Bone morphogenetic protein (BMP)-1 is a glycosylated metalloproteinase that is fundamental to the synthesis of a normal extracellular matrix because it cleaves type I procollagen, as well as other precursor proteins. Sequence analysis suggests that BMP-1 has six potential N-linked glycosylation sites (i.e. NXS/T) namely: Asn\textsuperscript{91} (prodomain), Asn\textsuperscript{142} (metalloproteinase domain), Asn\textsuperscript{332} and Asn\textsuperscript{363} (CUB1 domain), Asn\textsuperscript{599} (CUB3 domain), and Asn\textsuperscript{726} in the C-terminal-specific domain. In this study we showed that all these sites are N-glycosylated with complex-type oligosaccharides containing siaic acid, except Asn\textsuperscript{726} presumably because proline occurs immediately C-terminal of threonine in the consensus sequence. Recombinant BMP-1 molecules lacking all glycosylation sites or the three CUB-specific sites were not secreted. BMP-1 lacking CUB glycosylation was translocated to the proteasome for degradation. BMP-1 molecules lacking individual glycosylation sites were efficiently secreted and exhibited full procollagenase activity, but N332Q and N599Q exhibited a slower rate of cleavage. BMP-1 molecules lacking any one of the CUB-specific glycosylation sites were sensitive to thermal denaturation. The study showed that the glycosylation sites in the CUB domains of BMP-1 are important for secretion and stability of the molecule.

Bone morphogenetic protein-1 (BMP-1),\textsuperscript{1} also known as procollagen-C-proteinase-1 (PCP-1) was first identified in osteogenic extracts of bone (1). BMP-1 is a smaller splice variant of mammalian tolloid, which is the vertebrate ortholog of tolloid, in Drosophila. BMP-1, mammalian tolloid, and two highly homologous relatives tolloid-like 1 and 2 constitute the tolloid gene family (2). BMP-1 cleaves fibrillar procollagen type I, II, III (3, 4), and V (5–7), as well as type VII procollagen (8), prolysyl oxidase (9), probradykinin (10), and the y2 chain of prolammin 5 (11). BMP-1 also cleaves chordin (2) therefore affecting dorsal-ventral pattern formation in vertebrates (12). Similarly, tolloid cleaves the chordin homologue short gastrulation during Drosophila embryo development (13). Bmp1 homozygous null mice are perinatal lethal, with defects in ventral body wall closure and collagen fibrillogenesis (14), which illustrates the importance of BMP-1 in tissue assembly and development.

BMP-1 consists of a prodomain that is cleaved by a furin-like enzyme in the trans-Golgi network,\textsuperscript{2} an astacin-like zinc metalloproteinase domain (15), one epidermal growth factor-like domain, and three CUB domains. In other proteins, CUB domains mediate protein-protein interactions (16).

BMP-1 purified from mouse fibroblasts culture medium has been shown to be N-glycosylated (17). Sequence analysis reveals six potential N-glycosylation sites, one of which is located in the prodomain and five in the mature (active) molecule. However, no information is available on the structure and function of the glycosylation sites. Recent studies indicate that post-translational modification of proteins can have multiple roles including regulation of intracellular trafficking (18), stabilization of folded domains (19, 20), protection from proteolytic degradation of the core protein (21), and modulation of enzyme/hormone activities (22–24). Furthermore, high variability exists in the functions of N-glycan chains of proteins, which makes it difficult to predict with any confidence the functions of oligosaccharides on proteins (reviewed by Ref. 25).

In this study, we expressed FLAG-tagged BMP-1 in two different mammalian systems (HT1080 and 293-EBNA cells) and identified the type of glycosylation on BMP-1. By site-directed mutagenesis, we also established the role of the N-glycosylation in folding, secretion, and C-proteinase activity of BMP-1.

**EXPERIMENTAL PROCEDURES**

**Source of Materials**—PCR products were purified with Qiabquick kits (Qiagen). Plasmids were extracted with Qiagen spin miniprep kit (Qiagen). Prestained protein molecular weight standards (broad range) were from Bio-Rad. Full-length BMP-1 cDNA (GenBank\textsuperscript{\textregistered} accession number P13497) was cloned from a human placental cDNA library. The cDNA was inserted at the KpnI/XhoI sites of the expression vector pCNA3 (Invitrogen), thereby placing it under the transcriptional control of a cytomegalovirus promoter. A FLAG tag amino acid sequence (DYKDDDDK) recognized by a mouse monoclonal anti-FLAG M2 antibody (Sigma) was introduced into the BMP-1 sequence (BMP-1F) immediately 5’ of the stop codon. The cDNA encoding FLAG-tagged BMP-1 was subcloned into the epissomal expression vector pCEP4 (Invitrogen) and pCNA3, for heterologous protein expression studies in cultured cells. Previous studies have shown that the FLAG peptide at the C terminus of BMP-1 does not affect the procollagen C-proteinase activity of BMP-1 (2).

**Site-directed Mutagenesis**—Plasmids coding for the mutant BMP-1 proteins were produced by replacing wild-type fragments with the same fragments containing the desired mutations. These were generated by

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\textsuperscript{2} To whom correspondence should be addressed. Tel.: 44-161-275-6925; Fax: 44-161-275-1505; E-mail: laure.garrigue-antar@man.ac.uk.

\textsuperscript{\textregistered} The abbreviations used are: BMP-1, bone morphogenetic protein-1; BMP-1F, bone morphogenetic protein-1 containing a FLAG tag at the C terminus; PCP, procollagen C proteinase; CUB, complement subcomponents C1r/C1s Uegf and bone morphogenetic protein-1; ER, endoplasmic reticulum; PSPUII, boar seminal plasma protein; aSFP, bovine acidic seminal fluid protein; DMEM, Dulbecco’s modified Eagle’s medium; endoF/N, endopeptidase F/N-glycosidase F; endoH, endoglycosidase H; PBS, phosphate-buffered saline.
strand overlap PCR as described (26) using Pwo polymerase (Roche Molecular Biochemicals), a forward primer upstream and a reverse primer downstream, of unique restriction sites, respectively, and oligonucleotides containing the desired modification in both orientations (in bold, see below). For each mutation, the restriction sites used and their position on the nucleotide sequence are indicated. Briefly, a DNA fragment was amplified using a forward primer and the antisense mutant primer and an overlapping fragment was amplified using the sense mutant primer and a downstream reverse primer. Both fragments were gel-purified (Qiagen), mixed, and re-amplified with the Pwo enzyme with the forward and reverse primers. The product was digested with the appropriate enzymes, gel-purified, and ligated into the place of the corresponding wild-type fragment in BMP-1F. Mutagentic primers (mutation in bold) and restriction sites used to insert the segments into the wild-type sequence were as follows: N142Q, 5’-CTTGGGGGGTGATTCACTGGTA-3’, XcmI (position 383/BlpI (position 913); N332Q, 5’-CACGAGAAGCCGTGGTCTTCT-3’, BglII (position 913/PmlI (position 1416); N363Q, 5’-GACATCATTGCTTCGTCCTTGTAGTC (mutation in bold) and restriction sites used to insert the corresponding wild-type fragment in BMP-1F. Mutagentic primers (mutation in bold) and restriction sites used to insert the segments into the wild-type sequence were as follows: N142Q, 5’-CTTGGGGGGTGATTCACTGGTA-3’, XcmI (position 383/BlpI (position 913); N332Q, 5’-CACGAGAAGCCGTGGTCTTCTTCTTCTGACTCT-3’, the mutagentic primers, which also contained a restriction site (position 1503, N91Q/PmlI (position 1416); N726Q, XhoI (multicloning site of pcDNA3), and the FLAG tag (N726Q), italicized), were used in a single PCR reaction with reverse and forward primers, respectively. The two mutated PCR products were digested by PstI (position 251) and Apal (position 586) (N91Q) or BamHI (position 1391) and XhoI (N726Q). Pwo DNA polymerase was used to minimize base misincorporation during the polymerase chain reactions. DNA sequencing (ABI) was used to verify the mutations, and to ensure that the cDNA clones were error-free.

**Cell Culture and Transfection—Human fibrosarcoma HT1080 cells (ATCC CCL-121) and human embryonic kidney 293-EBNA cells (ECACC 85120602) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen) (complete DMEM) and for the 293 cells, with 0.25 mg/ml Geneticin (G418, Invitrogen) in a 37 °C incubator with 5% CO2. The recombinant wild-type and mutant BMP-1F proteins were expressed in stably transfected HT1080 cells. Transfections were made with Lipofectin reagent (Roche Molecular Biochemicals) and 4 μg of plasmid/T-25 flask. Cells were grown to ~50% confluency by overnight incubation in complete DMEM. After 2 rinses with Opti-MEM (Invitrogen), cells were transfected in serum-free DMEM (Invitrogen) following the manufacturer’s instructions and returned to the incubator. Twenty-four hours after transfection, medium was replaced by DMEM with 10% serum for a further 24 h. The cells were then trypsinized (Invitrogen) and diluted 1:15 for selection in 0.25 mg/ml Geneticin (G418, Invitrogen). 293-EBNA cells were transfected with the FLAG-tagged BMP-1 cloned into pCEP4 expression plasmid, following the procedure described above. Selection was applied by adding 0.25 mg/ml hygromycin B (Sigma).

**Preparation of the Medium—Stably transfected HT1080 or 293-EBNA cells were seeded in 100-mm dishes and grown to confluency. Cells were rinsed three times with phosphate-buffered saline (Invitrogen) and incubated in serum-free DMEM for 24 h (HT1080) or 48 h (293-EBNA cells), unless otherwise stated. The tissue culture media were collected, cleared of cell debris by centrifugation at 19,000 × g for 10 min, and in the case of HT1080-transfected cells, concentrated to 100 μl using Centriprep-30 and Microcon-10 concentrators (Amicon, Inc.). The samples were used immediately or stored at ~80 °C.

**Assay of Procollag en C-propeptidase—Recombinant BMP-1 was assayed for procollagen C-proteinase activity using human I, U-14C-type I procollagen substrate (0.4 μg). 14C-Labeled type I procollagen was obtained and purified from the medium of human skin fibroblasts (27). Analysis of the cleavage products on SDS gels (7% separating, 3.5% stacking) was performed as described (3, 28). The cleavage products were visualized by exposing dried gels to a phosphorimaging plate (Fuji, type BAS III) for phosphorimaging (Fujix BAS 2000). Bands corresponding to the pro-α1(I) and pα2(I) chains of type I procollagen and type I pα-collagen, respectively, were quantified using AIDA 2.0 software. The percentage of cleavage was calculated by multiplying the intensity of the pα2(I), corrected for molecular mass, by the initial concentration of procollagen (3, 28).

**Electrophoresis and Western Blotting—Cells were rinsed once with PBS, and incubated on ice for 15 min with occasional shaking with 500 μl of RIPA buffer (150 mM NaCl, 1% deoxycholate, 0.1% SDS, 10 mM Tris pH 7.6) containing 10 mM EDTA, and protease inhibitor mixture (Roche Molecular Biochemicals). Cells in RIPA buffer were scraped on ice and sonicated. Lysates were subjected to a 5-min centrifugation at 14,000 × g at 4 °C. Supernatants were retained and stored at ~80 °C until further analysis. The supernatants or cell lysates were resolved by electrophoresis on a 10% (w/v) SDS-Protein gel (Biothiokatt Molec. Biochem.) and electrophorograms were subsequently transferred to Western immunoblotting using the mouse monoclonal M2 antibody (Sigma) directed against the FLAG tag. Secondary antibody (anti-mouse peroxidase-conjugated IgG (Sigma)) was detected by the enhanced chemiluminescence method (SuperSignal West Dura extended duration, Pierce). The levels of BMP-1F were quantified by laser densitometry of enhanced chemiluminescence fluorograms exposed to pre-flashed films.

**Endoglycosidase Digestions—Endoprotease F/N’glycosidase F (endoF/NF), endoglycosidase H (endoH), and neuraminidase were from New England Biolabs, and endo-α-N-acetylglactosaminidase (O-glycosidase) was from Calbiochem. Culture medium (80 μl) (unconcentrated in the case of transfected 293-EBNA cells) from transfected HT1080s were incubated with the different glycosidases, in the buffer, detergents, and conditions recommended by the manufacturers. EndoF/NF (500 units), endoH (500 units), neuraminidase (50 units), or O-glycosidase (1 milliunit) were used per reaction, which were incubated for 18 h at 37 °C. Digestion by O-glycosidase was preceded by digestion with neuraminidase.

**Glycan Trimming and N-Glycosylations Inhibitors—Stably transfected HT1080 or 293-EBNA cells expressing BMP-1F were allowed to confluency on 100-mm dishes. Cells were preincubated in complete DMEM in the presence of 100 μg/ml castanospermine (Calbiochem), or 5 μg/ml swainsonine (Calbiochem) for 18 h. For tunicamycin (Sigma), cells were preincubated in the presence of 2 μg/ml of the inhibitor, for 5 h. Cells were then rinsed three times with PBS, and reincubated in the presence of the inhibitors for 24 h in serum-free DMEM, and for only 18 h in the case of tunicamycin. Medium was collected and treated as described above.

**Immunofluorescence Microscopy—pcDNA3, BMP-1F, and N4Q-transfected HT1080 were plated on glass coverslips in 6-well plates. After 24 h, cells were preincubated with or without 10 μl lactacytin (Calbiochem) for 1 h. Cells were rinsed three times with PBS and further incubated with or without lactacytin at the same concentration in serum-free medium for 4 h. Cells were washed three times with PBS and fixed and permeabilized with cold methanol (~20 °C) for 5 min. Fixed cells were washed three times with PBS and incubated for 20 min at room temperature with anti-FLAG mouse antibody or with anti-calreticulin rabbit antibody (Stressgen) in PBS supplemented with 1 mg/ml bovine serum albumin (Sigma). After washing, cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (Sigma) or with rhodamine-conjugated anti-rabbit IgG (Santa Cruz Bio-technology). Cells were washed with PBS and coverslips were mounted in Mowiol 4-88 (Calbiochem) and observed with a Bio-Rad MRC1000 laser confocal microscope.

**RESULTS**

**Characterization of the Post-translational Modification of BMP-1**—We transfected cultured HT1080 human fibrosarcoma and 293-EBNA human embryonic kidney cells with cDNAs encoding C-terminalflagged BMP-1 (BMP-1F). We chose to express BMP-1 in two different cell types to minimize the risk of cell type-specific post-translational modification. BMP-1F was stably expressed in HT1080 and 293-EBNA cells and the secreted protein was incubated with endoF/N, which cleaves all N-linked structures, regardless of their complexity, by hydrolyzing the asparagine-oligosaccharide bond, or endoH, which cleaves specifically high mannose-type structures. The proteins were also digested with neuraminidase, which removes sialic acids, and O-glycosidase, which specifically cleaves O-linked glycans. Proteins were separated by SDS-PAGE and immunoblotted with anti-FLAG antibody. Fig. 1 shows that BMP-1F is correctly processed and secreted in both HT1080 and 293-EBNA cells (lanes 2 and 9). No immunoreactive bands were found in medium from HT1080 cells transfected with pcDNA3 empty vector (lane 1). Complete deglycosylation of BMP-1F by endoF/N produced a decrease in the molecular mass of the BMP-1F molecule from ~75 to ~60 kDa (lane 3), strongly suggesting that most, or all, of the 5 potential N-glycosylation sites present in the active form had been stripped of oligosaccharides.
Wild-type BMP-1F was stably expressed in HT1080 and 293-EBNA cells. After reaching confluency, cells were conditioned in serum-free medium for 24 (HT1080 cells) or 48 h (293 cells). Cell medium from HT1080 cells was concentrated on YM membranes. Eighty μl of medium were treated with endoglycosidase F/N-glycosidase F (F/N, lane 3), with endoglycosidase H (H, lane 4), with neuraminidase (N, lanes 6 and 10), or with neuraminidase followed by O-glycosidase (N+O, lane 8). Proteins were separated on a 10% SDS-PAGE gel, in the presence of dithiothreitol. BMP-1F was detected by Western blot analysis using anti-FLAG M2 antibody. Control samples (C) were incubated without glycosidase (lanes 2, 5, and 9). Lane 1 (pCNA3) was medium from cells transfected with the empty vector.

Complex-type Glycosylation Is Not Required for Secretion and PCP Activity of BMP-1F—To determine the contribution of the complex-type N-linked oligosaccharides to secretion and enzymic activity of BMP-1F, two inhibitors of glycoprotein processing were used: castanospermine, which inhibits endoplasmic reticulum (ER) glucosidases I and II, thereby preventing the removal of the glucose residues of the Glc₃Man₉GlcNAc₂-N-linked glycoprotein (29), and swainsonine, which inhibits Golgi mannosidase II, therefore producing hybrid-type glycosylation (29). To do these experiments, stably transfected HT1080 cells were incubated in the presence of castanospermine or swainsonine, and the secreted BMP-1F was digested with endoF/N and endoH. The digested proteins accumulated within the cells. BMP-1F secreted in the presence of either inhibitor was not detected in the medium of tunicamycin-treated cells (lane 2), denoting the presence of both complex-type and high mannose-type structures. The digestion products had the same electrophoretic mobility (lanes 6 and 7). In the case of BMP-1F secreted in the presence of swainsonine, the digestion product by endoH (lane 10) migrated slower than the one obtained with endoF/N (lane 9), denoting the presence of both hybrid-type and high mannose-type structures.

To investigate the effects of high mannose- and hybrid-type oligosaccharides on PCP activity of BMP-1, we assayed the enzymes using type I procollagen as substrate. No significant difference of PCP activity was observed between BMP-1F control, BMP-1F containing high-mannose structures (synthesized in the presence of castanospermine), and BMP-1F with hybrid-type glycosylation (synthesized in the presence of swainsonine). Similar results were obtained with the 293-EBNA cells stably transfected with BMP-1F (data not shown). The results showed that complex-type oligosaccharide chains are not required for secretion and PCP activity of BMP-1.

N-Linked Oligosaccharides Are Necessary for Proper Folding and Secretion of BMP-1—To determine the role of N-linked sugars on folding and secretion of BMP-1, stably transfected 293-EBNA cells were incubated in the presence or absence of tunicamycin, which blocks transfer of N-acetylglucosamine onto the lipid carrier dolichol phosphate (30). Each medium and cell lysate were collected, the proteins were separated by SDS-PAGE and immunoblotted with anti-FLAG antibody (Fig. 3). Whereas BMP-1F was secreted from untreated cells (lane 1), BMP-1F could not be detected in the medium of tunicamycin-treated cells (lane 2). Latent BMP-1F was present in the cell lysates of treated and untreated cells. However, latent BMP-1F in the tunicamycin-treated cells was ~70 kDa, and therefore smaller than in control samples. The smaller molecular mass confirmed the presence of N-linked oligosaccharides on BMP-1. Furthermore, the ease of detection of the latent BMP-1F molecule in treated cells indicated that nonglycosylated BMP-1 accumulated within the cells.

The Absence of Asn332 and Asn599 Glycosylation Reduced Secretion of BMP-1—Although the tunicamycin experiments confirmed the importance of N-glycosylation for BMP-1 secretion, the contribution of individual sites within the molecule could not be assessed. BMP-1 contains 6 potential N-glycosylation sites (see Fig. 4): Asn⁹¹ (prodomain), Asn⁴⁴² (catalytic domain), Asn332 and Asn363 (CUB1 domain), Asn⁵⁹⁹ (CUB3 domain), and Asn⁷²⁶ (BMP-1 specific domain). To test the func-
Fig. 3. Effect of tunicamycin on BMP-1F secretion in 293-EBNA cells. Stably transfected 293-EBNA cells with BMP-1F were incubated in the absence (lanes 1 and 3 (C)) or presence of tunicamycin (2 μg/ml) (lanes 2 and 4 (T)) in serum-free medium for 18 h. Proteins from the cell medium (lanes 1 and 2) or from the cell lysates (lanes 3 and 4) were separated in reducing conditions on a 10% SDS-PAGE gel, followed by Western blot using the anti-FLAG M2 antibody.

Fig. 4. Schematic representation of BMP-1 domain structure with the positions of the six potential N-glycosylation sites. Domains are as follows: SS, signal sequence; Pro, prodomain; Protease, metalloproteinase domain; CUB 1, 2, and 3, C1r/C1s complement component; Uegf, BMP-1; E, epidermal growth factor-like domain; Spe, specific region. The glycosylation sites are numbered according to the amino acid positions in the latent protein. Below are the glycosylation mutants, with + indicating that the site is not mutated, and X that the site has been changed to glutamine.

Fig. 5. Expression of wild-type and mutant BMP-1F in HT1080 cells. Vectors containing cDNA-encoding wild-type and mutant BMP-1F were stably expressed in HT1080 cells. When confluent, cells were rinsed and conditioned in serum-free medium for 24 h. Culture supernatants (A and B) and cell lysates (C) were collected as described under “Experimental Procedures.” Protein samples were separated by SDS-PAGE (10%) in reducing conditions and detected by Western blot analysis using the anti-FLAG M2 antibody. A and B, the proteins were secreted as mature BMP-1. The N4Q and N6Q (lanes 10 and 11) were not detected. C, the protein present in the cell lysate corresponds to the latent form of BMP-1, pcDNA3, medium (lanes 1, panels A and B) and cell lysate (lane 1, panel C) from cells transfected with the empty vector.

N-Glycosylation of BMP-1

The single mutants N142Q, N332Q, N363Q, and N599Q were secreted, 72 °C secreted, the N332Q, N332Q/N363Q, and N599Q mutants were less well secreted, 72 °C, 60 °C, 15 °C (n = 3) of BMP-1F, respectively. The results showed that the N-glycosylation site at Asn91 and Asn589 are required for efficient secretion of the molecule.

All the Single Mutants Cleave Type I Procollagen But CUB Domain Glycosylation Is More Important Than Catalytic Domain Glycosylation for PCP Activity—All the single mutants exhibited PCP activity, in that they cleaved 14C-type I procollagen to completion in 18 h (Fig. 6). In contrast, the double mutant N332Q/N363Q was a weak C-proteinase. Minor differences between the mutants were detected when type I procollagen was incubated with the mutants for 4 h (Fig. 7). No difference was observed between BMP-1F and prodomain N91Q mutant. The single CUB mutants cleaved procollagen slower than BMP-1F. Furthermore, as shown above, the CUB1 double mutant (N332Q/N363Q) was much slower at cleaving procollagen than control samples. These results showed that the absence of single glycosylation sites has a minor effect on PCP activity of BMP-1. However, absence of two or more glycosylation sites decreases the PCP activity of the molecule.

Sensitivity of the CUB Glycosylation Mutants to Heat Inactivation—To evaluate the ability of the N-linked glycosylation sites to stabilize the structure of BMP-1, we incubated the secreted forms of the mutated proteins at 55 °C for up to 40 min, and assayed the molecules for PCP activity at 37 °C. The results are shown in Fig. 8. N91Q, which had an unaltered prodomain or change the electrophoretic mobility of the mature enzyme. By analyzing the corresponding cell lysates (where BMP-1F exists in the latent form, panel C, lanes 2 and 3), the N91Q mutant was shown to migrate faster than BMP-1F, indicating that the Asn91 site is glycosylated in latent BMP-1.

Whereas the N91Q, N142Q, and N363Q mutants were well secreted, the N332Q, N332Q/N363Q, and N599Q mutants were less well secreted, 72 °C, 60 °C, 15 °C (n = 3) of BMP-1F, respectively. The results showed that the N-glycosylation site at Asn91 and Asn589 are required for efficient secretion of the molecule.
HT1080 cells were transfected with vectors encoding wild-type and mutant BMP-1F for 4 h (reducing conditions). 

**FIG. 6.** Cleavage of type I procollagen by wild-type and mutant BMP-1F (18 h) (reducing conditions). 

**FIG. 7.** Cleavage of type I procollagen by wild-type and mutant BMP-1F for 4 h (reducing conditions). HT1080 cells were transfected with vectors encoding wild-type and mutant BMP-1F. The proteins in the culture medium were concentrated, and the levels of BMP-1F were quantitated by Western blot analysis using the anti-FLAG antibody. The PCP activity of the BMP-1F mutants, normalized for BMP-1 concentration, was assayed by cleavage of 14C-labeled type I procollagen alone. The proteins were separated in reducing SDS gels (7%) and detected using phosphorimaging. In samples containing wild-type and mutant BMP-1F, the procollagen was converted to pN-collagen, which is a normal intermediate in the conversion of procollagen to collagen containing the N-propeptides but not the C-propeptides. All the mutants were found to cleave completely and efficiently all the procollagen, except N332Q/N363Q.

was therefore fairly resistant to heat denaturation. However, the PCP activity of the CUB mutants N332Q/N363Q, N599Q, and N332Q decreased to zero in 5, 10, and 20 min, respectively. The N363Q mutant was partially resistant to heat denaturation. These results showed that the oligosaccharides in the CUB1 and -3 domains, and in particular those carried by Asn332 and Asn599, are important for the thermal stability of BMP-1. The greater susceptibility of the CUB mutants to thermal denaturation was also observed at 45 °C (data not shown).

**The N4Q Mutant Is Degraded by the Proteasome**—Recent work has shown that the ubiquitin-proteasome degradation pathway is not restricted to membrane proteins (31), but is also involved in the degradation of mutant secretory proteins (32, 33). To determine the fate of the N4Q mutant, immunofluorescence was performed on N4Q expressing cells (Fig. 9) in the presence and absence of lactacystin, which is an inhibitor of the proteasome. This inhibitor specifically blocks the three peptidase activities of the proteasome (34, 35), thereby preventing misfolded proteins from being deployed to distal compartments of the secretory pathway. In the absence of lactacystin, N4Q and calreticulin (an ER resident protein) did not co-localize, which indicated that N4Q was efficiently translocated out of the ER (Fig. 9A). However, in the presence of 10 μM lactacystin, the N4Q mutant accumulated in the ER (Fig. 9, C and D), as shown by its croissant-shaped co-localization with calreticulin. These results are consistent with translocation of the N4Q mutant of the ER and subsequent degradation by the proteasome.

**DISCUSSION**

In this study we have shown that BMP-1 is N-linked glycosylated at five sites within the molecule and that these sites are important for protein secretion and stability. Three of the sites occur within the protein interaction CUB domains and are important in stabilizing the structure of the BMP-1 molecule. Previous studies had shown that mouse BMP-1 (17) and a BMP-1 homologue in sea urchin (36) were N-glycosylated, but little was known about which of the six potential sites in BMP-1 were glycosylated and the nature of the glycosylation. Our studies show that 5 of the 6 consensus Asn-X-Ser/Thr sites of mouse BMP-1 had been expressed in HT1080 cells or 293-EBNA cells. We also showed that BMP-1 does not contain O-linked oligosaccharides.

As recombinant BMP-1 contained complex type N-glycosylation, it was of interest to evaluate the impact of these particular glycan chains on secretion and activity of BMP-1. Castanospermine and swainsonine have been widely used in a number of studies to determine whether changes in the structure of the N-linked sugars affects glycoprotein functions. Our results indicate that the complex-type glycosylation was not required for secretion and activity of BMP-1. Indeed, BMP-1F with high mannose-type glycans created by castanospermine, or with hybrid-type structures generated by swainsonine, were secreted as efficiently as untreated BMP-1F, and were equally
the intracellular trapping of the latent unglycosylated misfolded protein. However, the multiple mutants N4Q and N6Q could not be detected in the cell lysate of the transfected HT1080 cells either. This apparent contradiction could be explained by the fact that in tunicamycin-treated cells, all glycosylation on all glycoproteins are inhibited including those involved in the quality control and trafficking. In addition, tunicamycin has also been described as an inhibitor of protein synthesis, depending on the system used, some being more sensitive than others, and the nature of the proteins (30). In contrast, in the cells transfected with the multiple mutant constructs N4Q and N6Q, there is no change induced on proteins other than BMP-1F. Another possibility for this difference involves the level of BMP-1F production in HT1080 and 293-EBNA cells, which is much higher in 293-EBNA cells. Therefore, it is conceivable that the ER quality control could be overwhelmed in 293-EBNA cells, resulting in the accumulation of the protein inside the tunicamycin-treated cells, whereas the misfolded N6Q protein recognized as such in HT1080 cells would be rapidly degraded. Indeed, 293-EBNA cells have previously been suggested to have a reduced capacity to deal with improperly folded proteins (46).

Once we had established the implication of N-glycosylation in BMP-1 structure/function, our first question was to identify which sites were glycosylated, and which one(s) of these had a role in folding, secretion, and activity. We generated a series of single-glycosylation mutants of BMP-1, as well as multiple mutants. Our results showed that the individual elimination of any one site had no significant effect on the secretion of BMP-1F, except Asn<sup>332</sup> and Asn<sup>599</sup> from which the corresponding mutants N332Q and N599Q were slightly less secreted than the wild-type (72 and 86%, respectively). The double CUB1 mutant N332Q/N363Q was secreted to 60% of the control, indicating the significant contributions of the CUB glycan chains for efficient secretion, which was further confirmed by the absence of secretion of the multiple mutant N4Q. Furthermore, lactacystin allowed us to visualize this mutant accumulating in the ER, which showed that the N4Q mutant was degraded, at least in part, by the proteasome. Therefore, the N4Q mutant is recognized as misfolded by chaperones, which facilitate retrograde transport to the cytosol for degradation by the proteasome. This transport occurs through the Sec61p translocon (33). Numerous misfolded proteins have been described to be substrates of the proteasome, including a meprin A mutant lacking a MAM domain (47). Interestingly, a truncated meprin A mutant lacking all carbohydrates and expressed in 293-EBNA cells was not targeted for proteasomal degradation and was retained in the ER, implicating carbohydrates as essential factors in retrograde transport into the cytosol (22, 32). These results are in agreement with our observation, as the N4Q mutant still retains two glycosylation sites. Taken together, these results suggest that N-linked oligosaccharides participate in BMP-1 folding, probably with additive contributions of the carbohydrates chains.

We also noted that none of the single mutants had altered PCP activity. On the other hand, the low PCP activity of the double CUB-1 mutant N332Q/N363Q indicated that N-glycosylation plays an important role in BMP-1 PCP activity, either directly in enzyme-substrate interaction, or indirectly in domain-domain interactions. The metalloproteinase (N142Q) mutant exhibited a similar cleavage rate as BMP-1F. Indeed, the position of the glycosylation site, between β-strand I and helix A, is far from the active site (Fig. 10A). As this glycosylation site is highly conserved in mouse (48), Xenopus (49), and sea urchin (36) BMP-1, as well as in human tolloid-like 1 and 2 (2), and Drosophila tolink/tolloid-related-1 (50, 51), it is likely to
The N-glycosylation sites in the metalloprotease domain (A) and in CUB1 and -3 (B) of BMP-1. A. Schematic representation of the secondary structure elements of BMP-1, based on those of asactin, adapted to BMP-1 (59). Active site residues, zinc ion, and Met turn are indicated. β-Strands are shown as arrows, α-helices as cylinders, NT and CT, N and C termini, respectively. The BMP-1 N-glycosylation site Asn^{62} (red star) is located in the N-domain, between the β-I strand and helix A. Meprin A metalloprotease domain N-glycosylation sites are indicated as gray stars (22). B, Topological diagram of aSFP adapted to CUB1 and β-I (adapted from Ref. 53). BMP-1 CUB domains exhibit the same structure as aSFP and boar seminal plasma PSPI/II (52), which are built on a single CUB domain architecture. Conserved disulfide bridges are in green, glycosylation sites in CUB1 (Asn^{62} and Asn^{634}) are indicated as pink stars, and the one in CUB3 (Asn^{63}) as a red star. PSP-PSPI-II glycosylation sites are shown as gray stars. Numbering refers to amino acid sequence of aSFP. NT and CT, N and C termini, respectively.

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