Protease Inhibitor Cocktail, and 0.5 μM microcystin. Cellular extracts were then precleared on a rotator in the same RIPA buffer at 4 °C containing 50 μl IPA300 Protein A-conjugated resin for 30 min. Samples were centrifuged and the supernatant transferred to a fresh tube. 25 μl IPA300 Protein A-conjugated resin, and 2 μl anti-GC-B rabbit polyclonal primary antibody 6327 that was immunized against the last 10C-terminal amino acids of rat GC-B, were added to the samples and rotated over night at 4 °C. The resin was washed three times in RIPA buffer without NaCl or NaH_2PO_4, and then resuspended in protein sample buffer and boiled 5 min.

Immunocomplexes of GC-B were fractionated on an 8% SDS polyacrylamide gel, then the gel was sequentially stained with ProQ Diamond followed by SYPRO Ruby dyes as previously described [21,36]. Densitometry ratios were calculated by dividing the Pro-Q Diamond signal intensity (Phospho-GC-B) by the SYPRO Ruby signal intensity (Processed GC-B, which means processed in the ER by glycosylation) using the LiCor Image Studio software.

2.7. Phos-tag gel electrophoresis

For analysis of phosphorylation by Phos-tag, GC-B was immunoprecipitated as previously described [23]. Briefly, ~200–500 μg crude membrane protein was diluted to 0.5 or 1 ml in 50 mM Tris-HCl pH 7.5, 50 mM NaF, 10 mM NaH_2PO_4, 2 mM EDTA, 0.5% deoxycholate, 0.1% SDS, 1% NP-40, 100 mM NaCl, 10 mM NaH_2PO_4, 1 × Roche Protease Inhibitor Cocktail, and 1 μM microcystin. After adding 0.6 or 1 μl anti-GC-B rabbit polyclonal antiserum 6328, made against a C-terminal peptide of GC-B [35], samples were rotated at 4 °C for 1 h, then added to 25 or 50 μl Protein A/G magnetic beads (ThermoFisher Scientific) and rotated overnight at 4 °C. The beads were washed three times in the same buffer and protein was eluted for 10 min at 70 °C in protein gel sample running buffer with 75 mM dithiothreitol.

Phos-tag gel electrophoresis and western blotting were then performed as described, using a primary antibody made against the extracellular domain of GC-B [37]. For Fig. S1, the 6327 antibody against the C-terminus of GC-B was used. The blots were developed with WesternBright Sirius reagent (Advansta, Menlo Park, CA). For densitometry, the amount of staining for the upper, more phosphorylated