Cell- and Region-specific Expression of Biliary Glycoprotein and Its Messenger RNA in Normal Human Colonic Mucosa

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Abstract

The localization of biliary glycoprotein (BGP) and its mRNA in normal colonic mucosa was studied by immunohistochemistry and in situ hybridization. BGP mRNA was confined to columnar epithelial cells and expressed abundantly in the superficial mature cells and at low levels in differentiating cells in the upper crypts. Epithelial expression of BGP coincided with that of BGP mRNA. Ultrastructurally, BGP was localized to microfilaments of the fuzzy coat of the columnar cells at the luminal surface and the upper crypts. Additionally, BGP was found in crypt cavitated cells.

The results are consistent with primary transcriptional regulation of BGP production and suggest that BGP synthesis is controlled by the degree of cytodifferentiation. The fuzzy-coat localization of BGP implies a role in nonspecific defense mechanisms against pathogens.

Introduction

BGP is found in human bile discovered through its cross-reactivity with antibodies raised against carcinoembryonic antigen (1). BGP was characterized immunohistochemically and was shown to be present in bile canaliculi, the epithelium of bile ducts, the gall bladder, and small and large intestine (2). Molecular cloning and sequencing of cDNA for BGP (subsequently designated BGP or CD 66a) demonstrated that BGP belongs to the CEA subgroup of the CEA gene family, which in turn is a member of the immunoglobulin gene superfamily (3–6). In humans there is only one BGP gene (3, 4, 6, 7). However, by alternative splicing, at least 11 different mRNA species are generated, and 8 of them (BGPα–d, BGPx, x', y, and z) are considered to be expressed as BGP isoforms on the cell surface (4, 6, 9). The most complex isoforms, BGPα and BGPc, are composed of an IgV-like NH2-terminal domain, three IgC2-like domains (A1, B1, A2), a transmembrane region, and either a long (BGPα) or a short (BGPc) cytoplasmatic tail. BGP and BGPα lack the A2 domain, and BGP and BGPx lack all three constant domains, respectively. Two other isoforms (BGPβ and BGPz) carry a short non-immunoglobulin domain instead of the A2 domain (4, 6). It should be noted that all BGP isoforms identified thus far contain an IgV-like NH2-terminal domain. BGP is most closely related to CEA, NCA, and CGM1 (3, 7), displaying about 90% sequence identity at the amino acid level in the NH2-terminal domain (3, 7).

The expression pattern of BGP in human tissues is complex. BGP mRNA was found in a variety of locations: normal adult and fetal liver (4, 6, 10); normal colon mucosa (3, 11); normal placenta, stomach, mammary gland, pancreas, thyroid, spleen, prostate, uterus, lung, and kidney (6, 12); in granulocytes (8, 13) and B-lymphocytes (9); as well as in different tumor tissues and tumor cell lines (colon, pancreas, liver, lung, breast, endometrium, and acute myelogenous leukemia; Refs. 3, 4, 6, 9–16). Neumaier et al. (11) found that the expression of BGP transcripts was down-regulated in colorectal carcinoma as compared to normal colon. Similarly, a markedly decrease in rat and mouse BGP transcripts was seen in hepatocellular carcinomas (17) and in primary colon tumors (18), respectively. In contrast, in other studies (12, 16), BGP transcripts (BGPα, b, and probably c) were found to be expressed in human lung carcinoma, whereas they were not detected in adjacent normal tissue. Similarly, the short BGP forms (BGPx and x') appear to be expressed preferentially over the longer forms in transformed cells (6). On the other hand, Hinoda et al. (10) did not find any significant difference in BGP mRNA expression between malignant and nonmalignant tissues of human colon and liver. In these studies, BGP transcripts were analyzed by Northern blotting or by PCR performed on tissue extracts. These contradictory results may, therefore, be due to the fact that the source of BGP mRNA in the tissues was not identified. Moreover, BGP mRNA expression has not been correlated with the degree of differentiation of the malignant cells.

The function in vivo of human BGP isoforms is not known. However, some human and rodent BGP isoforms function in vitro as homotypic and heterotypic cell adhesion molecules (reviewed in Ref. 5). Human BGP has the ability to bind bacteria via its carbohydrate moiety and appears to regulate bacterial colonization in the gut (19). Moreover, mouse BGP was found to be the receptor for mouse hepatitis virus (20). Human BGPα and BGPc have been postulated to participate in signal transduction triggered by the binding of an unidentified ligand (6, 21). Rat BGP may also function as a liver ecto-ATPase and a bile transporter (3, 22). It is not known whether BGP in different species has different functions and/or whether individual isoforms of BGP have different functions at different sites in the body. Nor is it known whether some of the reported functions are simply in vitro artifacts.

In the present study, we investigated expression of the BGP mRNA in normal human colonic mucosa by in situ hybridization using a cRNA probe to the unique 3'-untranslated region of the BGP gene. Results were compared with those obtained by immunohistochemistry on the light and electron microscopic levels with the use of a BGP-specific mAb (13).

Materials and Methods

Tissue. Samples of adult human colonic mucosa were from five normal appearing specimens of colon obtained after surgical resections from patients with colon carcinoma.

Preparation of BGP Riboprobe. Analysis of the cDNA sequences of all 11 members of the CEA subgroup of the CEA gene family revealed that the nucleotide sequence at the 3'-untranslated region of BGP cDNA showed the...
lowest degree of homology with sequences from the other members of the subfamily (maximum 40.5% with CEA). This region was therefore used to construct the BGP riboprobe. The probe was synthesized by standard PCR technique from a cDNA library of adult human colon (HL 1034b; Clontech, Palo Alto, CA) with the use of primers corresponding to nucleotides 1740–1759 and nucleotides 1979–1988 of BGP cDNA (4). The pretreated template was subjected to 35 cycles of PCR amplification with the use of a Techne PHC-2 thermal cycler (Techne, Ltd., Cambridge, UK). Initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 1 min, and annealing at 56°C for 30 s and 72°C for 1 min. The final extension step lasted for 5 min at 72°C. The 259-bp PCR product was subcloned into a plasmid vector, pBlue-script SK II (+) (Stratagene, La Jolla, CA), amplified, purified, and sequenced as described previously (7). The vector allows in vitro transcription from each side of the polylinker region. The antisense and sense BGP cRNA were labeled with digoxigenin-UTP with the use of a Dig RNA-labeling kit (Boeringer Mannheim, Mannheim, Germany) according to the instructions of the manufacturer. The BGP cRNA probe is complementary to, at least, the four major splice variants of BGP mRNA, namely BGPα–d (6).

**In Situ Hybridization.** The in situ hybridization was performed according to the protocol of Schaeren-Wiemers and Gerfin-Moser (23) with the following modifications: (a) after the acetylation step, the sections were permeabilized in 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO) in PBS for 30 min at room temperature; (b) 4X Denhardt’s solution was used in the prehybridization and hybridization buffers; (c) after hybridization with probe, the sections were rinsed with 0.1 M Tris-HCl buffer (pH 7.5); (d) the sections were blocked with 0.1 M Tris-HCl buffer (pH 7.5) containing 0.5% (w/v) ovalbumin, 0.1% (v/v) fish gelatin, and 0.5% (v/v) FCS; (e) PBS alcohol (M, 31,000–50,000; Aldrich, Steinheim, Germany) was added to the alkaline phosphatase substrate solution at a final concentration of 5% to enhance the color reaction (24); and (f) the sections were counterstained with methyl green.

**Immunohistochemistry and Immunoelectron Microscopy.** Immediately after resection, tissue specimens were washed in cold HBSS for 15 min. Small pieces of colonic mucosa were fixed by immersion in a mixture of 4% paraformaldehyde (Merck, Darmstadt, Germany) plus 0.01% glutaraldehyde (Sigma) in 0.1 M phosphate buffer (pH 7.3) containing 0.05% saponin (Merck) for 4 h on ice. The fixed specimens were washed in the same buffer containing 3.5% sucrose and 0.05% saponin at 4°C overnight, frozen, and sectioned on a cryomicrotome. BGP was localized with the use of the BGP-specific mAb 4D1/2C12 (13) by the previously described indirect immunoperoxidase technique (25) with the following modifications: (a) instead of the free-floating cryosection, we used the cryosections collected on poly-L-lysine (Sigma)-coated Theranox coverslips (Nunc, Roskilde, Denmark); and (b) endogenous peroxidase activity was blocked by incubation in 0.02 M PBS containing 2 mM NaN₃ and 0.003% H₂O₂ for 30 min at 37°C. For light microscopy, the sections were mounted in glycerin-PBS solution. For electron microscopy, the sections were dehydrated with acetone and flat embedded in Epon-Araldite mixture (Fluka, Buchs, Switzerland). Ultrathin sections were examined without additional staining in a Zeiss EM 109 electron microscope. The specificity of immunocytochemical staining was confirmed by replacing the BGP-specific mAb with PBS.

**Results**

**In Situ Hybridization.** Initial experiments were conducted to assess the effect of PVA on the accuracy and intensity of the specific hybridization signal (blue color). For normal colonic mucosa, addition of 5% PVA to the substrate solution resulted in substantial enhancement of the signal in comparison with a PVA-free control (data not shown); therefore, PVA was selected for the experiments reported below.

BGP mRNA was identified in the tissue sections of normal colonic mucosa with the use of the BGP antisense riboprobe (Fig. 1, a and b). No hybridization signal and background staining were noticed with the control sense riboprobe (Fig. 1c). BGP mRNA was clearly localized in the epithelial layer, whereas the lamina propria and the muscularis mucosa were free of labeling (Fig. 1a). In the epithelial layer, BGP mRNA was present in the cytoplasm of the columnar absorptive cells and not in goblet cells (Fig. 1b). On the sections cut along the vertical axis of the crypts, a well defined gradient in the intensity of the BGP mRNA signal was observed. The undifferentiated cells lining the crypt base and the low differentiated cells of the middle part of the crypts did not contain BGP mRNA (Fig. 1b). The first faint cytoplasmic staining was seen in the columnar cells at the border between the middle and upper third of the crypts. The intensity of the diffuse cytoplasmic labeling increased rapidly from the differentiating cells at the upper one-third of the crypts to the fully differentiated, mature columnar cells at the crypt mouth and the free luminal surface, where the highest cytoplasmic labeling was observed (Fig. 1b). Interestingly, the surface epithelium in the middle of the narrow zone between two neighboring crypts usually exhibited patches of the mature columnar cells that showed a low cytoplasmic signal (Fig. 1, a and b).

**Immunohistochemical Analysis.** At the light microscopic level, BGP was found as a thin line along the apical surfaces (brush border) of the columnar cells as well as intracellularly in some of the cells (Fig. 1, d and e). Distinct and intense staining for BGP was seen in the brush border of all mature columnar cells at the luminal surface and the crypt mouth. These cells were also positively stained in their cytoplasm (Fig. 1, d and e). Cells at the middle and basal third of the crypts showed no detectable staining for BGP, whereas cells of the upper one-third of the crypts showed weak positive staining of both brush border and the cytoplasm (Fig. 1d). No BGP was found in goblet cells. Further, single cells scattered in the lamina propria were also stained by the reaction product.

The electron microscopic localization of BGP supported and extended the observations made at the light microscopy level. The reaction product revealed a thick coat situated on the apical microvillar surface of the mature columnar cells lining the free luminal surface and crypt mouth (Fig. 2a). At higher magnification, this coat was mainly seen in narrow spaces between adjoining microvilli and consisted of fine matted microfilaments distributed on the external side of the microvillus plasma membrane (Fig. 2b). Because the latter was also stained for BGP, it appears as if the majority of the microfilaments are intimately associated with the membrane. In the mature columnar cell the reaction product weakly stained numerous vesicles and rare profiles of granular endoplasmic reticulum located in the supranuclear cytoplasm (Fig. 2b). The differentiating columnar cells in the upper part of the crypts expressed less BGP on their sparse and short microvilli as well as intracellularly (Fig. 2c). Goblet cells, the columnar cells in the midregion and the base of the crypts, did not express BGP.

Individual cells in lamina propria exhibited staining of numerous cytoplasmic lysosome-like structures. This was probably due to inadequate suppression of endogenous peroxidase activity in macrophages and granulocytes because control sections also contained similar cells.

An unexpected observation was the presence of BGP in caveolae cells (26), which, although rare, were found in the crypts (Fig. 2d). These cells were identified by a narrow apical region displaying microvilli that were much longer than those of adjoining cryptal cells and stained well by the reaction product. The apical cytoplasm of the cells contained bundles of straight filaments and so-called “caveolae.” The latter were seen as membrane limited profiles or vesicles and were stained by the reaction product (Fig. 2d).

**Discussion**

In this study, the cellular and ultrastructural localization of BGP and its mRNA was investigated in normal human colonic mucosa. Immunohistochemistry with BGP-specific mAb and in situ hybridization with digoxigenin-labeled RNA probes complementary to the
Fig. 1. Detection of BGP mRNA (a, b, and c) and BGP (d and e) in normal colonic mucosa. a, in situ hybridization shows the presence of BGP mRNA (dark blue color and arrowheads) in columnar cells of the upper one-third of crypts (CR) and surface epithelium. Surface epithelium in the middle area between neighboring crypts exhibits groups of mature cells showing a low cytoplasmic signal (arrows). L, colon lumen. ×100. b, high-power view of surface epithelium and crypt (CR). The first low hybridization signal is registered in columnar cells at the border between the middle and upper one-third of the crypt (arrowheads). The highest cytoplasmic signal is observed in the cytoplasm of the columnar cells of the crypt mouth and surface epithelium (arrows). Thick arrows, some superficial mature cells showing a low cytoplasmic signal. L, colon lumen. ×160. c, no hybridization signal is observed in surface epithelium (arrowheads) and crypts (arrows) with a control sense probe. ×100. d, BGP-positive immunohistochemical staining appears dark brown, and osmium tetroxide counterstaining appears white-yellow. Apical positive staining is most intense on mature columnar cells at the luminal surface (thick arrowheads), less intense at the upper part of crypts (thin arrowheads), and absent at the crypt base (open arrow). Intracellular BGP is seen in mature cells at the free luminal surface (thick arrows). In lamina propria, numerous cells showing endogenous peroxidase activity are present (arrows). L, colon lumen. ×160. e, high-power view of surface epithelium and upper crypt. The intensity of brush border (thick arrowheads) and intracellular (arrows) staining of BGP is the highest on mature cells at the luminal surface and less on cells at the crypt top (thin arrowheads). Numerous goblet cells (G) are negative for BGP. ×240.

unique 3'-untranslated region of the BGP gene were used in the study. With these reagents we overcome potential problems of cross-reactivity with other members of the CEA family, notably CEA, NCA, and CGM1. The results demonstrate that it is possible to precisely visualize BGP transcripts in normal colonic epithelium. This finding shows that the BGP gene is efficiently transcribed in the adult human colon. Further, we have shown that BGP mRNA was not evenly distributed in the colonic epithelium. The hybridization signal was highest in the columnar cells at the free luminal surface, weaker in the cells at the upper one-third of the crypts, and absent in the cells at the middle and lower one-thirds of the crypts. Assuming that the strength of the hybridization signal in individual epithelial cells is directly
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proportional to the mRNA content, then BGP mRNA is predominately expressed by the columnar cells at the free luminal surface with virtually undetectable levels in the middle and lower one-thirds of the crypts. This pattern of luminal surface-crypt expression of BGP mRNA was compared to the pattern of BGP expression as demonstrated by immunohistochemistry. According to the immunohistochemical data, BGP was abundantly expressed at the apical plasma membrane and also in the cytoplasm of the columnar cells lining the luminal surface. The less differentiated columnar cells in the upper third of the crypts expressed less BGP on their microvilli and only occasionally in the cytoplasm. Finally, the columnar cells in the remaining parts of the crypts as well as all goblet cells did not express BGP. Thus, both BGP mRNA and BGP are detectable in equivalent areas of colonic epithelium and show almost identical patterns of heterogeneity in expression levels (i.e., more mRNA/more peptide and vice versa). Taken together, these results suggest that, in normal colonic epithelium, BGP production is restricted to the columnar absorptive cells and controlled at the transcriptional level.

Our results also indicate that the cell expression of BGP and BGP mRNA varied in one and the same columnar cell during its proliferation, differentiation, and migration along the crypt to the free luminal surface. Maximum expression of BGP and its mRNA was attained only when the columnar cell had migrated up to the level of the free luminal surface, that is, to the functional compartment of colonic mucosa. Thus, the expression of both BGP and BGP mRNA correlates inversely with cellular proliferation and directly with the degree of cytodifferentiation. There was only one contradiction with this conclusion: the mature columnar cells located at the middle of the area between two neighboring crypts were strongly positive for BGP but exhibited a low hybridization signal. The reason for this discrepancy may be that alterations in BGP mRNA synthesis or stability occur during terminal differentiation of the columnar cells, before their physiological extrusion from the free luminal surface.

Our immunoelectron microscopy findings are consistent with the presence of BGP in the glycoalyx or, more precisely, in the fuzzy coat of the normal colonic epithelium. The fuzzy coat, invisible in conventional electron microscopic specimens, was clearly stained by our BGP-specific mAb both on the mature columnar cells at the free luminal surface and the columnar cells at the upper one-third of the crypts. The BGP-positive material within the fuzzy coat consisted of microfilaments linked to the microvillus plasma membrane. Our previous electron microscopic investigations have demonstrated that CEA and NCA 50/90 were also present in the fuzzy coat (26). Thus, at least three members of CEA subgroup (CEA, NCA, and BGP) are

Fig. 2. Immunoelectron microscopy of BGP in normal human colon. a, low-power micrograph of the apical pole of mature columnar cells lining the free luminal surface. BGP is present on the microvilli (arrowheads) as well as in small and medium-sized cytoplasmic vesicles and vacuoles (arrows). L, colon lumen. ×8000. b, high-power micrograph of the apical part of a mature columnar cell. The BGP-positive filamentous material is seen between the sides and over the microvilli (arrowheads). Note the positive staining of the microvillus plasma membrane (thick arrowheads). A few of the BGP-containing vesicles (arrows) are present in the cytoplasm. L, colon lumen. ×42,000. c, micrograph of the apical part of differentiating columnar cells at the upper part of a colonic crypt. A delicate BGP-positive layer which consists of a matted microfilaments (arrowheads) is located between and over the short and wide microvilli (thick arrowhead). The contents of sparse cytoplasmic vesicles is occasionally stained by the reaction product (arrows). L, crypt lumen. ×25,000. d, micrograph of the apical part of a caveolated cell in the middle part of the crypt. The long microvilli are covered by the BGP-positive microfilaments (arrowheads). The plasma membrane of one microvilli is clearly stained by the reaction product (thick arrow), other microvilli are tangentially sectioned. In the cytoplasm, bundles of straight filaments (open arrows) and positively stained caveola (arrows) are seen. Note the absence of the BGP-positive material on the microvilli (thick arrowheads) of nearby epithelial cells (E). L, crypt lumen. ×24,000. All ultrathin sections were examined without any additional staining.
integrated glycoproteins of the fuzzy coat (the glycocalyx) in normal colonic mucosa. The luminal glycocalyx localization of BGP argues against a role in cell adhesion. That localization of BGP and its regulation by inflammatory cytokines such as IFN-γ (15) raise the possibility that BGP is involved in binding of pathogens and neutralization of their ability to enter cells and spread infection (15, 19, 25).

An exciting result of the present investigation was the finding that BGP was actively expressed by caveolated cells. This agrees with our previous observations of mouse BGP expression.4 The significance of this phenomenon and the function of caveolated cells are unclear. It was, however, suggested that these cells may function as chemoreceptors for the luminal milieu (26).

In conclusion, our findings provide the first precise localization of BGP and its mRNA in normal human colon. The results show that BGP is an integral component of the glycocalyx. BGP production is restricted to the columnar epithelial cells, is cytodifferentiation dependent, and is controlled at the transcriptional level. The apical glycocalyx localization of BGP is consistent with the hypothesis that BGP plays a role in preventing entry of pathogens.

References

V. Baranov, unpublished data.
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