Bone morphogenetic protein-15 (BMP-15) and growth and differentiation factor-9 (GDF-9) are oocyte-secreted factors that are critical local regulators of ovarian physiology. Recent studies have identified a number of mutations in these genes that cause increased fertility and infertility in heterozygous or homozygous ewes carrying the mutations, respectively. Interestingly, heterozygous ewes with a mutation in both BMP-15 and GDF-9 exhibit higher fertility than those having mutation in only one of the genes. Here, we have produced recombinant human BMP-15 and GDF-9 that carry the mutations identified in those sheep, i.e. I31D and S99I in BMP-15 and S77F in GDF-9. We found that when individually expressed, both BMP-15 mutations had no effect on the processing, secretion, and dimerization of the mature proteins or on the biological activity of the molecules. However, when mutant BMP-15 was co-expressed with wild-type GDF-9, the secretion of BMP-15 and GDF-9 was significantly reduced, suggesting that the mechanisms by which the BMP-15 mutations affect sheep fertility occurs at the level of protein secretion rather than dimerization and biological activity. Moreover, when mutant GDF-9 was co-expressed with mutant BMP-15, the secretion levels of both proteins were significantly lower than those of cells co-expressing wild-type GDF-9 and mutant BMP-15, suggesting a possible mechanism for the extreme fertility observed in the compound heterozygous mutant sheep.

Rapidly advancing recent research demonstrating the importance of the oocyte-secreted factors, BMP-15 and GDF-9, in the regulation of ovarian function has had an immediate impact on our understanding of female fertility (1, 2). These growth factors exhibit a high degree of structural homology with a notable similarity that they both lack the fourth of seven conserved cysteine residues that are characteristic features of the TGF-β superfamily (3–5). Our recent studies have shown that, although not covalently bound, both BMP-15 and GDF-9 do indeed exist as homodimers (6). Additionally, when BMP-15 and GDF-9 are co-expressed, BMP-15/GDF-9 heterodimers are formed; however, the physiological role of the heterodimer has yet to be established.

The sheep model has proven to be a particularly valuable tool in elucidating the role of these factors in determining the quota of oocytes ovulated in a given ovarian cycle, primarily because of the identification of naturally occurring mutations in the bmp15 and gdf9 genes that have profound effects on sheep fertility (7–9). Direct implication of the role of BMP-15 in sheep reproduction was provided when it was shown that two strains of highly prolific sheep, Inverdale and Hanna, carried causal point mutations in the bmp15 gene (7). Interestingly, female sheep that are heterozygous carriers of the mutations exhibit increased fertility because of an increase in their ovulation quota, whereas female sheep that are homozygous carriers of the mutations are infertile with an ovarian phenotype that resembles that of GDF-9 knock-out mice (10), i.e. a block in the primary stage of folliculogenesis. Because the Hanna mutation (called FecXIX), which induces a premature stop codon, results in a severely truncated mature protein, it has been postulated that the lack of BMP-15 bioactivity is responsible for causing the Hanna phenotypes. The mechanism by which the BMP-15 mutation found in Inverdale ewes (FecXIV) affects ovarian function is less clear. This mutation is a single point mutation that causes a substitution of valine with aspartic acid at residue 31 of the mature protein, a region that is highly conserved among TGF-β superfamily members. Our previous experiments have shown that when human BMP-15 with a mutation that mimics the Inverdale mutation (BMP-15I31D), is co-expressed with GDF-9 in vitro, the cleavage and secretion of BMP-15 is abolished, and the secretion of GDF-9 is impaired (6).

Recently, new distinct mutations in the BMP-15 gene that are associated with reproductive phenotypes resembling those of the Inverdale and Hanna mutations have been identified in other strains of sheep (9). Of these new mutations, one, designated FecXVII, causes the substitution of asparagine with aspartic acid at residue 31 of the mature protein, a region that is highly conserved among TGF-β superfamily members. Our previous experiments have shown that when human BMP-15 with a mutation that mimics the Inverdale mutation (BMP-15I31D), is co-expressed with GDF-9 in vitro, the cleavage and secretion of BMP-15 is abolished, and the secretion of GDF-9 is impaired (6).

Received for publication, January 30, 2004
Published, JBC Papers in Press, February 17, 2004, DOI 10.1074/jbc.M401050200

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Functional Characterization of BMP-15 and GDF-9 Mutations

Mutations in both BMP-15 and GDF-9 exhibit ovulation quotes that are higher than heterozygous carriers of either mutation separately (9), extending the concept that there are physiological or molecular interactions between BMP-15 and GDF-9 that have important consequences on ovarian processes (6, 11).

A critical challenge in this field is the elucidation of the molecular mechanisms underlying the impact that the mutations in these genes have on the progression of folliculogenesis and ovulation. A better understanding of such mechanisms will provide tremendous insight into the physiological basis of the local ovarian regulation of female fertility in mammals. In the present study, we have produced recombinant human BMP-15 and GDF-9 that contain the point mutations that affect sheep fertility to characterize the effects of these mutations on the processing, secretion, and biological activity of the proteins.

MATERIALS AND METHODS

Construction of Expression Plasmids—Full-length human GDF-9 cDNA was amplified by reverse transcription-PCR from ovarian RNA and cloned in-frame at the upstream site of the FLAG epitope tag present in pCMV-Tag4A (Stratagene, San Diego, CA). The GDF-9 cDNA with the FLAG tag was then released and subcloned into pcDNA3.1(zeo−) expression vector (Invitrogen) to form a plasmid designated as pGDF-9F. The plasmid pBMP-15F, described previously (12), was used in this study. Next we performed standard PCR-based site-directed mutagenesis using pBMP-15F and pGDF-9F to make plasmids having either the I31D or the S99I mutation in the gdf9 gene and the S77F mutation in the gdf9 gene as described previously (6); the resulting plasmids were designated as pBMP-15I31D, pBMP-15S99I, and pGDF-9F S77F, respectively.

Production and Analysis of Recombinant Proteins—For the purpose of producing sufficient amounts of wild-type and mutant BMP-15 and GDF-9 proteins for use in bioassay experiments, stable transfaction 293T cell lines were established as described previously (6). For transient transfections, 293T cells were transfected using FuGENE 6 (Roche Applied Science). Twenty-four h after transfection, the culture media were replaced with serum-free media for 3–4 days before the conditioned media were harvested for further analysis. Recombinant proteins were immunopurified by affinity column chromatography using anti-FLAG Ab-agarose as described previously (12). For chemical cross-linking, a non-cleavable cross-linker, bis(sulfosuccinimidyl) suberate (Pierce), was added at a final concentration of 0.625 mM to conditioned media or immunopurified protein solutions at a final concentration of 1–1.5 mg/ml growth factors. Western immunoblotting was performed as described previously (6) under reducing conditions using the anti-FLAG Ab-agarose beads yielded only the mature GDF-9 protein, not the proprotein (Fig. 1C, lane 1). Interestingly, immunopurification of wild-type GDF-9 and GDF-9 S77F with anti-FLAG-agarose beads yielded only the mature GDF-9 protein, not the proprotein, like the mature protein, contains the C-terminal FLAG epitope (Fig. 1A), it is likely that the folding of the proprotein causes the FLAG motif to be blocked.

No previous studies have investigated whether any of the mutations in BMP-15 and GDF-9 affect the biological activity of the proteins. For this purpose we developed stable transfector 293 cell lines expressing human wild-type BMP-15, BMP-15 I31D, BMP-15 S99I, wild-type GDF-9, and GDF-9 S77F. Then we used anti-FLAG-agarose to immunopurify the products from the conditioned media as described previously (12). Because the major consensus biological action shared by both BMP-15 and GDF-9 is the stimulation of GC mitosis, we tested the wild-type and mutant BMP-15 and GDF-9 preparations using our well established rat GC thymidine incorporation assay (12–14). In these biological assays the mutant proteins stimulated DNA incorporation with potencies and efficacies that were indistinguishable from their wild-type counterparts (Fig. 2).

Because BMP-15 and GDF-9 share a nearly identical expression pattern in the oocyte during folliculogenesis (2), we transiently transfected 293T cells with various combinations of wild-type and mutant BMP-15 and GDF-9 expression plasmids to evaluate whether intracellular interactions between BMP-15 and GDF-9 have any effect on the manifestation of the aberrancies caused by the mutations. Co-expression of GDF-9 with BMP-15 I31D or BMP-15 S99I caused a strong and significant decrease in the levels of mutant BMP-15 secreted into the media as compared with co-transfection with wild-type BMP-15 (Fig. 3, A and B; compare lane 2 with lane 1 for BMP-15 I31D and lane 3 with lane 1 for BMP-15 S99I). Likewise, when wild-type GDF-9 was co-transfected with either form of mutant BMP-15, the levels of GDF-9 secreted into the media also were significantly suppressed as compared with co-transfection with wild-type BMP-15 (Fig. 3, A and C; compare lane 2 with lane 1 for BMP-15 I31D and lane 3 with lane 1 for BMP-15 S99I). In contrast to the BMP-15 mutations, the FecG’ mutation in GDF-9 (GDF-9 S77F) did not cause any significant changes in the levels of GDF-9 or BMP-15 secretion when cells co-expressing GDF-9 S77F and wild-type BMP-15 were compared with those co-expressing wild-type GDF-9 and wild-type BMP-15 (Fig. 3, A–C; compare lane 4 with lane 1). Interestingly, however, when GDF-9 S77F was co-transfected with either BMP-15 I31D or BMP-15 S99I, there was a strong and significant reduction in the secretion of both BMP-15 and GDF-9 as compared with co-expression with wild-type BMP-15 (Fig. 3, A–C; compare lanes 5 and 6 with lane 4) or co-expression of wild-type GDF-9 and either BMP-15 I31D or BMP-15 S99I (Fig. 3, A–C; compare lane 5 with lane 2 for BMP-15 I31D and lane 6 with lane 2 for BMP-15 S99I).
These data demonstrate an additive effect of the BMP-15 mutations with the GDF-9 S77F mutation.

DISCUSSION

BMP-15 and GDF-9, oocyte-secreted factors that exhibit a high degree of homology, have proven to be critical local regulators of ovarian function (1, 2). The recent identification of naturally occurring mutations in BMP-15 and GDF-9 that cause reproductive aberrancies in sheep has provided a unique
FIG. 3. Production of BMP-15 and GDF-9 by singly transfected and co-transfected 293T cells. Three to four days after transient transfection of 293T cells with the indicated expression plasmids, conditioned media were collected and proteins were subjected to SDS-PAGE immunoblotting analysis using anti-FLAG Ab. A, representative immunoblot data. Lanes A–C and D and E show that each of the BMP-15 and GDF-9 plasmids, respectively, result in indistinguishable levels of secreted proteins, demonstrating equal transfection efficiency, promoter activity, transcription rates, and protein synthesis. Lanes 1–6 demonstrate changes in the levels of secreted protein as a result of different co-expression conditions. B, relative intensities of mature BMP-15 immunoreactivity averaged from three separate experiments. Lane numbers are representative of those in A. Data are expressed as relative to the band intensity of cells co-expressing wild-type BMP-15 and wild type GDF-9 (lane 1). All data are shown as the mean ± S.E.; different letters indicate significant differences at p ≤ 0.05. C, relative intensities of mature GDF-9 immunoreactivity averaged from three separate experiments and expressed as described for B.
opportunity to study the biochemical and molecular mechanisms involved in the ability of these factors to regulate mammalian reproduction (7, 9). To this end, we have produced recombinant human BMP-15 and GDF-9 with the missense mutations identified in the sheep. It is noteworthy that the mutated amino acids in BMP-15 and GDF-9 are conserved among various species (9). We used human BMP-15 and GDF-9 as templates because of the potential clinical relevance and because we have collected extensive data previously using recombinant human BMP-15 (12, 14–18).

BMP-15 and GDF-9 form a unique subset of the TGF-β superfamily in that they share the highest amino acid sequence homology with each other, they share a unique octapeptide-specific expression pattern, and they lack the cysteine that is responsible for forming the covalent bond between the subunits of the characteristic dimers. Although BMP-15 and GDF-9 bear many similarities to other members of the TGF-β superfamily, especially the BMPs, little is known about their functional structural features; thus, the identification of the distinct mutations that result in similar reproductive consequences allow for the investigation of structure-function relationships of these molecules. Also, because mutations in other members of the TGF-β superfamily are often lethal (19), investigation of these naturally occurring mutations in BMP-15 and GDF-9 may serve as a paradigm of the impact of mutations in these regions of the molecules of the TGF-β superfamily as a whole.

It was originally proposed that the I31D mutation of BMP-15 would prevent the formation of dimers, thus preventing the biochemical activity of BMP-15 (7). Likewise, it was predicted that the S99I BMP-15 mutation might affect the biochemical activity of BMP-15 (9), possibly by disrupting interactions with its receptor, based on the finding that mutating the corresponding serine in BMP-2 reduces affinity to the extracellular domain of BMP receptor-II (20). Our present findings that neither BMP-15 mutation affects BMP-15 dimerization, together with the findings that BMP-15^{I31D} and BMP-15^{S99I} stimulate GC thymidine incorporation to levels that are indistinguishable from that of wild-type BMP-15, suggest that the original predictions that these BMP-15 mutations could affect the ability of the secreted proteins to elicit proper biochemical activity may not be true. Indeed, it is more likely that defects in the secretion of mature forms of BMP-15 and/or GDF-9 are the mechanisms underlying the aberrant reproductive phenotype of the sheep carrying these mutations.

A recent study has shown that immunizing sheep against either BMP-15 or GDF-9 mimics the reproductive aberrancies of the Fec mutations (21, 22). Because immunization against either factor independently caused the reproductive abnormalities, it was proposed that BMP-15 and GDF-9 and/or the BMP-15/GDF-9 heterodimer each must be necessary for normal folliculogenesis in this species. Our data support this concept in that we predict that the FecX mutations would impair the secretion of both GDF-9 and BMP-15, thus affecting normal folliculogenesis regardless of whether one or both of the factors are necessary for the normal progression of folliculogenesis.

There have been reports of mutations in other TGF-β superfamily members that appear to have primary effects on the intracellular processing and secretion of the mature proteins (23–25). The effects of these mutations bear resemblance to the substitution mutations in BMP-15 reported here; however, our findings in BMP-15 are unique in that we cannot detect any defects in the processing, secretion, dimerization, or biological activity of BMP-15 caused by the FecX mutations unless the mutant BMP-15 is co-expressed with GDF-9.

Similar to the substitution mutations in BMP-15, the S77F mutation in GDF-9 did not affect homodimer formation, and GDF-9^{S77F} stimulation of GC thymidine incorporation was indistinguishable from that of wild-type GDF-9. However, in contrast to the pronounced effects of the BMP mutations on the processing and secretion of BMP-15 and GDF-9, no observable defects were caused by the S77F mutation in GDF-9, neither when GDF-9^{S77F} was singly expressed nor when co-expressed with wild-type BMP-15. An intriguing and surprising result from our present data was that when GDF-9^{S77F} was co-expressed with BMP-15^{I31D} or with BMP-15^{S99I}, there was an augmentation of the effects of the BMP-15 mutations. These results suggest that the further decrease in GDF-9 and BMP-15 secretion would be a likely mechanism to explain the increased ovulation rate observed in sheep that are heterozygous carriers of the Fec^{Xb} and Fec^{Gt} mutation (9). Although no reports have been published that describe the reproductive consequences of sheep that are compound heterozygous for the Fec^{Xb} and Fec^{Gt} mutations, based on our present data we predict that these sheep would phenocopy the compound heterozygous Fec^{Xb}/Fec^{Gt} sheep.

Our present study clearly demonstrates that complex intracellular interactions between BMP-15 and GDF-9 have important implications for the processing and secretion of the mature forms of the molecule. Further, our data indicate that the identified substitution mutations in BMP-15 (I31D and S99F) that cause reproductive aberrancies in sheep have primary effects on the secretion of mature BMP-15 and GDF-9 rather than on the biological activity of the molecules. Another important finding is that the S77I GDF-9 mutation augments the effects of the BMP-15 mutations on the secretion of BMP-15 and GDF-9, suggesting a mechanism for the additive effects that these mutations have in sheep in vivo. The mechanism by which the mutations inhibit the processing and secretion of BMP-15 and GDF-9 has not been resolved; however, it is possible that the mutant proteins form unstable heterodimers that are subjected to targeted degradation prior to completion of processing. In this regard there have been exciting recent developments in the study of protein folding that may provide some insight into the molecular basis of the observations that we have reported here. Specifically, there is a growing recognition that the misfolding of proteins during protein synthesis, often caused by gene mutations, can be a direct cause of intracellular targeted degradation. This phenomenon has been associated with many pathological conditions that were thought previously to be unrelated (26, 27). Thus, it is possible that the mutations that we have studied impair the proper folding of BMP-15 and GDF-9, leading to targeted degradation of the misfolded proteins. According to this model, because the aberrancies caused by the mutations only occur when BMP-15 and GDF-9 are co-expressed, it appears that the mutations would impair the folding of only the mutant heterodimers and not the homodimers of proteins carrying the mutations.

Consistent with the expression pattern of BMP-15 and GDF-9 that is primarily restricted to the oocytes of growing follicles in mammals, none of the mutations in these genes in sheep (7, 9), the deletion of these genes in mice (10, 11, 28), or the immunization of sheep against these factors (22) has resulted in any non-ovarian defects. Thus, these factors are extremely attractive targets for manipulating fertility with a low risk of non-ovarian side effects for the purpose of developing new clinical treatments for infertility, for developing new non-steroidal contraceptives, and for modulating fertility in agricultural settings. Our data indicate that regimens aimed at modulating the secretion of BMP-15 and GDF-9 from the oocyte in vivo may be effective for these purposes. Also, our data raise the possibility that aberrancies in the secretion rather than the expression or biological activity of BMP-15 and/or GDF-9 may...
play a causal role in the abnormal follicle growth in women with polycystic ovaries, the underlying cause of certain forms of unexplained infertility and possibly in the incidence of dizygotic twinning.

Acknowledgments—We thank Mei Wang for excellent technical assistance and Andi Hartgrove for editorial assistance.

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Functional and Molecular Characterization of Naturally Occurring Mutations in the Oocyte-secreted Factors Bone Morphogenetic Protein-15 and Growth and Differentiation Factor-9

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J. Biol. Chem. 2004, 279:17391-17396.
doi: 10.1074/jbc.M401050200 originally published online February 17, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401050200

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