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Human Biliary Glycoprotein Gene: Characterization of a Family of Novel Alternatively Spliced RNAs and Their Expressed Proteins

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Eight different human biliary glycoprotein (BGP) isoantigens, structurally related members of the carcinoembryonic antigen family, CD66/67 family, and immunoglobulin superfamily, are derived by alternative splicing from a single genomic transcription unit. Novel BGP isoforms have been identified by polymerase chain reaction amplification and by DNA sequencing of amplified cDNA segments. In addition to verifying previously documented BGPs, we describe four new forms, two of which have unusual nonimmunoglobulin exons contributed by inverted *Alu* repeats. Determination of the genomic DNA sequence encompassing most of the known extracellular and intracellular domains demonstrates that the translatable *Alu*-like sequences are encoded in bona fide exons. The third novel BGP isoform contains none of the extracellular disulfide-linked immunoglobulin-like domains typical of these molecules but retains N-terminal and intracellular domains, suggesting distinct functions for N-terminal versus other disulfide-linked domains. cDNAs coding for each identified isoform have been transfected into COS7 monkey cells, and the resulting polypeptides are heavily N glycosylated but can be deglycosylated to their expected primary sizes. Many of these deglycosylated forms can be correlated with unique patterns of BGP expression in different cell lines, while in granulocytes, some previously undescribed or alternatively modified forms may predominate. The BGP family represents a potentially large but unknown source of functional diversity among cells of epithelial and hematopoietic origin. The availability of a defined set of expressed of BGP cDNAs should permit critical definition of their function.

A large family of several immunoglobulin (Ig)-like genes resides on human chromosome 19. Among these are the subgroup of sequences related to the 180-kDa carcinoembryonic antigen (CEA), which itself has been used extensively as a diagnostic serum antigen for recurrence of colorectal cancer either at the primary site of surgical resection or at metastatic sites (28). The CEA family encompasses many members, including nonspecific cross-reacting antigens (NCAs), biliary glycoproteins (BGPs), and pregnancy-specific β_1-glycoproteins. Several of these have recently been placed into the cluster of differentiation classification as CD66/CD67 (36). These polypeptides are remarkably conserved in sequence, yet probably diverse in function and cellular expression, and include antigen forms that are either secreted by cells, tethered to cells by a phosphoinositol glycolipid moiety, or attached to cells by transmembrane (TM) and intracellular domains.

We are interested in defining the function of the molecules represented by the latter class of membrane-anchored molecules, the BGPs. These proteins were originally identified as circulating CEA-cross-reacting species in hepatic and gallbladder biles (33). Their inferred structure based on cloned cDNA has features resembling IgG and other related receptor molecules (5). We previously suggested that BGP isoantigens may exist as homodimers or heterodimers, based on the single unpaired cysteine found in an extracellular region of the molecules, just preceding the IIa domain that undergoes differential splicing in some BGP forms (5). By comparison with protein sequences from the extracellular Ig-like loop regions of CEA, NCA, and BGP, domain IIa is the only domain that diverges distinctly in amino acid sequence from other homologous family member loop domains (45% versus 75 to 80% [5]) while retaining the characteristic Ig-like folds. The overall Ig-like character imparted to BGP molecules suggests that they function as receptors, perhaps involved in adhesion (23, 37). However, the alternate splicing of the extracellular IIa domain, in combination with two possible intracellular forms, one of which may be phosphorylated (1), suggests that alteration in these molecular structures could play an important role in recognition and signal transduction. Similar observations gave been made for other Ig-like molecules, most notably the fibroblast growth factor receptor (15, 22) and N-CAM (12, 25, 29).

Defining the size and diversity of the BGP family is a first step in exploring a functional role. Toward this end, we surveyed the RNAs of many different normal tissues and transformed cell lines by polymerase chain reaction (PCR) amplification of the specific extracellular and intracellular regions of BGP mRNAs that are known to be alternatively spliced and code for one of several isoforms. By this process, we found additional size classes of amplified products that differ substantially from those previously described. Further characterization of these segments revealed that they code for two novel non-Ig-like extracellular domains derived by alternative splicing from *Alu*-like elements, as well as another form that codes for a BGP isoantigen with only an N terminus anchored via a TM domain to a cytoplasmic tail. Some of these forms appear to be represented more in RNAs from tumor-derived cells than in RNAs from normal tissue counterparts, suggesting that the transformed phenotype may contribute to the appearance of new BGP splice variants and their translated polypeptides.

The finding of additional novel forms of BGP extends the

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repertoire of transmembrane receptor-like BGP molecules to eight members and argues that adhesion mediated by interaction of a BGP isoform with one or more extracellular ligands, rather than by homotypic adhesion alone, may modulate their activity in vivo on a variety of cellular targets. Defining the nature and expression of the natural cellular ligand for each BGP isoform would provide insight into the normal function of CEA-like molecules. In addition, understanding the nature of these ligands could increase the utility of the CEA family in cancer monitoring and detection, as well as in determining whether alterations in their interactions contribute to the progression toward or maintenance of oncogenesis.

MATERIALS AND METHODS

Cells. Isolation and characterization of the transfectant 23.411$^+$ (a stable mouse fibroblast cell line containing human BGP genomic DNA) has been published elsewhere (15a). All other human cell lines were obtained from the American Type Culture Collection (Rockville, Md.) and propagated according to their recommendations. The cell lines included BT-20 (breast carcinoma; HTB 19), COLO 201 and COLO 205 (ascites fluid from colon adenocarcinoma; CCL 224 and CCL 222), HCMC (normal human colonic mucosal cells; CCL 239), MIA PaCa-2 (pancreatic carcinoma; CRL 1420), NIH:OVCAR3 (ovarian adenocarcinoma; HTB 161), BeWo (choriocarcinoma; CCL 98); KG-1 (acute myelogenous leukemia; CCL 246), U-937 (histiocytic lymphoma; CRL 1593), SW480 and SW620 (colon adenocarcinoma and lymph node metastasis of colon adenocarcinoma; CCL 228 and CCL 227), HT-29 (colon adenocarcinoma; HTB 38), and WIDR (colon adenocarcinoma; CCL 218). Normal human colonic mucosal tissue and MIP101 cells were obtained from Peter Thomas (New England Deaconess Hospital, Boston, Mass.); enriched granulocytes were prepared from human blood fractionated on Histopaque-1077 (Sigma).

RNAs. Total RNA from cell lines or tissues was prepared by the guanidinium thiocyanate-acid-phenol method of Chomczynski and Sacchi (10). Poly(A)$^+$ RNA was prepared from cellular extracts (5) or from total RNA by oligo(dT) fractionation. Some RNAs were purchased from Clontech (Palo Alto, Calif.).

cDNA preparation for PCR analysis. Two micrograms of total RNA was incubated in 20-μl reaction volumes with Moloney murine virus reverse transcriptase (GIBCO/BRL, Bethesda, Md.) to prepare first-strand cDNA. Five-microliter cDNA aliquots were used as the template in 50-μl DNA amplification reaction mixtures which contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 0.3 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Reaction cycles were as follows: cycle 1, 94°C for 5 min, 55°C for 1 min, and 72°C for 1.5 min; cycles 2 to 29 or 2 to 34, 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min; and cycle 30, 94°C for 1 min, 55°C for 1 min, and 72°C for 8 min. Similar conditions were used to amplify specific regions of bacteriophage lambda DNA containing the BGP gene (λ39 or λ55 [5]). Electrophoretic analysis of gel segments was on 1% agarose–2% NuSieve gels.

Several different oligonucleotides were used as PCR primers to amplify cDNA and genomic segments. In some cases, restriction sites were incorporated into the 5′ ends of the primers to facilitate subsequent cloning steps; these sites are underlined. The primers used were as follows:

PCR-1	5′-GGAATTCTAGAATAAGTAACTTCATTCATCTTGT
PCR-2	5′-GGAATTCAATAGTGGATCCTATACCTGCCACGCC
CEA+NCAF	5′-GTCATAAAGTCAGATCTTGTGAATGAA
M	5′-CAGGTTTCATTACTGCTTTTTTAC
DIIIFOR	5′-CTTGAAATCACCTAGTCCTA
DIIIREV	5′-TTTGCACCACTGCACTCCAGTCT
DIVFOR	5′-ATAAATTTTAGAATGAATT
DIVREV	5′-TAATCAAAACAGTATGGTA
TMF1	5′-AAACCCTCTCAATTCATCTG
TMF2	5′-CACGCCTGTAACCAGCAGTT
TMR1	5′-TCCACTTGGAATCTTCTTCT
TM2R1	5′-ATACAGCTGGTGACTTCAGA
TM10F1	5′-CAACAGAGCAAGACTCTGTC
TM10R1	5′-AGAAACCAGTGGAATAAAAT
TM18F1	5′-CTATATAATTTTAGAATGAA
HDFOR	5′-AACCCAATCAGTAAGAACCAAAGC
HDREV	5′-TGTGGTCTTGCTGGCTTTGATTTG
START	5′-AGCCCAAGCTTTCCTCCACAGGTGAAGACAGGGCCA
END	5′-TGAAGCGGCCGCTCATTACTGCTTTTTTACTTCTGAATAAAT

DNA sequence analysis. DNA segments obtained by PCR were cleaved with restriction endonucleases, gel purified, and cloned into Bluescript I KS+ (Stratagene, San Diego, Calif.). Nested deletions within subcloned molecules were prepared by the exonuclease III method (13a), and DNA sequencing was performed by the dideoxy method (24a), using T7 DNA polymerase (Pharmacia, Piscataway, N.J.) and [α-^{35}S]dATP (Amersham Corp.). DNA sequences were analyzed by the University of Wisconsin Genetics Computer Group computer programs described by Devereux et al. (11).

Expression and characterization of BGP isoantigens. The coding regions of BGP cDNAs were inserted as *Hin*dIII-*Not*I segments into the mammalian expression vector pcDNA I NEO (Stratagene). Uptake of plasmid DNAs into COS7 monkey kidney cells was facilitated by incubation with DEAE-dextran (24). After 48 h, COS cell transfectants or stable cells from stable lines were counted, pelleted, resuspended at 10^7 cells per ml of lysis buffer (10 mM $NaPO_4$ [pH 7.5], 140 mM NaCl, 10 mM EDTA, 10 mM benzamidine, 1% Nonidet P-40, 10 μg each of chymostatin, leupeptin, pepstatin A, and 4-aminophenylmethylsulfonyl fluoride per ml), incubated on ice for 1 h, and then centrifuged for 15 min at 15,000 × *g*. To 100-μl aliquots of supernatants was added 10 μl of 20% sodium dodecyl sulfate (SDS)–100 mM 2-mercaptoethanol, after which the mixtures were heated for 10 min at 95°C. After cooling, 110 μl of digestion buffer (40 mM $NaPO_4$ [pH 8.5], 3% Nonidet P-40, 10 mM EDTA, 10 μg each of chymostatin, leupeptin, pepstatin A, and 4-aminophenylmethylsulfonyl fluoride per ml) and 2 U of *N*-glycosidase F (peptide-N^4-[*N*-acetyl-β-glucosaminyl]asparagine amidase; Boehringer Mannheim Biochemicals) were added. Reactions were usually carried out for 8 h or overnight, with no difference in pattern. As a control for digestion, α_1-acid glycoprotein (Sigma) was sometimes included in samples and detected in its deglycosylated form on Western immunoblots by a polyclonal antibody (Sigma). Samples were prepared for electrophoresis on denaturing SDS–4 to 12% polyacrylamide gels (Novex, San Diego, Calif.) by adding 5× Laemmli sample buffer and boiling for 10 min at 95°C. Deglycosylated polypeptides were transferred to polyvinylidene difluoride membranes in buffer containing 10% methanol, and BGP isoforms were detected by DAKO rabbit anti-human CEA polyclonal antibody (DAKOPATTS, Carpenteria, Calif.). Verification of specificities was by CEA, NCA, and BGP class-specific mouse

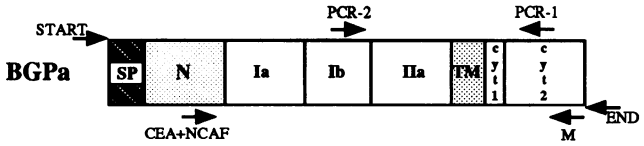

FIG. 1. Schematic diagram of BGPa cDNA showing positions of encoded polypeptide domains (SP, signal polypeptide; N, N-terminal domain; Ia, Ib, and IIa, domains with disulfide-linked loops; TM, transmembrane domain; cyt1 and cyt2, intracellular domains) and of oligonucleotide pairs (PCR-1 and PCR-2; CEA+NCAF and M; and START and END) used in PCR amplification of cDNAs (see text for details).

monoclonal antibodies obtained from J. Elting. Antibody reactivities were monitored by using a mouse anti-rabbit IgG conjugated with alkaline phosphatase (Promega). Signals were detected by autoradiography of filter-bound immune complexes after incubation with the chemiluminescent substrate [3-(2′-spiroadamantane)-4-methoxy-4-(3′-phosphoryloxy)-phenyl-1,2-dioxetane] (Bio-Rad).

Nucleotide sequence accession numbers. Sequences included in this report can be accessed through the GenBank data base by using the following codes: M76741 (BGP genomic DNA), M76742 (BGPx cDNA), M76743 (BGPy cDNA), and M76744 (BGPz cDNA).

RESULTS

PCR identification and cloning of alternatively spliced mRNAs. Two oligonucleotide primers, PCR-1 and PCR-2, were used to amplify first-strand cDNA prepared from various cellular RNA samples. The locations of these primers within the cDNA of the largest BGP isoantigen, BGPa, are shown in Fig. 1. The primers were designed to flank extracellular and intracellular domains that had previously been demonstrated to be involved in alternative splicing. Figure 2 shows the result of PCR amplification. For each of the cell lines, multiple bands are present in each lane, suggesting the presence of several different cDNAs corresponding to different BGP isoantigens. We previously demonstrated by DNA sequencing of gel bands cloned from a PCR amplification of SW620 cDNA that the two largest (~650- to 700-bp) and two smallest (~230- to 290-bp) fragments correspond to BGPa/c and BGPb/d, respectively (bars, Fig. 2A; see reference 6 for nomenclature). The less prominent bands of ~300 and ~350 bp (arrows) were unexpected from combinatorial splicing. One of these size classes was also present in the 23.411$^+$ transfectant line (Fig. 2B). Bands corresponding to 300- to 350-bp amplified cDNAs derived from BT-20, MIA PaCa-2, and transfectant 23.411$^+$ cell lines were excised from a preparative agarose gel and cloned. DNA sequencing of representative clones δ6, 3.3, and TM-X2.2 from each cell line, respectively, indicated that among them, these new segments contained two previously unidentified extracellular domains, designated IIy and IIz (Fig. 3A and B). The BGP isoantigens from which they are likely derived are named BGPy and BGPz.

For PCR characterization of BGP mRNAs present in the transfectant 23.411$^+$ line, a second primer set, CEA+NCAF and M (Fig. 1), was designed to detect transcripts from alternatively spliced products that included both extracellular and intracellular regions. This process gave rise to two new products, BGPx and BGPx′, that could be detected in several different colorectal tumor cell samples (Fig. 2C). After excision and cloning of the larger (BGPx; ~300 bp) of the two bands from amplified 23.411$^+$ cDNA, DNA sequencing of representative clone X-6.1.PCR revealed a product of alternative splicing that contained as its only extracellular region a variable (V)-like Ig domain corresponding to the N terminus of BGP, while retaining TM as well as both Cyt1 and Cyt2 cytoplasmic domains. The second, smaller band of about 250 bp detected in the 23.411$^+$ line and other cells, BGPx′, has not been cloned, but its characteristics and size are consistent with a splice form generated without the 53-bp Cyt1 exon (as for BGPc/d [5]) (Fig. 1). Figure 2D demonstrates that when 23.411$^+$ cDNA is amplified with START and END primers (Fig. 1; Materials and Methods), an abundant full-length transcript corresponding to BGPx can be detected in the RNA population and thus is not likely generated by PCR from a random cDNA fragment.

Since the results described above were derived from RNAs of transformed cells, we addressed the possibility that they may not be representative of RNA expression in cells of normal tissues. We prepared random first-strand cDNA from normal cell RNA and then amplified it either with PCR-1 and PCR-2 or with CEA+NCAF and M primers. The results for the latter primer set, which detects all BGP isoforms, are shown in Fig. 2E. The results indicate that BGPy and BGPz splice variants do not appear to be limited to either the transformed or normal cellular state. However, we detect no or relatively little BGPx and BGPx′ RNAs in normal tissues, implying the possibility of transformation-enhanced expression of some BGP isoforms. It should be noted, however, that the source RNAs in the two experiments are different; cultured transformed cells, unlike normal tissues, are not likely to be composed of or infiltrated by other cell types that may invalidate this interpretation. On the other hand, it appears that for both transformed and normal cell RNAs, there appears to be equivalent, perhaps coordinate, expression of BGPa and BGPc RNAs that include domain IIa and, likewise, of BGPb and BGPd RNAs that exclude IIa. Coordinate expression could imply heterotypic interactions among isoforms with identical extracellular but different intracellular regions.

Sequences of BGPx, BGPy, and BGPz cDNAs. The derived DNA sequences and corresponding amino acid translation of the amplified BGP cDNAs described above are shown in Fig. 3. The size difference between the BGPy (Fig. 3C) and BGPz (Fig. 3D) cDNA clones can be accounted for by the fact that BGPy is 53 bp longer in its intracellular domain than BGPz because it contains both intracellular splice domains of the BGPa/b isoforms (5); the shorter cytoplasmic domain of BGPz derives from the alternative splice characteristic of BGPc/d.

The novel feature of BGPy/z cDNAs is the presence of previously unidentified 31-amino-acid regions that substitute for the 297-bp extracellular domain IIa of BGPa/c. The 93-bp sequences and their amino acid translation bear no resemblance to any other domain within either the CEA gene family or the Ig gene superfamily. However, a GenBank/EMBL data base search reveals significant homology over 91 bp with an equivalent region of an inverted and truncated human *Alu* repeat sequence (data base accession number M21204 [17]). It is of interest that these *Alu*-derived domains, like the splice of domain IIa, retain a conservative Glu or Asp residue at their 5′ splice junctions.

Domains IIy and IIz demonstrate considerable nucleotide homology with each other (78%; Fig. 4A), as might be expected for related *Alu* elements. Their exact alignment, however, requires spacing of two nucleotides at each of two different sites within the IIy and IIz domains. A two-

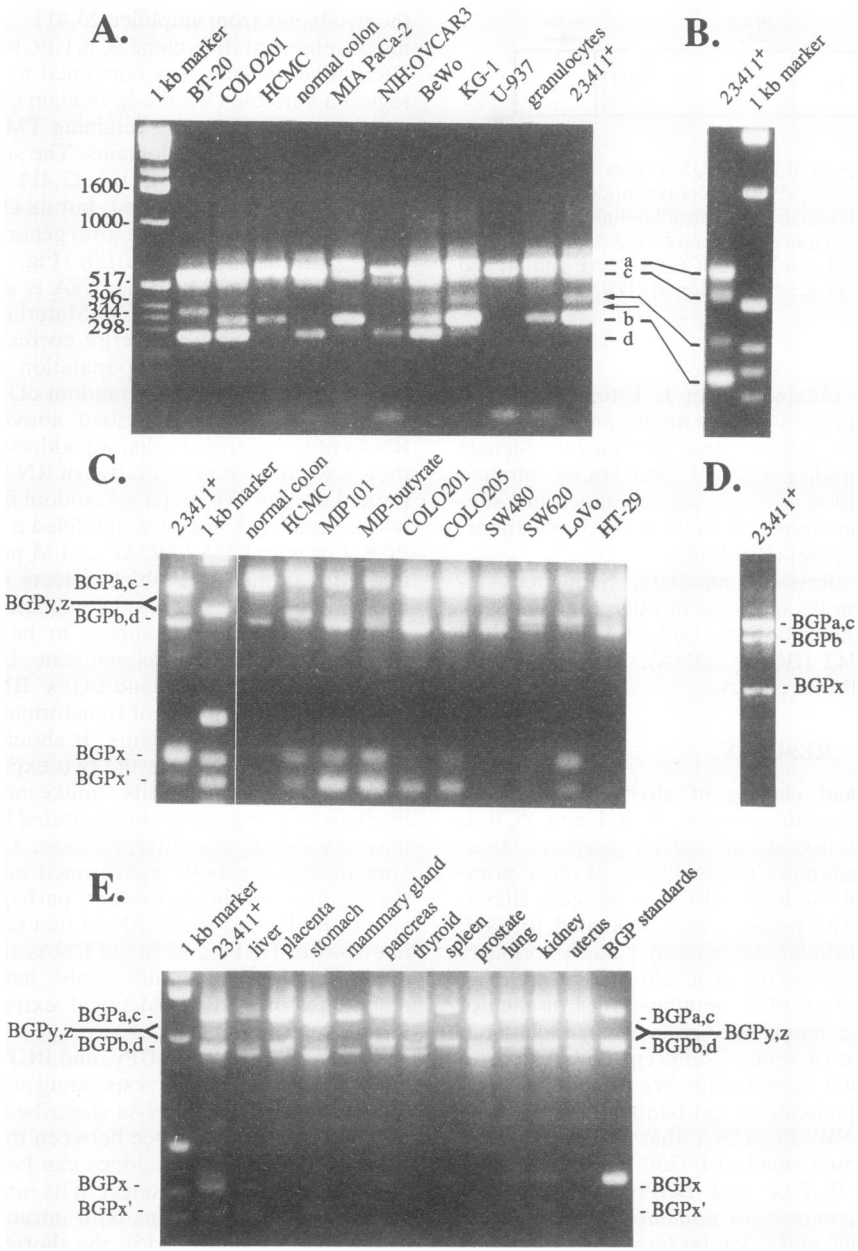

FIG. 2. Gel electropheretograms of PCR products derived from amplification of reverse-transcribed cDNAs with the oligonucleotides noted in the legend to Fig. 1. Cellular RNAs were prepared from cell lines, normal tissues, or fractionated normal granulocytes. PCR products in short (A) and long (B and E) gel runs were derived by priming with oligonucleotides PCR-1 and PCR-2. Fragments marked a, b, c, and d are derived from BGPa, -b, -c, and -d RNAs, respectively, and arrows show the positions of new fragments derived from BGPy (upper arrow) and BGPz (lower arrow) RNAs. In panel B, the arrowed line marks the position of the BGPy fragment. In panel E, the region containing both BGPy and BGPz is marked by an arrow. Marker fragments in base pairs are from the 1-kb DNA ladder (GIBCO/BRL). PCR products in panels C and E were derived by priming with oligonucleotides CEA+NCAF and M, while products in panel D were derived by priming with oligonucleotides flanking the 5′ ATG and 3′ TAA codons of BGPa (START and END). The most obvious bands and their derivation are marked adjacent to lanes. For panel C, the region containing both BGPy and BGPz is marked by an arrow; the DNA marker is identical to that used above. In panel D, full-length BGP coding regions present in the 23.411^+ cell line are shown. In panel E, BGP standards are PCR products from (top to bottom) BGPa, BGPb, and BGPx cDNAs, using oligonucleotides CEA+NCAF and M as primers.

nucleotide deletion at the 5′ end of the IIz exon translates into a considerable amino acid sequence dissimilarity for the first 22 amino acids of each polypeptide, where a two-nucleotide insertion restores the same reading frame as for IIy (Fig. 4B). The BGP IIz exon appears to be derived from the first monomer region of an inverted *Alu*-like sequence (26), while the derivation of the IIy exon from the first or second monomer region is not clear, largely because of their high sequence similarity.

The apparent contribution to topological structure of these two non-Ig-like exons to transmembrane BGPs is not clear. Two Pro residues are juxtaposed in the IIy exon but divide the IIz exon into two regions; however, neither exon contains the extensive homology with the proline-rich Ig hinge

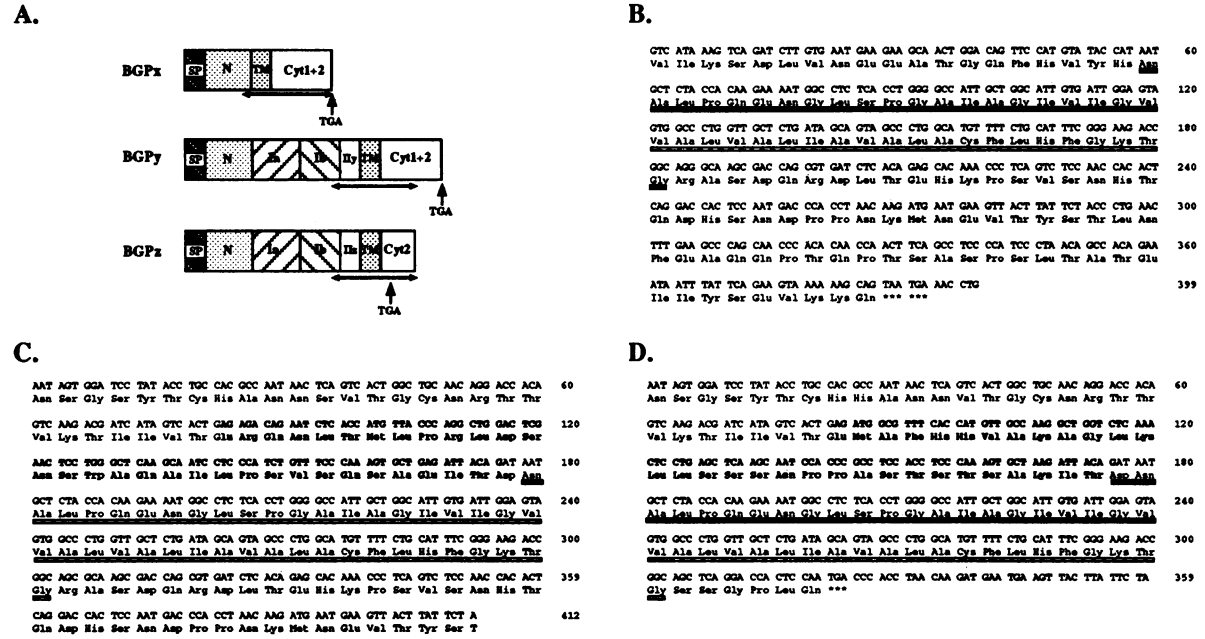

FIG. 3. Diagrammatic organization of domains in BGPx, BGPy, and BGPz based on cDNA and derived amino acid sequences of representative clones. (A) Proposed primary domain structure of BGPx, BGPy, and BGPz. Abbreviations of domains are as described in the legend to Fig. 1. Double-headed arrows define the location of the product derived from PCR amplification of RNA and cDNA and show their inferred domain structure (see Fig. 1). For BGPx (clone X-6.1.PCR), oligonucleotides used are CEA+NCAF and M; for BGPy (clone TM-X2.2) and BGPz (clone 3.3), oligonucleotides used are PCR-1 and PCR-2. TGA shows the site of translation termination. (B to D) Sequences of representative cloned X-6.1.PCR (segment of BGPx; B), TM-X2.2 (segment of BGPy; C) and 3.3 (segment of BGPz; D) cDNAs and translation of their open reading frames. Boldface type shows *Alu*-derived DNA and amino acid sequences; the transmembrane exon is underlined.

regions that has been described for the 65-amino-acid membrane-proximal domain of CD8 (32), for example. On the other hand, each of the 31-amino-acid domains is enriched in Thr/Ser residues (8 of 31 amino acids) or repeats thereof, suggesting the potential for O-linked glycosylation or other posttranslational modifications. Detailed analysis of the cellular expression of BGPy and BGPz polypeptides awaits the use of domain-specific antibodies.

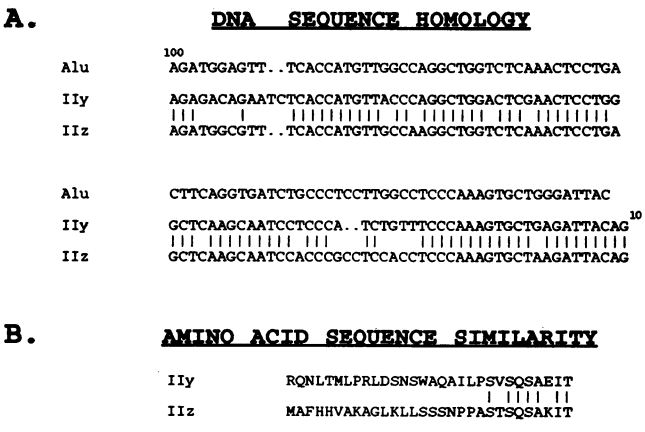

FIG. 4. Sequence relationships of exons IIy and IIz. (A) An inverted DNA sequence from positions 10 to 100 of a cloned *Alu* element (17) is compared with DNA sequences of exons IIy and IIz. Double dots mark the positions of dinucleotide differences among the three sequences. (B) Amino acid translation of DNA sequences from exons IIy and IIz.

By contrast, the finding of spliced exons representing the N-terminal V-like domain proximal to the membrane-spanning and intracellular regions in BGPx is unusual. It is not likely that this product is simply the result of random splicing patterns, since its presence accounts for a significant fraction (~10%) of the five different types of BGP mRNAs detected by the PCR in 23.411^+ cellular RNA; it is also found with various degrees of abundance as two different intracellular splice forms in some, but not all, tumor cell line poly(A)$^+$ RNAs and to a much lesser degree in the normal tissue RNAs that we examined (Fig. 2E). Whether or not specific transcription factors account for the differential appearance of some BGP mRNAs in neoplastic compared with normal cells is unknown, although precedent for this exists in the case of CD44 variants, some of which contribute directly to the metastatic phenotype (16). To our knowledge, this unusual splice form has not been described for other Ig-like integral membrane proteins, in which the disulfide-linked loops likely constitute the interactive portion of the molecule (e.g., I-CAM, CD4, and CD8). Despite the fact that no Cys residues are present in the BGP N-terminal domain (5), its structural features include Ig V-like β-sheet folds and remnants of the Ig-like consensus sequence (C . . . W . . .G.Y.C), but without Cys residues. This finding suggests the potential to form a noncovalent loop-like structure. A second feature of BGPx is the absence of the membrane-proximal Cys residue normally found in BGPa, -b, -c, -d, -y, and -z, making covalent interchain dimer formation unlikely.

Cloning and sequencing of BGP genomic DNA. With the characterization of two novel domains in BGP cDNAs, we wished to determine their organization within genomic DNA and to examine whether other related domains might be

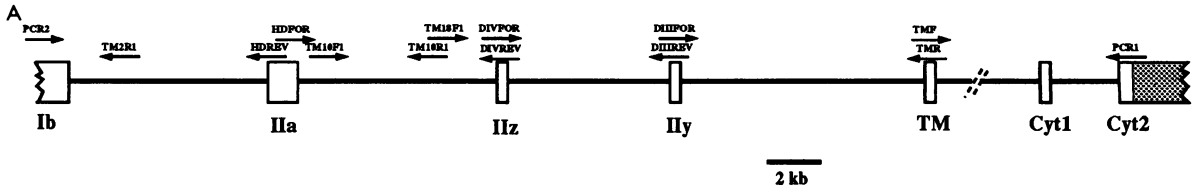

FIG. 5. Organization of alternatively spliced exons in the BGP genomic gene and DNA sequence of the region. (A) Linear organization of BGP-derived exons determined from PCR products of cloned λ39 and λ55 DNAs (5). Ragged ends indicate additional flanking sequences not shown. (B) DNA sequence derived from part of exon Ib and all of exons IIa, IIz, IIy, and TM defined by primers PCR-2 and TMR in panel A. Intron sequences having homology to the $TCTCA_n$ 3′ tail of most *Alu* elements are underlined. Numbers on the right indicate base pairs.

represented within the BGP gene. We previously identified, by exon-specific hybridization to a genomic 23.411$^+$ DNA library, two recombinant bacteriophages that together spanned the region from the Ib exon to the 3′ end of the cDNA (5). These clones, bacteriophages λ11 and λ55, were used as PCR templates with primer sequences shown in Materials and Methods. Our approach used primers PCR-2 and PCR-1 in combination with a specific primer from within domains IIa (HDFOR and HDREV), IIy (DIIIFOR and DIIIREV), and IIz (DIVFOR and DIVREV) or the TM domain (TMF and TMR) to generate specific DNA fragments. Gel analysis of DNA from combinatorial primer amplification provided an overall exon/domain organization of Ib-IIa-IIz-IIy-TM-Cyt (Fig. 5A). Specific PCR products were cloned and sequenced by the exonuclease III method (13a). The entire derived sequence of BGP genomic DNA from exon Ib to the exon coding for the TM domain is shown in Fig. 5B. This sequence confirms the exon organization deduced from direct PCR analysis of genomic DNA and demonstrates that the unusual IIy and IIz domains are bounded by consensus sequences usually found near splice junctions. Extensive computer searching for additional exons related to IIy and IIz within the genomic sequence was largely unsuccessful. Some intron-embedded *Alu*-like sequences yielded very short open reading frames with similarities to exons IIy and IIz but otherwise are not likely to be included as alternatively spliced regions within BGP (data not shown).

Expression of BGP isoantigens. *N*-Glycosidase F treatment of glycosylated BGP polypeptides from 23.411$^+$ cells previously revealed a few discrete polypeptide bands that could be resolved by gel fractionation and detected by immunological means (5). We have not been able to make an assignment of a particular BGP isoantigen to a particular polypeptide detected in 23.411$^+$ BGP transfectant cells based simply on size. The finding of additional spliced RNAs, as described in this report, might account for most, if not all, BGP isoantigens. Since BGP isoantigen-specific antibodies are not available, we used transient expression of discrete BGP cDNAs in COS cells, followed by deglycosylation, to generate individual antigen sizes. Where full-length coding sequences were not available, such as for BGPy and BGPz, cDNAs were reconstituted by replacing the IIa domain with IIy or IIz and eliminating the long 3′ untranslated region (UTR). For BGPx, the upstream N-terminal region was added by cloning the partial segment shown in Fig. 3B into the appropriate sites of BGPa. Full-length coding regions were prepared as *Hin*dIII-*Not*I restriction segment cassettes in the mammalian expression vector pcDNA I NEO.

Western blot detection of BGP polypeptides expressed transiently by COS cells transfected with individual cDNA templates or from stably transfected 23.411$^+$ cells is shown in Fig. 6. In Fig. 6A, the expressed BGP isoantigens appear as high-molecular-weight smears as a result of their extensive N-linked glycosylation. In Fig. 6B, samples were denatured and treated with *N*-glycosidase F. In each case, the isoantigen form can be reduced to a single major band consistent in size with that expected from the primary amino acid sequence of each (BGPa, 58 kDa; BGPb, 47 kDa; BGPc, 51 kDa; BGPd, 40 kDa; BGPy, 50 kDa; BGPz, 43 kDa; and BGPx, 21 kDa). This finding suggest that the major source of heterogeneity seen in untreated samples is likely due to asparagine-linked *N*-acetylglucosamine. Interestingly, BGPy and BGPz do not have the same degree of band sharpness displayed by the other deglycosylated antigens, tempting us to speculate that such heterogeneity could be due to uncleaved *N*-acetylgalactosamine linked via some Ser/Thr-rich residues of the IIy and IIz domains described above. The low-molecular-weight bands detected in some lanes are presumed to be incompletely glycosylated or processed polypeptides, since these bands are present both before and after *N*-glycanase treatment.

We also detected deglycosylated BGP isoantigens prepared from extracts of cells known to produce BGP RNAs (5, 14). From the results of the PCR analysis of BGP transcripts in 23.411$^+$ amplificants (Fig. 2), we expected to resolve bands corresponding at least to BGPa, -b, -c, -y, and -x. While BGPa, -b, and -c of the 23.411$^+$ cell line are similar to those expected from transient transfectants, BGPy is not easily determined and BGPx cannot be detected, possibly because they do not constitute substantial fractions of the protein population as do other forms (Fig. 6B). Examination of other BGP-producing cell lines indicates that BGPa is not the major component of the isoantigen population as it is for 23.411$^+$ cells (Fig. 6B). KG-1 (acute myelogenous leukemia; lane 11), HepG2 (hepatocellular carcinoma; lane 12), COLO 201 (colonic carcinoma ascites fluid; lane 13) cells and normal granulocytes (lanes 14 and 15) display a major BGP size more consistent with that of BGPc than with that of BGPa; normal granulocytes also display some BGP antigens that are more similar in size to BGPy and -z or may represent novel or modified antigen forms.

DISCUSSION

We have previously characterized three different members of the BGP antigen family and provided evidence for a fourth (5). The work presented in this study confirms the proposed organization of these four members and extends the size of the family to include two new members with novel non-Ig-like extracellular domains and two additional forms that lack C2-like disulfide-linked loops but that are characterized by single V-like extracellular domains. In each case, attachment

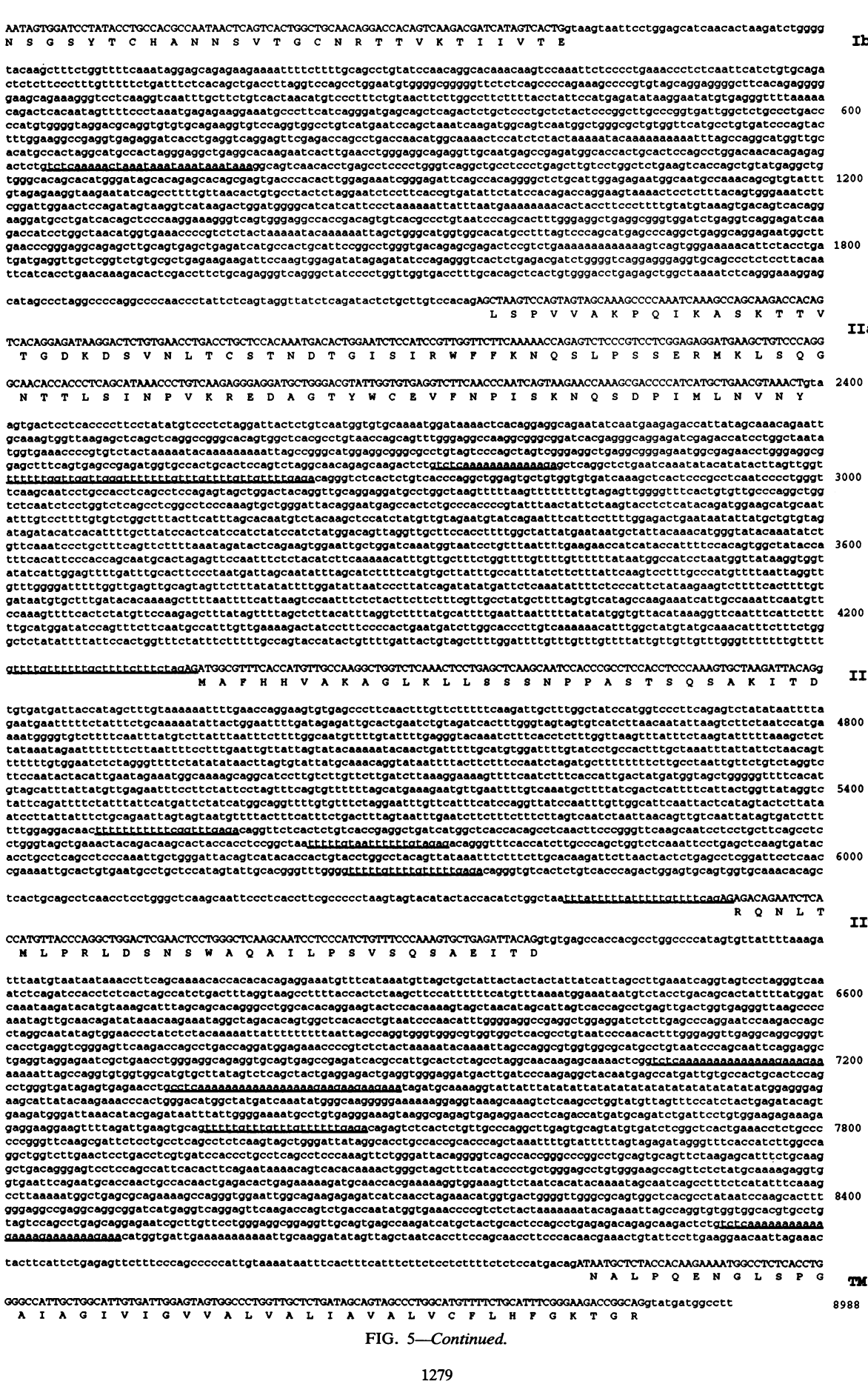

FIG. 5—*Continued.*

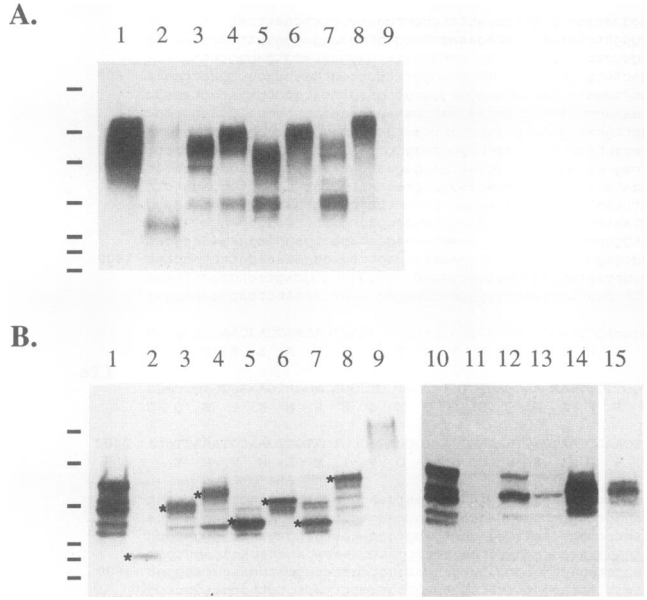

FIG. 6. Western immunoblot analysis of BGP isoantigens expressed by COS cells transfected with specific cDNAs or from cell lines. Lysates from COS7 cells expressing BGP antigens for 72 h posttransfection or from cell lines were either untreated (A) or treated with *N*-glycanase (B), analyzed on 4 to 12% gradient gels, and detected on Western blots by a DAKO polyclonal antibody (see Materials and Methods). Transfected samples: lanes 1 and 10, 23.411$^+$ cells; lane 2, BGPx; lane 3, BGPz; lane 4, BGPy; lane 5, BGPd; lane 6, BGPc; lane 7, BGPb; lane 8, BGPa; lane 9, vector pcDNA I only. Nontransfected cell lines: lane 11, KG-1; lane 12, HepG2; lane 13, COLO 201; lanes 14 and 15 (shorter exposure of lane 14), normal granulocytes. In panel B, the expected primary polypeptide sizes after deglycosylation are indicated by asterisks (BGPa, 58 kDa; BGPb, 47 kDa; BGPc, 51 kDa; BGPd, 40 kDA; BGPy, 50 kDa; BGPz, 43 kDa; and BGPx, 21 kDa). Lines on the left side of each panel represent the apparent molecular sizes of prestained protein markers (GIBCO/BRL): myosin (221 kDa; panel A only), phosphorylase *b* (105 kDa), bovine serum albumin (75 kDa), ovalbumin (46 kDa), carbonic anhydrase (28 kDa), β-lactoglobulin (19 kDa), and lysozyme (16 kDa). The nature of the additional lower-molecular-weight bands detected in panel B is not known.

to the cell membrane appears to occur via one of two possible intracellular regions, similar to that described for Ig superfamily member CD8 (32). On the basis of analyses of both cDNAs and the genomic gene, we suggest that the BGP family size encompasses the eight isoantigen forms reported here.

The in vivo function of human BGP, or any other CEA-related antigen, is unknown. The most suggestive indication that BGP functions as a homotypic cell adhesion molecule comes from studies on the glycoprotein analog of human BGPa, cell-CAM 105, isolated directly from rat hepatocytes (22a) and equivalent to rat hepatic ecto-ATPase (2, 18). Several in vitro studies using cells transfected with BGPa and BGPb cDNAs suggest that, like CEA and NCA, the BGPs function as class-specific homotypic adhesion proteins (23). The interesting feature of the latter work derives from the Ca^{2+}- and temperature-dependent adhesion of BGP isoforms, not displayed by CEA or NCA but similar to that described for other integral membrane adhesion proteins, like E-cadherin (35) and PECAM-1 (1a). This dependency may be related to cytoplasmic domain-mediated signal transduction that is different from the signalling by extracellular molecules linked via phosphoinositol glycan moieties, like CEA and NCA. These adhesion dependencies are also displayed by a cell line, COLO 201, derived from the ascites fluid of a patient with colonic adenocarcinoma (23). A related cell line, COLO 205, that is derived from the same patient as is COLO 201 does not demonstrate these adhesive properties even though both lines produce identical patterns of BGP isoform transcripts (Fig. 2C and unpublished data). Whether or not the additional expression of CEA by COLO 205 cells negatively influences their ability to adhere in vitro is not known, but we suspect that factors other than BGP alone may be required to mediate aggregation.

The in vitro adhesion studies have been extended to include integral membrane proteins expressed by BGPx and BGPy transfectants (31), and their adhesion patterns are similar to those of BGPa and BGPb. This finding suggests that the in vitro adhesive properties of these molecules minimally derive from the N-terminal domain (since BGPx has only an N-terminal domain) and are independent of the presence or absence of extracellular domains immediately distal to the N terminus. We interpret these results to indicate that the different BGP isoforms probably have additional and diverse in vivo functions that are directly influenced by domains IIa, IIy, and IIz.

Recently, Williams et al. (38) and Yokomori and Lai (39) have shown that a murine analog of human BGP acts as a liver receptor for mouse hepatitis virus, a member of the *Coronaviridae* family. Even if human BGP isoantigens, like several other Ig superfamily members, are viral receptors (13, 20), this is probably a usurped function. BGP transcripts are present in a wide variety of cell sources, from human liver to placenta. The occurrence of multiple protein forms with specific extracellular regions (domains IIa, IIy, and IIz) bearing distinct cytoplasmic domains that are phosphorylated Tyr (1, 5) suggests that these molecules are coupled to functions involving ligand recognition and signal transduction. The detection of short, 31-amino-acid non-Ig-like domains present as alternatively spliced protein domains within the transmembrane forms of the CEA family suggests that there are additional functions for these molecules that may reflect their ability to bind selectively to as yet unidentified cellular targets. The recent description of two different fibroblast growth factor binding specificities determined by two alternative splices involving a short extracellular loop domain of the fibroblast growth factor receptor gene (22) substantiates the possibility that subtle differences detected in BGP extracellular domains could translate into distinct ligand binding properties. Likewise, the finding that small differences in exon selection by the hyaluronate receptor, CD44, can result in either a metastatic or nonmetastatic cellular phenotype is intriguing (16). Preliminary evidence (3) suggests that the extracellular domains of BGPa fused to human IgG1 Fc recognize a non-CEA-related membrane-bound molecule on some cell types, while a similar BGPb fusion (lacking the IIa domain) does not.

It is of interest that *Alu*-like sequences are incorporated into RNA and translated as protein. In the case of soluble decay-accelerating factor, the C-terminal domain is derived by translation of an unspliced *Alu*-containing intron (9). Similar short sequences (~32 to 50 amino acids) translated from *Alu*-related elements have also been described as extracellular domains of human complement component 5a (19), human B-cell growth factor and interleukin-1β (27), and the human *rel* proto-oncogene (8). We believe that BGPy and BGPz may be among the very few examples in which splicing selectively includes *Alu*-like exons into mature

translatable RNA, rather than being derived from an incompletely spliced, *Alu*-embedded intron.

The latter observation could easily account for the recent identification by Kuroki et al. (16) of several unusual BGP molecules which they isolated from a human leukocyte cDNA library. Comparison of our DNA genomic sequence (Fig. 5B) with the regions surrounding the novel splice junctions that they present suggests that their cDNAs are derived more likely from incompletely spliced RNA transcripts rather than from processed RNA, a situation that we have also found and which has been described for nerve growth factor receptor RNA (2a). Our conclusion is based on several pieces of evidence. While their BGP-like cDNA clone, W233, has a sequence identical to that of BGPa for 1,053 nucleotides and 260 amino acids, the adjacent new amino acid and 3′ UTR sequences in their clone can be accounted for by translational read-through of a bona fide exon into adjoining intron sequence (compare our genomic DNA sequence positions 83 to 813 in Fig. 5 with the C-terminal and 3′ UTR sequences of W233 cDNA presented in Fig. 1 of reference 16). In addition, the 3′ poly(A) tail of clone W233 coincides exactly at the probable initiation site of oligo(dT) priming for reverse transcription (our sequence position 813). Likewise, cDNA clone W211 can be accounted for by read-through into the unspliced *Alu*-embedded intron starting at our sequence position 2398 and terminating within an *Alu*-like element at position 2958. Because all of their cDNAs end within a dA-rich region often derived from *Alu* elements, they lack the expected consensus splice junction signals. Kuroki et al. (16) have not detected W233, W239, or W211 RNA or polypeptides in leukocyte extracts. While their cloned DNAs could be expressed as polypeptides in vitro, the corresponding translation products would nonetheless be artifactually derived. For evaluation of such unusual cDNA forms, comparison with genomic DNA is essential.

The novel forms of BGP mRNA described here are not limited to cells that have undergone transformation, since normal tissues also display these transcripts, albeit at lower abundance (Fig. 2). Whether this lower abundance reflects the presence of a small number of BGPx, -y, or -z molecules on all cells or a larger number on a defined subset of cells, perhaps related to the degree of cellular differentiation or other cellular state, is not yet known. In addition, there is no obvious correlation between tissue type and expression of BGP isoforms, although it appears that cells do display selectivity for one or another set of BGP mRNAs. An associated correlation between expression of BGPa and -c RNAs and between BGPb and -d RNAs in both normal and transformed cells could be functionally significant and could imply, for example, unusual heterotypic associations between BGP molecules that have otherwise identical extracellular domains but distinct intracellular domains (like BGPa and -c). Functionally important heterotypic interactions are common features of several Ig family members (e.g., CD2-LFA-3 [21], CD8-CD1 [30], and CD3-TCR [7]).

It is clear from the work presented here that there is differential expression of BGP antigen forms on cells (for example, 23.411$^+$ versus HepG2; Fig. 6). On the basis of size, it appears that some BGP forms with the short (7-amino-acid) intracellular domain, like BGPc, may predominate on cells compared with those with long (77-amino-acid) intracellular domains. This view supports the observations of Svenberg et al. (34), who showed that washing human liver sections with saline prior to fixation largely reduced BGP immunodetection, suggesting reduced anchoring of the short form on cells. The functional significance of long (Cyt1/Cyt2) versus short (Cyt1) intracellular domains remains elusive. The specific juxtaposition by splicing of domain IIy with the Cyt1/Cyt2 domain and domain IIz with the Cyt2 domain (Fig. 3) may be functionally significant, while for some molecules, like BGPx, the lack of disulfide-linked loop domains altogether suggests specific functions attributed to certain domains (4). The diversity of topological architecture likely imparted by inclusion or exclusion of specific extracellular domains (IIa, IIy, and IIz) suggests that there are multiple cellular targets for interaction with the BGP antigen repertoire.

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