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Activation of Latent Human GDF9 by a Single Residue Change (Gly^{391}Arg) in the Mature Domain

Courtney M. Simpson, Peter G. Stanton, Kelly L. Walton, Karen L. Chan, Lesley J. Ritter, Robert B. Gilchrist, and Craig A. Harrison

Prince Henry's Institute of Medical Research (C.M.S., P.G.S., K.L.W., K.L.C., C.A.H.), Clayton, Victoria 3168, Australia; Department of Biochemistry and Molecular Biology (C.M.S.), Monash University, Clayton, Victoria 3800, Australia; and Robinson Institute (L.J.R., R.B.G.), Research Centre for Reproductive Health, School of Paediatrics and Reproductive Health, University of Adelaide, Adelaid, South Austarlia 5005, Australia

Growth differentiation factor 9 (GDF9) controls granulosa cell growth and differentiation during early ovarian folliculogenesis and regulates cumulus cell function and ovulation rate in the later stages of this process. Similar to other TGF-β superfamily ligands, GDF9 is secreted from the oocyte in a noncovalent complex with its prodomain. In this study, we show that prodomain interactions differentially regulate the activity of GDF9 across species, such that murine (m) GDF9 is secreted in an active form, whereas human (h) GDF9 is latent. To understand this distinction, we used site-directed mutagenesis to introduce nonconserved mGDF9 residues into the pro- and mature domains of hGDF9. Activity-based screens of the resultant mutants indicated that a single mature domain residue (Gly^{391}) confers latency to hGDF9. Gly^{391} forms part of the type I receptor binding site on hGDF9, and this residue is present in all species except mouse, rat, hamster, galago, and possum, in which it is substituted with an arginine. In an adrenocortical cell luciferase assay, hGDF9 (Gly^{391}Arg) had similar activity to mGDF9 (EC_{50} 55 ng/ml *vs.* 28 ng/ml, respectively), whereas wild-type hGDF9 was inactive. hGDF9 (Gly^{391}Arg) was also a potent stimulator of murine granulosa cell proliferation (EC_{50} 52 ng/ml). An arginine at position 391 increases the affinity of GDF9 for its signaling receptors, enabling it to be secreted in an active form. This important species difference in the activation status of GDF9 may contribute to the variation observed in follicular development, ovulation rate, and fecundity between mammals. **(*Endocrinology* 153: 1301–1310, 2012)**

Oocyte-derived growth differentiation factor 9 (GDF9), a member of the TGF-β superfamily, is essential for mammalian ovarian folliculogenesis (1). GDF9 controls both early follicular maturation and the number of ovulating follicles in each estrus cycle (2). Female mice deficient in GDF9 have no antral follicles, no ovulations, and no pregnancies. Organogenesis of the *GDF9*$^{-/-}$ ovary is morphologically normal with large numbers of primordial follicles, many of which advance to the primary type 3b stage (*i.e.* the point at which the oocyte is fully grown with a single layer of cuboidal granulosa cells) (1). In the absence of GDF9, however, folliculogenesis does not progress beyond this point. The arrested follicles have abnormal granulosa cells and they fail to acquire a theca layer, indicating that GDF9 exerts paracrine actions on the surrounding somatic cells (1). The block in normal follicular growth is followed by degeneration of the oocyte and the formation of abnormal nests of luteinizing granulosa cells (1).

Studies in sheep have identified two point mutations in GDF9 that result in sterility. The Ser^{395}Phe mutation in Belclare sheep (3) and the Ser^{427}Arg mutation in Thoka sheep (4) are predicted to disrupt type I and type II receptor interactions, respectively, and ewes homozygous for these

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Abbreviations: AC, Adrenocortical cell line; BMP, bone morphogenetic protein; FCS, fetal calf serum; GDF, growth differentiation factor; h, human; HEK-293 T, human embryonic kidney 293, human renal epithelial cell; IMAC, immobilized metal affinity chromatography; KGN, human granulosa cell line; m, murine; mAb, monoclonal antibody; mhGDF9, mouse/human GDF9 chimera; RP-HPLC, reversed-phase HPLC; Smad, phosphorylated mothers against decapentaplegic; TLD, tolloid.

inactivating mutations are infertile with ovaries that resemble *GDF9* $^{-/-}$ mice. Interestingly, reduced GDF9 activity in ewes heterozygous for these mutations may increase the FSH sensitivity of secondary follicles, leading to increased numbers of antral follicles that undergo precocious maturation and ovulate at smaller stages (3–5). Thus, a reduction in GDF9 activity is actually associated with increased fecundity, promoting the concept of a dose-dependent effect of GDF9 on ovulation rate.

The vital importance of GDF9 in maintaining female reproductive homeostasis implies that any alteration in this gene might lead to infertility and ovarian pathology. Thus, screening for mutations in GDF9 in women with premature ovarian failure (*i.e.* women under 40 yr of age who experience approximately 6 months of amenorrhea and elevated serum FSH levels) identified several missense mutations not found in control women (6–8). Mutations in the *GDF9* gene have also been identified in the mothers of dizygotic twins (9, 10) and in women with polycystic ovary syndrome (11), a common condition (in 5–10% of women) of ovarian dysfunction linked to aberrant folliculogenesis (12).

Although GDF9 is crucial for the maintenance of female reproductive homeostasis in murine, ovine, and human systems, recent studies have indicated that the activity of GDF9 is differentially regulated across species (13, 14). Similar to other TGF-β ligands, GDF9 is synthesized as a precursor molecule consisting of an N-terminal prodomain and a C-terminal mature domain (15). During synthesis, the prodomain interacts noncovalently with mature GDF9, maintaining the molecule in a conformation competent for dimerization (13). Dimeric precursors are cleaved by proprotein convertases and GDF9 is secreted from the oocyte noncovalently associated with its prodomain. The continued presence of the prodomain does not suppress the biological activity of murine GDF9, which can directly regulate the growth and differentiation of granulosa cells by binding to cell surface receptors (13). Human GDF9, in contrast, is secreted in a latent complex with its prodomain (13) and presumably requires activation before receptor binding. This is the first observed species difference in the activation status of a TGF-β ligand, and it may contribute to the variation observed in follicular development, ovulation rate, and fecundity between mammals.

In this study, we have characterized the molecular interactions that confer latency to human (h) GDF9. Using site-directed mutagenesis, we introduced nonconserved murine (m) GDF9 residues into the pro- and mature domains of hGDF9, with the aim to activate this latent growth factor. Of the mutants generated, only hGDF9 (Gly^{391}Arg) was active in both an adrenocortical cell luciferase assay and a mouse granulosa cell proliferation assay. These findings indicate that Gly^{391} is responsible for maintaining hGDF9 in a latent complex with its prodomain.

Materials and Methods

Reagents

mGDF9 and the polyhistidine monoclonal antibody were purchased from R&D Systems (Minneapolis, MN). GDF9 antibody (mAb53) was purchased from AbD Serotec (Oxford, UK), whereas horseradish peroxidase-conjugated antimouse IgG was from GE Healthcare (Buckinghamshire, UK). Lumilight chemiluminesence Western blotting substrate was obtained from Roche (Basel, Switzerland), BioXact short DNA polymerase from Bioline (Taunton, MA), PCR/plasmid purification kits from Promega (Madison, WI), and DMEM, DMEM:F12, Opti-MEM, and SeeBlue Plus2 from Invitrogen (Carlsbad, CA).

Production and purification of recombinant GDF9

The development of a stable human embryonic kidney 293 (HEK-293) T cell line expressing mGDF9 has been described previously (16). To produce large amounts of hGDF9, we generated a stable HEK-293E cell line. Briefly, the hGDF9 gene with an N-terminal His-tag was generated by overlap PCR and cloned into the *Xba*I and Sse8387I sites of pAPEX3P. HEK-293 E cells, plated at 8×10^5 cells in six-well plates, were transfected with hGDF9/pAPEX3P (5 μg) using polyethyleneimine (1 mg/ml; Sigma-Aldrich, St. Louis, MO). Cells were incubated for 24 h in culture medium [DMEM/10% fetal calf serum (FCS)/50 mM HEPES/Pen-Strep/250 μg/ml geneticin] before the addition of puromycin (2 μg/ml; Sigma-Aldrich). Successfully transfected cells, which expressed resistance to puromycin, were expanded forming a stable cell population (referred to as 293E hGDF9 cells). To produce hGDF9, 293E-hGDF9 cells were transferred into production media [DMEM:F12 medium containing L-glutamine, 0.02% BSA, and 0.01% heparin (Sigma-Aldrich)] and cultured for 4 d. The resultant conditioned medium was centrifuged, filtered through a 0.45-μm filter, concentrated (Centricon Plus-70; Millipore, Billerica, MA), and resuspended in binding buffer (50 mM phosphate buffer; 0.5M NaCl, pH 8.0). The concentrated media were then subjected to immobilized metal affinity chromatography (IMAC) using Ni-NTA Agarose (Invitrogen) prepacked in an HR10/10 column (GE Healthcare). His-tagged hGDF9 was eluted from the Ni-NTA Agarose using elution buffer (50 mM phosphate buffer; 0.5M NaCl; 0.5M imidazole, pH 8.0) before final purification by reverse-phase HPLC. To determine the purity and mass of hGDF9 after the two chromatography steps, protein fractions (reduced with 5% β-mercaptoethanol) were fractionated on 10% SDS-PAGE gels and either silver stained or subjected to Western blot. After electrophoresis, samples were transferred onto enhanced chemiluminescence Hybond membranes (GE Healthcare) and probed with the GDF9-specific monoclonal antibody (mAb)-53 (1:5,000) (17) or anti-His primary antibody (1:1,000) and a secondary antibody, horseradish peroxidase-conjugated antimouse IgG (1:10,000). Immunoreactive proteins were detected using Lumilight chemiluminesence reagents (Roche). Mass estimates for hGDF9 throughout the purification were determined by Western blot using recombinant mGDF9 (R&D Systems) as a reference.

Production of hGDF9 point mutants

Point mutations in the pro- and mature regions of hGDF9 were introduced using the QuikChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA). pCDNA3.1 (Invitrogen) vectors containing full-length hGDF9 cDNA served as the template in these reactions. For each construct, the mutated region was confirmed by DNA sequencing. Wild-type and mutant hGDF9 proteins were produced by transient transfection in HEK-293 T cells using Lipofectamine 2000. Briefly, the HEK-293 T cells were plated at 9×10^5 cells/well in a six-well plate. Wild-type or mutant hGDF9 DNA (5 μg) was combined with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 20 min incubation, DNA-Lipofectamine complexes were added directly to the plated cells and incubated in DMEM/10% FCS medium for 24 h at 37 C in 5% CO_2. After 24 h, the medium was removed and replaced with production media and incubated for a further 48 h at 37 C in 5% CO_2. The amount of hGDF9 variants in the conditioned medium was determined by Western blotting, as described above.

Generation of a mouse/human GDF9 chimera

To test whether differences within the prodomains of mouse and hGDF9 contributed to their bioactivity, we generated a mouse/human GDF9 chimera (mhGDF9). mhGDF9 was engineered by fusing the prodomain of mGDF9 (residues 1–306) to the mature domain of hGDF9 (residues 320–454). mhGDF9 was generated by overlapping PCR (mGDF9 prodomain sense primer: 5′-caccatcatcatcatcaccacgaagaatcccagagtggagccagtg-3′; antisense primer: 5′-tcgacggcgccggggagatc-3′; hGDF9 mature domain sense primer: 5′-gatctccccggcgccgtcgaggtcaggaaactgtcagttctg-3′; antisense primer: 5′-atcgggatccttaacgacaggtgcactttg-3′) and cloned into the *Bam*HI and *Hin*dIII sites of the pCDNA3.1(−) vector (Invitrogen). mhGDF9 was produced by transient transfection in HEK-293 T cells using Lipofectamine 2000 (Invitrogen), as described above.

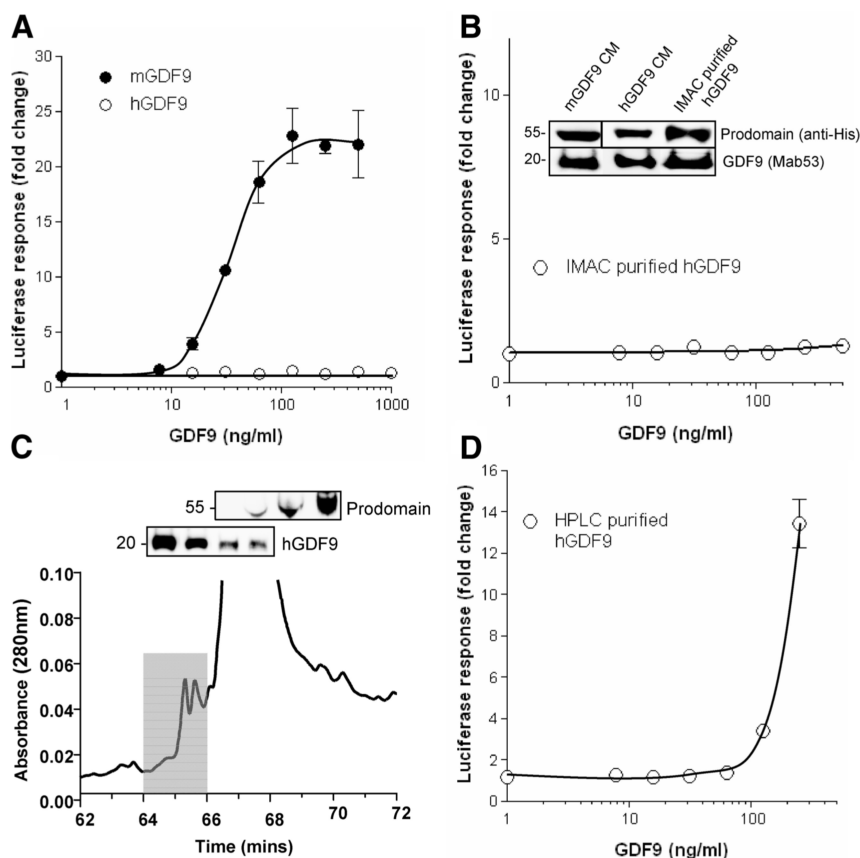

FIG. 1. Human GDF9 is secreted in a latent complex with its prodomain. A, Adrenocortical cells transfected with a Smad2/3-responsive luciferase reporter were stimulated with increasing concentrations (7.8–1000 ng/ml) of either mGDF9- (●) or hGDF9 (○)-conditioned medium. Luciferase activity was measured as described in *Materials and Methods*. B (*inset*), Western blot analysis of hGDF9 in conditioned medium and after IMAC purification, using mature domain (mAb53) or prodomain (anti-His) antibodies. B, Adrenocortical cells transfected with a Smad2/3-responsive luciferase reporter were stimulated with increasing concentrations (7.8–500 ng/ml) of IMAC-purified hGDF9 (○). C, hGDF9 eluted from the IMAC column was further purified by RP-HPLC using a Vydac Jupiter column (250 × 4.6 mm, 5 μm particle size) on a gradient of 20–70% acetonitrile in 0.1% heptafluorobutyric acid at 1 ml/min over 90 min. HPLC fractions 64–69 were analyzed by SDS-PAGE and Western blot for the presence of the pro- and mature domains of hGDF9 (*inset*), and fractions 64–65 (containing only mature hGDF9) were pooled. D, Adrenocortical cells transfected with a Smad2/3-responsive luciferase reporter were stimulated with increasing concentrations (7.8–250 ng/ml) of HPLC-purified mature hGDF9 (○). For each assay, the values are the means ± SD from two representative experiments.

In vitro bioassay

Wild-type and mutant hGDF9 were compared with mGDF9 for their ability to stimulate a luciferase response in a mouse adrenocortical cell line (AC) (18) or in a human granulosa cell line (KGN). Briefly, AC or KGN cells were plated in 48-well plates at 114,000 cells/well or 40,000 cells/well, respectively. After 24 h incubation, cells were transfected with an activin responsive luciferase reporter construct (pGRAS) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The cells were washed 24 h after transfection with complete medium (DMEM/10% FCS) and treated for 16 h with either mGDF9 or hGDF9 variants (7.8–500 ng/ml). The medium was aspirated, and the cells were solubilized in solubilization buffer ([25 mM glycylglycine (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA, 1% Triton X-100, and 1 mM dithiothreitol], and luciferase reporter activity was measured.

Receptor binding studies

Carrier-free mGDF9 (1 μg; R&D Systems) in 15 μl 0.5 M sodium phosphate buffer, pH 7.5, was labeled with Iodine-125 (Perkin Elmer, Boston, MA). The stability of ^{125}I-GDF9 was limited, with all binding assays performed on the day of iodination. AC cells were plated at 2×10^5 cells/well in DMEM/10% FCS in 24-well plates coated with poly-D-lysine. The following day, the cells were washed in a binding buffer (DMEM/0.1% BSA) and incubated for 4 h at room temperature with ^{125}I-GDF9 (40,000 cpm/well) together with increasing concentrations of GDF9 variants (0.03–20 nM). The cells were

washed in cold 10 mM PBS (pH 7.4) and solubilized in 1% Triton X-100. Radioactivity was measured using a γ-counter. The binding data were analyzed using the Prism program (version 5.0; GraphPad Software Inc., San Diego, CA) and by Scatchard analysis.

Murine granulosa cell proliferation assay

Mice were maintained in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes and with the approval of the Adelaide University Animal Ethics Committee. This mitogen bioassay was performed as previously described (17, 19, 20). In brief, mural granulosa cells were recovered from 21- to 26-d-old 129/SV mice, 44–46 h after administration of 5 IU of equine chorionic gonadotropin (5 IU; Folligon; Intervet, Castle Hill, Australia). After recovery, mural granulosa cells were washed in B-TCM-199 medium with supplements (19). Assays were conducted with 25,000 granulosa cells per well in a 96-well plate (Falcon; Becton Dickinson, Franklin Lakes, NJ) in a final volume of 125 μl. Cells were cultured in the presence of increasing concentrations (3.9–500 ng/ml) of wild-type or mutant hGDF9 (recombinant mGDF9 was included as a positive control) in an atmosphere of 37 C, 96% humidity in 5% CO_2 in air for 18 h, followed by a further 6-h pulse of 15.4 kBq [^{3}H]thymidine (MP Biomedicals, Solon, OH) under the same conditions. After the culture, mural granulosa cells were harvested, and the incorporated [^{3}H]thymidine was quantified using a scintillation counter as an indicator of the proportion of cells in S phase, hence providing an indication of the level of mural granulosa cell DNA synthesis and proliferation. Each treatment was performed in duplicate and the experiment repeated three times.

Results

Human GDF9 is secreted in a latent complex with its prodomain

Previous studies have suggested that mGDF9 is secreted in an active form, whereas hGDF9 is latent (13). To confirm this, we assessed the ability of conditioned medium from mGDF9 or hGDF9 expressing cells to activate a phosphorylated mothers against decapentaplegic (Smad)-2/3-responsive luciferase reporter (pGRAS) in a murine adrenocortical cell line (18). Treatment of cells with mGDF9 resulted in a dose-dependent increase in luciferase activity (EC_{50} 28ng/ml) (Fig. 1A). In contrast, hGDF9 was inactive at all doses tested (Fig. 1A). The lack of bioactivity of hGDF9 was not due to defective production or processing of the precursor molecule (Fig. 1B, lane 2, *inset*). For some other TGF-β ligands (TGF-β1, -β2, -β3, myostatin, and GDF11), latency is conferred by high-affinity interactions with their prodomains. The placement of a His6 tag at the N terminus of the hGDF9 prodomain enabled us to use Ni_2+ based IMAC chromatography to show that hGDF9 was secreted in a latent complex with its prodomain (Fig. 1B and lane 3, *inset*). mGDF9 was also secreted noncovalently associated with its prodomain (Fig. 1B, lane 1, *inset*); however, the affinity of this interaction was insufficient to suppress biological activity. The separation of mature hGDF9 from its prodomain by reversed-phase HPLC (RP-HPLC) (Fig. 1C) activated the growth factor (Fig. 1D), indicating that the prodomain plays a role in conferring latency to hGDF9.

The human GDF9 prodomain limits growth factor expression

The prodomains of mouse and human GDF9 share 64% amino acid identity, whereas the mature domains are 90% identical (Fig. 2). Based on this analysis, we predicted that differences in the prodomains of mouse and human GDF9 would determine the biological activity of the ma-

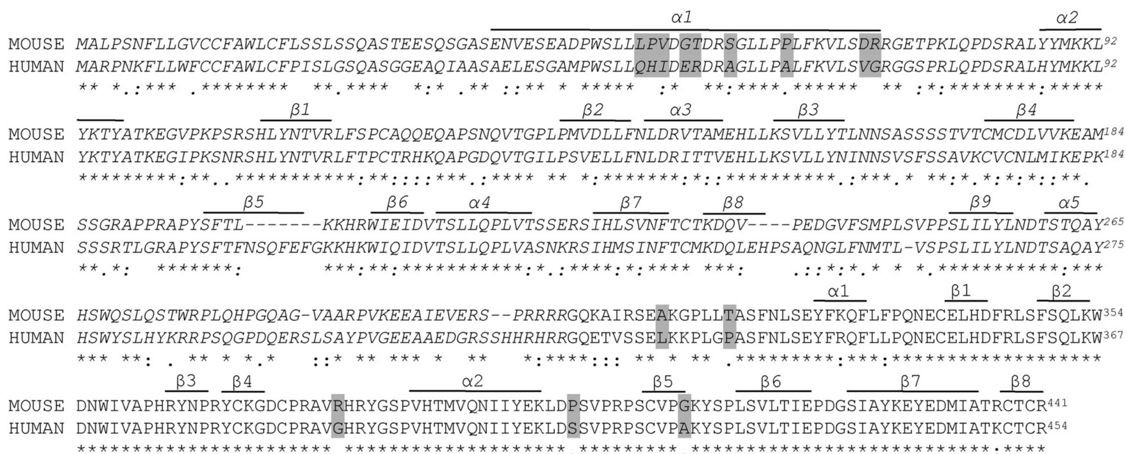

FIG. 2. Sequence alignment of mouse and human GDF9. The pro- (*italics*) and mature domains of mouse and human GDF9 were aligned using ClustalW (Conway Institute, University College Dublin, Ireland). Secondary structure elements (β-sheets and α-helices) as determined by QuickPhyre (Quick Protein Homology/analogy Recognition Engine, Structural Bioinformatics Group, Imperial College, London, UK) are depicted above the sequences. Nonconserved hGDF9 residues in the α1-helix of the prodomain and throughout the mature domain predicted to influence growth factor expression and activity are indicated (*shaded gray*). In this study, these hGDF9 residues were substituted for the corresponding residues from mGDF9.

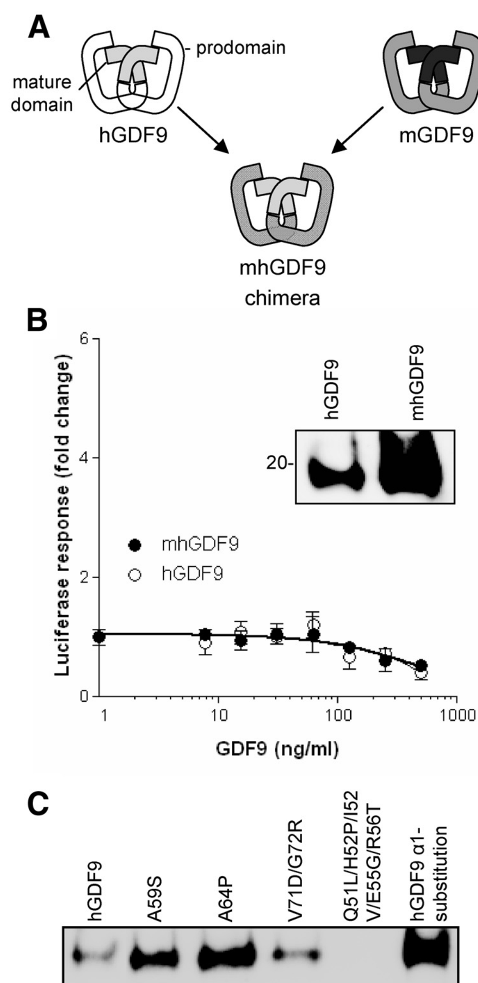

FIG. 3. Specific residues within the prodomain of hGDF9 limit mature growth factor expression but do not confer latency. A, The prodomain (residues 1–306) of mGDF9 was fused to the mature domain (residues 320–454) of hGDF9 to generate a mhGDF9 chimera. B, Adrenocortical cells transfected with a Smad2/3-responsive luciferase reporter were stimulated with increasing concentrations (7.8–500 ng/ml) of either mhGDF9- (●) or hGDF9 (○)-conditioned medium. Luciferase activity was measured as described in *Materials and Methods*. B (*inset*), The expression of the mhGDF9 chimera, relative to hGDF9, was determined by Western blot using the mature domain antibody (mAb53). C, To identify the regions of the mGDF9 prodomain that contributed to increased expression of hGDF9, residues in the α1-helix of hGDF9 were substituted with the corresponding residues from mGDF9, and effects on growth factor expression were determined by Western blot analysis.

ture growth factors. This concept was supported by studies showing that prodomains coordinate the formation and stability of other latent TGF-β complexes (21–24). Therefore, we generated a mhGDF9 chimera by linking the prodomain of mGDF9 (residues 1–306) to the mature domain of hGDF9 (residues 320–454) (Fig. 3A). Expression of the mhGDF9 chimera was 10-fold higher than wild-type hGDF9 (Fig. 3B, *inset*); however, activity was not restored (Fig. 3B). To identify the regions of the mGDF9 prodomain that contributed to increased expression of hGDF9, we referred to the recent crystal structure of TGF-β1 bound to its prodomain (25). In this structure, residues within the N-terminal α-helix (α1) of the prodomain mediate high-affinity interactions with mature TGF-β1. Major differences exist within the α1-helix of mouse and human GDF9 (Fig. 2). We substituted eight hGDF9 residues through this region for the corresponding residues from mGDF9, either alone or in combination. Of the single- or double-point mutants generated, hGDF9 (Ala^{59}Ser) and hGDF9 (Ala^{64}Pro) were expressed at 3-fold elevated levels compared with wild-type hGDF9 (Fig. 3C). More strikingly, when all eight mGDF9 α1-helix residues were incorporated into hGDF9 (termed hGDF9 α1-substitution) expression was increased 8-fold (Fig. 3C). Together, these results indicate that specific residues within the prodomain of hGDF9 limit mature growth factor expression; however, they do not confer latency.

A single mature domain residue confers latency to hGDF9

The mature domains of mouse and human GDF9 are 90% identical, with most differences occurring at the disordered N terminus or being conservative in nature (Fig. 2). We substituted five nonconserved hGDF9 mature domain residues for the corresponding residues from mGDF9 (Fig. 2) and assessed their effects on expression and biological activity. Of the five mutants tested (Leu^{328}Ala, Pro^{334}Thr, Gly^{391}Arg, Ser^{412}Pro, and Ala^{422}Gly) all expressed at levels comparable with wild-type hGDF9, except Ala^{422}Gly, which was significantly reduced (Fig. 4A, *inset*). Similar to wild-type hGDF9, the Leu^{328}Ala, Pro^{334}Thr, and Ser^{412}Pro variants were inactive in an adrenocortical cell luciferase assay (Fig. 4A). In contrast, hGDF9 (Gly^{391}Arg)-conditioned medium induced a dose-dependent increase in luciferase activity (Fig. 4, A and B). Similar to wild-type hGDF9, the Gly^{391}Arg variant was secreted noncovalently associated with its prodomain (data not shown); however, the affinity of this interaction was not strong enough to inhibit biological activity (Fig. 4C). In a more physiologically relevant system, hGDF9 (Gly^{391}Arg) also stimulated a luciferase response in the human KGN granulosa cell line (Fig. 4D).

hGDF9 (Gly^{391}Arg) has higher affinity for its signaling receptors than wild-type hGDF9

We reasoned that hGDF9 remains associated with its prodomain in a latent complex because it has intrinsically low affinity for its signaling receptors. Indeed, when we separated wild-type and mutant hGDF9 from their prodomains by RP-HPLC, isolated mature hGDF9 (Gly^{391}Arg) was 8-fold more potent than the corresponding mature region of wild-type hGDF9 (Fig. 5A), suggesting that a substituted arginine at position 391 significantly increases

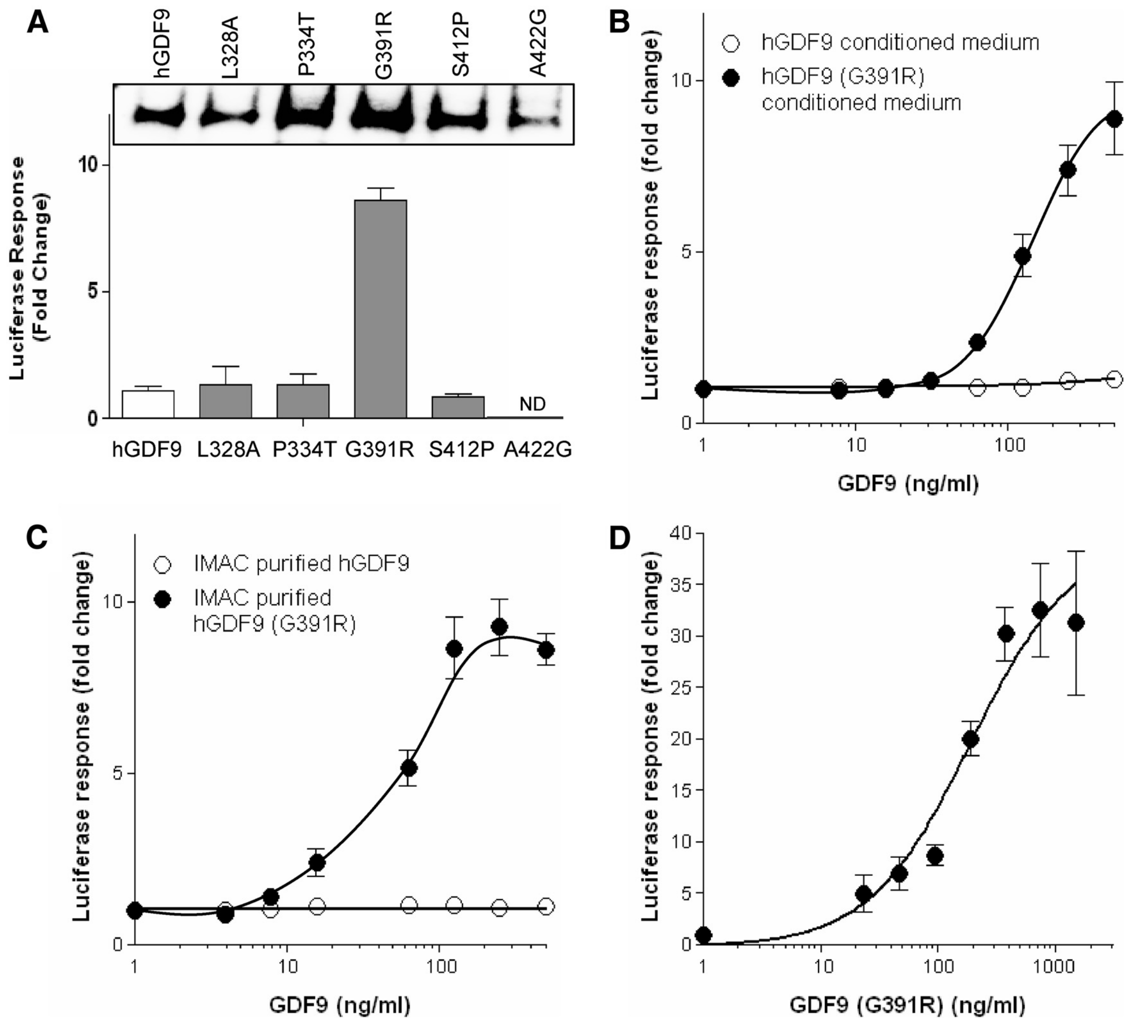

FIG. 4. A single mature domain residue confers latency to hGDF9. Selected mature domain residues of hGDF9 were substituted with the corresponding residues from mGDF9, and the effects on growth factor expression and activity (100 ng/ml) were determined by Western blot (A, *inset*) and in an adrenocortical cell luciferase assay (A). Adrenocortical cells transfected with a Smad2/3-responsive luciferase reporter were stimulated with the indicated concentrations of hGDF9 (○) or hGDF9 ($Gly^{391}Arg$) (●) in conditioned medium (B) or after IMAC purification (C). hGDF9 ($Gly^{391}Arg$) (●) also induced a luciferase response in the KGN cells transfected with a Smad2/3-responsive luciferase reporter (D). ND, Not determined.

the affinity of hGDF9 for its signaling receptors. To test this, we determined the ability of mature hGDF9 variants to block the binding of ^{125}I-mGDF9 to its receptors on the surface of AC cells (Fig. 5B). In this assay, hGDF9 ($Gly^{391}Arg$) displayed a significantly higher affinity for its signaling receptors (K_d 0.4 nM) than wild-type hGDF9 (K_d 1 nM) (Fig. 5C). Gly^{391} forms part of the predicted type I receptor binding site on mature hGDF9 (26, 27), and this residue is present in all species except mouse, rat, hamster, greater galago, and possum, in which it is substituted with an arginine (Fig. 6). To determine the requirement for an arginine at position 391 to activate hGDF9, we generated two other hGDF9 variants, $Gly^{391}Leu$ and $Gly^{391}Ala$. Although expressed, both these mutants were inactive in the adrenocortical cell luciferase assay (data not shown). Thus, the bioactivity of GDF9 across species is dependent on the nature of the amino acid at position 391.

hGDF9 ($Gly^{391}Arg$) is a potent stimulator of granulosa cell proliferation

Previous studies have shown that mGDF9 is a potent oocyte-secreted granulosa cell mitogen (17, 20). In these experiments we assessed the effect of hGDF9 variants on murine granulosa cell proliferation. Wild-type hGDF9 and hGDF9 ($Gly^{391}Arg$), purified via the His-tag within their prodomains, were added to mural granulosa cells in culture. Treatment of cells with hGDF9 ($Gly^{391}Arg$) led to a potent, dose-dependent stimulation of granulosa cell DNA synthesis (EC_{50} 55 ng/ml), as assessed by [^{3}H]thymidine incorporation (Fig. 7). Wild-type hGDF9, in con-

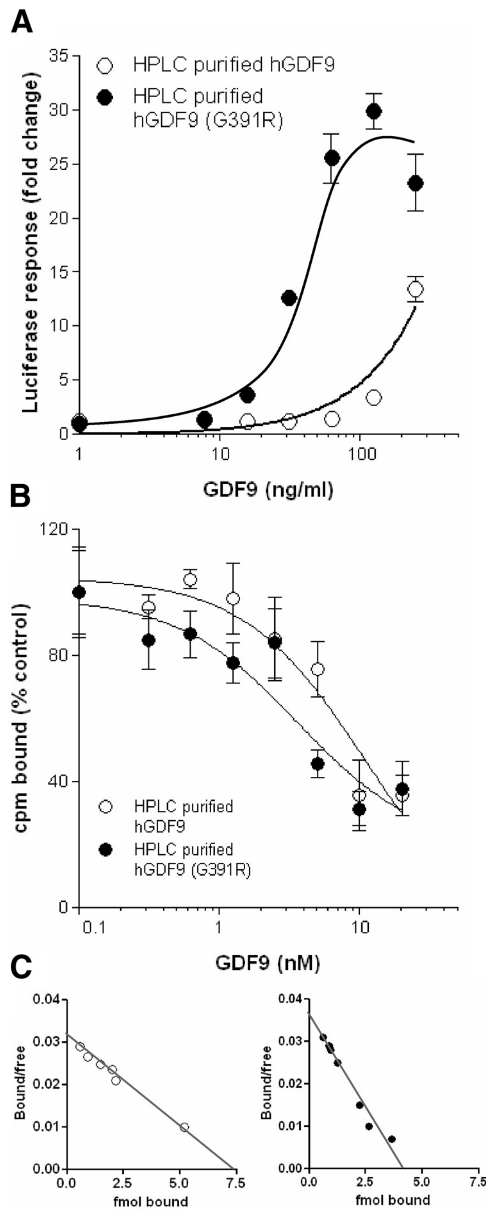

FIG. 5. hGDF9 (Gly³⁹¹Arg) has high affinity for its signaling receptors. A, Adrenocortical cells transfected with a Smad2/3-responsive luciferase reporter were stimulated with the indicated concentrations of HPLC-purified hGDF9 (○) or hGDF9 (Gly³⁹¹Arg) (●). B, GDF9-responsive AC cells were subjected to competition binding as described in *Materials and Methods*. Displacement curves for HPLC-purified hGDF9 (○) or hGDF9 (Gly³⁹¹Arg) (●) are shown. The amount of bound ^{125}I-mGDF9 was determined in triplicate for each experiment, and the values are the mean ± SD. C, Scatchard plots of hGDF9 (○) or hGDF9 (Gly³⁹¹Arg) (●) binding to AC cells are shown.

trast, had no effect on granulosa cell proliferation. Thus, a glycine at position 391 prevents hGDF9 from directly mediating an important physiological function. Interestingly, the extent of granulosa cell proliferation in response to hGDF9 (Gly^{391}Arg) was 4.5-fold lower than that observed with recombinant mGDF9 (EC_{50} 12 ng/ml) (Fig. 7).

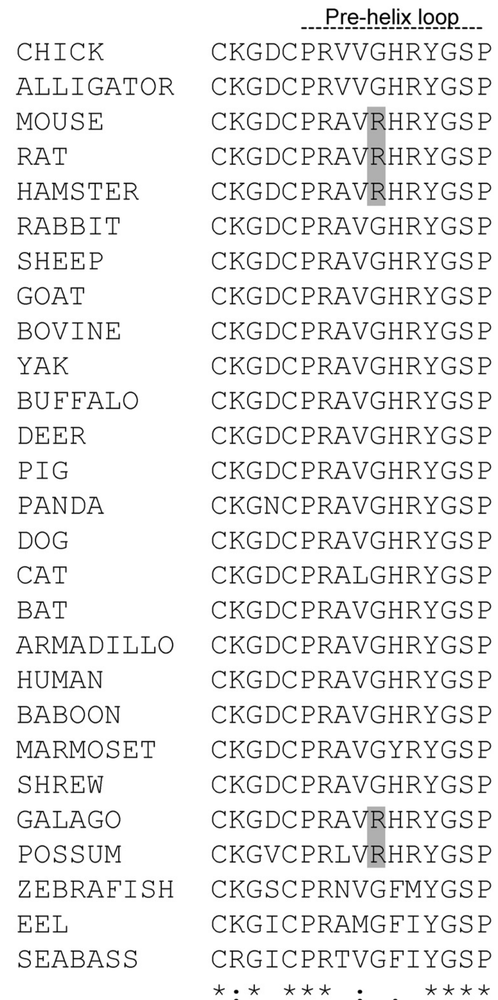

FIG. 6. Sequence alignment of GDF9 across species. GDF9 sequences were obtained from the UniProt database (www.uniprot.org/) and the prehelix loop region was aligned using ClustalW. Note that Gly³⁹¹ in hGDF9 is conserved in all species except for mouse, rat, hamster, greater galago, and possum, in which it is substituted with an arginine.

Discussion

The mechanism of ovarian folliculogenesis in mammals is reasonably well understood, involving a complex exchange of endocrine signals between the pituitary gland and the ovary and the actions of autocrine and paracrine factors secreted by the oocyte and its surrounding somatic cells (28–30). GDF9 is one of the most important oocyte-derived factors that regulate folliculogenesis, controlling multiple aspects of this process, including granulosa cell growth and differentiation (1, 31, 32). Studies in sheep have also shown that GDF9, together with the related TGF-β ligand, bone morphogenetic protein (BMP)15, is a primary determinant of ovulation rate within species (*i.e.* the number of mature oocytes released during one reproductive cycle) (5, 29, 33). What has been less clear is whether GDF9, given its high evolutionary conservation, could contribute to the marked differences in ovulation

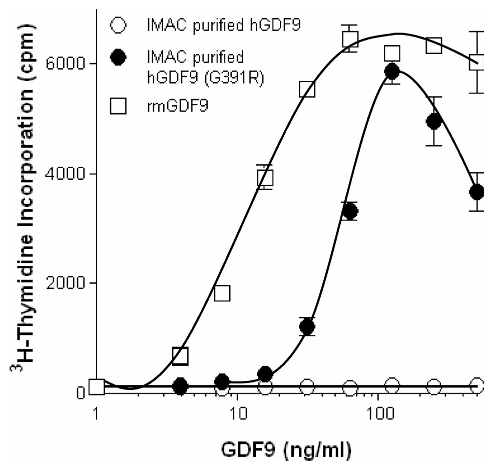

FIG. 7. hGDF9 (Gly^{391}Arg) stimulates granulosa cell proliferation. Mural granulosa cells were cultured with increasing concentrations (3.9–500 ng/ml) of recombinant mGDF9 (■), IMAC-purified hGDF9 (○), or IMAC-purified hGDF9 (Gly^{391}Arg) (●). After 24 h of culture, the labeled thymidine incorporated into cells was counted. Points are mean ± SEM from duplicate wells from a representative experiment. The experiment was performed three times.

rate between species. In this study, we have identified specific residues in the pro- and mature domains of hGDF9 that limit growth factor expression and activity, relative to mGDF9. These adaptations may contribute to the variation observed in ovulation rate and fecundity between mammals.

The recent elucidation of the pro-TGF-β1 crystal structure (25) provides some insights into why mouse and human GDF9 are expressed differently and have disparate bioactivities. Within this structure, described as a straight-jacket, the two prodomains form the neck, shoulders, and upper arms and connect at the elbows to the crossed forearms formed by the two TGF-β1 monomers (25). Prodomain dimerization occurs between the eighth and ninth β-strands and links the prodomains in a bowtie at the neck. Residues within the α1- and α2-helices and the intervening latency lasso of the prodomains encircle the fingers of each TGF-β1 monomer and are likely to be important in directing the folding of the growth factor domain (25). Significant differences exist in the prodomain α1-helices of mouse and human GDF9, particularly in the region predicted to form intimate contacts with the mature growth factor domains. Substituting mGDF9 residues across this region into the hGDF9 prodomain increased expression 8-fold, indicating that the hGDF9 prodomain is designed to limit mature growth factor expression.

TGF-β1 is secreted in a latent form because of two major adaptions within its prodomain: 1) cysteine residues (Cys^{223} and Cys^{225}) within the bowtie region, which covalently link the two prodomain chains; and 2) fastener residues (Lys^{27}, Tyr^{74}, Tyr^{75}, Ala^{76}, and Arg^{238}), which intimately connect the straight-jacket and arm regions of the prodomains (25). Mutation of these latency-conferring residues results in the spontaneous activation of TGF-β1 (25, 34). Interestingly, the two prodomain chains of hGDF9 are not covalently linked, nor do they show conservation of fastener residues, suggesting that a separate mechanism confers latency to hGDF9. In support, linking the prodomain of active mGDF9 to the mature domain of hGDF9 was insufficient to restore biological activity.

The mature domains of mouse and human GDF9 are 90% identical, with most differences occurring at the disordered N terminus. Within the structurally ordered regions of hGDF9, only four amino acids are not conserved (Gly^{391}, Ser^{412}, Ala^{422}, and Lys^{450}). Of these, Gly^{391} was of most interest because it is located in the prehelix loop, a region of other TGF-β ligands critical for type I receptor interactions (26, 27). Significantly, mutation of the corresponding residues in BMP4 (Ala^{346}Val) and GDF5 (Arg^{438}Leu) cause the diseases nonsyndromic orofacial cleft type 11 and multiple synostoses syndrome type 2, respectively (35, 36). In addition, Gly^{391} is present in all species except mouse, rat, hamster, greater galago, and possum, in which it is substituted with an arginine. Incorporation of an arginine at position 391 resulted in an hGDF9 variant that was active in two separate bioassays, indicating that Gly^{391} is necessary and sufficient to confer latency to hGDF9. Gly^{391} reduces the affinity of hGDF9 for its signaling receptors (37), ensuring that it remains noncovalently associated with its prodomain. Because Gly^{391} is conserved in most other species (Fig. 6), it is likely that GDF9 latency is a common phenomenon. In support, ovine GDF9 is also secreted in a latent form (14).

For latent TGF-β ligands to signal via specific complexes of type I and type II serine/threonine kinase receptors, they must be separated from their prodomains. The mechanism of activation differs for individual ligands according to prodomain affinity, cell type, and context, but all known activating mechanisms directly target prodomains (38). One common mechanism of activation is via members of the BMP1/tolloid (TLD) family of extracellular metalloproteases. Wolfman *et al.* (24) showed that BMP1 can cleave the myostatin prodomain between Arg^{98} and Asp^{99}, just upstream of the fastener, and thereby activate latent myostatin. Mice carrying a myostatin (Asp^{99}Ala) point mutation, which renders the prodomain protease resistant, exhibit increases in muscle mass similar to those seen in mice lacking myostatin (39, 40). GDF11, which plays important roles in anterior-posterior patterning of the axial skeleton (41) and the inhibition of neurogenesis (42), also requires activation by BMP1/TLD proteases both *in vitro* and *in vivo* (21, 43). In addition,

BMP1/TLD proteases can activate the latent BMP10 complex (44) and cleave latent TGF-β binding proteins at two sites to facilitate the subsequent activation of TGF-β1 (45). A recent study demonstrated that BMP1 is expressed by granulosa cells at all stages of follicular development (46), identifying this protease as a potential activator of oocyte-secreted hGDF9. Further studies are required to determine whether BMP1 can cleave and activate hGDF9.

The differences in expression and bioactivity of GDF9 across species have implications for the broader TGF-β superfamily. Previous studies have shown striking differences in the reproductive phenotypes of BMP15 knockout mice (subfertility) and BMP15-mutant sheep (sterility) and have identified defects in production of mBMP15 as a likely cause (3, 29, 47, 48). However, murine and ovine BMP15 also differ at the residue corresponding to Gly^{391} in hGDF9 (Pro in mBMP15; His in ovine BMP15), suggesting that BMP15 bioactivity could also be differentially modulated across species. Sequence analysis indicates that other TGF-β ligands with reproductive roles (*e.g.* anti-Mullerian hormone, inhibin-α, BMP8B) also have significant differences in the structurally important regions of their pro- and mature domains. Further studies are required to determine whether these differences contribute to the variation observed in follicular development, ovulation rate, and fecundity between mammals.

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Address all correspondence and requests for reprints to: Craig A. Harrison, Prince Henry's Institute of Medical Research, 246 Clayton Road, Clayton, Victoria 3168, Australia. E-mail: craig.harrison@princehenrys.org.

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