Structural Characterization of the Fibroblast Growth Factor-binding Protein Purified from Bovine Prepartum Mammary Gland Secretion*

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A novel heparin-binding protein was purified to homogeneity from bovine prepartum mammary gland secretion using heparin-Sepharose chromatography and reverse-phase high performance liquid chromatography successively. Structural information obtained by N-terminal amino acid sequencing of a series of proteolytically generated peptides permitted the cloning of the corresponding cDNA. The isolated cDNA was 1170 base pairs long and consisted of an 83-base pair 5'-untranslated region followed by a 702-base pair coding region and a 385-base pair 3'-untranslated region. The open reading frame resulted in a protein comprising 234 amino acid residues, including a signal sequence. Instead of Lys24 as the predicted N terminus, Edman degradation of the native protein revealed N-terminal processing at two sites as follows: a primary site between Arg31–Gly32 and a secondary site between Arg51–Ser52. The amino acid sequence showed a significant similarity with that of human (60%) and mouse (53%) fibroblast growth factor-binding protein (FGF-BP). Accordingly, ligand blotting experiments revealed that bovine FGF-BP bound FGF-2. The theoretical mass of the protein predicted from the cDNA sequence is 22.5 kDa. However, the molecular mass of the purified protein was estimated to 28.6 kDa by mass spectrometry and 36 kDa by electrophoresis. The apparent molecular weight differences are most likely due to post-transcriptional modifications, shown to involve N- and O-glycosylation of Asn155 and Ser172, respectively. All 10 cysteine residues in the protein participated in disulfide bonds, and the pattern was identified as Cys71–Cys88, Cys97–Cys130, Cys106–Cys142, Cys198–Cys234, and Cys214–Cys222. As the 10 cysteines of the three known FGF-BPs are positionally conserved, the disulfide bond pattern of bovine FGF-BP may be regarded as representative for the FGF-BP family.

The fibroblast growth factor (FGF)1 family, with its prototype members FGF-1 and FGF-2, comprises structurally related heparin-binding proteins involved in a variety of biological processes including morphogenesis, angiogenesis, and tissue remodeling (1–6). Signaling by members of the FGF family is dependent upon a dual-receptor system, consisting of four high affinity tyrosine kinase receptors, termed fibroblast growth factor receptors (FGFRs), and of low affinity heparan sulfate proteoglycan (HSPG) that enhances ligand presentation to the FGFRs (7–9). Despite the ubiquitous presence of FGFs throughout the body, their interaction with the signal-transducing receptors is tightly controlled. Sequestration of FGF by the heparan sulfate-rich extracellular matrix (ECM) seems to fulfill this role by inhibiting a local differential distribution of FGFs and FGFRs. Furthermore, the binding and release from ECM seem important as means of protection and optimizing the biological effect of FGF-2 (10, 11). Several mechanisms have been proposed for the release of active FGFs from the ECM reservoir. The classical route is release of the growth factors upon digestion of HSPG by glycosaminoglycan-degrading enzymes or protease activity (9, 13–15). However, recent transfection studies have revealed the existence of an alternate mode that does not require matrix degradation but instead involves an FGF-binding protein (FGF-BP). The heparin-binding protein FGF-BP is distinct from cellular receptor molecules and binds to FGF-1 and FGF-2 in a non-covalent, reversible manner. Besides decreasing the affinity or accessibility of FGF for ECM-HSPGs, FGF-BP has been shown to protect FGF-2 from degradation and preserves its mitogenic activity (16–20).

In rodents in situ hybridization and Northern analysis have shown that FGF-BP expression is prominent in skin and intestine during the perinatal growth (19). The down-regulation of FGF-BP in the adults is, however, reversed in tumor samples, cell lines derived from squamous cell carcinomas (SCC), and some colon cancers (16, 20, 21). The involvement of FGF-BP in skin cancer is supported by the findings that the chemotherapeutic agent all-trans-retinoic acid reduces FGF-BP expression in SCC xenografts, inhibits their angiogenesis, and leads to a decrease of the tumor growth rate (22, 23). Moreover, FGF-BP

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF271896.

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1 The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor; FGF-BP, fibroblast growth factor binding protein; BPMS, bovine prepartum mammary gland secretion; ECM, extracellular matrix; MALDI-TOF-MS, matrix assisted laser desorption ionization-time of flight-mass spectrometry; FAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; SCC, squamous cell carcinomas; HSPG, heparan sulfate proteoglycan; PVDF, polyvinylidene difluoride; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
mRNA expression is up-regulated by direct transcriptional mechanisms in phorbol ester-promoted skin cancer (19, 24). The significance of FGF-BP expression in tumors has also been assessed in vitro and in situ by transfection studies. Overexpression or conversely reduced expression by ribozyme targeting suggests that FGF-BP mobilizes and activates extracellular stored FGF-2 and promotes angiogenesis and growth of xenografted tumors in mice (16, 17, 19).

Previously, FGF-BP cDNAs from human and mouse have been cloned, and their predicted open reading frames encode proteins of 234- and 251-amino acid residues, respectively. A comparison of their deduced amino acid sequences shows 63% similarity, including a conserved location of all cysteine residues (19, 20). The purified form of FGF-BP is a 17-kDa heparin-binding protein (HBP17), derived from culture medium conditioned by the human epidermal carcinoma cell line A431. HBP17 binding of FGF-1 and FGF-2 can be reversed by heparin, which in turn binds a cluster of basic amino acids in the C-terminal half of the molecule (20, 25). Because of its scarcity, very little information is available regarding structural features of FGF-BP so far.

In bovine mammary gland the growth-promoting activity peaks in the early stage of the last trimester of gestation. Accordingly, mammary gland secretion drawn from this period is a rich source of different growth-promoting substances (26, 27). In the present study, we report the purification and structural characterization of bovine FGF-BP recovered from bovine prepartum mammary gland secretion (BPMS). Peptide mapping, cDNA cloning, and N-terminal amino acid sequencing allowed disclosure of the primary structure of a member of the FGF-BP family for the first time.

**Experimental Procedures**

**Miscellaneous—**FGF-1 and FGF-2 (purified from bovine brain) were from R & D Systems (Minneapolis, MN). Trypsin (1-tosylamido-2-phenylethyl chloromethyl ketone-treated), Staphylococcus aureus V8 protease, and chymotrypsin were all from Worthington. Polyvinylidene difluoride (PVDF) filters were from Millipore (Bedford, MA). Proteins were isolated with NaOH using chloramine T as the oxidizing agent. The reaction was stopped with meta- bisulfite, and the ligands were desalted on a PD10 column (Amersham Pharmacia Biotech) equilibrated with 50 mM NH4HCO3 and packed on a column (diameter 50 mm). The column was developed with a linear gradient of 0 to 60% of acetonitrile in 0.1% trifluoroacetic acid at 25 °C (flow 0.15 ml/min). Selected tryptic peptides were subdigested with chymotrypsin (3 h) at 37 °C, and the generated peptides were purified using the described conditions. Cystine-containing fractions were selected by amino acid analysis of aliquots treated with performic acid prior to hydrolysis. N-terminal amino acid sequences were obtained by sequential Edman degradation as described above. Carbohydrate composition analysis was carried out using a Dionex pulsed-electrochemical detector and a polymeric anion-exchange CarboPac PA1 column (Dionex Corp., Sunnyvale, CA) as described (28). Molecular mass was determined by matrix-assisted laser desorption ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) on a Bruker Biflex mass spectrometer (Bremen, Germany).

**Screening of Mammary Gland cDNA Library—**The amino acid sequences QPTNYP, KGKFTV, FTTGNW, and YYXGTGW obtained from the peptide mapping were used to design the following degenerated oligonucleotide probes: A, 5'-CA/G/C/A/G/C/T/A/A/C/G/T/AA/A/G/T/C/T/C; B, 5'-AA/G/G/G/C/T/A/G/T/G/T/A/G/T/G/C/G/T/A/A/G/T/C/T/C/D; C, 5'-TT/C/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T/T
with a linear gradient of 0.05–1.5 M ammonium bicarbonate, pH 8.1. The column was eluted described under “Experimental Procedures.” The column was eluted 3 8.1, BPMS was applied to a heparin-Sepharose column (5 dialysis against buffer A containing 0.05 M ammonium bicarbonate, pH 8.1, agarose chromatography of BPMS. Following removal of debris and dried, and processed by reverse-phase HPLC (Fig. 1 further, the 36-kDa enriched fractions were pooled, freeze-dried for further fractionation. B, reverse-phase HPLC of the selected fractions. The freeze-dried proteins were re-disolved in 0.1% trifluoroacetic acid, and aliquots were rechromatographed on a Resource RPC column (1 ml). After the wash with 0.1% trifluoroacetic acid, the column was eluted with a linear gradient of 0–55% 2-propanol in 0.1% trifluoroacetic acid, the column was eluted with a linear gradient of 0–55% 2-propanol in 0.1% trifluoroacetic acid for the initial 65 min, followed by a linear 65–80% gradient for 7 min. The resulting peaks were collected at a flow rate of 1 ml/min and evaporated under vacuum. The fraction containing the purified FGF-BP homolog eluted at approximately 38% 2-propanol (boldface bar). By using a colorimetric assay, we estimated that about 30 μg of purified FGF-BP were extracted from 300 ml of BPMS. cDNA Cloning and Sequencing—To obtain the complete protein sequence, bovine FGF-BP was cleaved using trypsin, and/or chymotrypsin, and the amino acid sequences of reverse-phase HPLC-purified peptides were determined. A bovine mammary gland cDNA library was screened using degenerated oligonucleotides based on the amino acid sequence derived from the bovine FGF-BP digests. The resulting 1170-bp cDNA and its derived amino acid sequence are shown in Fig. 2. The deduced amino acid sequence was verified by the finding that all proteolytically generated peptides analyzed by N-terminal amino acid sequencing were encompassed in the open reading frame. A putative initiation codon was found at position 85, representing the first in-frame ATG codon in the 5-prime region. Two consecutive stop codons commenced from position 787. Assuming the methionine codon to be the initiator, the open reading frame encodes 234 amino acids with a calculated molecular mass of 22,648 Da. Only one potential N-glycosylation site with the canonical (Asn-X-Ser/Thr), X-Pro) sequence was found (residues 155–157). A prediction of the secretory signal sequence using the neural network-based SignalP program (30) suggests that the bovine FGF-BP has a cleavage site between Ala23 and Lys 24. However, instead of the predicted signal sequence, FLSMVQGSSC, represents the C-terminal amino acid sequence. The 36-kDa protein and its 34-kDa N-terminal truncated derivative coeluted in the peak fraction at approximately 38% solvent B (Fig. 1B). By using a colorimetric assay, we estimated that about 30 μg of purified FGF-BP were extracted from 300 ml of BPMS. The cDNA sequence and the deduced amino acid sequence of bovine FGF-BP. The shaded residue indicates the predicted N-terminal amino acid following cleavage of the signal sequence. Boldface double underlined residues indicate the encountered N-terminal amino acids of isolated FGF-BP. The single potential N-linked glycosylation site is shown in boldface italic. Asterisks mark the consecutive termination codons at the end of the reading frame. Amino acid sequence obtained by peptide mapping and Edman degradation is underlined.

Fig. 1. Purification of bovine FGF-BP from BPMS. A, heparinagarose chromatography of BPMS. Following removal of debris and dialysis against buffer A containing 0.05 M ammonium bicarbonate, pH 8.1, BPMS was applied to a heparin-Sepharose column (5 × 25 cm) as described under “Experimental Procedures.” The column was eluted with a linear gradient of 0.05–1.5 M ammonium bicarbonate, pH 8.1. Fractions of 10 ml were collected at a flow rate of 50 ml/h. Aliquots of every peak and shoulder were pooled, and their proteins were identified. Based on their content of the bovine FGF-BP homolog, the heparin-binding proteins in the 1.0–1.2 M ammonium bicarbonate interval (boldface bar) were selected and freeze-dried for further fractionation. B, reverse-phase HPLC of the selected fractions. The freeze-dried proteins were re-disolved in 0.1% trifluoroacetic acid, and aliquots were rechromatographed on a Resource RPC column (1 ml). After the wash with 0.1% trifluoroacetic acid, the column was eluted with a linear gradient of 0–55% 2-propanol in 0.1% trifluoroacetic acid for the initial 65 min, followed by a linear 65–80% gradient for 7 min. The resulting peaks were collected at a flow rate of 1 ml/min and evaporated under vacuum. The fraction containing the purified FGF-BP homolog eluted at approximately 38% 2-propanol (boldface bar). C, SDS-PAGE of fractionated BPMS. Lane 1, crude BPMS extract. Lane 2, total protein eluted from the heparin affinity chromatography. Lane 3, purified bovine FGF-BP. Proteins were resolved in 10–20% polyacrylamide gels under nonreducing conditions and stained with Coomassie Blue. To show each lane 4 μg of protein was applied. Molecular mass markers are shown on the left.

N-terminal amino acid sequence analysis. Homology searches based on the retrieved amino acid sequences revealed that the predominant 83-kDa protein recovered was lactoferrin. Based on spectrophotometric scanning, we estimated that lactoferrin and lactoferrin-derived fragments comprised more than 90% of all the heparin-binding protein isolated from the secretion (Fig. 1C). Among the minor protein components, eluting in the 1.0–1.2 M ammonium bicarbonate interval, was a previously unidentified protein migrating as a 36-kDa band in SDS-PAGE. The 36-kDa band resembled the amino acid sequence of FGF-BP as previously deduced from human and mouse cDNA (19, 20). Thus, to purify the putative bovine FGF-BP homolog further, the 36-kDa enriched fractions were pooled, freeze-dried, and processed by reverse-phase HPLC (Fig. 1B). As revealed by SDS-PAGE and confirmed by N-terminal amino acid sequencing, the 36-kDa protein and its 34-kDa N-terminal truncated derivative coeluted in the peak fraction at approximately 38% solvent B (Fig. 1B). By using a colorimetric assay, we estimated that about 30 μg of purified FGF-BP were extracted from 300 ml of BPMS. The cDNA sequence and the deduced amino acid sequence of bovine FGF-BP. The shaded residue indicates the predicted N-terminal amino acid following cleavage of the signal sequence. Boldface double underlined residues indicate the encountered N-terminal amino acids of isolated FGF-BP. The single potential N-linked glycosylation site is shown in boldface italic. Asterisks mark the consecutive termination codons at the end of the reading frame. Amino acid sequence obtained by peptide mapping and Edman degradation is underlined.
submitted to a database search, only human and mouse FGF-BP showed close resemblance (Fig. 3). Similar to the bovine sequence the open reading frame of human FGF-BP translates to 234 residues (19), and the mouse protein was extended with an additional 17 residues (19). The amino acid sequence of the bovine protein was 60 and 53% identical to that of FGF-BP from human and mouse, respectively. Notably, the positions of all the 10 cysteines present in each of the three proteins are conserved.

Analysis of Disulfide Bridges—To localize disulfide bridges in bovine FGF-BP, the purified protein was cleaved with trypsin, and the resulting fragments were separated by reverse-phase HPLC. Aliquots of the eluted peptide fractions were subjected to amino acid analyses, and retrieved fractions containing cysteine were analyzed by N-terminal sequencing and MALDI-TOF-MS (Table I). Sequence analysis revealed the tryptic peptides T6 and T9 to coelute. Taking into account that both peptides contain only one cysteine, the finding suggests that T6 and T9 are covalently connected, via a disulfide bond between Cys71 and Cys88. Mass spectrometry confirmed this conclusion, as the obtained mass peaks at $m/z$ 1160.2, 734.8, and 1893.6 agree with the calculated weight of the T6 and T9, and a disulfide-linked complex between the two peptides. Trypsin digestion also produced a cysteine-containing fraction that comprised T10, T15, and T17+T18. Subdigestion with chymotrypsin followed by reverse-phase HPLC resulted in a chromatogram where T15 (containing Cys130) coeluted with and a new peptide T10.I containing Cys97. Mass spectroscopy of the fraction revealed a mass corresponding to the size of T15 linked to T10.I, demonstrating that Cys97 is bound to Cys130. The chymotrypsin cleavage also resulted in the formation of a T10.II peptide, which contains Cys106. N-terminal sequencing revealed that T10.II coeluted with the tryptic peptide T17+T18, which includes Cys142. In support of a disulfide bond between Cys106 and Cys142, the detection of the molecular mass of 1571.7 Da corresponded well to the size of a disulfide-linked complex between T10.II and T17+T18. Both amino acid sequencing and mass spectrometry showed the presence of T29.I.

**Table I**

Peptide mapping analysis of bovine FGF-BP using N-terminal sequencing and MALDI-TOF-MS

| Peptide no. | Residue no. | Peptides | Obs. | Calc. |
|-------------|-------------|----------|------|-------|
| Tryptic cleavage sites in bovine FGF-BP | | | | |
| T1 | 32–34 | GSK | | |
| T2 + T3 | 35–51 | ASADESLALGKPKEPR | 1726.9 | 1726.9 |
| T4 | 52–60 | SQPTNYPIK | 1047.2 | 1047.5 |
| T5 | 61–62 | GK | | |
| T6 | 63–72 | FVTDPHDACR | 1160.3 | 1160.3 |
| T7 | 73–77 | WAVTK | 604.6 | 604.7 |
| T8 | 78–85 | QEEGIVLJK | 915.9 | 915.5 |
| T9 | 86–91 | VECQTQR | 734.8 | 734.8 |
| T10 | 92–111 | DNTFSCFTGNTSCLELHK | 2260.0 | 2260.0 |
| T11 | 112–117 | NAYWK | 795.3 | 795.8 |
| T12 | 118–121 | QGR | | |
| T13 | 122–124 | NRL | | |
| T14 | 125–127 | SQR | | |
| T15 | 128–134 | VIGCDAK | 705.5 | 704.8 |
| T16 | 135–138 | SVLK | | |
| T17 + T18 | 139–143 | TRVCR | 634.4 | 634.7 |
| T19 + T20 + T21 | 144–152 | KKFPESNL | 1091.4 | 1091.3 |
| T22 | 153–160 | LVSNTLIR | 2790.2 | 916.1 |
| T23 | 161–162 | IK | | |
| T24 | 163–187 | KPSQELMEPSMDITVEVTSSPEK | | |
| T25 | 188–194 | TQTMATK | | |
| T26 | 195–204 | DPQCEEEDL | | |
| T27 | 205–207 | NQR | | |
| T28 + T29.1 | 208–224 | KAALEYCGETWGLCNF | 1890.7 | 1891.1 |
| T29.I | 209–224 | AAEYCGETGSLCNF | 1763.3 | 1764.0 |
| T29.II | 225–234 | FLSMVQGSSC | | |

**Isolated disulfide-linked tryptic peptides**

| Peptide no. | Residue no. | Peptides | Obs. | Calc. |
|-------------|-------------|----------|------|-------|
| T6-T9 | 63–72/86–91 | FVTDPHDACR/VECTQR | 1893.6 | 1893.6 |
| T10-T15-T17 + T18 | 92–112/128–134/139–143 | DNTFSCFTGNTSCLELHK/VIGCDAK/TRVCR | 3339.7 | 3339.8 |

**Isolated disulfide-linked chymotryptic subdigests**

| Peptide no. | Residue no. | Peptides | Obs. | Calc. |
|-------------|-------------|----------|------|-------|
| T10.I–T15 | 95–97/128–134 | FSC/VIGCDAK | 1059.0 | 1058.9 |
| T10.II–T17 + T18 | 99–107/129–143 | FNGTSCLELHK/VIGCDAK/TRVCR | 1571.8 | 1573.8 |
| T29.I | 214–224 | CGETWGLCNF | 1215.8 | 1215.3 |

*Non-tryptic peptide derived from endogenous cleavage of the purified binding protein.

*Peptides isolated from digests of unreduced bovine FGF-BP.*
which originates from a tryptic cleavage following Lys208 and an intrinsic C-terminal processing between Phe224 and Phe225. The existence of an internal disulfide bond between the included Cys214 and Cys222 was demonstrated by identification of a di-phenylthiohydantoin-cystine in the 14th cycle of the Edman degradation of T29.III. This also agrees with the finding that reduction of T29.II resulted in a mass increase from m/z 1890.7 to 1893.5, corresponding to the reduction of one cystine for two cysteines. The disulfide bond between Cys198 and Cys234 explains why the observed background sequence of T29.II accompanies the N-terminal amino acid sequence of unreduced bovine FGF-BP (see Fig. 2).

Glycosylation—Tryptic peptide fragments purified by reverse-phase HPLC were analyzed for the presence of the amino sugars GalNAc and GlcNAc. GalNAc was detected in the tryptic fraction T22, which contained the only consensus sequence for N-glycosylation in the binding protein. A carbohydrate composition analysis of T22 performed by high pH anion-exchange chromatography showed the presence of GlcNAc, GalNAc, galactose, mannose, and sialic acid in a molar ratio of 2:1:1:5:1 (results not shown). In favor of N-glycosylation, amino acid sequencing of T22 did not detect an asparagine in the third step, and finally, instead of the calculated 915.1-Da molecular mass of T22, mass spectrometry revealed a peak at m/z 2790.2 (Table I). Collectively our results warrant the attachment of a carbohydrate to Asn150.

In contrast to N-glycosylation, no consensus amino acid sequence for O-glycosylation exists. However, evidence for an O-linked glycosylation on Ser172 was found. Following digestion with S. aureus V8 protease, GalNAc was observed in a fraction containing the octapeptide LMEPSPMD175. Thus, no Ser172 was detected upon Edman degradation, and the mass spectrum of the octapeptide showed a peak at m/z 2121.9. Since the calculated mass of the unmodified peptide was 920.1 Da, this suggests that the carbohydrate moieties amount to 1201.8 Da.

Binding of Bovine FGF-BP to FGF—The previously reported presence of an FGF binding domain within human FGF-BP prompted us to investigate the binding specificity of bovine FGF-BP. We tested the ability of FGF-BP to bind FGF-1 and FGF-2, using ligand blotting analysis. Following electrophoresis in 16% Tris-Tricine gels. After electrophoresis, the proteins from a crude preparation of BPMS. The samples were resolved by SDS-PAGE in 16% Tris-Tricine gels. After electrophoresis, the proteins were electrotransferred from the gel onto PVDF membrane. The filters were incubated with 30 μl of bovine 125I-FGF-BP with (A) or 30 μl of bovine 125I-FGF-BP supplemented with 1.5 μM unlabeled bovine FGF-BP (B). Molecular mass markers are shown on the left.

![Fig. 4. Ligand blotting analysis of the binding of bovine FGF-BP to FGF. Lane 1, 1 μg of bovine FGF-1; lane 2, 1 μg of bovine FGF-2; lane 3, 1 μg of bovine serum albumin; and lane 4, 50 μg of proteins from a crude preparation of BPMS. The samples were resolved by SDS-PAGE in 16% Tris-Tricine gels. After electrophoresis, the proteins were electrotransferred from the gel onto PVDF membrane. The filters were incubated with 30 μl of bovine 125I-FGF-BP with (A) or 30 μl of bovine 125I-FGF-BP supplemented with 1.5 μM unlabeled bovine FGF-BP (B). Molecular mass markers are shown on the left.](image)

![Fig. 5. Schematic representation of the distribution of disulfide bonds and glycosylation sites in bovine FGF-BP.](image)

DISCUSSION

This paper describes the purification and characterization of a novel heparin-binding protein secreted from the bovine mammary gland. Partial amino acid sequences obtained from purified material enabled isolation of a full-length cDNA from a mammary gland library and deduction of the amino acid sequence of bovine FGF-BP. The derived amino acid sequence was confirmed by peptide mapping, covering 74% of the purified protein. Glycosylation sites and disulfide bridges were also assigned (Fig. 5).

Inspection of the cDNA sequence predicts that the unprocessed bovine FGF-BP is 234 residues long, which is equivalent to the human counterpart. Based on the known structural requirements it is expected that signal peptidease would cleave between residues 23 and 24 during the secretion process, resulting in Lys30 at the N terminus. Nevertheless, the predominant forms of the purified protein observed in the present study started at Gly22 and Ser22, respectively. The enzyme responsible for this processing remains to be elucidated. However, it is noteworthy that a dibasic sequence is found in the C terminus of the removed peptides (Arg30–Arg31 and Lys30–X–Arg31), suggesting that one or more substilin-like endopeptidases may be responsible for the processing (31). A similar phenomenon is seen in human FGF-BP, where it has been reported that the purified protein starts with Lys24 (20). However, no dibasic sequence prior to an eventual processing site is found in the human precursor. Whether secondary modification of the N-terminal is a common phenomenon linked to FGF-BPs must await further investigations as well as elucidation of the proteases involved.

Applied methods for estimating the molecular weight of the isolated FGF-BP gave different results. Based on the amino acid composition, the molecular mass of the predominant 203-residue form was calculated to 22,648 Da. Measurement by mass spectrometry estimated the molecular mass to 28,549 Da, whereas the protein migrates as a 36-kDa protein in SDS-polyacrylamide gels. The discrepancy in mass between the isolated protein and the translated cDNA sequence was at least partly due to post-translational modifications. A search for glycosylation sites revealed the presence of glycans on Asn155 and Ser172, resulting in an additional mass of about 3074 Da. The collected data do not explain the remaining difference between the calculated and observed molecular weights. However, the apparently anomalous behavior of the bovine FGF-BP in SDS-polyacrylamide gels is most likely a result of the basic nature of the protein (estimated pI is 9.3) and the attached carbohydrate. The literature provides no data on glycosylation of the other FGF-BPs; however, the bovine N-linked glycosylation site has no counterpart in the predicted amino acid sequences of human and mouse FGF-BP. Although the O-glycosylated residue in the bovine protein is conserved in human

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**FIG. 5.** Schematic representation of the distribution of disulfide bonds and glycosylation sites in bovine FGF-BP.
and mouse FGF-BP, a prediction of the presence of O-linked glycosylation in the two proteins is currently not possible.

Contrary to the bovine homolog, human FGF-BP purified from A431 epidermoid carcinoma cells migrates notably faster in SDS-PAGE than the molecular mass prescribed by its translated cDNA sequence, i.e. 17 versus 22.7 kDa (20). It is unclear whether the apparent molecular mass of the human FGF-BP reflected an electrophoretic artifact or is due to the lack of protein sequence per se. Considering the similarity in overall amino acid composition, charge, and hydrophobic character between the predicted human and bovine molecule, the difference in SDS-PAGE migration pattern is unexpected. Whereas protein sequencing of the purified bovine protein identifies the predicted C terminus unambiguously, this is not so for the human counterpart, and the precise position of its C-terminal end is unknown. Hence, our observations support the notion by Wu and co-workers (20) that the purified form of human FGF-BP could result from proteolytic C-terminal processing.

Cleavage using a combination of trypsin and chymotrypsin allowed us to solve the organization of the disulfide bridges for bovine FGF-BP. All cysteine residues are involved in disulfide bonds, and the pattern is as follows: Cys130–Cys106–Cys142, Cys198–Cys234, and Cys214–Cys222. Care was taken to reduce the probability of disulfide bond shuffling, and indeed no alternate bonding patterns were identified. No information is available regarding the three-dimensional structure or the disulfide bridge pattern of human or mouse FGF-BP, but the finding that all cysteines are positionally conserved in the three proteins suggests that they are structurally alike.

The principal heparin-binding site of human FGF-BP purified from A431-conditioned medium has recently been localized to residues Arg110–Phe143 (25). This sequence does not include the principal heparin-binding site of human FGF-BP purified from A431 epidermoid carcinoma cells (20, 25). In the present work, we found that expression of FGF-BP is not restricted to perinatal and pathological conditions. A physiological role for FGF-BP in pregnant mammary gland is not known. However, it is notable that the importance of FGF-2signaling in pregnancy-dependent lobuloalveolar development of the mammary gland recently has been reported (12, 33, 34). The accessibility of purified bovine FGF-BP opens for future investigations concerning the involvement of FGF-BP in physiological and pathological processes.

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Primary Structure of FGF-BP
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