

# Glycosylation-dependent Cell Adhesion Molecule 1 (GlyCAM 1) Mucin Is Expressed by Lactating Mammary Gland Epithelial Cells and Is Present in Milk

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## Abstract

Glycosylation-dependent cell adhesion molecule 1 (GlyCAM 1) is a mucinlike endothelial glycoprotein that acts as an adhesive ligand for L selectin by presenting one or more O-linked carbohydrates to the lectin domain of this leukocyte cell surface selectin. The GlyCAM 1 glycoprotein has been previously shown to be expressed specifically by the endothelial cells of peripheral and mesenteric lymph nodes and in an unknown site in lung. Here we report that this protein is also expressed during lactation by mammary epithelial cells. Northern blot analysis has shown that the mRNA for GlyCAM 1 appears to be induced during pregnancy in a manner similar to that previously described for hormonally induced milk proteins. In situ hybridization analysis reveals that the site of GlyCAM 1 synthesis in the mammary gland is in the epithelial cells that produce these same milk proteins. Immunohistochemistry of mammary glands using antisera directed against GlyCAM 1 peptides demonstrates that these epithelial cells contain GlyCAM 1 protein, and that this protein is also found lumenally in the milk of the secreting mammary gland. Analysis of murine milk shows that immunoreactive GlyCAM 1 is found in the soluble whey fraction. Finally, labeling analysis of milk GlyCAM 1 has demonstrated that this form of the glycoprotein lacks the sulfate-modified carbohydrate that has recently been shown to be required for the ligand binding activity to L selectin. The nonsulfated mammary GlyCAM 1 is unable to interact with L selectin, consistent with the hypothesis that milk GlyCAM 1 has a different function than endothelial GlyCAM 1. These data thus suggest that milk GlyCAM 1 is a hormonally regulated milk protein that is part of the milk mucin complex. In addition, the finding that the mammary form of GlyCAM 1 contains different carbohydrate modifications than the endothelial form suggests that this glycoprotein may be a scaffold for carbohydrates that mediate functions in addition to cell adhesion. (*J. Clin. Invest.* 1993. 92:952-960.). Key words: mucin • lactation • mammary glands • milk • protein scaffold

## Introduction

Glycosylation-dependent cell adhesion molecule 1 (GlyCAM 1)<sup>1</sup> is a recently described sulfated glycoprotein that appears to

mediate leukocyte-endothelial cell adhesion by presenting carbohydrate ligands to the lectin domain of L selectin (1-5). GlyCAM 1 appears to be a mucinlike glycoprotein, since ~70% of its native molecular weight is contributed by carbohydrates that are found in two serine/threonine rich O-linked domains (5). The tissue specific expression of this mucin on the luminal surface of the high endothelial venules (HEV) of peripheral lymph nodes (PLN) and mesenteric lymph nodes is consistent with a role for GlyCAM 1 in the regional trafficking of lymphocytes to these lymphoid organs. In addition to the expression of GlyCAM 1 in these lymphoid locations, mRNA for this glycoprotein has been demonstrated in lung, although the anatomic localization of lung GlyCAM 1 has not been described. The interaction between GlyCAM 1 and L selectin is dependent upon the O-linked carbohydrate side chains that are presented by the mucin to the leukocyte selectin. These carbohydrates have been shown to contain a sialic acid component that is critical for the recognition of these carbohydrate ligands by the L selectin lectin domain (2). In addition, recent data have demonstrated that the sulfate modification of the carbohydrates attached to GlyCAM 1 is also required for the adhesive recognition of this glycoprotein by L selectin (3, 6). Finally, the expression of GlyCAM 1 mRNA and protein appear to be regulated by afferent lymphatic flow, since deafferentiation of peripheral lymph nodes results in a loss of expression of these components as well as in a profound decrease in the trafficking of lymphocytes to these treated sites (7, 8, Mebius, R., L. Lasky, and S. Watson, unpublished observations). This result suggests that the tissue-specific expression of GlyCAM 1 in the PLN lymphoid compartment is defined by a potentially unique regulatory mechanism.

Mucins other than GlyCAM 1 are expressed in a number of organs and fulfill a variety of functions. For example, mucins that are expressed in the gastrointestinal and respiratory tracts and the vagina function as lubricants and to protect the epithelium (9-14). Another mucinlike molecule, ZP3, is expressed by mammalian eggs and appears to function as a sperm receptor (15, 16). Yet another interesting and important site of mucin biosynthesis is in milk (17, 18). Milk has a number of functions in addition to nutrition, and one of the most important ones is to protect the offspring from gut and respiratory pathogens (19-21). This protection is, in part, elicited by secretory IgA antibodies (22), but an additional component of this protective response appears to be derived from milk mucins (19-21). Work from Yolken and colleagues has demonstrated that the milk mucin complex, a high molecular weight aggregate of several mucinlike glycoproteins that is associated with the milk fat globule, has antiviral activity against rotaviruses, which are gut pathogens that elicit diarrhea in infants (20, 21). The mechanism for this antiviral activity is hypothesized to be competitive inhibition of viral binding to its cellular receptor, since the virus can be demonstrated to bind directly to the partially purified milk mucin complex. The antiviral effect appears to be due to carbohydrate side chains on the milk

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1. Abbreviations used in this paper: GlyCAM 1, glycosylation-dependent cell adhesion molecule 1; HEV, high endothelial venules; PLN, peripheral lymph nodes.

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mucins, since removal of the sialic acid from these mucins by enzymatic or chemical treatment appears to eliminate the antiviral effect (20, 21). Other investigators have demonstrated that a similar high molecular weight mucin complex from milk also inhibits the replication of respiratory syncytial virus (19). These data are consistent with the hypothesis that some mucins in milk may function as naturally occurring antibiotics that prevent respiratory and/or gastrointestinal pathogenesis in immunologically naive offspring.

To examine if GlyCAM 1 has the potential to function as a scaffold for presentation of carbohydrates for other functions in tissues such as the mammary gland, we have examined the expression of this mucin in murine milk. Here we describe data demonstrating that a form of GlyCAM 1 that does not bind to L selectin is expressed as a hormonally induced protein in the whey fraction of murine milk, and we offer some speculations as to the possible functional significance of the regulated expression of this mucin.

## Methods

**RNA analysis.** Total RNA from either mammary tissues at various times during pregnancy and lactation or other organs was purified as previously described (5). Northern blot analysis using equivalent amounts of total RNA from each tissue was performed using a GlyCAM 1 cDNA that was <sup>32</sup>P-labeled using the random priming method. Filters were washed and exposed to x-ray film as previously described (5, 23).

**In situ hybridization analysis.** Mammary tissues from lactating or virgin female mice were sectioned and prepared for in situ hybridization as previously described for PLN tissue. Antisense and sense probes were produced using the previously described GlyCAM 1 subclone in pSKII (Stratagene Inc., La Jolla, CA). Sections were processed and exposed as previously described (5, 24).

**Immunohistochemistry.** Mammary tissues from lactating and virgin mice were dissected out and immersed in buffered 4% paraformaldehyde. The tissues were hydrated with a series of ascending ethanol steps and substituted with xylol and embedded in paraffin. 3- $\mu$ m thick paraffin sections were cut and mounted on albumen-coated slides. Deparaffinized sections were rinsed with PBS and then incubated with PBS/5% normal goat serum. The sections were then stained with the previously described anti-GlyCAM 1 peptide antisera for 1 h. Sections were washed and then incubated with biotin anti-rabbit IgG for 30 min. The sections were washed and then incubated with horseradish peroxidase-conjugated streptavidin and diaminobenzidine substrate. The sections were finally counterstained with hematoxylin or methyl green and photographed.

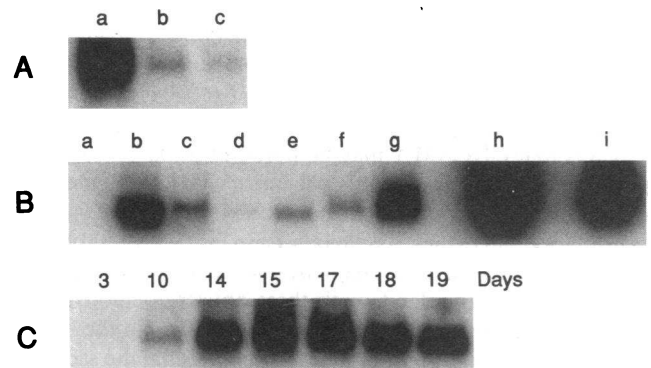
**Western blot analysis.** The whey fraction of murine milk was isolated by centrifugation of either frozen or fresh murine milk (21), and this fraction was boiled for 5 min and centrifuged to remove denatured proteins. Various amounts of the boiled whey fraction were run on 4–20% acrylamide gradient gels after boiling in SDS-mercaptoethanol and transferred to a ProBlot (Applied Biosystems, Inc., Foster City, CA) membrane electrophoretically (25). The blots were incubated with a 2% solution of bovine skim milk, and then they were reacted with a 1:1,000 dilution of anti-peptide antiserum overnight. The blots were washed and then incubated with protein G gold reagent until a signal could be visualized, after which the blots were enhanced with silver. A GlyCAM 1 IgG fusion protein was produced in transfected 293 cells as previously described for L selectin (26) and purified by protein A sepharose chromatography. Crude quantitation of the amount of GlyCAM 1 in milk was performed by including a known quantity of the recombinant GlyCAM 1 IgG fusion protein on the same blots.

**In vitro labeling in organ culture.** Mesenteric or peripheral lymph nodes and mammary glands were dissected out of pregnant and virgin animals and labeled in organ culture with Na<sup>35</sup>SO<sub>4</sub> or [<sup>3</sup>H]serine/

threonine as previously described (2, 3). Solubilized proteins were precipitated with either an L selectin IgG (21) chimera or with anti-peptide antisera as previously described (2, 3, 5). The precipitated proteins were run on 4–20% acrylamide gradient gels, treated with Amplify (Amersham, Inc., Amersham, UK), and autoradiographed.

## Results

**Expression of GlyCAM 1 mRNA during pregnancy.** During the DNA sequence analysis of the genomic region that encodes murine GlyCAM 1 (27), a search of the GenBank revealed that this region of the murine genome had been previously isolated and was shown to encode an mRNA that was expressed in mammary glands during pregnancy and lactation but not in virgin mammary glands (28, 29). To determine if this mammary gland mRNA encoded GlyCAM 1, we analyzed the expression of mRNA encoding this mucin during pregnancy and lactation using the isolated GlyCAM 1 cDNA. As can be seen in Fig. 1, the mRNA for GlyCAM 1 does indeed appear to be expressed in a regulated fashion in mammary glands during pregnancy. Panel A illustrates that a very high level of GlyCAM 1 mRNA is found in mammary glands from lactating females (day 19 of pregnancy), and that the level of this mRNA is much higher than that found in mesenteric lymph nodes. As can be seen from this panel, the size of mammary GlyCAM 1 mRNA appears to be the same as PLN GlyCAM 1 mRNA, and cloning of the mammary GlyCAM 1 cDNA by PCR revealed that it had the exact same sequence as the PLN form (Young, P., and L. Lasky, data not shown). In addition, this panel shows that the mRNA in mammary glands falls to very low levels when pups are removed from the mother (~ 10 d after weaning of pups), suggesting that the regulation of GlyCAM 1 mRNA in mammary glands is similar to other milk proteins in that it requires exogenous stimulation by feeding pups for continued expression (30–33). Panel B illustrates that the level of GlyCAM 1 mRNA is increased when compared with the level in virgin mammary glands. In addition, this panel also illustrates that the level of this mRNA in the inguinal lymph nodes adjacent to the lactating mammary



**Figure 1.** Northern blot analysis of GlyCAM 1 mRNA from various tissues. 10  $\mu$ g of total RNA was run per lane on denaturing gels, transferred to nitrocellulose, and probed with <sup>32</sup>P-labeled GlyCAM 1 cDNA (A) a, Lactating mammary gland; b, mesenteric lymph node; c, mammary gland prepared 10 d after weaning of pups. (B) a, Spleen; b, peripheral lymph node; c, mesenteric lymph node; d, Peyer's patch; e, lung; f, virgin mammary gland; g, virgin inguinal lymph node; h, lactating mammary gland; i, inguinal lymph node from a lactating animal. (C) Time course of induction of GlyCAM 1 mRNA during pregnancy. Shown are the days after conception.

glands also increases, although the relative degree of enhanced expression is only a fewfold and is, therefore, far less than that seen for lactating versus virgin mammary glands (> 100-fold). Panel C illustrates the time course of induction of the expression of GlyCAM 1 mRNA during pregnancy. As has been found for a number of other milk proteins that are regulated by levels of progesterone, prolactin, and insulin, the expression of GlyCAM 1 mRNA begins on day 10 of pregnancy, reaches a peak on day 15, and begins to fall slightly until the completion of gestation. These data clearly demonstrate that GlyCAM 1 mRNA is expressed in murine mammary glands in a manner that appears identical to that previously described for milk proteins that are hormonally regulated during pregnancy and lactation.

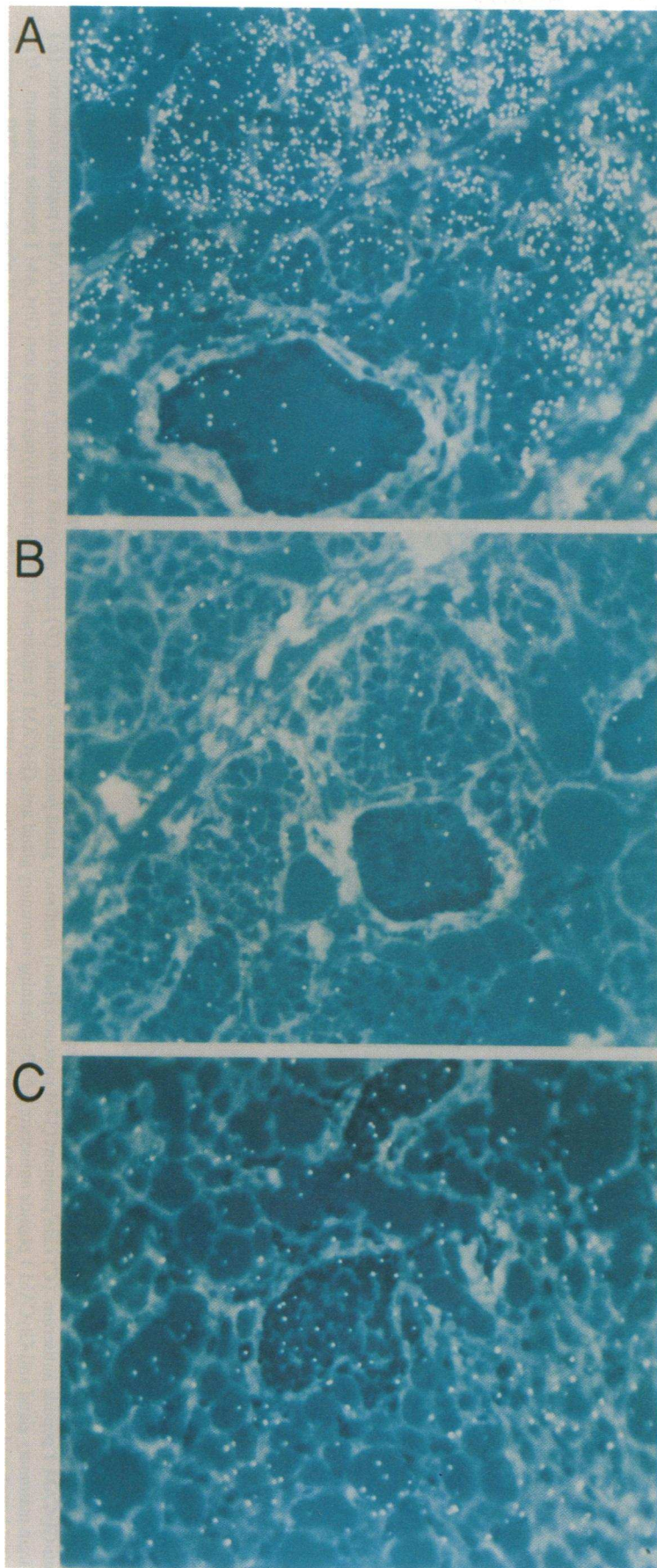
*In situ hybridization analysis.* Although the expression of GlyCAM 1 during pregnancy and lactation appeared to mimic that seen for the milk proteins that are produced by the mammary secretory epithelial cells, it was possible that the elevated expression of this mucin was in a mammary vascular site analogous to the HEV. Such vascular expression of GlyCAM 1 during lactation might be expected if this L selectin ligand was involved in the trafficking of, for example, IgA-secreting B cells to the mammary glands (22). To examine the anatomical location of GlyCAM 1 mRNA synthesis, *in situ* hybridization was performed. Previously, we demonstrated that this technique revealed the expression of GlyCAM 1 mRNA in the HEV of peripheral lymph nodes (5). Fig. 2, however, clearly illustrates that GlyCAM 1 mRNA was expressed in the epithelial cells of the mammary gland during lactation. As can be seen from panel A, strong hybridization of the antisense probe was seen directly over the mammary epithelial cells that produce the milk. In agreement with the Northern blot analysis described above, the virgin mammary glands showed only a low level of specific hybridization over these epithelial cells. Analysis of the sections revealed no obvious hybridization over any of the vascular sites. While these data do not totally rule out the possibility that GlyCAM 1 is expressed in mammary gland vascular sites, they are consistent with the hypothesis that GlyCAM 1 is expressed in a hormonally dependent manner in the epithelial cells that produce milk proteins.

*Immunohistochemical analysis of GlyCAM 1 expression.* The *in situ* hybridization analysis suggested that GlyCAM 1 protein may be expressed by the epithelial cells during pregnancy and lactation. A further possibility raised by these findings was that GlyCAM 1 may be found in milk. To examine these possibilities, immunohistochemistry was performed using anti-peptide antisera specific for GlyCAM 1 (5) and various mammary gland tissues. As can be seen in Fig. 3, immunohistochemical analysis of 17-d pregnant mammary glands and 4-d lactating mammary glands with these antisera demonstrated that immunoreactive material could be observed in the mammary epithelial cells and in the lumen containing the secreted milk. The 4-d lactating glands revealed a high level of staining directly over the cells and in the lumen, consistent with the expression of GlyCAM 1 mRNA at this time. The 17-d pregnant sample revealed a high level of staining of the non-milk fat globule fraction of the luminal milk, but a somewhat lower level of staining over the cells. In agreement with the Northern blot and *in situ* analyses, this figure also illustrates that virgin and postweaning mammary glands show no immunoreactive material. Finally, panel H of this figure clearly shows the simultaneous expression of GlyCAM 1 in the epithelial cells and

associated lumens of the lactating mammary glands as well as in the HEV cells of the associated inguinal lymph nodes. In summary, these data demonstrate that GlyCAM 1 protein is expressed by the mammary epithelium during pregnancy and lactation and that this glycoprotein is secreted into the luminal milk.

*Western blot analysis of GlyCAM 1 in milk.* Because it demonstrated that most of the luminal milk GlyCAM 1 was soluble and not associated with the prominent milk fat globules, the immunohistochemical analysis of pregnant and lactating mammary glands with GlyCAM 1 antisera suggested that the glycoprotein would be found in the soluble whey fraction of milk. Previously, we demonstrated that GlyCAM 1 was completely resistant to denaturation by organic solvents, such as chloroform methanol, and to boiling (5). We therefore isolated the whey fraction of murine milk by centrifugation and boiled this fraction to eliminate many of the contaminating proteins. The boiled whey fraction was run on SDS acrylamide gels and analyzed by Western blot using the anti-peptide antisera described above. As can be seen from Fig. 4, immunoreactive material migrating at ~ 45 kD and ~ 35 kD could be detected in this fraction of milk. The 45-kD band is apparently artifactual, since it is not seen with some of the anti-peptide antisera and is occasionally seen with nonimmune sera. In addition, the ~ 35-kD band, which is only observed with immune sera, appears to migrate with the same mobility as the GlyCAM 1 band produced in radioactively labeled mammary gland organ cultures (see below). Comparison of the Western blot signal obtained with various quantities of whey and that obtained with a standard quantity of recombinant GlyCAM 1 suggest that this protein is present at only a few micrograms per milliliter of milk (Fig. 4). These data also suggest that, since the native molecular weight of GlyCAM 1 derived from the cDNA sequence is ~ 14 kD (5), there must be a large amount of O-linked carbohydrate on the milk form of the glycoprotein. These data are entirely consistent with the immunohistochemistry data and suggest that a highly glycosylated form of GlyCAM 1 is found in the soluble whey fraction of murine milk.

*Analysis of labeled milk GlyCAM 1.* To analyze the potential functional differences between the mammary form of GlyCAM 1 and the endothelial form of the mucin, radioactive labeling and immunoprecipitation experiments were performed. Previously, we demonstrated that the HEV form of GlyCAM 1 could be readily labeled with inorganic sulfate in organ culture (2, 3). We therefore determined if the mammary form of GlyCAM 1 contained this sulfate modification. Fig. 5 shows that, while the PLN HEV form of GlyCAM 1 could be readily labeled with sulfate, the peptide antisera directed against GlyCAM 1 could not immunoprecipitate any sulfate labeled material from late pregnancy mammary glands, in spite of the fact that total mammary gland sulfate-labeled protein contained a heterogeneous species that appeared to migrate at approximately the same molecular weight as lymph node GlyCAM 1. As has been previously described (2, 3), the higher molecular weight fraction of total sulfate-labeled PLN GlyCAM 1 appears to react preferentially with L selectin-IgG. These results suggest that the sulfate type of sugar modification that the GlyCAM 1 polypeptide backbone undergoes during synthesis in HEV does not occur when the mucin is produced in mammary epithelial cells. One of the results of this lack of sulfation is shown in Fig. 5. The sulfate modification of GlyCAM 1 has been previously shown to be an absolute require-



*Figure 2.* In situ hybridization analysis of mammary glands probed with GlyCAM 1  $^{35}\text{S}$ -RNA. (A) Lactating mammary gland probed with antisense RNA. (B) Lactating mammary gland probed with sense RNA. (C) Virgin mammary gland probed with antisense RNA. Magnification, 300.

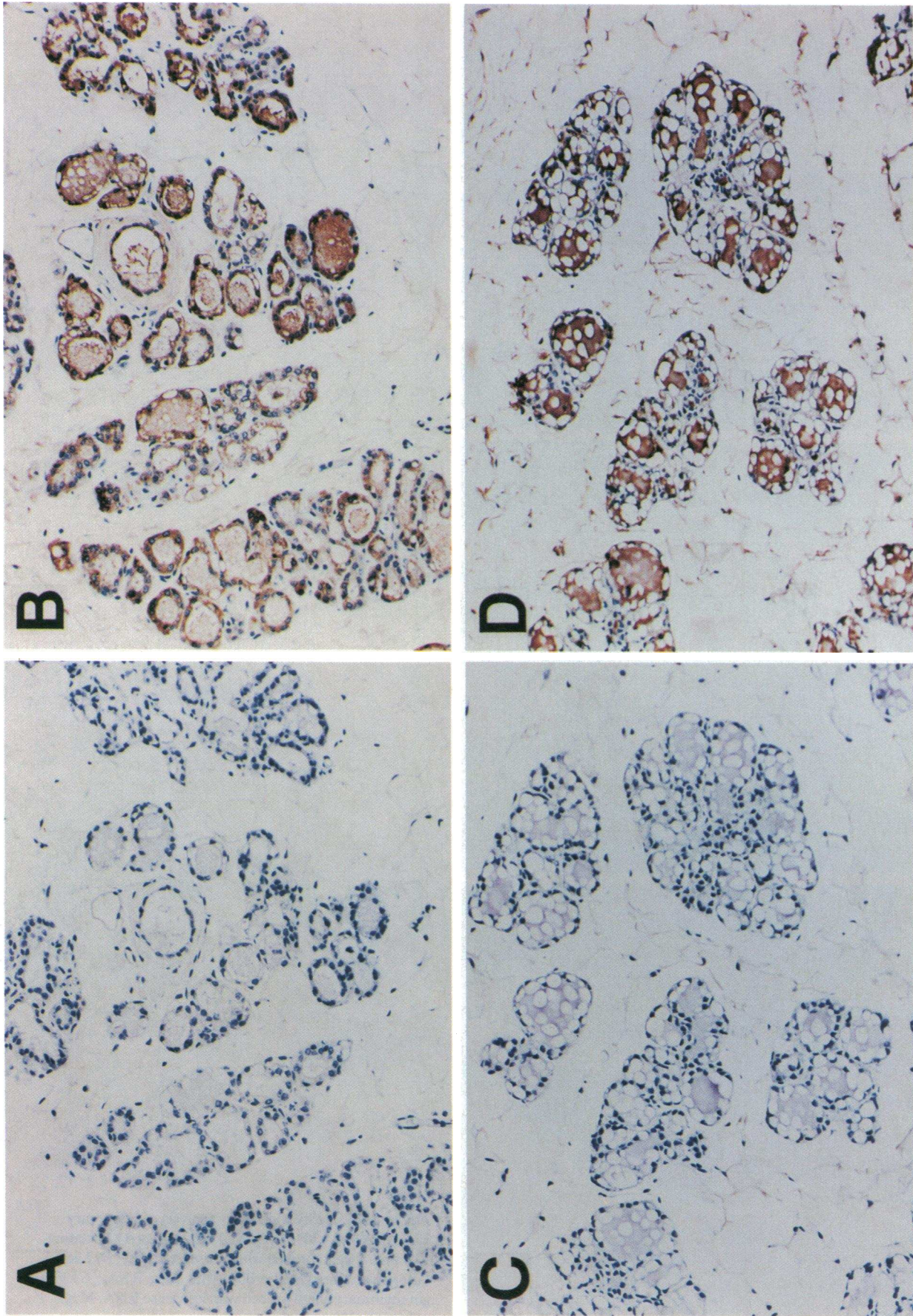


Figure 3. Immunohistochemistry of GlyCAM 1 expression analyzed with antipeptide antisera. (A) Lactating (4 d postpartum) mammary gland preimmune serum. (B) Lactating mammary gland anti-GlyCAM 1 peptide antiserum. (C) Late pregnant (17 d postconception) mammary gland preimmune serum. (D) Late pregnant mammary gland anti-GlyCAM 1 peptide antiserum. (E) Virgin mammary gland anti-GlyCAM 1 peptide antiserum. (F) Postweaning mammary gland anti-GlyCAM 1 peptide antiserum. (G) Peripheral lymph node anti-GlyCAM 1 peptide antiserum. (H) Inguinal lymph node and mammary gland anti-GlyCAM 1 peptide antiserum. The arrows illustrate the HEV of the inguinal node staining with the antiserum. All photographs, except G, which is  $\times 400$ .

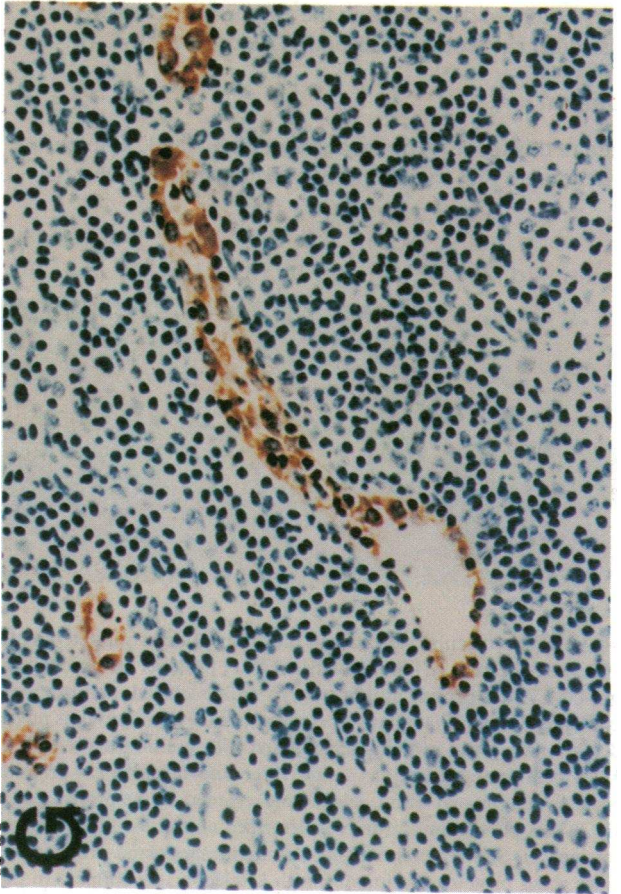
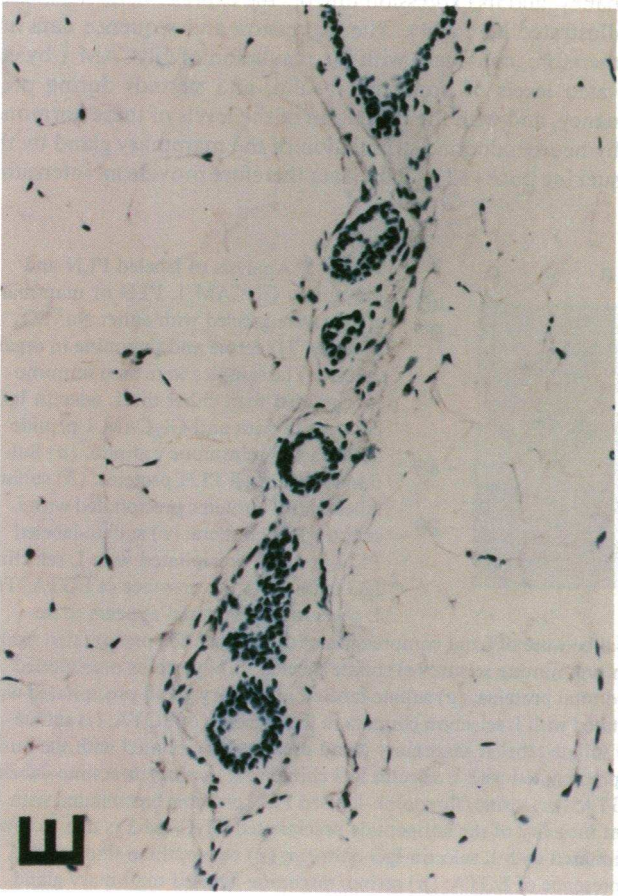
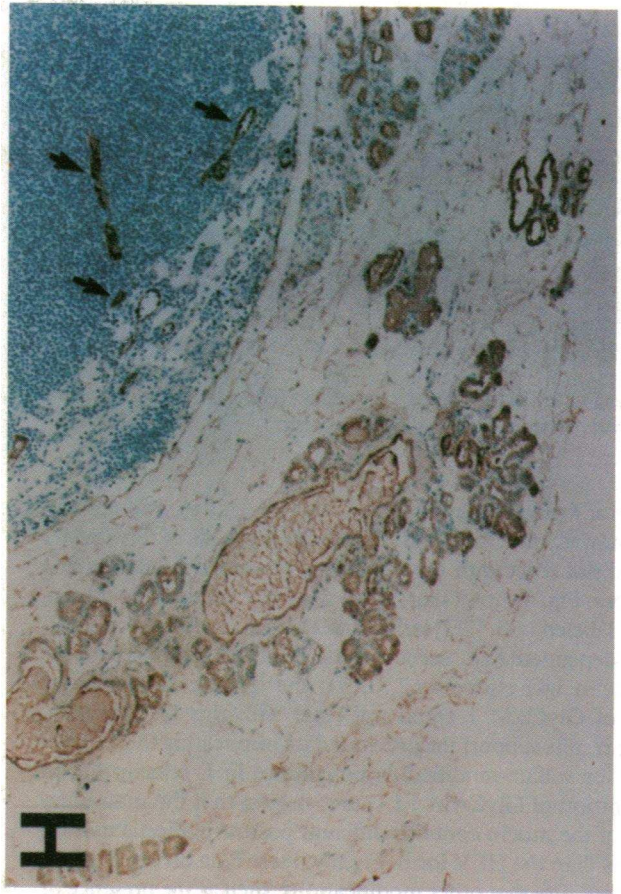
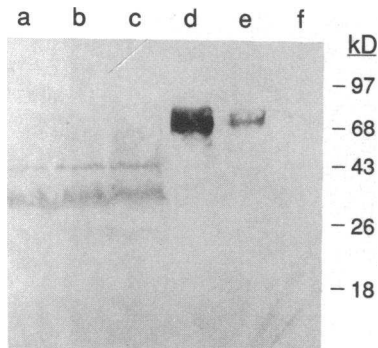


Figure 3 (Continued)



**Figure 4.** Western blot analysis of GlyCAM 1 in milk. 2.5 (a), 5 (b), and 10 (c)  $\mu$ l of the boiled whey fraction of milk was run with 500 ng (d), 50 ng (e), or 5 ng (f) of purified recombinant GlyCAM IgG chimera on an SDS 4–20% acrylamide gradient gel after boiling in SDS mercaptoethanol. The proteins were

transferred to ProBlott, stained with anti-peptide antiserum, and visualized with protein G gold and enhancement.

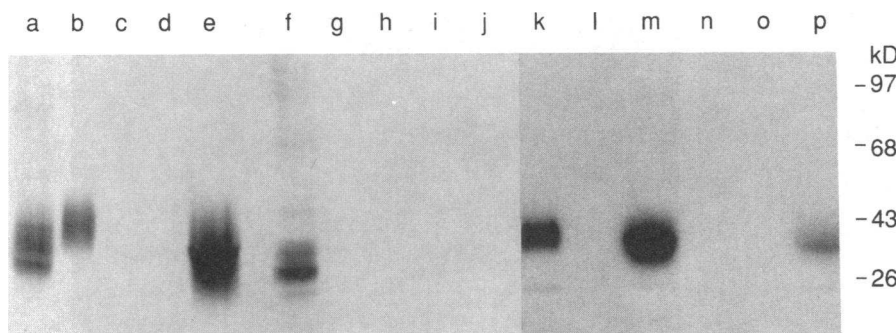
ment for ligand recognition by L selectin (3, 26), and the prediction was, therefore, that the mammary form of GlyCAM 1 should not react with the previously described L selectin IgG chimera. Fig. 5 shows that, while the HEV form of the mucin, when labeled with [ $^3$ H]serine and threonine, reacted in a calcium-dependent (i.e., carbohydrate-directed) manner with the L selectin IgG chimera (2, 3, 5), the  $^3$ H-labeled mammary form of GlyCAM 1 could not react with this IgG chimera. These results support the previous data implicating the requirement for sulfate-modified carbohydrates in L selectin ligand recognition of GlyCAM 1 (3) and suggest that the mammary form of the mucin contains different posttranslational modifications than the HEV form. Finally, these data do not rule out the possibility that other modifications, such as the presence or absence of sialic acid or fucose in the appropriate linkages (2), may also differ between the milk and PLN forms of GlyCAM 1.

## Discussion

The data in this report demonstrate that GlyCAM 1, a mucin-like glycoprotein that was previously demonstrated to be a tis-

sue-specific adhesion ligand for L selectin, is also expressed during pregnancy and lactation in milk. The data also demonstrate that the form of GlyCAM 1 that is expressed in milk appears to lack the sulfate modification that is found in the endothelial form, and that this mammary form is unable function as a ligand for L selectin. These results are consistent with the possibility that the mammary form of GlyCAM 1 has a function other than cell adhesion, a hypothesis that suggests that the GlyCAM 1 polypeptide may be a scaffold that presents diverse carbohydrates for different tissue-specific functions.

Previous work investigating the expression of GlyCAM 1 in mammary glands suggested that the regulation of the expression of this protein was under hormonal control during pregnancy (28, 29). A number of observations in this paper support this supposition. For example, previous data have indicated a requirement for continued suckling by pups for the expression of milk proteins regulated by prolactin, insulin, and steroids (30–33), and we found here that removal of pups from lactating mothers resulted in the downregulation of the expression of the mRNA for GlyCAM 1. In addition, we found that the mRNA for GlyCAM 1 is induced during pregnancy with kinetics that mimic those seen with other milk proteins (31, 32). These data suggest that the mammary expression of GlyCAM 1 is under hormonal control, and they are supported by the sequence analysis of the GlyCAM 1 genomic fragment (27–29). This analysis revealed several potential glucocorticoid receptor binding elements and a number of sequences potentially capable of binding the mammary gland transcription factor (27, 34). The mammary gland transcription factor has been shown to be regulated by hormones during pregnancy, and its expression mirrors the expression of GlyCAM 1 illustrated here (34). The expression and sequence data are, therefore, consistent with the regulation of GlyCAM 1 by elevated levels of prolactin, insulin, and steroids during pregnancy, and with the regulation of the levels of these hormones by neuroendocrine stimulation of the mammary gland by the sucking pups (33). These data therefore provide an interesting



**Figure 5.** Analysis of labeled PLN and mammary GlyCAM 1. PLN or mammary glands were labeled with either  $\text{Na}^{35}\text{SO}_4$  or with [ $^3$ H]serine and threonine in organ culture. The samples were then immunoprecipitated with either the L selectin IgG chimera or with anti-GlyCAM 1 peptide antisera or preimmune antisera. (a) Sulfate-labeled total PLN proteins; (b) sulfate-labeled PLN proteins precipitated with L selectin IgG chimera; (c) sulfate-labeled PLN proteins precipitated with L selectin IgG chimera in the presence of EGTA. The L selectin IgG material appears to be

somewhat higher molecular weight than the anti-peptide precipitated material because of band compression of the anti-peptide precipitated ligand by the IgG heavy chain. (d) Sulfate-labeled PLN proteins precipitated with preimmune serum; (e) sulfate-labeled PLN proteins precipitated with anti-GlyCAM 1 peptide antiserum; (f) sulfate-labeled mammary gland total proteins; (g) sulfate-labeled mammary gland precipitated with L selectin IgG chimera; (h) sulfate-labeled mammary gland proteins precipitated with L selection chimera in the presence of EGTA; (i) sulfate-labeled mammary gland proteins precipitated with preimmune serum; (j) sulfate-labeled mammary gland proteins precipitated with the anti-GlyCAM 1 peptide antiserum; (k) serine/threonine-labeled PLN proteins precipitated with L selectin IgG chimera; (l) serine/threonine-labeled PLN proteins precipitated with L selectin IgG chimera in the presence of EGTA; (m) serine/threonine-labeled PLN proteins precipitated with anti-GlyCAM 1 peptide antiserum. As described above, the higher apparent mobility of the anti-peptide precipitated GlyCAM 1 is due to band compression. (n) Serine/threonine-labeled mammary gland proteins precipitated with L selectin IgG chimera; (o) serine/threonine-labeled mammary gland proteins precipitated with L selectin IgG chimera in the presence of EGTA; (p) serine/threonine-labeled mammary gland proteins precipitated with anti-GlyCAM 1 peptide antiserum.

example of differential gene regulation in two different tissues: the HEV of PLN and mammary glands. It will, therefore, be of great interest to examine the mechanisms by which the GlyCAM 1 gene is regulated at these two divergent sites.

A second interesting aspect of the work described here is the question of the function of milk GlyCAM 1. The data reported here are consistent with a nonadhesive function for mammary GlyCAM 1, since this form of the mucin lacks the sulfate modification required for L selectin binding and has been shown to not interact with L selectin-IgG. A number of possible functions may be performed by milk GlyCAM 1. For example, the protein may function in the gastrointestinal tract of the pup as a lubricant or to protect the lining of these organs (9–14). An argument against this hypothesis is that it might be expected that large quantities of a mucin might be required for this protective/lubrication function, but the Western blot analysis shown above suggests that relatively low levels ( $\sim$  micrograms per ml) of GlyCAM 1 glycoprotein appear to be present in milk. Mucins have also been shown to function as adhesive ligands for sperm binding to mammalian eggs (15, 16), but it is unlikely that milk GlyCAM 1 plays any such adhesive function. An alternative hypothesis is that GlyCAM 1 may be another mucin whose role is to inhibit the replication of pathogenic organisms in the respiratory and gastrointestinal tracts. Previously published data suggest that the mucins in the high molecular weight mucin complex associated with the milk fat globules have antirotaviral activity at relatively low concentrations ( $\sim$  0.1  $\mu$ g/ml) (21). This inhibition appears to be mediated by carbohydrate side chains, particularly sialic acids, that are attached to these mucins and the mechanism of inhibition appears to be due to competitive blocking of viral binding to the cell surface (21). The mucinlike structure of GlyCAM 1 (35–38) is consistent with the possibility that it too may function as a naturally occurring antiinfection agent, and we are currently attempting to purify sufficient quantities of this glycoprotein from milk to analyze this prospect.

In addition to differences in sulfation, another apparent major difference between HEV GlyCAM 1 and mammary GlyCAM 1 is in their relative degrees of cell association. HEV GlyCAM 1 appears to be associated lumenally with HEVs, a result that is expected in view of its presumed role as an adhesion molecule (1–3, 5). The immunohistochemical data described here suggest that mammary GlyCAM 1 is readily secreted into the luminal milk, and the direct demonstration of this mucin in milk supports this result. Interestingly, low levels of sulfated, active GlyCAM 1 can also be demonstrated to be shed into the circulation in vivo (39). The physiological reasons for this vascular shedding are not clear, but one possibility is that GlyCAM 1 must be weakly associated with the HEV surface to allow for ready extravasation of the recirculating lymphocyte into the lymph node. Because GlyCAM 1 does not have a transmembrane domain or phosphatidyl-inositol-type linkage, the mechanism by which HEV GlyCAM 1 is bound to the cell surface can only be speculated upon. Various possibilities include association with a transmembrane protein or peripheral insertion into the membrane through the COOH-terminal amphipathic helix (40, 41). The results reported here may be interpreted to suggest that, since GlyCAM 1 was forced to evolve for dual functions in the vasculature and in milk, a creative mechanism for HEV surface association was derived to allow the mammary form of the protein to be easily secreted. Of course, this conundrum might have been solved by alterna-

tive splicing, but, since the sequence of mammary GlyCAM 1 is identical to HEV GlyCAM 1 (Young, P., and L. Lasky, data not shown), this mechanism is clearly not used. It will, therefore, be of great interest to determine the mechanism by which GlyCAM 1 associates with the HEV luminal surface.

In summary, the data reported here imply that GlyCAM 1 can perform at least two functions: a known function as an adhesive ligand for L selectin in PLN HEV and an unknown function in milk. These data provide the first example to our knowledge of a mucinlike molecule that appears to be used as a scaffold for the presentation of tissue-specific carbohydrate residues for functionally different reasons. Because of the apparently nonadhesive function of the milk form of this mucinlike molecule, the GlyCAM 1 nomenclature (5) will, of course, have to be reassessed. Finally, a thrombospondin-binding nonmucin adhesion molecule, CD36 or PAS IV, is also expressed in a hormonally regulated manner in mammary glands as well as constitutively in a number of endothelial sites as well as in platelets (42–44). As with GlyCAM 1, the function of the CD36 adhesion molecule in mammary glands or milk has also not been demonstrated. It will therefore be of great future interest to isolate the milk forms of these types of adhesive glycoproteins in sufficiently pure form to analyze their potential functions.

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