Biosynthesis, Post-translation Modification, and Functional Characterization of Drm/Gremlin*

Lilia Z. Topol‡, Boris Bardot§§, Qingyun Zhang¶, James Resau**, Emmanuelle Huillard§, Maria Marx§§, Georges Calothy§§¶, and Donald G. Blair∥∥

From the ‡Intramural Research Support Program, SAIC Frederick, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201, §Basic Research Laboratory, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201, ¶Unité Mixte de Recherche 146 du CNRS, Institut Curie, Centre Universitaire, 91405 Orsay, France, and ∥∥Advanced BioScience Laboratories, Inc. Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201

Down-regulated by mos (Drm)/Gremlin is a highly conserved protein whose properties and expression pattern suggest a role in early development, tissue-specific differentiation, and cell transformation. We have investigated the biosynthesis and processing of Drm expressed endogenously in rat fibroblasts or overexpressed following transient or stable transfection. Analysis of metabolically labeled cells revealed that Drm exists in secreted and cell-associated forms that exhibit similar mobilities in SDS-polyacrylamide gel electrophoresis. Protein analysis indicated that Drm is present in two major species: a slow migrating glycosylated form and a non-glycosylated form. Both forms of Drm are able to undergo phosphorylation. Drm is released into the media within 30 min of synthesis and is detectable for up to 4–5 h, whereas the cell-associated form has a half-life of about 1 h. Confocal immunofluorescent microscopy indicates that Drm is present both on the external surface of expressing cells, as well as within the endoplasmic reticulum and the Golgi. Both glycosylated and nonglycosylated forms of Drm exhibit identical distributions and are able to antagonize bone morphogenetic protein signaling. Like the soluble form, the cell-associated forms are capable of binding 125I-bone morphogenetic protein-4. These properties are consistent with a role for Drm in interfering with signaling and indicate that Drm may act at the cell surface during tissue development and transformation.

The down-regulated by mos (drm) gene was originally isolated during a differential screen of a transformation-resistant revertant of v-mos-transformed rat fibroblasts (1). Drm was identified on the basis of its high level of expression in non-transformed fibroblasts and the loss of expression following transformation by a variety of viral oncoproteins, including v-mos, v-raf, and v-ras. Whereas a number of genes have been identified whose expression is suppressed by oncogene-mediated transformation (2, 3), analysis of the cDNA sequence of drm revealed that it represented a novel gene containing a cysteine-rich repeat region (1). This motif is also found in DAN, a previously identified tumor suppressor (4), the Xenopus gene cerberus (5), as well as the human Muc2 (6). A portion of this conserved repeat structure has been termed a cystine knot (7–9) and is also shared by members of the tumor growth factor-β family, platelet-derived growth factor, nerve growth factor, and other secreted proteins (10).

Interestingly, the Xenopus homolog of drm, designated Gremlin, was isolated during a screen for proteins, which would act on Xenopus explants as dorsalizing factors (11), and was shown, along with cerberus and DAN, to antagonize bone morphogenetic protein (BMP) function. Both XeGremlin/drm and DAN appeared to bind BMP and act in a fashion similar to the previously identified pattern-inducing genes noggin and chordin (12, 13). It was thus suggested that Gremlin/drm, DAN, and cerberus made up a family of related secreted proteins whose members functioned during differentiation to interfere with the interactions of specific tumor growth factor-β ligands with their receptors (9, 11). The peptide sequences of Xenopus, rat, human, mouse, and chicken drm revealed a high degree of identity across species (11), suggesting that they play an important role in conserved cell structures or functions. Consistent with this hypothesis, Drm/Gremlin has recently been reported to function in limb bud development in both mouse (27) and chicken (28). However, in addition to its possible role as a regulator of early development, our previous results indicated that Drm expression is highly regulated in various adult rat tissues and is particularly expressed in terminally differentiated cells (1). Taken together, these results suggest drm is likely to play an important role in regulating multiple cell functions both during early development and in adult tissues.

These properties and potential functional significance led us to undertake the biochemical characterization of the Drm gene product. Analysis of the predicted amino acid sequence indicated the presence of several significant features, including potential nuclear localization signals near the C terminus, potential N-linked glycosylation sites, and multiple potential

* This work was supported in whole or in part with Federal funds from NCI, National Institutes of Health, Contract No1-CO-56000, and was sponsored in part by the NCI, National Institutes of Health, Department of Health and Human Services, under contract with Advanced Bioscience Laboratories, Inc. Part of this work was funded by the Center National de la Recherche Scientifique and the Institut Curie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

∥∥ Supported by a doctoral fellowship from the Ministère de l’Éducation Nationale et de la Recherche.

†† Recipient of a NATO Collaborative Research Grant.

‡‡ To whom correspondence should be addressed: NCI-Frederick Cancer Research and Development Ctr., P. O. Box B, Bldg. 468, Rm. 102, Frederick, MD 21702-1201. Tel.: 301-846-1319; Fax: 301-846-6164; E-mail: blair@ncicfr.gov.

§§ The abbreviations used are: Drm, down-regulated by mos; BMP, bone morphogenetic protein; ER, endoplasmic reticulum; GST, glutathione S-transferase; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; wt, wild type, PBS, phosphate-buffered saline; AP, alkaline phosphatase.

1 The abbreviations used are: Drm, down-regulated by mos; BMP, bone morphogenetic protein; ER, endoplasmic reticulum; GST, glutathione S-transferase; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; wt, wild type, PBS, phosphate-buffered saline; AP, alkaline phosphatase.

2 L. Topol, and M. Marx, unpublished results.
sites for phosphorylation. Our results indicate that many of these features are active in regulating the processing and localization of the Drm protein. Specifically, we show that Drm is a glycosylated, phosphorylated, secreted protein present both on the external cell surface and within the ER-Golgi compartments. We demonstrated that Drm is secreted via the constitutive secretory pathway in both glycosylated and nonglycosylated forms and that both forms of Drm can antagonize BMP-4 in C2C12 differentiation assays. In addition, we show that cell surface-associated Drm is capable of binding specifically to 125I-BMP-4, indicating that it also has the potential to interfere with signaling. This report describes the first detailed characterization of the processing and localization of a member of the Drm/Gremlin family and suggests properties of potential functional significance.

**EXPERIMENTAL PROCEDURES**

**Preparation of GST-Drm Polyclonal Antibodies**—The entire coding region of rat *drm* cDNA was cloned in frame fused to GST sequences of the pGEX-4T1 bacterial vector (Amersham Pharmacia Biotech) by polymerase chain reaction, according to the Expand long template polymerase chain reaction system (Roche Molecular Biochemicals). The *drm* cDNA (1) was used as template. The region encoding the Drm protein was amplified using 5′- and 3′-primers P1 (5′-CCGGGAATTCTGACGAGGAGAAGAGAAAAGAAA-GGGTCCAAAGGACATCCACCCATCTGAGCAGCAGG-3′) and P2 (5′-CCGGACTTAATGCGATG-3′), respectively. Amplified DNA was sequenced, digested with EcoRI, and inserted into pGEXT1 between the SalI and PstI sites, respectively, to generate the pGEXT1-DRM plasmid. Amplified DNA was digested, ligated and used to transfect CHO cells, and the product formed by ligation links this sequence in frame at the EcoRI and SalI sites. Recombinant plasmids were transfected into *Escherichia coli* in solid box, and NLS, nuclear localization signal sequence; *+,* phosphorylation site; solid box, cysteine-rich motif. The position of the HA tag is indicated. *aa,* amino acid.

**Fluorescence Microscopy**—To observe living cells, cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) and examined with a Nikon E800 fluorescence microscope (Nikon, Tokyo, Japan) equipped with a 100× oil immersion objective. Photographs were captured with a CoolSNAP fx camera (Photometrics, Tucson, AZ). For high magnification, images were captured with a Zeiss 710 confocal microscope with a 100× oil immersion objective. Images were processed with Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA).

**Preparation of GST-Drm Polyclonal Antibodies**—The entire coding region of rat *drm* cDNA was cloned in frame fused to GST sequences of the pGEX-4T1 bacterial vector (Amersham Pharmacia Biotech) by polymerase chain reaction, according to the Expand long template polymerase chain reaction system (Roche Molecular Biochemicals). The *drm* cDNA (1) was used as template. The region encoding the Drm protein was amplified using 5′- and 3′-primers P1 (5′-CCGGGAATTCTGACGAGGAGAAGAGAAAAGAAA-GGGTCCAAAGGACATCCACCCATCTGAGCAGCAGG-3′) and P2 (5′-CCGGACTTAATGCGATG-3′), respectively. Amplified DNA was sequenced, digested with EcoRI, and inserted into pGEXT1 between the SalI and PstI sites, respectively, to generate the pGEXT1-DRM plasmid. Amplified DNA was digested, ligated and used to transfect CHO cells, and the product formed by ligation links this sequence in frame at the EcoRI and SalI sites. Recombinant plasmids were transfected into *Escherichia coli* in solid box, and NLS, nuclear localization signal sequence; *+,* phosphorylation site; solid box, cysteine-rich motif. The position of the HA tag is indicated. *aa,* amino acid.

**Fluorescence Microscopy**—To observe living cells, cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) and examined with a Nikon E800 fluorescence microscope (Nikon, Tokyo, Japan) equipped with a 100× oil immersion objective. Photographs were captured with a CoolSNAP fx camera (Photometrics, Tucson, AZ). For high magnification, images were captured with a Zeiss 710 confocal microscope with a 100× oil immersion objective. Images were processed with Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA).
buffer. Cellular lysates were normalized and electrophoresed on Tris-glycine SDS-PAGE (Novex) and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech). The membranes were processed as described previously (1).

Tunicamycin and Glycosidase Treatment—Cells grown on 100-mm dishes were treated with 1–2 mg/ml tunicamycin for 16 h and lysed with 2× SDS-loading buffer. Total unlabeled cell lysates or 35S-labeled immunocomplexes were treated with peptide N-glycosidase F (PNGF) (0.4 units/10 µg) at 37 °C for 15–20 h as described previously (17).

In Vitro Phosphatase Treatment—35S-labeled immunoprecipitates were washed three times in radioimmune precipitation buffer, resuspended in dephosphorylation buffer (50 mM Tri-HCl, pH 8.5, 0.1 mM EDTA) with 40–60 units of calf intestinal alkaline phosphatase (Roche Molecular Biochemicals), and incubated at 37 °C for 60 min. After the addition of 2× SDS-loading buffer, samples were boiled and analyzed by SDS-PAGE (Novex).

Acidic Treatment—Cells grown on 100-mm dishes were washed with PBS and treated (two times with 2 ml for 5 min each) with an acidic buffer (50 mM glycine, pH 3.0, 100 mM NaCl) (25, 26). Viability of the cells was controlled by staining with trypan blue. Where indicated, 4 ml of acidic wash were clarified by centrifugation 2000×g for 10 min at 4 °C and precipitated by the addition of 10 volumes of ice-cold acetone.

Immunofluorescence—Cells cultured on glass coverslips were fixed for 15 min in buffered 3% formaldehyde solution and washed for 30 min.

Fig. 2. drm encodes multiple forms of protein. In A and B cell lysates were resolved on Novex 16% SDS-PAGE gels and were analyzed for Drm expression using Drm antibody in Western blotting. C shows analysis of the product of in vitro translation of wt and mutant Drm-expressing constructs. A, lysates of Cos-7 cells transfected with pMEX vector (lane 1), pMEX-DRM (lane 2), pHA-DRM (lane 3), or pSVL vector (lane 4). B, lysates from rat primary fibroblasts (lane 1) and DTM cells (lane 2). C, in vitro translation of wt drm (lanes 1 and 2) and the pHA-GDRM mutant (lanes 3 and 4). Drm proteins labeled in vitro with [35S]L-cysteine were synthesized in the absence (lanes 1 and 3) or presence of canine microsomal membranes (lanes 2 and 4) (see “Results”). The position of markers (A and B, Novex; C, New England Biolabs) are shown.

Fig. 3. Drm is glycosylated and phosphorylated. Drm-expressing and control cells were subjected to Western blot analysis using Drm-specific antibodies. A, Cos-7 cells transfected with the pHA-DRM construct. Lane 1, untreated control lysates; lane 2, the same lysates treated with PNGaseF. Prior to lysis, one population of transfected cells were treated with tunicamycin (1 µg/ml, 16 h), lane 4. Lane 3 is untreated control lysates for the samples shown in lane 4. B, Cos-7 cells transfected with pMEX-DRM and treated in culture with tunicamycin (lane 2) as described above. Lane 1 represents an untreated sample. Lanes 3 and 4 represent rat primary fibroblasts untreated (lane 3) or treated with tunicamycin (lane 4). C, Cos-7 cells transfected with pHA-DRM (lanes 1 and 2) or pHA-DRM-21N (lanes 3 and 4) and treated with tunicamycin (lanes 2 and 4). D, Cos-7 cells transfected with pHA-DRM (lane 1), pHA-GDRM (lane 2), and pMEX-DRM (lane 3). E, primary rat fibroblasts metabolically labeled with [35S]L-cysteine and Drm were immunoprecipitated from cell lysates with anti-Drm antibodies. Precipitates were treated with calf intestinal alkaline phosphatase (+) or left untreated (−). F, cells labeled for 5 h with [33P]orthophosphate. After cell lysis immunoprecipitations were performed with preimmune (lane 1) or anti-Drm (lane 2) serum using the same number of trichloroacetic acid precipitable counts.
were washed in PBS and lysed by sonication in 300 mM Tris, pH 7.5. The activity of the cells was assayed using a colorimetric kit (Sigma). Cells were washed twice in PBS and then incubated in DMEM (H-G) supplemented with 20% FCS (Life Technologies, Inc.). Cells were incubated at 37 °C for 30 min. After stopping with 0.1 N NaOH, absorbance at 405 nm was measured and compared with a standard curve of p-nitrophenol (20).

RESULTS

Drm Undergoes Post-translational Modification—To characterize the Drm gene product, we immunized rabbits against purified GST-Drm fusion protein and assessed the ability of immune sera to specifically recognize Drm proteins. In Western blots the sera detected two bands with apparent mobilities of 22 and 26 kDa in COS cells transfected with pMEX-DRM (Fig. 2A, lane 2). HA-tagged Drm migrated with a slightly reduced mobility (Fig. 2, lane 3), consistent with the presence of the tag, and these bands were also detected using anti-HA monoclonal antibody (data not shown). Major bands migrating at about 22 and 26 kDa, and an intermediate band migrating slightly above the 22-kDa band (Fig. 2, lane 1), were also detected in normal primary rat fibroblasts that express Drm (24, 25, 26). C2C12 cells transfected with a pAKA-GDRM construct (Fig. 1), in which the presumptive 42-NDSE-45 glycosylation site was changed to 42-IEAE-45 (Fig. 1), were also transfected with pHA-DRM-21N (Fig. 2C, lane 3). The size of the lower 20-kDa band is likely to arise through glycosylation. As seen in Fig. 2, lane 3), consistent with the presence of the tag, and these bands were also detected using anti-HA monoclonal antibody (data not shown). Major bands migrating at about 22 and 26 kDa, and an intermediate band migrating slightly above the 22-kDa band (Fig. 2, lane 1), were also detected in normal primary rat fibroblasts that express Drm (24, 25, 26). C2C12 cells transfected with a pAKA-GDRM construct (Fig. 1), in which the presumptive 42-NDSE-45 glycosylation site was changed to 42-IEAE-45 (Fig. 1), were also transfected with pHA-DRM-21N (Fig. 2C, lane 3). The size of the lower 20-kDa band is likely to arise through glycosylation.

To verify that glycosylation also takes place in vivo, we transfected Cos cells with wt drm or the pKA-GDRM mutant (Fig. 2C, lane 4). The size of the lower 20-kDa form is compatible with the product generated by removal of the predicted signal peptide sequence, whereas the 24-kDa band is likely to arise through glycosylation.
and analyzed protein expression in control cells and cells treated with tunicamycin, which prevents cotranslational N-glycosylation (22). Following tunicamycin treatment the slower migrating band was no longer detectable in cells transfected with wt drm (Fig. 3, A, lanes 3 and 4; and B, lanes 1 and 2). Similarly, this band was not visible in normal rat fibroblasts treated with tunicamycin (Fig. 3B, lanes 3 and 4). A similar effect was seen (Fig. 3A, lanes 1 and 2) if cell extracts were treated with the deglycosylating enzyme peptide N-glycosidase F (17). These results indicated that the more slowly migrating form of the protein (26 kDa) observed in vivo represented the glycosylated form of Drm and that the faster migrating (22 kDa) form was generated by signal peptide cleavage. This possibility was verified by analyzing Cos cells transfected with the pHA-DRM-21N mutant, which encodes a protein lacking 21 of the presumptive 24-amino acid signal peptide. These cells exhibited only the faster migrating band (Fig. 3C, lane 3), and the mobility of this band was not affected by treatment with tunicamycin (Fig. 3C, lane 4). Finally, we could not detect the 26-kDa band in cells transfected with the pHA-GDRM mutant lacking the glycosylation site (Fig. 3D, lane 2), in agreement with results of in vitro translation. Analysis of the predicted Drm sequence also showed the presence of potential phosphorylation sites by various kinases, such as protein kinase C, cAMP- and GMP-dependent kinases, and casein kinase II (Fig. 1). We examined in vivo phosphorylation by metabolically labeling primary rat cells with [35S]-cysteine, immunoprecipitating the Drm protein, and treating the immunocomplexes with calf intestine alkaline phosphatase before electrophoretic resolution. We observed an overall increased mobility in phosphatase-treated samples (Fig. 3E, lanes 1 and 2), consistent with the removal of phosphate residues from all Drm forms. Direct confirmation of in vivo phosphorylation was obtained by metabolic labeling with [32P]orthophosphate and immunoprecipitation of Drm. All detectable forms of Drm appeared to be labeled (Fig. 3F), consistent with the results observed following phosphatase treatment. Thus, our analysis indicates that the multiple forms of Drm protein detected in vivo probably result from a combination of signal peptide cleavage, glycosylation, and phosphorylation.

**Drm Secretion**—The presence of a putative N-terminal secretory signal sequence (1, 11), together with previous reports (11), strongly suggested that Drm was a secreted protein. Therefore, to further analyze the distribution of inter- and extracellular Drm, we performed indirect immunofluorescence microscopy, as shown in Fig. 4. In Fig. 4, A and G, fixed, permeabilized Cos-7 cells transiently transfected with HA-DRM exhibited a diffuse, fiber-like network of staining suggestive of a localization in the endoplasmic reticulum-Golgi complex. Some cells also exhibited a distinct perinuclear staining, which may indicate that this is the site of Drm synthesis (Fig. 4, A and G). This pattern is analogous to that observed for proteins known to be located in the ER, such as calnexin (23), and we confirmed this localization using monoclonal antibodies directed against the Golgi-specific p58K protein (Fig. 4F), a marker of the ER-Golgi intermediate compartment (24). Our results showed that both Drm (Fig. 4G) and p58K (Fig. 4F) colocalized in the Golgi stacks (Fig. 4H).

In contrast to the permeabilized cells, nonpermeabilized cells showed a clumped, punctate pattern that appeared to surround the outer surface of the cell membrane (Fig. 4B), indicating the presence of Drm on the external cell surface. Analysis of live, unfixed cells showed a similar pattern (Fig. 4D). We observed a similar subcellular distribution of Drm in COS cells by using anti-HA antibodies and in rat cells expressing the endogenous protein (data not shown).

These data indicate that Drm is accumulated in the ER and after release from the ER transverses the Golgi apparatus to the outer surface of the cell. To confirm that the hydrophobic region was necessary for the entrance of Drm into the secretory pathway, we transfected Cos-7 cells with pHA-DRM-21N, which lacked the signal sequence. The truncated protein was exclusively intranuclear (Fig. 4C), in agreement with the fact that the protein contains two nuclear localization signals (amino acids 147–150 and 168–171). As expected, we did not observe surface staining in these live or nonpermeabilized cells (data not shown).

Bands corresponding to glycosylated and unglycosylated Drm, as well as minor bands consistent with phosphorylated forms, could be detected in immunoprecipitates from the supernatants of [35S]-labeled Drm expressing cells using Drm-specific antibodies (Fig. 5A, lane 1), confirming that Drm could be secreted into the medium. Preimmune serum failed to detect Drm-specific bands (Fig. 5A, lanes 2 and 4). The mobilities of the secreted and cell-associated forms (Fig. 5A, lanes 1 and 3) appeared to be identical, suggesting all forms of Drm were
released into the supernatant. Interestingly, cells expressing Drm containing a mutation that destroyed the glycosylation site still secreted the protein. The supernatant form exhibited a mobility consistent with a cleaved nonglycosylated form of tagged Drm (Fig. 5A, lane 6 compared with lane 5), indicating that glycosylation was not necessary for the processing and release of Drm from expressing cells.

To elucidate the kinetics of Drm synthesis, release, and degradation, we performed pulse-chase experiments using HT1080 cells transfected with a drm expression construct and stably expressing high levels of exogenous Drm. As shown in Fig. 5B, lane 1, both the glycosylated and nonglycosylated forms could be detected in the cell lysates after a 30-min pulse. The intensity of these cell-associated bands decreased during the first 30-min chase period and could not be detected after 6 h. Densitometer analysis of this and other similar experiments suggested that the half-life of cell-associated Drm was between 45–60 min. Both glycosylated and nonglycosylated forms were lost at equivalent rates, indicating that glycosylation did not influence protein stability.

We also observed a consistent mobility shift of all Drm bands during the chase (Fig. 5B, compare lane 1 with lanes 2 and 3), suggesting that phosphorylation might be involved in degradation. To confirm that the shift involved phosphorylation, we treated cell extracts after a 30-min pulse and after a 2.5-h chase period with alkaline phosphatase. All shifted Drm bands exhibited increased mobility following alkaline phosphatase treatment, indicating that the shift seen during the chase period was likely to be because of phosphorylation (data not shown).

In contrast to the rapid appearance of cell-associated Drm, we were able to detect Drm in the medium only after a 30-min chase (Fig. 5B, lane 2). The level in the supernatant increased during the first 2 h of the chase and then rapidly decreased over the next 4 h (Fig. 5B, lanes 3–7). In the experiment shown in Fig. 5B, the Drm band represents the immunoprecipitate of the total labeled cell extract and supernatant from a single 60-mm dish. Comparison of the amount of secreted Drm to the amount found in cells suggests that only a small amount is actually released in a soluble form and that the majority of the protein remains cell-associated.

**Cell-associated Drm Binds BMP—Soluble secreted, Myc-tagged Gremlin/Drm, overexpressed in COS cells, was shown to block the activity of purified BMP-2 (11). Because our data indicated that Drm was present both on the external cell surface and in the culture supernatant, we examined the function of both forms in a biological assay, measuring the ability of Drm to interfere with the osteogenic differentiation of C2C12 mouse myoblastic cells induced by BMP-4 (20). In our assays C2C12 cells cultured in the presence of BMP-4 expressed high levels of alkaline phosphatase (AP) activity within 24 h, and this induction was not affected when BMP-4 was preincubated with culture medium from control HT1080 cells or of cells transfected with vector (Fig. 6A, vector alone). In contrast, preincubation with culture supernatants from cells expressing Drm, HA-DRM, or HA-GDRM proteins blocked AP induction in C2C12 cells over a range of BMP from 1 to 5 nM (Fig. 6A). The supernatant from cells expressing Drm was still active when diluted 4–10-fold (Fig. 6B), consistent with the fact that a 5-fold increase in BMP concentration only restored about 50% of the maximal activity in the absence of Drm (Fig. 6A). Culture supernatants from rat primary fibroblasts expressing endogenous Drm could also antagonize BMP-4, demonstrating that endogenous Drm is also released from fibroblasts in a biologically active form. However, as shown in Fig. 6A, the inhibitory activity of this supernatant was much weaker than that of supernatants from Drm expressing HT1080 cells, which may be related to the total level of Drm protein detected in these cells (Fig. 6A, inset). Cocultivation of C2C12 cells with cells expressing different forms of Drm also interferes with the osteogenic differentiation of mouse myoblastic cells (data not shown). The results shown in Fig. 6A also demonstrate that glycosylation has no apparent effect on the ability of Drm to block BMP-4 activity. Thus, neither secretion nor BMP antagonism appears to be affected by the failure of the mutant GDRM protein to be glycosylated.

Although soluble forms of Drm interact with BMP and interfere with its activity (Fig. 6 and Ref. 11), a large fraction of secreted Drm appears to remain cell-associated (see above), and this form, if biologically active, could be of functional significance. We therefore investigated whether the cell-bound Drm was able to bind BMP directly. We incubated 125I-BMP-4 with HT1080 cells expressing Drm or vector alone, then extensively washed the cells, lysed them, and treated the lysates with anti-Drm serum. We found that 125I-BMP was present in the anti-Drm immunoprecipitate (Fig. 7, lanes 1, 2, and 4) but not when preimmune serum was used (Fig. 7A, lane 3) or when
vector-transfected cells were analyzed (Fig. 7A, lane 7). Binding of labeled BMP could be competed with an excess of cold BMP (Fig. 7B), indicating that the Drm-BMP interaction was specific. Furthermore, when cells exposed to 125I-BMP were treated with a bifunctional cross-linker (bis[sulfosuccinimidyl] suberate) before lysis, Drm precipitates contained two labeled bands, one of about 20 kDa consistent with the size of free BMP and a second 44-kDa band of the size expected for a Drm-BMP complex (Fig. 7A, lane 1). The relative intensity of the 44-kDa band was reduced when cold BMP was added to the cells prior to washing and cross-linking (Fig. 7A, lane 2), further supporting the hypothesis that it represented a cross-linked BMP-Drm complex. These binding studies indicate that cell surface-associated BMP-4 in the presence of different molar excesses (100–400-fold) of cold BMP-4. Cells were thoroughly washed, lysed, and immunoprecipitated with Drm-specific antibodies (lanes 1, 2, 4, 5, 6, and 7) or preimmune serum (lane 3) and separated on SDS-PAGE. B, competition analysis of BMP-4 binding. HT1080 cells expressing Drm were incubated with 125I-BMP-4 in the presence of Drm or vector alone were incubated with 125I-BMP-4 in the presence (lanes 2 and 6) or absence (lanes 1, 3, 4, 5, and 7) of cold BMP, washed, and cross-linked before immunoprecipitation (lanes 1, 2, 3, 5, and 6) or left without cross-linking (lanes 4 and 7). Cellular lysates were immunoprecipitated with Drm-specific antibodies (lanes 1, 2, 4, 5, 6, and 7) or preimmune serum (lane 3) and separated on SDS-PAGE. B, competition analysis of BMP-4 binding. HT1080 cells expressing Drm were incubated with 125I-BMP-4 in the presence of different molar excesses (100–400-fold) of cold BMP-4. Cells were thoroughly washed, lysed, and immunoprecipitated with Drm antibodies. Immunocomplexes were separated on SDS-PAGE.

**DISCUSSION**

We previously described the isolation of the rat drm gene (1), also known as Gremlin (11), and its expression pattern in tissues and cell lines. Drm/Gremlin is a member of a family of related BMP antagonists (1, 11, 26) whose critical functions in embryonic patterning (11) and limb development (27, 28) are becoming increasingly clear. Here we have reported the first biochemical characterization of the Drm/Gremlin gene product, showing that it is a glycosylated, phosphorylated, and secreted protein. Following secretion, we have shown that Drm protein is de-processed at the cell surface and in the extracellular medium and that both forms bind BMP-4. Our data also demonstrate that glycosylated and nonglycosylated forms of Drm can antagonize its ability to induce osteogenic differentiation of mouse C2C12 cells.

The drm gene was initially identified in phenotypically normal fibroblasts and was found to be repressed when these cells were transformed by oncogenes, which constitutively activate the extracellular signal-regulated kinase pathway (1). Down-regulation of Drm expression is seen in a wide range of human cancer cell lines, and we also have reported expression of Drm in differentiated cells from various adult tissues (1). These observations underlined the importance of Drm expression in adult tissues and raised the possibility that loss of Drm expression may be involved in regulating the emergence or maintenance of the transformed phenotype.

The Drm/Gremlin amino acid sequence contains multiple sites for potential post-translational modifications, and consistent with these structures, we have shown that the two major forms of Drm/Gremlin detected in primary rat fibroblasts and cells overexpressing drm cDNA are generated through protein cleavage and glycosylation. Tunicamycin and peptide N-glycosidase F treatments demonstrate that the two bands represent glycosylated and nonglycosylated protein, respectively, and this is further supported by the absence of the higher molecular weight band in cells transfected with a drm construct containing a mutant glycosylation site. Moreover, the smaller form of the protein comigrates with the single band of Drm protein detected in cells overexpressing the HA-DRM-21N mutant (which lacks the first 21 amino acids of the presumptive signal peptide), indicating that all Drm forms detected in vitro are cleaved. Similar forms have been seen in cells from various species and tissues.

In agreement with previous reports (11), this study shows that secreted Drm antagonizes the ability of BMP-4 to induce osteogenic differentiation of C2C12 mouse myoblasts, placing drm within the family of BMP antagonists that includes DAN, cerberus, noggin, and chordin. These proteins comprise an evolutionarily conserved, functionally related gene family (26), and although, as in the developing limb bud, they are frequently expressed in the same tissues and developing structures, their expression pattern suggests that they act in a functional manner to regulate specific developmental processes.

---

3 Topol, L., Modi, W. S., Koochekpour, S., and Blair, D. G. (2000) Cytogenet. Cell Genet., in press.

4 L. Topol, unpublished observations.
complementary rather than a redundant fashion (27, 28). Thus, it is likely that the various family members exert specific functions and might be expected to express unique properties.

Consistent with this, we observed that Drm/Gremlin exhibited several potentially significant differences in comparison to other related BMP antagonists. We found that, in contrast to DAN and Cerberus, two other secreted members of this protein family, only a small fraction of Drm is glycosylated. Preventing glycosylation did not alter its secretion or its ability to antagonize BMP signaling. Thus, glycosylation does not appear to be required for the interaction of Drm with BMP-4, although the functional role of Drm glycosylation remains unclear.

We also show that Drm is a phosphoprotein by \textit{in vivo} labeling with \[^{32}P\]orthophosphate, as well as by demonstrating changes in protein electrophoretic mobility after treatment with phosphatase. Phosphorylation may be unique to Drm, because none of the other members of the Drm/DAN family are known to be phosphorylated. Interestingly, besides potential sites for phosphorylation by kinases such as casein kinase II, cAMP-dependent, and protein kinase C, Drm/Gremlin also contains two potential targets for serine kinases that phosphorylate secreted proteins (29). The Ser\(^{\alpha}\) Glu-Ser motif is repeated twice in Drm at positions Ser\(^{77}\)GluGlu\(^{79}\) and Ser\(^{140}\)CysSer\(^{142}\) but are not conserved within the cysteine repeat domains of the other family members.

Immunofluorescent microscopy indicated that intracellular Drm is localized in the ER-Golgi intermediate compartment. It appears to enter the ER upon synthesis, where it is posttranslationally modified and moved through the secretory pathway. The half-life of cell-associated Drm is relatively short (less than 1 h), and the lysosomal and proteosomal pathways appear to be involved in Drm degradation. The phosphorylation of Drm that we have detected could indicate its involvement in targeting the protein for ubiquitination and subsequent degradation via the proteosomal pathway (30).

Consistent with previous reports (11), we found that Drm is released into the extracellular medium, although a significant fraction of secreted Drm appeared to remain noncovalently bound to the outer cell surface. Besides immunofluorescent data, this localization of the protein was supported by experiments showing treatment of Drm-expressing cells with trypsin or acidic buffer (25) significantly reduced the amount of cell-membranes showing treatment of Drm-expressing cells with trypsin bound to the outer cell surface. Besides immunofluorescent fraction of secreted Drm appeared to remain noncovalently released into the extracellular medium, although a significant amount of the protein is detectable outside the cells could reflect an intermediate stage before release in a fully soluble form, perhaps in response to appropriate external or internal signal. Alternatively, the fact that only a small amount of the protein is detectable outside the cells could suggest that cell association, which would allow Drm to regulate signaling at the cell surface in cis, is functionally important. Based on co-immunoprecipitation and cross-linking experiments, our results indicate that Drm can bind BMP when exposed on the cell surface. This raises the possibility that Drm/Gremlin could exert antagonistic effects on BMP-mediated signaling both in cis and in trans. Co-culture experiments suggested that cell surface-associated forms of DRM could also antagonize BMP-4, although we could not rule out the possibility that this inhibition is because of a combination of biological activities of the secreted and cell surface-associated forms.

Understanding the structure and mechanisms of action of BMP antagonists such as Drm/Gremlin is important, because these molecules have been shown to play critical roles in several BMP-mediated functions. In addition to its initially reported role in early embryonic patterning (11), Drm/Gremlin has been reported to control the Sonic Hedgehog/FGF4 feedback loop in murine limb buds (27). Similarly, it has been shown to perform multiple functions in avian limb bud development, including the inhibition of BMP-induced apoptosis during limb outgrowth (28). However, BMP molecules are also known to be involved in an increasingly broad range of tissue-specific functions, including those in the brain (31–33) and ovaries (34) and to effect the properties of neuroectodermal tumors (35). Because expression of Drm is high in the brain and other differentiated adult tissues (1), the function of this protein is likely to be important outside embryonic development. We have reported that Drm is down-regulated in transformed cells (1), and, interestingly, the related DAN gene has been characterized as a tumor suppressor (4). Furthermore, a recent report showed that Drm is up-regulated by the homeobox gsh-1 gene (36), consistent with an involvement of Drm in the negative control of cell proliferation.

Drm/Gremlin shares structural and functional properties with other members of the Drm/Gremlin/DAN family of BMP antagonists that are consistent with its important, defined role as a regulator of BMP action in embryonic development. Our results describe the basic mechanisms of synthesis and processing of Drm/Gremlin, adding to our understanding of this important class of proteins. In addition, our results show that Drm/Gremlin has unique features that could suggest specific roles and functional mechanisms for this protein in embryonic as well as adult tissues.

Acknowledgments—We thank Rebecca Allen for technical assistance. We are also grateful to Drs. M. Jeffers, S. Koohcehpour, and Han-Mo Ko for supplying us with reagents and helpful suggestions, and for critically reviewing the manuscript and to Karen Cannon for final preparation of the manuscript.

REFERENCES

1. Topol, L. Z., Marx, M, Laugier, D., Bogdanova, N. N., Boubnov, N. V., Clausen, P. A., Caloathy, G., and Blair, D. G. (1997) \textit{Mol. Cell. Biol.} 15, 4801–4810
2. Lin, X., Nelson, P. J., Frankfurter, B., Tumbler, E., Johnson, R., and Gelman J. H. (1995) \textit{Mol. Cell. Biol.} 15, 2754–2762
3. Coutente, S., Kenyon, K., Rimoldi, D., and Friedman, R. M. (1990) \textit{Science} 249, 797–798
4. Ozaki, T., and Sakiyama, S. (1993) \textit{Proc. Natl. Acad. Sci. U. S. A.} 90, 2593–2597
5. Bronner-Fraser, M., Kim, H. S., Sasaki, Y., Lu, B., and De Robertis, E. M. (1996) \textit{Nature} 382, 592–595
6. Gumi, J. R., Hicks, J. W., Toribara, N. W., Rothe, E. M., Lagace, R. E., and Kim, Y. S. (1992) \textit{J. Biol. Chem.} 267, 21375–21383
7. Meining, T., Meinde, A., Bork, P., Rost, B., Sander, C., Haasemann, M., and Murken, J. (1993) \textit{Nat. Genet.} 5, 376–380
8. Biben, C., Stanley, E., Fabri, L., Kotecha, S., Rhinn, M., Drinkwater, C., Lah, M., Wang, C-C., Nash, A., and Hilton, D. (1998) \textit{Dev. Biol.} 194, 135–151
9. Stanley, E., Biben, C., Kotecha, S., Fabri, L., Tajakhabab, S., Wang, C-C., Hatziavtarv, T., Roberts, B., Drinkwater, C., Lah, M., Buckingham, M., Hilton, D., Nash, A., Mohun, T., and Harvey, R. P. (1998) \textit{Mech. Dev.} 77, 173–184
10. Isaacs, N. W. (1995) \textit{Curr. Opinn. Struct. Biol.} 5, 391–395
11. Hsu, D. R., Economides, A. N., Wang, X., Eimon, P. M., and Harland, R. M. (1997) \textit{Mol. Cell} 1, 673–680
12. Smith, W. C., and Harland, R. M. (1992) \textit{Cell} 70, 829–840
13. Piccolo, S., Agius, E., Lu, B., Goodman, S., Dale, L., and De Robertis, E. M. (1997) \textit{Cell} 91, 407–416
14. Frangioni, J. V., and Neel, B. G. (1993) \textit{Anal. Biochem.} 210, 179–187
15. Grehalde, J., Gegenone, A., Pogone, P., Derins, D., Leprince, D., and Steln, D. (1986) \textit{Proc. Natl. Acad. Sci. U. S. A.} 83, 1714–1718
16. Hersh, E., and Lane, D. (1988) \textit{The Antibody Labmanual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY}
17. Nunez, E., and Aragon, C. (1994) \textit{J. Biol. Chem.} 269, 16920–16924
18. Frolik, C. A., Wakefield, L. M., Smith, D. M., and Sporn, M. B. (1984) \textit{J. Biol. Chem.} 259, 10965–11000
19. Nishohoto, H., Ishioh, H., Kimura, M., Matsutomo, T., Makishima, F., Yamaguchi, A., Yamashita, H., Enomoto, S., and Miyazono, K. (1996) \textit{J. Biol. Chem.} 271, 21345–21352
20. Katagiri, T., Yamaguchi, A., Ikeda, T., Yoskii, S., Wozney, J. M., Rosen, V., Wang, E. A., Tanaka, H., Omura, S., and Suda, T. (1990) \textit{Biochem. Biophys. Res. Commun.} 173, 289–299
21. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) \textit{Protein Eng.} 10, 1–6
22. Dukusin, D., and Maloney, W. C. (1982) \textit{J. Biol. Chem.} 257, 3105–3109
23. Hohman, T. C., Zhao, B., Chan, H., and Fargulski, M. G. (1998) \textit{Cell} 9, 8792
24. Saraste, J., and Kuismanen, E. (1992) *Semin. Cell Biol.* 3, 343–355
25. Haigler, H. T., Maxfield, F. R., Willingham, M. C., and Pastan, I. (1980) *J. Biol. Chem.* 255, 1239–1241
26. Pearce, J. J., Penny, G., and Rossant, J. (1999) *Dev. Biol.* 209, 98–110
27. Zuniga, A., Haramis, A. P., McMahon, A. P., and Zeller, R. (1999) *Nature* 401, 594–602
28. Merino, R., Rodrigues-Leon, J., Macias, D., Ganan, Y., Economides, A. N., and Hurle, Y. M. (1999) *Development* 126, 5515–5522
29. Sherr, C. J. (1996) *Science* 274, 1673–1677
30. Fuchs, S. Y., Fried, V. A., and Zeev, R. (1998) *Oncogene* 17, 1483–1490
31. Mehler, M. F., Mahie, P. C., Zhang, D., and Kessler, J. A. (1997) *Trends Neurosci.* 20, 309–317
32. Ebendal, T., Bengtsson, H., and Soderstrom, S. (1998) *J. Neurosci. Res.* 51, 139–146
33. Golden, J. A., Brasilovic, A., McFadden, K. A., Beesley, J. S., Rubenstein, J. L. R., and Grinspan, J. B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 2439–2444
34. Shimasaki, S., Zachow, R. J., Li, D., Kim, H., Iemura, S-I., Ueno, N., Sampath, K., Chang, H. J., and Erickson, G. F. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 7282–7287
35. Iantosca, M. R., McPherson, C. E., Ho, S.-Y., and Maxwell, G. D. (1999) *J. Neurosci. Res.* 55, 248–258
36. Li, H., Schrick, J. J., Fewell, G. D., MacFarland, K. L., Witte, D. P., Bodenmiller, D. M., Hsieh-Li, H.-M., Su, C.-Y., and Potter, S. S. (1999) *Dev. Biol.* 211, 64–76
Biosynthesis, Post-translation Modification, and Functional Characterization of Drm/Gremlin

Lilia Z. Topol, Boris Bardot, Qingyun Zhang, James Resau, Emmanuelle Huillard, Maria Marx, Georges Calothy and Donald G. Blair

*J. Biol. Chem.* 2000, 275:8785-8793.
doi: 10.1074/jbc.275.12.8785

Access the most updated version of this article at [http://www.jbc.org/content/275/12/8785](http://www.jbc.org/content/275/12/8785)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 13 of which can be accessed free at [http://www.jbc.org/content/275/12/8785.full.html#ref-list-1](http://www.jbc.org/content/275/12/8785.full.html#ref-list-1)