Investigation of the mechanism of tumor inhibition by biliary glycoprotein 1

by

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Abstract

Biliary glycoproteins (BGPs), members of the carcinoembryonic antigen (CEA) family, have been observed to be down-regulated at the mRNA and protein levels in a number of epithelial malignancies. To investigate the potential tumor suppressive role of BGP, two murine *Bgp1* cDNA isoforms were introduced into a mouse colonic carcinoma cell line. Transfected cells bearing either Bgp1 isoform exhibited a reduced growth rate in culture and a reduced ability to form colonies in soft agar. However, when cells transfected with the Bgp1 isoform bearing the longer cytoplasmic domain (73 amino acids) were injected into syngeneic mice, tumor formation was inhibited by 80%. Transfectant cells expressing the Bgp1 isoform with the shorter cytoplasmic domain (10 amino acids) did not inhibit tumor formation. Since the long cytoplasmic domain appeared to be mediating the tumor inhibition phenotype *in vivo*, the identification of cytoplasmic binding proteins was pursued. SHP-1 (SH2-containing protein tyrosine phosphatase 1) was identified to bind the long, but not the short, cytoplasmic domain of Bgp1. This binding was dependent on tyrosine phosphorylation of Bgp1: mutation of one tyrosine residue or the other, within the cytoplasmic tail, abrogated SHP-1 binding. The presence of either SH2 domain of SHP-1 was sufficient to mediate binding to Bgp1 *in vitro*. The significance of SHP-1 association to Bgp1 *in vivo* has yet to be formally addressed.

List of Abbreviations

aa	amino acid(s)
Ab	antibody
ADNc	acide désoxyribonucléique complémentaire
Ala	alanine
Arg	arginine
ARNm	acide ribonucléique messager
ATP	adenosine triphosphate
BCR	B cell receptor
BGP	biliary glycoprotein
Bgp1	mouse biliary glycoprotein 1
Bgp2	mouse biliary glycoprotein 2
BHK	baby hamster kidney
bp	base pair
cAMP	cyclic adenosine monophosphate
CBP	calcium binding protein
C-CAM	cell-cell adhesion molecule
cDNA	complementary deoxyribonucleic acid
CEA	carcinoembryonic antigen
CGM	CEA gene family member
CHO	Chinese hamster ovary
CSF-1	colony-stimulating factor 1
dpc	days *post coitum*
DPP IV	dipeptidyl peptidase IV
EC	endothelial cell
ELAM-1	endothelial leucocyte adhesion molecule-1
EPO	erythropoietin
EPO-R	erythropoietin receptor
fMLP	formly-methionine-leucine-phenylalanine
Gly	glycine

Chapter 1

Introduction

I. Discovery of CEA and BGP

Tumor-specific antigens provide significant clinical tools for identifying and treating malignant cancers. In an attempt to isolate such antigens, Gold and Freedman raised antisera to human colonic carcinoma cells that did not recognize antigens from normal colonic tissues (28). They subsequently demonstrated the presence of these antigenic determinants in carcinoma of the esophagus, stomach, duodenum, pancreas, and rectum. Identical antigens were also found in human embryonic tissues such as, gut, liver, and pancreas (29). Hence, these antigens were termed carcinoembryonic antigens. Despite the subsequent identification of carcinoembryonic antigen (CEA) in normal plasma (13) and normal adult colonic mucosa (26), it continues to be a significant clinical tool for monitoring patients with colorectal cancer and other tumors of epithelial origin, such as breast, lung, and ovarian (reviewed in 99, 98, 83).

Shortly after the initial discovery of CEA, it became apparent that many cross-reacting antigens existed in both cancerous and normal tissues. Although a few of the antigens turned out to be degradation products of CEA itself, most of them were distinct, but related protein products (reviewed in 83). One such distinct antigen was isolated from human bile by Svenberg (94). The isolated species, biliary glycoprotein I (BGP I), exhibited unique immunological determinants not found in CEA and differed from CEA in several physicochemical properties, such as electrophoretic mobility and migration through a gel filtration column (94). The discovery of other CEA-related antigens, such as, non-specific cross-reacting antigen (NCA), tumor-extracted antigen (TEX), normal

fecal antigen (NFA), and others are described in a review by Shively and Beatty (83).

II. Cloning of human CEA, BGP, and related genes

To definitively characterize CEA and its related antigens, it was necessary to identify the genes encoding for these protein products. CEA was eventually cloned in 1987 by several groups (5, 70, 110). The authenticity of the cDNA clones was confirmed by comparison with amino acid sequence data available for CEA (72). CEA is synthesized as a 702 amino acid (aa) precursor protein. The N-terminal 34 aa comprise a leader sequence, while the C-terminal 26 aa are strongly hydrophobic and were thought to constitute a transmembrane domain. It was later determined that this hydrophobic sequence was actually a signal for glycosyl-phosphatidylinositol (GPI) anchorage to the plasma membrane (30). Hence, the precursor protein of 702 aa is reduced to a mature protein of 642 aa with a predicted molecular weight of 73 kDa. The observed molecular weight of CEA is 180 kDa. This discrepancy is due to the extensive glycosylation of CEA; in fact, 28 N-linked glycosylation sites were identified from the predicted CEA amino acid sequence (5).

CEA was immediately recognized to be a member of the immunoglobulin supergene family (106). The N-domain of 108 aa resembles an immunoglobulin variable (Ig V) domain. The rest of the protein consists of 6 repeats that resemble immunoglobulin C2-set (Ig C2-set) domains. Each repeat contains a pair of invariant cysteine residues that are postulated to form disulfide bridges (5).

NCA was simultaneously cloned by Thompson *et al.* (100). This gene is also a member of the immunoglobulin supergene family. It consists of one Ig V domain and only two Ig C2-set domains (64); it is also bound to the plasma membrane by means of a GPI-anchor (41).

The third CEA-related antigen to be cloned was BGP I (32). Similar to CEA and NCA, the BGP I protein consisted of a 34 aa leader peptide and a 108 aa N-terminal Ig V domain. The N-terminal domain was followed by three Ig C2-set domains. In contrast to CEA and NCA, BGP I was predicted to have a transmembrane domain followed by a cytoplasmic domain. BGP I is also highly glycosylated with 20 N-linked glycosylation sites identified from the predicted amino acid sequence (32). The *BGP I* gene was later shown to undergo extensive alternative splicing to give rise to 12 different transcripts (3, 4). Most notably, the transcripts differ in the number of Ig C2-set domains and in the length of the intracytoplasmic domain. The inclusion of a 53 bp-exon after the transmembrane domain exon causes a shift in the reading frame and consequently increases the length of the cytoplasmic tail from 10 aa to 71 aa (4). Since additional BGP-like genes were not found in the human genome, the *BGP I* gene was renamed simply *BGP*.

The CEA gene family continued to expand at a rapid pace (37, reviewed in 99). It became apparent that the CEA family could be divided into two subfamilies; the CEA-like subfamily and the PSG-like subfamily. The pregnancy-specific glycoproteins (PSGs) emerged as a distinct subgroup within the CEA gene family (38). The PSGs could be distinguished from the other CEA-like family members based on sequence homology and

on patterns of tissue expression. There is 80%-95% sequence identity of the N-domain exons within the CEA-like or PSG-like subgroups, but this conservation drops significantly to 65%-75% identity when the two subgroups are compared to each other (99). In addition, the PSGs are mainly expressed in placental trophoblasts during pregnancy (44), while the CEA-like genes have a wide range of temporal and spatial tissue expression (reviewed in 101). Another distinguishing feature of the PSGs is their lack of membrane anchorage; PSGs are secreted, while most CEA-like family members are membrane-bound (99).

To date, 29 CEA gene family members have been identified (71, 96). They have all been mapped to a 1.3 Mb region on the long arm of chromosome 19; 19q13.2 (103, 9, 96, 71). The 29 genes consist of 11 active PSG-like genes, 12 CEA-like genes (7 are transcriptionally active), and a third subgroup of 6 pseudogenes that lack N-terminal Ig V exons (96). The 7 active CEA-like genes can be further subdivided into members that are GPI-linked (*CEA*, *NCA*, *CGM2*, and *CGM6*; CGM = CEA gene family member) and members that possess transmembrane and cytoplasmic domains (*BGP*, *CGM1*, and *CGM7*) (71). Interestingly, the *CGM1* gene also contains an alternatively spliced 53 bp-exon that gives rise either to a short (31 aa) or long (71 aa) cytoplasmic tail (57).

It is worth noting that many of the CEA-like gene products have been identified to be members of the cluster of differentiation 66 (CD66) (105, 88). The nomenclature in this field is as follows: CD66a = BGP, CD66b = CGM6, CD66c = NCA, CD66d = CGM1, and CD66e = CEA. Each of the above CD66 antigens is expressed in granulocyte lineages, except CD66e (CEA).

III. Cloning of the mouse and rat BGP homologs

Establishing a rodent model to investigate the *in vivo* function of CEA and its role in carcinogenesis required the identification of CEA-like genes in the mouse and the rat. A mouse homolog of the human *CEA* gene was identified by screening a CD-1 mouse colon cDNA library with a human *CEA* cDNA probe under low stringency conditions (6). Further characterization of the gene revealed that outbred CD-1 mice possess two allelic variants of the murine CEA-like gene, differing in the N-terminal domain (53), and that the gene can undergo alternative splicing to produce four different transcripts per allele (52). Analysis of the deduced amino acid sequence predicted that the protein product possessed a transmembrane domain with a cytoplasmic tail and 16 potential N-linked glycosylation sites in the extracellular domain (53). In addition, the murine sequence exhibited higher homology to the human *BGP* gene than to the human *CEA* gene. The mode of membrane-anchorage and sequence homology indicated that the murine CEA-like gene was actually a *BGP* homolog; hence, the mouse gene was eventually named *Bgp1* and the two alleles of the CD-1 mouse were referred to as $Bgp1^a$ and $Bgp1^b$.

The *Bgp1* gene was extensively characterized in the inbred BALB/c mouse at the genomic level (62). This gene was virtually identical to the $Bgp1^a$ allele of the CD-1 mouse. The exon-intron structure of *Bgp1* was perfectly conserved with the human *BGP* gene (3). The *Bgp1* gene consists of 9 exons and 8 introns (Figure 1 (a), p.8). The exons roughly divide the gene into the respective domains of the protein product. The Ig C2-set exons A1 and B1 are alternatively spliced as a unit to give rise to two extracellular

domain isotypes (Figure 1, p.8): (i) one Ig V domain and one Ig C2-set domain (BgpC and BgpG) or (ii) one Ig V domain and three Ig C2-set domains (BgpA and BgpD). The 53 bp-exon 7 is also alternatively spliced to give rise to a short (10 aa) or long (73 aa) cytoplasmic tail (*c.f.* human *BGP* and *CGM1* genes). The combination of these two variable splicing events results in a total of four transcripts (Figure 1 (b), p.8). The $Bgp1^b$ allele also undergoes the same splicing events that result in four transcripts of its own (52).

A second BGP-like gene was isolated from the BALB/c mouse, *Bgp2* (61). Although the exon-intron structure was very similar to the *Bgp1* gene, alternative splicing has yet to be observed for *Bgp2*. The only observed *Bgp2* transcript corresponded to the BgpC splice variant of the *Bgp1* gene (see Figure 1 (b), p.8). The A1 and B1 exons were identified in the *Bgp2* gene at the genomic level, but they do not appear to be used. The conserved 53 bp-exon 7 is also present, but long-tail *Bgp2* cDNAs have not been isolated to date. In addition to the apparent lack of alternative splicing, the *Bgp2* gene differs most markedly from the *Bgp1* gene in the amino acid sequence of the entire N domain and in the middle third of the A2 domain (61).

The mouse also has several PSG homologs (80). To date, eleven murine CEA-related genes have been reported in the literature; 4 genes in the CEA-like subgroup and 7 genes in the PSG-like subgroup. This is considerably less than the 29 genes identified for this family in *Homo sapiens* (71, 96). The *Bgp1* gene has been mapped to the proximal region of mouse chromosome 7 which is syntenic to the part of human chromosome 19 containing the human CEA gene family cluster (77). Other murine CEA

Figure 1 (a)

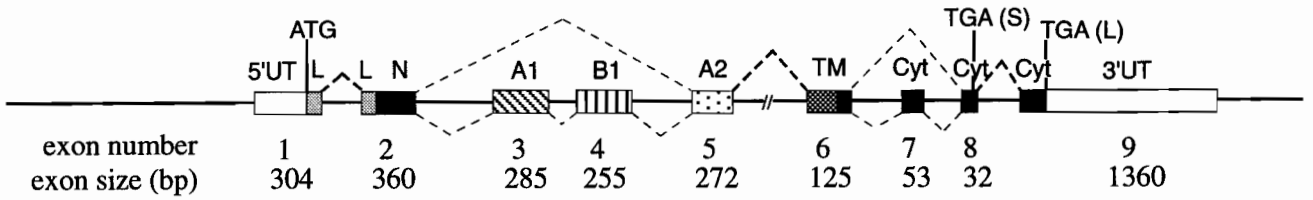

Figure 1 (b)

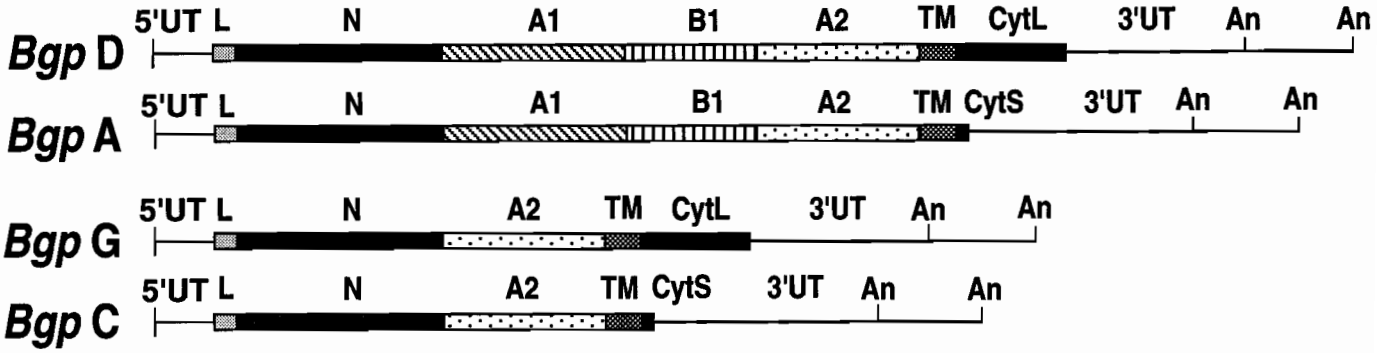

Figure 1: (a) **Genomic exon-intron structure of the mouse *Bgp1* gene.** Exons are indicated by boxes. The size and number of each exon is indicated. Exons 3 and 4 (A1 & B1) are alternatively spliced as a unit to give rise to two different extracellular domains. Exon 7 (53 bp Cyt) is also alternatively spliced to give rise to either a short (CytS) or long (CytL) cytoplasmic domain. Alternative and constitutive splicing events are denoted by thin and thick dashed lines, respectively. L, leader sequence; N, N-terminal IgV-like domain; A1, B1, A2, IgC2-set-like domains; TM, transmembrane domain; Cyt, cytoplasmic domain; 5'UT, 3'UT, 5' and 3' untranslated regions, respectively; ATG, start codon; TGA(S), TGA(L), stop codons used to generate the short and long cytoplasmic domains, respectively. (b) ***Bgp1* transcripts.** BgpD, BgpA, BgpG, and BgpC represent the four different transcripts produced from alternative splicing *in vivo*. CytL, long cytoplasmic tail (73aa); CytS, short cytoplasmic tail (10aa); An, polyadenylation signal.

Adapted from: Nédellec *et. al.* (1995) *Eur. J. Biochem.* **231**, 107

family members have also been mapped to this region, suggesting that clustering is also occurring in the mouse (80). The presence of more than twice as many CEA family members in the human than in the mouse genome and the fact that these genes are tightly linked suggests that this family may have arisen by gene duplication. In addition, the apparent lack of GPI-linked CEA family members in mice and their presence in humans suggests that GPI-linkage was a recent evolutionary event (91).

The most recently characterized murine CEA-like gene, *cea10*, exhibits a unique domain organization (36). It is composed of a leader sequence followed by two Ig V domains separated by a half-leader sequence; it lacks Ig C2-set domains. In addition, there is a lack of a membrane-anchorage sequence and hence, the protein product is secreted. In this respect, *cea10* appears to be a member of the PSG-like subfamily. However, sequence analysis of the two Ig V domains and the observed expression patterns of *cea10* actually place it in the CEA-like subfamily (36).

The *BGP* homolog in rat was cloned through an attempt to characterize the rat liver plasma membrane ecto-ATPase (48). Further characterization of the gene revealed it also conserved the exon-intron structure of the human *BGP* gene (58). Although the rat *BGP* homolog has carried many names (pp120, HA4, and ecto-ATPase), it is now referred to as cell-cell adhesion molecule (C-CAM). Two variants of *C-CAM* were identified, *C-CAM1* and *C-CAM2* (16). They differed at 16 aa residues in the N-terminal Ig V domain and were both shown to undergo alternative splicing of a 53 bp-exon to give rise to two isoforms that differ in the length of their cytoplasmic domains (10 aa versus 71 aa) (50, 22, 58). The respective long and short tail isoforms are referred to as C-

CAM1a and C-CAM1b, and C-CAM2a and C-CAM2b. Whether *C-CAM1* and *C-CAM2* represent different alleles (*c.f.* $Bgp1^a$ and $Bgp1^b$) or different genes has yet to be unambiguously resolved. Alternative splicing of the internal Ig C2-set domains of *C-CAM* was not observed, as it was for the mouse *Bgp1* gene (52). The conservation of alternative splicing mechanisms for the 53 bp-exon 7 (in human, mouse, and rat) suggests it may be of functional importance.

IV. Expression patterns of BGP in normal tissues

BGP is widely expressed in many normal tissues. In particular, BGP can be found in many cells of epithelial, endothelial, and myeloid origin (reviewed in 99, 56). Since BGP was first identified in hepatic bile (94), it is not surprising that this protein is highly expressed in human (31), rat (66), and mouse (43) hepatocytes. More specifically, BGP was localized to the canalicular (apical) surface of hepatocytes (43). BGP was also found to be apically expressed in epithelia of the colon and small intestine (74, 27, 43). Detailed analysis of BGP expression in normal colonic mucosa revealed that the protein was expressed on the microvilli of columnar epithelial cells located in the upper third of colonic crypts and in the free lumen (25). The less differentiated columnar cells of the lower two-thirds of colonic crypts do not express BGP protein or mRNA (25). Characteristic apical staining of BGP was also observed in epithelia of the esophageal glands, gallbladder, renal proximal tubules, prostate, pancreatic and mammary ducts, the endometrium, and others (74, 27, 67).

BGP expression was observed in endothelial cells of many tissues. Glomerular vessels, arterioles, and venules in the cortical and medullary regions of the kidney were positive for BGP (74, 27). Blood vessels of the placenta, endometrium, prostate, and pancreas also expressed BGP (74, 27, 67). Large veins and arteries were BGP-negative (67). Connective tissues, testes, distal and collecting tubes of the kidney, peripheral nerves, and neuronal and glial cells of the central nervous system also lacked BGP expression (27, 67).

Several cell types of the myeloid lineage express BGP; they include granulocytes, megakaryocytes, macrophages, and B lymphocytes (74, 15, 67). Purified platelets also exhibited detectable levels of BGP which were up-regulated upon platelet activation and aggregation (67). Resting T lymphocytes (15), resting natural killer (NK) cells (54), and erythrocytes (67) were BGP-negative. However, stimulated T cells and a sub-population of NK cells exhibited an up-regulation and/or *de novo* expression of BGP (54).

The highly promiscuous expression of BGP in various adult tissues is not immediately suggestive of a single particular function for this protein. The possibility of BGP undertaking different functions in different tissues is interesting and will be addressed later.

The expression of Bgp1 during mouse embryogenesis has been well documented by *in situ* hybridization (34, 17) and immunostaining techniques (17). The first embryonic tissues to express Bgp1 were the primitive gut epithelium and the surface ectoderm at 8.5-10.5 days *post coitum* (dpc). Early mesenchyme did not exhibit Bgp1 expression until periods of active mesenchymal-epithelial interactions (15.5 dpc); positive

staining was observed in mesenchymal cells of the meninges, dermis, lung, pancreas, and salivary glands (17). Hepatocytes began to express Bgp1 in the precursor bile canaliculi at 18.5 dpc. Interestingly, a Bgp1 epitope recognized by one antibody, but not others, was observed during active myogenesis. The expression was restricted to secondary myotube formation at sites of terminal differentiation (13.5 dpc - 17.5 dpc) (17). This is yet another example of the presence of BGP in terminally-differentiated cells (*c.f.* the columnar epithelial cells of upper colonic crypts (25) and the suprabasal cells of stratified squamous epithelia (67)). Bgp1 expression also appeared in developing craniofacial tissues starting at 16 dpc and peaking at 18 dpc. The epithelia of the oral cavity exhibited higher Bgp1 expression than the epithelia of the nasal cavity (75). The dynamic and highly specific expression of Bgp1 in many embryonic tissues is suggestive of an important, but as yet undefined role during embryogenesis.

V. Down-regulation of BGP in various cancers

Since CEA was discovered by virtue of its overexpression in colorectal carcinomas (28), it was of interest to investigate if other members of the CEA family also exhibited alterations of expression during carcinogenesis. Before any of the CEA family members were cloned, alterations in their protein expression in malignancy was detected by immunochemical means. The expression of the rat BGP homolog, C-CAM1 (previously named cell-CAM 105), was investigated in 13 transplantable hepatocellular carcinomas (33). When compared to normal rat hepatocytes, all hepatocellular carcinoma lines

exhibited a complete loss of two acidic glycoproteins that were reactive to anti-C-CAM1 antiserum (33).

Once the *BGP* genes from various species were cloned (32, 6, 48), both the mRNA and protein levels in malignant *versus* normal tissues could be investigated. Neumaier *et al.* observed a down-regulation of human *BGP* mRNA in 21 colorectal carcinoma tissues specimens. The *BGP* mRNA levels were reduced to 20% of the BGP expression in adjacent normal mucosae (63). Mouse Bgp1 expression was also shown to be down-regulated in malignant tissues at the protein and mRNA levels (79). Primary adenocarcinomas from CD-1 mice exhibited a reduction in *Bgp1* mRNA by Northern analysis and a loss of Bgp1 protein expression as shown by immunochemical staining of normal and malignant colon sections (79). Several colon carcinoma cell lines and a transformed liver cell line also lacked Bgp1 expression; however, a rectal carcinoma cell line, CMT-93, retained Bgp1 expression (79). Prostate carcinomas also exhibited a down-regulation of BGP expression in comparison to normal prostate epithelia (40). Twenty-two moderately and poorly differentiated human prostate tumors were completely BGP-negative (40).

By contrast, Ohwada *et al.* observed an increase in *BGP* mRNA in primary and metastatic lung cancer cells when compared to normal adjacent lung tissue (68). Another report also demonstrated an increased expression of BGP in lung carcinoma and in several colorectal carcinoma cell lines (39). Finally, Hinoda *et al.* could not observe any difference in *BGP* transcription in malignant *versus* normal human liver tissues (31). These conflicting reports might be explained by differences in assay procedures, different

tissue types (i.e. liver *vs* colon carcinoma), or perhaps differences in the stage or advancement of the cancer. In fact, a study by Ilantzis *et al.* (35) revealed a two-fold reduction in total BGP in well-differentiated tumors, while late stage tumors exhibited a two-fold increase in BGP levels.

Although the precise mechanism of BGP down-regulation during malignant transformation is not known, some evidence suggests that DNA methylation may be playing a role. The upstream region of the mouse *Bgp1* gene is less methylated in several colon carcinoma cell lines when compared to that of normal colon DNA (79). Moreover, Bgp1 down-regulation appears to be an early event in carcinogenesis since, (i) early stage mouse colonic tumors (equivalent to stage A human colonic tumors) already experienced a loss of Bgp1 expression (79), and (ii) Kleinerman *et al.* observed a loss of BGP expression in the well-differentiated foci within moderately-differentiated human prostate tumors (40). The observed down-regulation of BGP in many epithelial malignancies warrants further investigation of a possible tumor-suppressive function of this protein.

The overexpression of CEA and NCA in colorectal cancer (14, 35) is in direct contrast to the observed down-regulation of BGP (33, 63, 40, 79). Since CEA and NCA are GPI-linked proteins and BGP and its splice variants are transmembrane-linked proteins, it has been postulated that their mode of membrane-anchorage may be a determining factor in the observed differences in expression during cancer progression (90). This hypothesis was also based on other observed differences between BGP and CEA, such as the different mechanisms of adhesion and the opposite effects on myogenic differentiation (reviewed in 90). The expression patterns of a recently identified GPI-

linked CEA family member, CGM2, appears to contradict the above hypothesis. Thompson *et al.* observed an 80%-90% reduction in *CGM2* mRNA levels in 11 colonic adenocarcinomas when compared to normal adjacent mucosae (102). In addition, they found that 60% of 71 breast tumors examined were negative for CGM2 expression. The authors suggest that CGM2, like BGP, may be playing a possible tumor suppressor role in colorectal cancer (102). The up-regulation and down-regulation of various CEA gene family members during carcinogenesis cannot be predicted in a simple manner. Investigations to determine if these alterations in expression are functional or consequential will provide insight into the mechanisms giving rise to epithelial malignancies.

VI. Adhesion properties of BGP

The first function ascribed to any member of the CEA gene family was that of intercellular adhesion *in vitro*. Ocklind *et al.* (66) demonstrated that a specific 105 kDa glycoprotein was responsible for initiating the adhesion process between rat hepatocytes. They also showed that antibodies specific to the 105 kDa glycoprotein could effectively inhibit hepatocyte aggregation *in vitro* (66). The protein was named cell-cell adhesion molecule 105 (cell-CAM 105) and later renamed C-CAM1 (the rat BGP homolog). Structural and functional analysis of the rat BGP protein revealed which domains were important for adhesion. Mutational analysis of BGP demonstrated that the N-terminal Ig V domain was necessary and sufficient to mediate intercellular adhesion when transfected

into Sf9 insect cells (12). Moreover, the naturally-occurring short isoform of rat BGP, possessing a 10 aa-cytoplasmic tail, was able to support adhesion when introduced into Chinese hamster ovary (CHO) cells (50). In addition, deletion of the majority of the long cytoplasmic domain of BGP to 10 aa also did not affect intercellular adhesion (49). Interestingly, expression of an anomalous rat BGP isoform (C-CAM3) in Sf9 cells did not support intercellular adhesion (11). C-CAM3 was encoded by a transcript that contained an unspliced intron; the protein product had a cytoplasmic domain of only 6 aa (11).

Adhesion studies on human BGP corroborate the observations from rat BGP. Human BGP functioned as an *in vitro* adhesion molecule when transfected into CHO cells or a CHO-variant cell line, LR-73 (69, 78). Two studies found the adhesion properties to be dependent on cations (Ca^{2+} or Mg^{2+}) and physiological temperatures (78, 97), while another study found the adhesion activity to be completely Ca^{2+}-independent (69). This discrepancy may be due to different levels of BGP expression in the cell lines used for the adhesion assays or differences in assay procedures. Indeed, Rojas *et al.* (78) observed that cells expressing more BGP were able to form more aggregates, while cells expressing less BGP formed fewer and smaller aggregates. However, alterations in calcium dependency of the high and low-expressing transfectants were not investigated. In concordance with rat BGP, domain analysis of human BGP revealed that the N-terminal domain was essential for homotypic binding, while the long-intracytoplasmic tail was dispensable (97); further deletions of the short (10 aa) cytoplasmic tail were not investigated.

Mouse Bgp1 also exhibited *in vitro* adhesion properties when transfected into a

mouse LTA fibroblast cell line (104) or NIH 3T3 fibroblasts (53). The adhesion activity of Bgp1 was determined to be dependent on calcium and temperature for one allele, $Bgp1^b$ (formerly known as mmCGM2 and mmCGM1b) (104) and to be Ca^{2+} and temperature-independent for the other allele, $Bgp1^a$ (formerly known as mmCGM1 and mmCGM1a) (53). The cation and temperature dependency of BGP-mediated adhesion continues to remain a subject of controversy.

Unlike BGP, other members of the CEA gene family, such as CEA and NCA, clearly exhibit Ca^{2+} and temperature-independent intercellular adhesion *in vitro* (7, 109). The observed adhesion can be inhibited by either monovalent Fab fragments (109) or domain-specific peptides that correspond to known adhesion contact points (108). This mechanism of adhesion is reminiscent of another well-studied cell adhesion molecule of the immunoglobulin superfamily, neural-cell adhesion molecule (N-CAM). N-CAM is also known to mediate intercellular adhesion in a Ca^{2+}-independent manner (20).

The specificity of adhesion for some CEA family members has been investigated. CEA and NCA transfectants formed heterogeneous aggregates when mixed together, but when these transfectants were individually mixed with N-CAM transfectants, only homogeneous aggregates resulted (109) indicating that CEA nor NCA were unable to bind N-CAM. BGP was also shown to exhibit heterotypic adhesion with CEA and NCA, but not with CGM6 (69).

The proposed adhesion function of BGP and other members of the CEA family has come under some scrutiny lately. Since BGP, NCA, and CEA are expressed at the apical surfaces of simple epithelia, it has been argued that they are not appropriately

the above point mutations and PMA or staurosporine treatments had no effect on the ecto-ATPase activity of BGP (85). Although it has been demonstrated that the bile acid efflux activity of rat BGP is not directly dependent upon its ecto-ATPase activity, it still remains possible that regulation may be mediated indirectly by changes in the net ATP concentrations in the extracellular space (i.e. the bile canaliculi) (86). Theories on how these two activities relate to the observed adhesion properties of this glycoprotein (66) have not been put forth. At least the bile efflux activity is consistent with the expression pattern of BGP on the canalicular membranes of hepatocytes where bile salt extrusion is known to occur (60 for review). However, numerous extrahepatic tissues have also been demonstrated to express BGP (see above: Expression patterns of BGP in normal tissues). Whether BGP is functioning as an efflux pump for bile salts or other substrates in these various cell types has not been investigated. Alternatively, evidence is accumulating that BGP is a multi-functional protein, taking on different roles in different tissues.

BGP, NCA, and CEA have been reported to bind *Escherichia coli* of human origin and several species of *Salmonella* (46, 45). The binding was mediated by the type 1 fimbriae on the bacteria and was inhibited by derivatives of α-D-mannose (45). Inhibition of bacterial binding to CEA family members was also accomplished by the addition of purified type 1 fimbriae (45). Since type 1 fimbriated bacteria bind colonic epithelial cells and granulocytes via D-mannosyl structures (82 for review), recognition of CEA family members is likely mediated by their high-mannose-type structures. However, an additional level of specificity is required, since ovalbumin, a protein also possessing high-mannose-type structures does not bind *E. coli* or salmonellae (45). Bacterial binding is

thought to be mediated by the N-terminal Ig V domain, since site-directed mutagenesis of the first two N-linked glycosylation sites of NCA reduced binding of type 1 fimbriae by nearly 100% (81). The functional significance of bacterial binding to CEA family members has been hypothesized to be involved in the normal colonization of the gastrointestinal tract and/or in the neutralization of pathogenic bacteria (45, 25). The expression of BGP and other CEA family members in the lumen of the gut is consistent with its potential role in regulating bacterial colonization. In addition, the expression of BGP (CD66a) and NCA (CD66c) on neutrophils and other granulocytes (42, 74) and the up-regulation of BGP and CEA expression in response to the immune-modulator, γ-interferon (95), is suggestive of a role in host defense mechanisms.

It is still unclear whether the various *in vitro* functions of BGP (adhesion, ecto-ATPase, bile acid transporter, and bacterial binding) reflect its *in vivo* role(s) or whether some reported functions represent *in vitro* artefacts. It is also not known if BGP performs different functions in different tissues (or even different species) nor whether the functions are modified from embryonic to adult life.

VIII. Mouse hepatitis virus receptor

The mouse *Bgp1* gene product has been identified as the cellular receptor of the mouse hepatitis virus (MHV) (19). Transfection of the mouse *Bgp1* cDNA into resistant baby hamster kidney (BHK) cells rendered them susceptible to MHV infection (19). The infection was blocked by pretreatment of the cells with the monoclonal anti-MHV

receptor (MHVR) antibody, CC1 (19). Different splice variants of *Bgp1* also served as functional MHV receptors (107, 18). This was the first reported example of multiple splice variants functioning as receptors for a single virus.

The MHV-resistant mouse strain, SJL/J, lacked the *Bgp1*^{a} allele, but expressed the *Bgp1*^{b} allele (18). This implied that the *Bgp1*^{a} allele conferred MHV-susceptibility, while the *Bgp1*^{b} allele conferred MHV-resistance. However, transfection of the *Bgp1*^{b} cDNA into BHK cells rendered them susceptible to MHV infection and moreover, this infection could not be blocked by the monoclonal Ab, CC1 (18). It has been postulated that the involvement of a second factor may be determining the resistant or susceptible phenotype.

The mouse *Bgp2* gene product has also been demonstrated to function as a MHVR in *Bgp2*-transfected BHK cells, but not in the resistant SJL/J mouse (61). As for the Bgp1^b receptor, Bgp2-mediated MHV infection of transfected BHK cells was not blocked by the monoclonal Ab, CC1 (61). Interestingly, the mouse rectal carcinoma cell line, CMT-93, expressing both Bgp1^a and Bgp2, experienced a CC1-mediated block in MHV infection (61). This indicated that Bgp1^a, and not Bgp2, was the only functional receptor in this cell line.

The complexities of MHV infection have yet to be completely elucidated. It is clear so far that Bgp1^a can act as a receptor in all contexts, while Bgp1^b and Bgp2 can only act as receptors in particular contexts; perhaps they require the presence or absence of a secondary factor to efficiently mediate MHV infection.

IX. Phosphorylation of BGP

Several reports have described the phosphorylation of BGP (2, 89, 51). Afar *et al.* (2) and Skubitz *et al.* (89) found BGP to be phosphorylated on serine, threonine, and tyrosine residues following vanadate treatment of transfected CHO cells (2) or the human colon cancer cell lines, COLO 201, COLO 205, and KM12 (89). Both groups also observed basal levels of BGP serine phosphorylation in untreated cells (2, 89).

Since vanadate treatment is non-specific, the identification of specific BGP kinases would lend validity to the argument that BGP is phosphorylated *in vivo*. BGP was first demonstrated to be an endogenous substrate of the insulin receptor tyrosine kinase (76). BGP (therein termed pp120) was observed to be specifically phosphorylated in response to insulin treatment of rat liver preparations (76). As observed for the untreated and vanadate-treated cells, a basal level of phosphoserine was present in untreated hepatocytes, with a rapid increase in tyrosine phosphorylation upon insulin treatment (73). However, in contrast to the vanadate-treated cells, phosphothreonine was not observed upon treatment with insulin (73). Insulin-mediated phosphorylation of BGP was only demonstrated in hepatocytes and could not be observed in other tissues (1). Analysis of the BGP amino acid sequence revealed two tyrosine residues that could be potentially phosphorylated (48). Site-directed mutagenesis of one tyrosine (Tyr^{488}), but not the other (Tyr^{513}), resulted in a complete loss of insulin-stimulated tyrosine phosphorylation (59). Other studies also revealed that Tyr^{488} is the only tyrosine residue phosphorylated, even upon vanadate treatment (49). Interestingly, site-directed mutagenesis of Ser^{503} within the

potential cAMP-dependent protein kinase site (KRPTS^{503}), completely eliminated the previously observed basal serine phosphorylation and inhibited the ability of Tyr^{488} to be phosphorylated upon insulin treatment (59). By contrast, deletion of the cAMP-dependent protein kinase site continued to permit the phosphorylation of Tyr^{488} in vanadate-treated cells (49). These results indicate that Ser^{503} may regulate phosphorylation of Tyr^{488} *in vivo* in a unique and novel manner. The importance of Ser^{503} was further exemplified by a report demonstrating that an Ala^{503} mutation completely abrogated bile acid efflux activity (85).

Recent evidence has implicated BGP in playing a potential role in the receptor-mediated internalization of insulin (24). Co-transfection of the insulin receptor and BGP in NIH 3T3 fibroblasts resulted in a 2-fold increase in receptor-mediated internalization when compared to cells expressing the insulin receptor alone (24). Moreover, mutagenesis of Tyr^{488} or Ser^{503} abolished this effect, while mutagenesis of Tyr^{513} did not alter the rate of internalization (24). This data suggested that BGP may play a regulatory role in the receptor-mediated internalization of insulin and that its function is critically dependent on Tyr^{488} and Ser^{503} and their ability to become phosphorylated (24).

Other tyrosine kinases have been implicated in phosphorylating the intracytoplasmic tail of BGP. Skubitz *et al.* (88) demonstrated that the Src-like kinases, Lyn and Hck, specifically associated with BGP, CGM6, and NCA in human neutrophils. Active Lyn and Hck kinases were presumed to be responsible for the observed tyrosine phosphorylation of BGP in neutrophils (88).

The recombinant cytoplasmic domain of BGP was shown to be an *in vitro*

substrate of $pp60^{c\text{-}src}$ (10). It also specifically bound to the c-Src SH2 domain *in vitro* and not to the C-terminal SH2 domain of the p85 subunit of phosphatidylinositol-3'-kinase nor to the N-terminal SH2 domain of phospholipase Cγ (10). However, Skubitz *et al.* did not detect the association of c-Src to BGP or other CEA family members in neutrophils (88).

The regulation of BGP phosphorylation is mediated by several candidate kinases and some as yet unidentified phosphatases. The state of BGP phosphorylation has been shown to be important for receptor-mediated internalization of insulin (24) and for bile acid transport activity (85). However, it still remains possible that this putative multi-functional glycoprotein is capable of signal transduction via its phosphorylated residues.

X. BGP-associating proteins

In addition to the Src-like kinases described above, two different calcium binding proteins have been shown to associate with BGP; annexin VI (47) and calmodulin (8). By immunofluorescence analysis, annexin VI (calcium binding protein (CBP) 65/67) was observed to co-localize with rat BGP in the bile canalicular region of hepatocytes (47). In addition, BGP-annexin VI complexes were isolated by affinity chromatography with either anti-annexin VI antibodies or anti-BGP antibodies (47). Moreover, covalently crosslinked BGP-annexin VI complexes were isolated from membranes treated with a heterobifunctional crosslinking agent (47). These results suggest a direct association between annexin VI and BGP, but the physiological function of this interaction has not

been determined.

The intracellular calcium binding protein, calmodulin, was demonstrated to bind rat BGP by two different techniques; a dot-blot assay and a gel overlay assay (8). This binding was shown to be calcium-dependent and specific for the cytoplasmic domain of rat BGP (8). Surprisingly, calmodulin was able to bind both isoforms of rat BGP (C-CAM1 and C-CAM2) bearing either the long (71 aa) or short (10 aa) cytoplasmic domains (23). The precise calmodulin-binding sequences in the cytoplasmic domains of both BGP isoforms has been determined by using synthetic decapeptides scanning the respective cytoplasmic domains (21). A strong membrane-proximal calmodulin-binding site was observed for both the short-tail and long-tail isoforms of BGP in rat, mouse, and man (21). A weak calmodulin-binding sequence was identified in a membrane-distal region of the long-tail BGP isoforms of rat and mouse, but not man (21). Interestingly, calmodulin association in the presence of Ca^{2+} significantly reduced the homophilic binding properties of BGP *in vitro* (21). Edlund *et al.* speculated that a potential ligand-receptor interaction of BGP or another cell surface molecule could trigger an increase in intracellular Ca^{2+} concentration, thereby increasing the association of calmodulin with BGP and consequently reducing its homophilic binding properties (21).

XI. BGP ligands/counter-receptors

Although BGP has been shown to interact with itself, CEA, and NCA (69), the only other molecule reported to interact with the extracellular domain of BGP is E-

selectin (previously known as endothelial leucocyte adhesion molecule-1 (ELAM-1)) (42). The vascular selectins, E-selectin and P-selectin, are known to recognize glycoconjugates containing sialyl Lewis^x (sLe^x) groups. Since BGP (CD66a) was determined to be the major protein carrier of sLe^x in neutrophils (92), it was a good candidate counter-receptor for the selectins. Kuijpers *et al.* demonstrated that the rolling adhesion of neutrophils on cytokine-activated endothelial cells (EC) was inhibited by CD66 antibodies or by soluble CEA-like antigens (42). In addition, the binding of soluble CEA-like antigens to activated EC was blocked by anti-E-selectin antibodies. Neutrophils lacking GPI-linked proteins from paroxysmal nocturnal hemoglobinuria (PNH) patients were also tested. PNH neutrophils also exhibited a significant, but limited ability to bind activated EC in a manner that was inhibited by CD66 antibodies (42). Since BGP (CD66a) is the only CD66 antigen recognized on PNH neutrophils, this glycoprotein is capable of mediating adhesion to endothelial cells. The other GPI-linked CD66 antigens also appear to contribute binding activity to neutrophils, since the PNH cells exhibited a somewhat diminished adhesion capacity (42).

In addition to contributing to the rolling adhesion of neutrophils, CD66 antigens have been implicated in priming the cells for their eventual arrest and extravasation (93). Engagement of CD66 antigens with specific antibodies caused the neutrophils to undergo an activation of $ß_2$-integrins (required for strong adhesion and arrest), shape change, and a priming of the respiratory burst (93). CD66-primed neutrophils experienced a significantly increased respiratory burst in response to the chemotactic factor, formyl-Met-Leu-Phe (fMLP), when compared to unprimed cells (93). The observed CD66-induced

phenomena could be triggered by E-selectin binding *in vivo* and the required signals could be subsequently transduced by some of the Src-like kinases described earlier (88).

Soluble ligands or counter-receptors for BGP in other tissues, such as colon or liver, have not been discovered. Their identification would provide great insight into the various *in vivo* functions of BGP in the numerous cell types in which it is found.

XII. Objectives

The down-regulation of BGP has been observed in a number of epithelial malignancies (43, 40, 33) and epithelial tumor-derived cell lines (79). The first objective of this thesis endeavoured to investigate the effects of re-introducing Bgp1 isoforms into a colon carcinoma cell line that has lost endogenous Bgp1 expression. *In vitro* and *in vivo* transformation assays were employed to detect any alterations in growth or tumorigenic characteristics of the transfectant cells.

(Objectives continued on p.54)

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Inhibition of colonic tumor cell growth by biliary glycoprotein

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Biliary glycoproteins (BGPs) are members of the carcinoembryonic antigen (CEA) family. These glycoproteins function *in vitro* as intercellular adhesion molecules and, in the mouse, serve as receptors for the mouse hepatitis viruses. In previous studies, BGP expression has been reported to be generally down-regulated in colon and liver carcinomas of human, rat and mouse origins. We now demonstrate that introduction of murine *Bgp*1 cDNA isoforms into a mouse colonic carcinoma cell line, negative for endogenous Bgp1 expression, significantly alters the growth properties of these cells. Cells bearing two Bgp1 isoforms were growth-retarded and exhibited a reduced ability to form colonies in an *in vitro* transformation assay, when compared to parental or control neo^r cells. Furthermore, tumor formation was inhibited by 80% when cells bearing a full-length Bgp1 isoform were injected into BALB/c syngeneic mice, while cells expressing a Bgp1 isoform lacking most of the intracytoplasmic domain produced tumors as readily as the parental cells. These results indicate that a biliary glycoprotein isoform is involved in negative regulation of colonic tumor cell growth, by a process which requires its intracytoplasmic domain. The precise mechanisms causing Bgp-dependent tumor growth inhibition remain, however, to be defined.

Keywords: biliary glycoprotein; BGP; carcinoembryonic antigen; CEA; growth inhibitor; tumor; colon carcinoma

Introduction

The biliary glycoproteins (BGPs) are members of the carcinoembryonic antigen (CEA) family, which is part of the immunoglobulin (Ig) superfamily (Thompson *et al.*, 1991). CEA is widely used to detect recurrences and progression of gastro-intestinal tumors in patients (Gold and Freedman, 1965). The human *BGP* gene, located in the same chromosomal cluster as the *CEA* gene (19q13.1-3) (Thompson *et al.*, 1991), is subjected to alternative splicing mechanisms generating 12 mRNAs (Barnett *et al.*, 1989; 1993). The encoded proteins exhibit heavily glycosylated extracytoplasmic Ig-like domains with a N-terminal domain resembling the Ig variable region and one or three Ig C2-set constant regions (Barnett *et al.*, 1989). Amongst CEA family members, BGPs are unique in that they possess cytoplasmic domains that are either short (10 amino acids) or long (71–73 amino acids) (Barnett *et al.*, 1989; McCuaig *et al.*, 1993). Inclusion of a 53 bp exon between exons 6 and 8 of the mouse *Bgp*1 gene shifts the open reading frame of the encoded cytoplasmic domain and an additional 63 amino acids is inserted into the protein (Nédellec *et al.*, 1995). Investigations on the expression patterns of the mouse Bgps have revealed that these glycoproteins are expressed in epithelial cells of many different tissues, in endothelial cells of large blood vessel walls and in B cells, macrophages, monocytes, platelets and granulocytes (Öbrink, 1991; McCuaig *et al.*, 1992; Coutelier *et al.*, 1994). They are however, absent in T lymphocytes (Coutelier *et al.*, 1994). Although they are abundant in normal colon and liver (McCuaig *et al.*, 1992; 1993), these proteins are generally down-regulated in colonic and hepatic tumors (Neumaier *et al.*, 1993; Rosenberg *et al.*, 1993). Rat Bgp homologs, called C-CAMs, are also decreased in expression in primary and transplantable hepatocarcinomas (Hixson *et al.*, 1985). The *Bgp*1 transcriptional block is most likely an early event in the progression to malignancy, since in the mouse, this glycoprotein is absent from colonic stage A tumors (Rosenberg *et al.*, 1993).

These glycoproteins behave as intercellular adhesion molecules in *in vitro* aggregation assays; at low levels of expression in transfected cells, Bgps require calcium and physiological temperature for aggregation (Rojas *et al.*, 1990; Turbide *et al.*, 1991; Oikawa *et al.*, 1992), whereas high levels of Bgp1 expression abrogate the calcium-dependency (Öbrink, 1991; McCuaig *et al.*, 1992). The adhesion function is postulated to be instrumental in hepatocyte aggregation during embryonic development and for adoption of colonic tissue architecture (Ocklind and Öbrink, 1982; Benchimol *et al.*, 1989). Mouse Bgps have also been recognized as the receptors for colono, hepato- and meningo-tropic strains of mouse hepatitis viruses (Dveksler *et al.*, 1991; Yokomori and Lai, 1992). Rat Bgps have been shown to function as ecto-ATPases (Lin and Guidotti, 1989) and the 71 amino acid Bgp cytoplasmic tail is known to be responsible for bile acid efflux from hepatocytes (Sippel *et al.*, 1993). Bgps may also be involved in signal transduction events since they are phosphorylated on either serine/threonine and tyrosine residues subsequent to activation of either protein kinase C (PKC) or the insulin receptor (Rees-Jones and Taylor, 1985; Lin and Guidotti, 1989; Afar *et al.*, 1992).

To understand the role played by these proteins in tumorigenesis, we have inserted two cDNA isoforms of the mouse *Bgp*1 gene bearing either a 10 or 73 amino acid intracytoplasmic domain into mouse colon carcinoma cells, negative for endogenous Bgp1 expression, and evaluated the properties of the resulting transfectant cells. Expression of either the short or the long-tailed Bgp1 protein in these cells reduced their rate of proliferation and their ability to form colonies in clonogenic assays, as compared to

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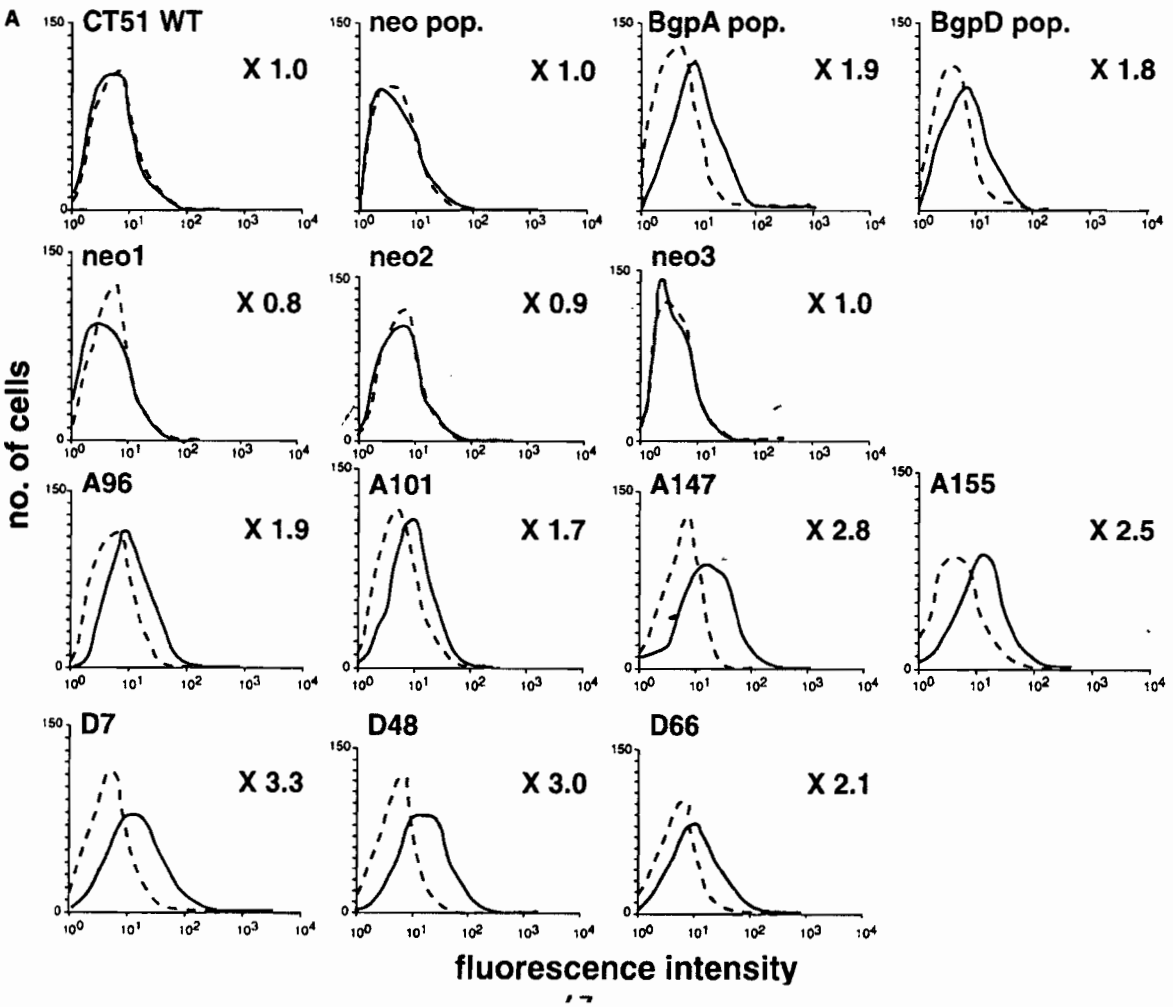
parental or control infected cells. Furthermore, expression of the long-tailed Bgp1 variant inhibited tumor formation in syngeneic mice by 80%. Notably, the short-tailed Bgp had no inhibitory effect *in vivo*. This data indicates that Bgps may play a role in growth and/or differentiation of colonic epithelial cells.

Results

Expression of Bgp1 proteins in colon carcinoma cells alters their growth properties

We have previously described that several mouse *Bgp1* splice variants (Nédellec *et al.*, 1995) are expressed in normal colon and liver, but are absent from colon tumors or colonic and hepatic tumor cell lines (Rosenberg *et al.*, 1993). A survey of expression patterns in mouse tissues has also shown that, as in colon, the long intracytoplasmic tail (73 amino acids)-bearing variant (named BgpD) is always expressed in a lower ratio than the short-tailed variant (named BgpA) produced by the same *Bgp1* gene (McCuaig *et al.*, 1993; Nédellec *et al.*, 1995). To understand the role played by each of these glycoproteins in tumorigenesis, we introduced their respective cDNAs into BALB/c-derived CT51 mouse colon carcinoma cells (Brattain *et al.*, 1980) using retroviral-mediated infections. As shown in Figure 1A, the parental (CT51) or the control infected cell population (neo pop.) or clones (neo1, neo2, neo3) expressing the empty vector exhibited undetectable expression of the Bgp1 proteins at the cell surface. The shoulder observed in these cytofluorometric profiles is a consequence of the two types of epithelial cells which are present in this clonal cell line. As reported by Brattain *et al.* (1980) attempts to resolve the heterogeneity of this cell line by cloning have not been successful and the heterogeneity may be due to the state of differentiation of the cells or the phase of the cell cycle of particular cells. The parental cell line (Figure 1B, CT51) or control infected cell population or clones (Figure 1B, neo pop., neo1, neo2, neo3) did not express detectable amounts of Bgp1 by immunoblot analyses. We have, however, detected low transcription of the *Bgp1* gene in CT51 cells using nested reverse transcription-polymerase chain reaction (RT-PCR) methods with *Bgp1*-specific primers (data not shown).

Bgp-positive cell clones were isolated by immunoselection with an anti-Bgp polyclonal antibody (Ab 231) and Dynabeads. The Bgp-expressing populations as well as derived clones were evaluated for Bgp1 expression by both cytofluorometric analyses and immunoblotting (Figures 1A and B). The BgpA protein exhibiting a short intracytoplasmic domain migrates as a broad 120 kDa band while the BgpD protein migrates slightly slower due to the 8 kDa provided by the additional 63 amino acids in the



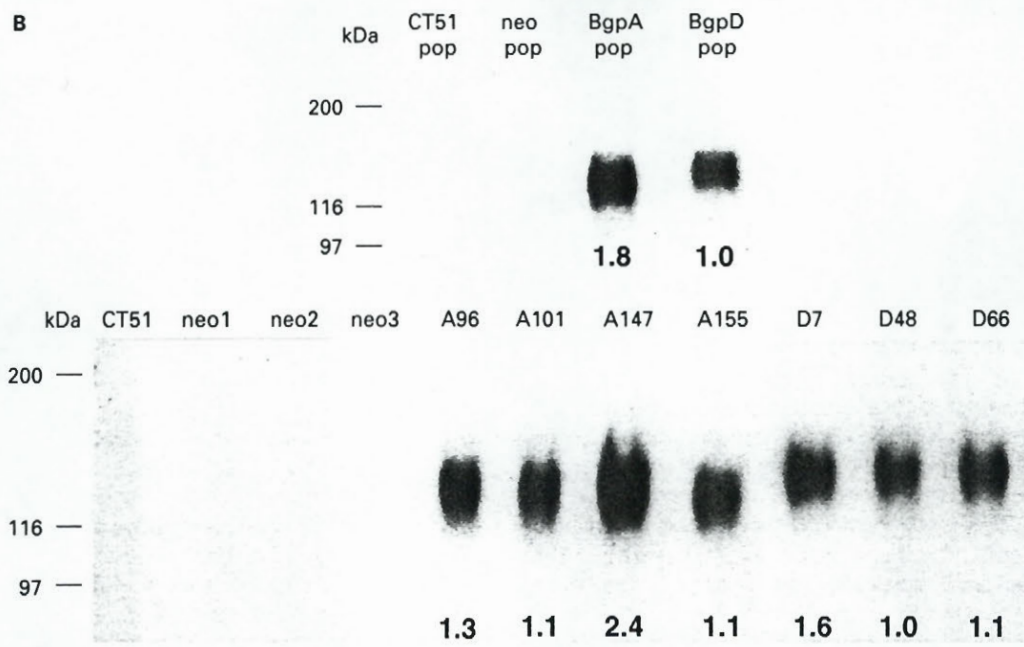


Figure 1 Expression of Bgp1 in CT51 parental and infected cells. CT51 parental, neo^r control or Bgp-expressing infected cell populations or clones described in Materials and methods were analysed for Bgp expression by cytofluorometric analysis using a Bgp1-specific B10 MAb. (**A**). Fluorescence was evaluated on a log scale. CT51 parental (CT WT). neo pop. or neo1, neo2 and neo3 represent CT51 populations and clones infected with an empty virus while BgpA pop., A96, A101, A147 and A155 are populations or clones expressing the short-tailed BgpA protein. BgpD population (pop.) or D7, D48 and D66 clones express the long-tailed BgpD protein. Dashed lines depict cells incubated with the secondary fluorescein-labeled antibody only and solid lines represent cells incubated with both primary (B10) and secondary antibodies. Fold expression of the clones over background is indicated in the top right corner of each profile and represents the ratio of the positive over negative medians. (**B**) Cells were lysed and 150 μg of total cellular proteins were separated on 7.5% SDS–PAGE gels. After transfer of the proteins, the membranes were incubated with a rabbit anti-mouse Bgp polyclonal antibody (serum 231). The radioactive bands were quantified on a Bas 2000 BioAnalyzing system and are reported as fold expression relative to the faintest band on each immunoblot. Molecular weight markers are indicated on the left of the autoradiograms

intracytoplasmic tail. As shown in Figure 1A, Bgp-infected populations or clones selected in this study over-expressed Bgp1 at the cell surface by a factor of 1.7–3.3-fold over background, while quantification of the radioactive bands on the immunoblots (Figure 1B) indicate that Bgp was overexpressed by factors of 1.1–2.4-fold relative to the band of lowest intensity on each immunoblot. Although the two methods of calculating fold expression over background are not directly comparable (see Materials and methods), the values calculated from the immunoblots were in the same relative range as those from the cytofluorometric profiles (except that of the A155 clone). The few discrepancies found between the amount of Bgp1 expressed at the cell surface and the total amount found in cell lysates could indicate that not all of the Bgp1 proteins synthesized in these cells reached the cellular membrane. However, the amount of Bgp1 expressed in these infected cells corresponds to approximately the concentration of Bgp1 found in normal mouse colon (McCuaig *et al.*, 1993).

As cell clones were being established, we observed that most Bgp-expressing cells grew more slowly than the parental cells or the neo^r control cells. To quantify this observation, equal numbers of CT51 parental or infected cells were plated and growth curves were derived. Typical experiments are shown in Figure 2. Significant differences in growth were not observed between three neo control clones (Figure 2A) suggesting that the differences seen with the BgpA- or BgpD-infected clones were not likely due to clonal variability. Significant growth differences of the BgpA- or BgpD-expressing cells were, however, observed by day 6 when compared to the CT51 parental cells or the neo3 control clone (Figure 2B and C). Moreover, the differences in growth between the parental and control cells and the Bgp-expressing cells became more apparent over the course of the next 4 to 6 days due to a lower saturation density of the BgpA- and BgpD-expressing cells. The parental cells and neo^r transfectant control cells tended to stack up whereas the Bgp1 transfectant cells demonstrated a reduced ability to do so; they either reduced their growth rate or sheded off and died. Furthermore, growth retardation of Bgp clones was somewhat correlated with the amount of Bgp1 protein expressed at the cell surface; the A147 and A155 clones, expressing 2.5–2.8-fold of BgpA over background, grew significantly slower than the the A96 or A101 clones expressing 1.7–1.9-fold, respectively (Figures 1A and 2B). The D7 and D48 clones were similarly growth-retarded relative to the control cells (Figure 2C). The D66 clone was not used in this assay. This suggested a lower saturation density for cells expressing a higher Bgp1 concentration at the cell surface. Similarly, the growth rate of the Bgp-positive cells, calculated by deriving the slope of the growth curve drawn on a semi-logarithmic scale (data not shown), was reduced when compared to that of the parental cells in the first 6–8 days (1.05×10^5 cells/day for BgpA155-expressing cells, 1.53×10^5 cells/day for BgpD7-expressing cells *vs* 3.13×10^5 cells/day for parental CT51 cells) ($P < 0.05$). These data indicate

that the presence of Bgp1 at the surface of colonic carcinoma cells affects the growth rate and the saturation density of these cells.

Bgp1 expression inhibits anchorage-independent growth of CT51 cells

Since the growth of Bgp-bearing CT51 cells was affected, we investigated if Bgp1 expression had effects on anchorage-independent growth of CT51 cells. Significant differences in the ability to form colonies in soft agar were not observed when either neo control populations or BgpA- and BgpD-expressing populations were compared (data not shown). When three neo^r control clones were analysed and compared to the parental cells (Figure 3A and B), the neo1 clone exhibited the same properties as the parental cells, while the neo2 clone showed a 10% increase relative to the parental cells. The neo3 clone also behaved like the parental cells and was able to form a significant number of colonies in soft agar (Figure 3B). Bgp-positive cells, as shown in Figure 3B, irrespective of whether the protein included a short or a long intracytoplasmic tail, formed a reduced number of colonies in soft agar (35–83% inhibition) when compared to the CT51 parental or neo3 control cells. In addition, the colonies that did form with the Bgp-positive clones contained fewer cells (data not shown). Furthermore, cells expressing higher amounts of BgpA at the cell surface produced a greater inhibition (Figure 3C, A147 and A155 *vs* A96 and A101), suggesting that this phenotype was in fact due to the presence of Bgp1. Decreasing the serum concentration from 10 to 2.5% had no effect on anchorage-independence (data not shown). These results suggest that the relatively abundant expression of Bgp1 in normal colon epithelial cells could be instrumental in normal cellular growth and/or differentiation, since Bgp1 expression in colon carcinoma cells considerably reduced their transformed properties in *in vitro* assays.

Expression of the Bgp1 isoform bearing a long intracytoplasmic domain inhibits tumor formation in syngeneic mice

To corroborate these *in vitro* results, either the parental CT51, neo^r control or Bgp-bearing cell populations and clones were injected into BALB/c syngeneic mice. When CT51 parental, neo^r control or BgpA-expressing cell populations were used, a statistically significant number of mice (8/10, 9/10 or 10/10) developed tumors within a 2 week period and these tumors increased in size until the end of the experimental period ($P>0.1$). However, only 4/10 mice developed tumors when BgpD-expressing cell populations were injected into mice, which was statistically significant when compared to that of the control neo^r population ($P<0.02$). The average size of the tumors from the BgpD-injected populations was not significantly decreased relative to that of the neo^r populations. This result indicated that the Bgp1 cytoplasmic domain influenced the *in vivo* growth properties of these colonic carcinoma cells.

To corroborate the results obtained with the cell populations, cell clones were used in a similar experiment (Table 1). Two cell concentrations were used for this assay, as done previously (Brattain *et al.*, 1980) and the length of the experimental period was prolonged until the tumors had reached approximately 1.0–1.5 cm in diameter. When 4×10^6 CT51 parental cells or neo^r control clones were injected s.c., 82% of the mice developed tumors that reached 1.0–1.5 cm in diameter in 31.4 ± 7.2 (Table 1, CT51 cells) or 34.0 ± 5.3 days (Table 1, neo3 clone). When a lower cell concentration was used (1×10^6 cells/injection),

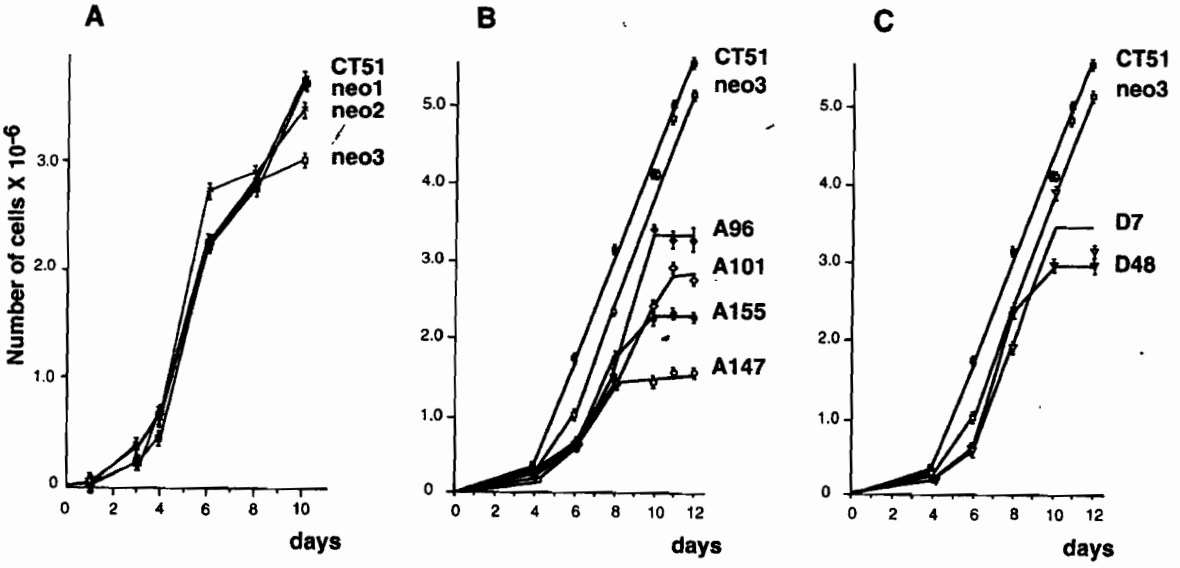


Figure 2 Growth curves of CT51 parental, Neo^r control and Bgp1 transfectant cells. 10^4 cells of CT51 parental (CT51), control (neo^r), Bgp short-tailed (A) or long-tailed (D) derived cells were plated in duplicate 9.64 cm^2 wells and grown for 10–12 days. Cells were collected at various time points with trypsin-EDTA and counted. Expression of Bgp was verified by cytofluorometric analysis at the beginning and the end of every growth curve. Experiments were repeated four times and representative experiments are plotted. Experiments in (**B**) and (**C**) were performed tandemly but were plotted separately for clarity. Variability indicated by the standard error which is smaller

XII. Objectives

(continued from p.30)

Since the long-cytoplasmic domain of BgpD appeared to be mediating an *in vivo* tumor inhibition phenotype, a search for downstream effectors of this phenotype, such as cytoplasmic Bgp1-binding proteins, was initiated. The presence of two tyrosine residues in the cytoplasmic domain of BgpD directed efforts toward SH2 domain-containing proteins. The ability of BgpD to be tyrosine-phosphorylated was investigated first, followed by a mutational analysis. The binding of SH2 domain-containing proteins was evaluated by co-immunoprecipitation analyses. Interactions identified by this method were subjected to mutational analyses and *in vitro* binding studies.

Biliary glycoprotein (Bgp) is a member of the immunoglobulin superfamily and the carcinoembryonic antigen family. Previous studies have shown that Bgp functions as an intercellular adhesion molecule and a canalicular bile salt transporter. Moreover, we and others demonstrated that Bgp can inhibit colonic and prostatic tumor cell growth *in vivo*, through a mechanism which depends on sequences present in its cytoplasmic domain. In this study, we have examined the possibility that the cytoplasmic domain of Bgp can interact with signal transduction molecules. We showed that tyrosine phosphorylated Bgp, expressed in mouse colon carcinoma CT51 cells, could reversibly associate with protein tyrosine phosphatase SHP-1. Mutation of either of two tyrosine residues present in the cytoplasmic domain of Bgp abrogated SHP-1 binding, suggesting that this association was mediated by both tyrosine residues. Similarly, we noted that either of the two SH2 domains of SHP-1 could bind tyrosine phosphorylated Bgp *in vitro*. In combination, these results implied that, upon tyrosine phosphorylation, Bgp can associate with and regulate the function of SHP-1. It is therefore conceivable that some of the functions of Bgp are mediated through its ability to induce intracellular protein tyrosine dephosphorylation.

Keywords: biliary glycoprotein, Bgp, C-CAM, colon carcinoma, SHP-1, PTP1C, HCP.

Introduction

Biliary glycoprotein (Bgp[1]) is a cell-surface immunoglobulin-like protein and a member of the carcinoembryonic antigen (CEA) family (Thompson *et al.*, 1991). Bgp is well conserved throughout evolution. The human, mouse and rat Bgp proteins exhibit very similar extracellular domains (Barnett *et al.*, 1989; Lin and Guidotti, 1989; Öbrink, 1991; McCuaig *et al.*, 1992). However, their most conserved feature is in their cytoplasmic domains, which exhibit 77-92% similarity at the amino acid level. Bgp proteins possess cytoplasmic domains of either 10 or 71 (73 in mouse) amino acids which are produced by alternative splicing of a conserved 53 bp exon (Barnett *et al.*, 1989; Nédellec *et al.*, 1995). They are mainly expressed in epithelial cells of the gastro-intestinal tract, endothelial cells and hematopoietic cells, particularly B cells, neutrophils and macrophages (Hinoda *et al.*, 1988; Barnett *et al.*, 1989; Öbrink, 1991; Coutelier *et al.*, 1994; Nédellec *et al.*, 1995).

Bgp is multifunctional. First, it behaves as intercellular adhesion molecules as demonstrated by *in vitro* aggregation assays (Ocklind and Öbrink, 1982; Rojas *et al.*, 1990; McCuaig *et al.*, 1992). This function is postulated to be important for maintenance of tissue architecture during embryonic development (Ocklind and Öbrink, 1982; Daniels *et al.*, 1996). Second, Bgp can also promote extrusion of bile salts from cells, suggesting

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[1]The abbreviations used are: Bgp, biliary glycoprotein; BgpS, Bgp with a 10 amino acid-cytoplasmic domain; BgpL, Bgp with a 73 amino acid-cytoplasmic domain; CEA, carcinoembryonic antigen; C-CAM, cell-cell adhesion molecule; GST, glutathione-S-transferase; PKC, protein kinase C; FACS, fluorescence-activated cell sorting; ITAM, Immunoreceptor Tyrosine-based Activation Motif; ITIM, Immunoreceptor Tyrosine-based Inhibition Motif.

to ITAMs, in which both conserved tyrosines are phosphorylated, only one tyrosine of Bgp (Tyr^{488}) was convincingly shown to undergo phosphorylation in cells treated with insulin (Najjar *et al.*, 1995) or with the protein tyrosine phosphatase inhibitor vanadate (Lin *et al.*, 1995). To address the function provided by the cytoplasmic domain of Bgp, we have examined its ability to associate with tyrosine phosphorylated cellular proteins. In cells treated with the protein tyrosine phosphatase inhibitor pervanadate, we observed that Bgp was specifically complexed with a 66 kDa-tyrosine phosphorylated protein. Immunoblot analyses showed that this protein was SHP-1 (also known as PTP1C, HCP, SH-PTP1, SHP), a cytosolic protein tyrosine phosphatase containing two SH2 domains in its amino-terminal region (Matthews *et al.*, 1992; Plutzky *et al.*, 1992; Adachi *et al.*, 1996). Structure-function analyses showed that this association required tyrosines 488 and 515 of Bgp. Moreover, *in vitro* binding studies indicated that either of the two SH2 domains of SHP-1 could bind tyrosine phosphorylated Bgp. Together, these results suggested that part of the role of the Bgp cytoplasmic domain may be to mediate recruitment of SHP-1 and regulate intracellular protein tyrosine phosphorylation.

Results

Tyrosine Phosphorylation of Bgp in Pervanadate-Treated Colonic Epithelial Cells

To characterize the role of the cytoplasmic domain of Bgp in epithelial cells, we first focused on the potential functions of its tyrosine phosphorylation. Since the mechanisms regulating tyrosine phosphorylation of Bgp have not been clearly defined, we chose to artificially induce Tyr phosphorylation by treatment with pervanadate, a potent inhibitor

of cellular protein tyrosine phosphatases (O'Shea *et al.*, 1992). This choice was based on the observation of others, which showed that vanadate, a less active protein tyrosine phosphatase inhibitor was efficient at provoking phosphorylation of Bgp at Tyr^{488} (Lin *et al.*, 1995).

Derivatives of CT51 colon carcinoma cells expressing various forms of Bgp proteins were incubated with pervanadate, lysed in non-ionic detergent-containing buffers and Bgp was immunoprecipitated from cell lysates using anti-Bgp polyclonal antibodies. Tyrosine phosphorylated proteins were detected by immunoblotting with anti-phosphotyrosine antibodies (Figure 2A), while the abundance of Bgp was monitored by immunoblotting with anti-Bgp antibodies (Figure 2B). As parental CT51 cells do not express Bgp (Rosenberg *et al.*, 1993; Fig. 2B, lane 1), anti-Bgp immunoprecipitates from pervanadate-treated cells did not contain any tyrosine phosphorylated proteins other than non-specific contaminating proteins (Figure 2A, lane 1). Similarly, cells expressing the short cytoplasmic domain variant of Bgp (BgpS, Figure 2A, lanes 2 and 3) did not exhibit Bgp Tyr phosphorylation, therefore implying that the Tyr residue present in this variant (Figure 1A) was not a target for pervanadate-induced Tyr phosphorylation. However, the long cytoplasmic domain version of Bgp underwent a noticeable increase in Tyr phosphorylation following pervanadate treatment. Similar results were observed in all three clones tested (Figure 2A, lanes 4-9).

We then wished to determine the sites undergoing tyrosine phosphorylation in pervanadate-treated cells. Since both Tyr^{488} and Tyr^{515} are potential Tyr phosphorylation sites, they were mutated to Phe either individually or in combination, and the resulting

Bgp mutants (Y488F, Y515F and Y488,515F) were expressed in CT51 cells through retrovirus-mediated gene transfer. Monoclonal cell lines expressing these various Bgp mutants were isolated by limiting dilutions and identified by FACS analysis and immunoblotting (data not shown). We observed that replacement of Tyr^{488} (Y488F) abrogated pervanadate-induced Bgp Tyr phosphorylation (Figure 2B, lanes 12 and 13). However, it should be noted that the amount of Y488F Bgp expressed in this or other cell lines was lower than that found in cells expressing wild-type Bgp (Figure 2B, lanes 12 and 13 *vs* lanes 4 and 5). Nonetheless, as longer exposures of the immunoblots failed to reveal Tyr phosphorylation of Y488F Bgp, we felt confident that this mutant underwent little, if any, tyrosine phosphorylation in response to pervanadate treatment. Mutation of Tyr^{515} (Y515F) did not prevent pervanadate-induced Bgp tyrosine phosphorylation. However, this polypeptide was tyrosine-phosphorylated with a lower efficiency than wild-type Bgp (Figure 2A, lanes 10 and 11). These results suggested that Tyr^{488} was the major site of tyrosine phosphorylation in Bgp. Finally, mutation of both tyrosines (Y488,515F) completely abolished Bgp Tyr phosphorylation (Figure 2A, lanes 14 and 15). In combination, these results indicated that Tyr^{488} was the major site of tyrosine phosphorylation in pervanadate-treated cells. However, they also suggested that Tyr^{515} was either a minor phosphorylation site, or was required for maximal phosphorylation of Tyr^{488}.

Association of Bgp with Tyrosine Phosphorylated SHP-1 in Pervanadate-Treated Cells

To investigate whether Tyr phosphorylated Bgp could bind to other cellular proteins, Bgp

was immunoprecipitated with anti-Bgp antibodies and Bgp-associated proteins were detected by anti-phosphotyrosine immunoblotting. In pervanadate-treated cells, immunoprecipitation of Bgp with either polyclonal or monoclonal antibodies consistently led to the detection of another Tyr phosphorylated-protein of approximately 66 kDa (Figure 3, lane 2). A second phosphorylated protein of approximately 100 kDa was also detected. But since this last protein was also recovered from lysates of parental CT51 cells (Figure 2A, lane 1), it was not considered to be associated with Bgp.

Two cytosolic protein tyrosine phosphatases, SHP-1 and SHP-2, each carrying two N-terminal SH2 domains are known to associate with tyrosine phosphorylated receptors (Matthews *et al.*, 1992; Plutzky *et al.*, 1992; Vogel *et al.*, 1993; Feng *et al.*, 1993; Adachi *et al.*, 1996). SHP-1 is a 66-68 kDa-protein abundantly expressed in hemopoietic cells and in various epithelial cell types (Matthews *et al.*, 1992; Plutzky *et al.*, 1992). In contrast, SHP-2, a 70 kDa polypeptide, is ubiquitously expressed (Vogel *et al.*, 1993). The molecular weight of the Bgp-associated protein prompted us to test whether it could represent SHP-1 or SHP-2. To this end, lysates from pervanadate-treated CT51 cells expressing Bgp were submitted to immunoprecipitation with an antibody specific to the carboxy-terminus of SHP-1. This antiserum reacts against SHP-1, but not SHP-2. Immunoblotting with anti-phosphotyrosine antibodies revealed that two major Tyr phosphorylated proteins of either 66 kDa or 120 kDa were present in SHP-1 immunoprecipitates from pervanadate-treated cells (Figure 3, lane 4). The migration of the 120 kDa protein coincided with that of Bgp (Figure 3, lane 2), whereas the 66 kDa product was consistent with SHP-1. Similar results were obtained with an antibody raised

against the SHP-1 phosphatase domain (data not shown). Immunoblotting with anti-SHP-2 antibodies failed to document expression of this phosphatase in CT51 cells (data not shown).

To demonstrate that the 66 and 120 kDa polypeptides represented SHP-1 and Bgp respectively, Bgp was first immunoprecipitated with a Bgp-specific monoclonal antibody, and Bgp and SHP-1 were detected by immunoblotting with the appropriate antibodies (Figure 4A, top and bottom). While SHP-1 was not present in the Bgp immune complexes from untreated cells (Figure 4A, lane 1), it was detectable in Bgp immunoprecipitates from pervanadate-treated cells (Figure 4A, lane 2). These results were also confirmed by the reverse experiment. When SHP-1 was immunoprecipitated from pervanadate-treated cells, Bgp was detected by anti-Bgp immunoblotting (Figure 4B, lanes 7 and 8). These results suggested that Tyr phosphorylated Bgp protein was associated with SHP-1.

To determine whether tyrosine phosphorylation of Bgp was needed for association with SHP-1, the ability of the various Y→F mutants to bind SHP-1 in pervanadate-stimulated cells was examined. Mutations of either Tyr^{488}, Tyr^{515} or both completely abrogated this binding (Figure 4A, lanes 3-5; Figure 4B, lanes 9-14). It is noteworthy that, although Bgp was phosphorylated at Tyr^{488} in pervanadate-treated cells (Figures 2A and 5C), SHP-1 was not associated with these mutants under the conditions used. Moreover, Bgp-SHP-1 association was not detected in transfectant cells expressing Bgp bearing the naturally-occuring short cytoplasmic domain (Figure 4B, lanes 4 and 5). The results of these experiments suggested that, although Tyr^{488} of Bgp was phosphorylated

in these experimental conditions, association of Bgp with SHP-1 also required either the phosphorylation of Tyr^{515} or a particular conformational folding of the Bgp cytoplasmic domain mediated by the presence of Tyr^{515}.

Binding of SH2 domains of SHP-1 to Tyr phosphorylated Bgp

To define which domains of the SHP-1 phosphatase were contacting Bgp, *in vitro* binding assays were performed with bacterially-produced glutathione-S-transferase (GST) fusion proteins encompassing various domains of SHP-1. Lysates from CT51 Bgp-expressing cells treated or not with pervanadate were adsorbed onto GST fusion proteins bound to agarose glutathione beads. After several washes, binding proteins were eluted, resolved by electrophoresis and detected by immunoblotting with anti-Bgp antibodies. No Tyr phosphorylated protein bound to GST alone (Figure 5A, lanes 1 and 2). However, a 120 kDa polypeptide consistent with Bgp bound to a GST fusion protein containing full-length SHP-1 (Figure 5A, lanes 9 and 10) or bearing one or both SHP-1 SH2 domains (Figure 5A, lanes 3-8). This binding occurred only when Bgp was Tyr phosphorylated (Figure 5C, lane 2). This result suggested that either SHP-1 SH2 domain could associate with Bgp Tyr residues *in vitro*. In order to examine whether the presence of Tyr^{488} was sufficient for binding to the SHP-1 SH2 domains, the ability of GST-SHP-1 fusion proteins to associate with tyrosine phosphorylated Y515F Bgp was tested. We found that Y515F Bgp did not bind to either SHP-1 SH2 domain or to full-length SHP-1 (Figure 5B, lanes 2-5). Similar results were obtained with the Y488F Bgp mutant (data not shown). The results from the *in vitro* experiments concurred with those obtained *in vivo* (Figure

4A), suggesting that Bgp-SHP-1 association required contact with more than Tyr^{488}.

Discussion

In this study, we provided evidence that Tyr phosphorylated Bgp interacted with SHP-1, a protein tyrosine phosphatase expressed in cells of hemopoietic and epithelial lineages. This was demonstrated using combinations of immunoprecipitation and immunoblotting of Bgp and SHP-1 proteins expressed in CT51 colon epithelial cells. This interaction was seemingly mediated by the tyrosine phosphorylated form of Bgp and the SH2 sequences of SHP-1.

Mutational analyses of Tyr residues in the cytoplasmic domain of Bgp provided evidence that Tyr^{488} of Bgp was the only site of Bgp tyrosine phosphorylated in pervanadate-treated conditions. This result was in agreement with previous reports that utilized phosphopeptide mapping to reveal Tyr^{488} as the only tyrosine phosphorylated in insulin-treated cells (Sippel *et al.*, 1994; Najjar *et al.*, 1995). Therefore, the Bgp-SHP-1 complex was presumably mediated by Tyr^{488} of Bgp. However, it should be noted that mutation of Tyr^{515} also abrogated the interaction between Bgp and SHP-1, suggesting that Bgp-SHP-1 binding may actually be mediated by both tyrosine residues. It remains possible that Tyr^{515} may also be transiently phosphorylated *in vivo*. Our results also indicated that one or the other SH2 domains of SHP-1 bound to tyrosine phosphorylated Bgp. However, the affinity of each SH2 domain for binding to phosphorylated tyrosine of Bgp may be somewhat different and will need to be evaluated.

Several models can be envisaged for the Bgp-SHP-1 association. Phosphorylated

Tyr^{488} may be the sole binding site for one or the other SHP-1 SH2 domains. In this regard, our results were consistent with a recent report from Pei *et al.* (1996) demonstrating that both SHP-1 SH2 domains can associate independently with the same Tyr residue of FcγRIIB1, albeit with a five fold difference in the K_D of their binding. The authors of this report proposed a role for the SHP-1 N-SH2 domain in regulation of SHP-1 enzymatic activity as well as recruitment to the receptor phosphotyrosine residue, while the C-SH2 domain would primarily be involved in recruitment (Pei *et al.*, 1996). On the other hand, it is possible that *in vivo*, transient phosphorylation of Tyr^{515} of Bgp may enhance Tyr^{488} phosphorylation; the lower level of Tyr^{488} phosphorylation detected in the Y515F mutant when compared to wild-type Bgp argued in favour of this event. This could then promote association of the two SHP-1 SH2 domains with the two phosphorylated Tyr residues of Bgp.

We found that Bgp was Tyr phosphorylated only once cellular protein tyrosine phosphatases were inactivated through pervanadate treatment. Although enhancement of Tyr phosphorylation using this inhibitor may not be considered to faithfully mimic physiological situations, it should be noted however, that Bgp was demonstrated to be Tyr phosphorylated in other situations. Tyr^{488} of Bgp is phosphorylated in response to activation of the insulin receptor (Margolis *et al.*, 1988; Najjar *et al.*, 1995) and phosphorylation of this residue as well as Ser^{503} phosphorylation correlate with efficient bile salt extrusion and insulin receptor endocytosis (Sippel *et al.*, 1994; Formisano *et al.*, 1995). Similarly, Bgp was shown to be Tyr phosphorylated by $pp60^{c\text{-}src}$ in human colonic carcinoma cells (Brümmer *et al.*, 1995) and by $p53/56^{lyn}$ in human neutrophils (Skubitz

et al., 1995).

Our study provides the first report of association of SHP-1 with a cell surface protein in epithelial cells. Other cell surface receptors bind to SHP-1 in hemopoietic cells (Yi *et al.*, 1993a; Klinmuller *et al.*, 1995; Pani *et al.*, 1995; Burshtyn *et al.*, 1996), such as the erythropoietin receptor (Klinmuller *et al.*, 1995), the IL-3 receptor, Kit (Yi *et al.*, 1993a, 1993b), FcγRIIB1, the natural killer receptor p58 and B cell antigen receptor (D'Ambrosio *et al.*, 1995; Pani *et al.*, 1995; Burshtyn *et al.*, 1996). These receptors exhibit a so-called Immunoreceptor Tyrosine-based Inhibition Motif (ITIM) (D'Ambrosio *et al.*, 1995; Burshtyn *et al.*, 1996). In this respect, the first half of the Bgp motif centered around Tyr^{488} coincides perfectly with the ITIM motif (Figure 1C). Binding of SHP-1 to this sequence in all these cell surface proteins terminates signalling with profound effects on cellular proliferation.

To investigate the possibility that Bgp-dependent inhibition of epithelial tumor cell proliferation is mediated by the binding of SHP-1, we assessed whether Bgp mutants, incapable of binding SHP-1, were defective in their ability to mediate tumor inhibition *in vivo*. We found no evidence that SHP-1 association altered the inhibitory effect of Bgp in CT51 cells (Turbide *et al.*, unpublished). Therefore, the association of Bgp and SHP-1 in epithelial cells may not be able to inhibit cell proliferation, at least in the system tested.

Bgp was implicated in many cellular events such as intercellular adhesion, bile salt transport, insulin receptor internalization and inhibition of tumor cell growth (Ocklind and Öbrink, 1982; Rojas *et al.*, 1990; McCuaig *et al.*, 1992; Sippel *et al.*, 1993; Kunath *et al.*,

1995; Formisano *et al.*, 1995; Hsieh *et al.*, 1995). All of the above functions, except adhesion (Rojas *et al.*, 1990; McCuaig *et al.*, 1992) depend on the presence of the longer cytoplasmic domain of Bgp (Lin *et al.*, 1995). Bgp may also act as a cell surface receptor and while Bgp ligands have not yet been identified, it is conceivable that engagement of Bgp by its ligand(s) results in Bgp tyrosine phosphorylation. Alternatively, rat Bgp has been shown to be Tyr phosphorylated by the insulin receptor following insulin treatment (Margolis *et al.*, 1988; Najjar *et al.*, 1995). Since SHP-1 associates with the insulin receptor *via* its carboxy-terminal end (Uchida *et al.*, 1994), it is tempting to speculate that it could mediate interactions between the insulin receptor and Bgp. Association of SHP-1 with Tyr phosphorylated Bgp may be required to facilitate the recruitment of the tyrosine phosphatase to the cell membrane, possibly within macromolecular complexes (such as that found with the insulin receptor). This may then possibly lead to termination of insulin receptor endocytosis or other events by dephosphorylation.

Further functional studies will be needed to investigate the respective functions of Bgp and SHP-1 in epithelial cells. These can be pursued in the SHP-1-deficient *motheaten* mouse (Tsui et al., 1993) and in a bgp1-/- mouse.

Material and methods

Cell culture

Growth of CT51 mouse colon carcinoma cells, generously provided by Dr. Michael Brattain (Medical College of Ohio, Toledo, Ohio), and insertion of *bgp*1 cDNA constructs

by retroviral-mediated infection have previously been described (Kunath *et al.*, 1995). Cell clones were manually selected from G418-resistant populations (G418: 1.5 mg/ml) after FACS sorting using a Bgp-specific mAb AgB10 (Kuprina *et al.*, 1990). All cell clones were tested for Bgp cell surface expression and shown to be positive. All experiments were performed with a minimum of two clones for each transfectant cell line. Pervanadate treatment of cells was performed by incubating CT51 transfectant cells in α-minimal essential medium for 10 min at 37°C with a 10 mM H_2O_2 and 100 μM sodium vanadate solution (O'Shea *et al.*, 1992). Cells were collected from the dishes by scraping followed by centrifugation (1500 rpm) at 20°C for 3 min and subsequent washes in the same medium containing pervanadate.

Antibodies

A polyclonal antibody to the purified mouse colon p120 Bgp protein was raised in rabbits (Ab 231) (McCuaig *et al.*, 1992). Similarly, a rabbit polyclonal antibody was raised to the longer cytoplasmic domain of Bgp cleaved from a GST-fusion protein overexpressed in bacteria (Ab 836) (Rosenberg *et al.*, 1993). A monoclonal antibody to Bgp (CC1), recognizing an epitope in the N-terminal domain (Dveksler *et al.*, 1993), was a generous gift from Dr. K.V. Holmes (Smith *et al.*, 1991). A rabbit polyclonal antibody specific to the SHP-1 protein was raised to a GST-fusion protein encompassing the last C-terminal 85 amino acids of SHP-1 (Ab 838). The *Msc*I SHP-1 cDNA fragment [nt. 1682-1962 (Matthews *et al.*, 1992)] was cloned into a pGEX2T plasmid which was transformed into

1161 *E. coli* bacteria and IPTG-induced. The SHP-1 cDNA was generously provided by Dr. W. Muller (McMaster University, Hamilton, Ont.). This antibody (838) does not recognize SHP-2, a highly related protein tyrosine phosphatase (Feng *et al.*, 1993; Vogel *et al.*, 1993). Anti-SHP-2 antibodies were obtained from Santa Cruz Biotechnology and Upstate Biotechnology Incorporated. Anti-phosphotyrosine antibodies used were either a 1:1 mixture of anti-pY72 and pY20 hybridomas, generously provided by Dr. Bart Sefton (The Salk Institute, La Jolla CA), or clone 4G10.

Generation of Bgp mutants

Mutations were introduced in the cytoplasmic domain of the *bgp*D cDNA (McCuaig *et al.*, 1993) by single-strand excision with M13K07 helper virus of a uracyl-containing BlueScript SK$^-$ plasmid (Smith, 1985) containing a 1.1 kb *Sac*I-*Hind*III fragment of the *bgp*D cDNA (the *Hind*III site is from a Blue-Script SK+ vector containing the full length *bgp*D cDNA). Two oligonucleotides leading to Y488F and Y515F mutations as well as mutations abolishing an *Aat*II restriction site for Y488F or inserting a *Sty*I restriction site for Y515F without changing the encoded amino acids were synthesized: BC1, 5'GGTGGATGACGTGGCATTCACTGTCCTGAA3', BC2, 5'TCTCCAAGGGCCACAGAAACAGTTTTTTCAGAAG3'. These oligonucleotides were used to prime the polymerization of a second strand which was ligated by T4 DNA ligase and the resulting plasmid was transformed into *E. coli* JM109 cells. Mutations were identified by restriction digests and confirmed by DNA sequencing (Sanger *et al.*, 1977). The mutated *Sac*I-*Hind*III fragments were reintroduced into the pLXSN vector (Miller and

Rossmann, 1989) encompassing the *bgp*1 cDNA construct to produce a full length cDNA. Double Y488,515F mutants were generated by priming the synthesis with the two oligonuclotides.

Immunoprecipitation and immunoblotting

Cells were scraped from dishes, collected by centrifugation and lysed in a cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1% Nonidet NP-40 or 1% Brij, 10 μg/ml each of the protease inhibitors (leupeptin, aprotinin, phenylmethylsulfonyl fluoride, N-α-p-tosyl-1-lysine chloromethyl ketone, N-tosyl-1-phenylalanine chloromethyl ketone), 50 mM sodium fluoride and 1 mM sodium orthovanadate. Protein determinations were performed using a BCA protein assay (Pierce Chemicals) on total cell lysates after discarding cellular debris by centrifugation (5 min at 4°C, 11,000g). Either 1 mg or 2 mg of total cell lysates were immunoprecipitated with indicated antibodies for 2 h at 4°C and immune complexes were collected on protein A or G Sepharose beads. Proteins were separated on 8% SDS-PAGE gels, transferred to Immobilon filters and immunoblotted as described previously (Kunath *et al.*, 1995). Immune complexes were visualized by incubation of the membranes with either [^{125}I]-labelled protein A or an [^{125}I]-labelled goat anti-mouse IgG antibody. Quantification of the radioactive bands was performed on a Fuji BioAnalyzing System 2000.

Generation of GST-SHP-1 fusion proteins and in vitro binding assays

GST-SHP-1 fusion proteins were prepared by cloning SHP-1 cDNA fragments into a

pGEX2TK plasmid and overexpression in JM109 *E.coli* bacteria by 0.3 mM IPTG-mediated inductions for 3-12 h (Guan and Dixon, 1991). cDNA fragments were amplified by PCR using Vent DNA polymerase and the following oligonucleotides, designed to the SHP-1 sequence (Matthews *et al.*, 1992): SH2-N domain (nt. 113-403), 5'TGGTTTCACCGGGACC3' and 5'CAGTGGGTACTTAAGG3'; SH2-C domain (nt 431-742), 5'TGGTACCACGGCCACA3' and 5'GTAAGGCTGCCGCAGG3'; both SH2 domains (nt 113-742), first and last oligonucleotides mentioned above; and SHP-1 (nt 113-1897), the first oligonucleotide and 5'CCCAGATCACTTCCTC3'. The oligonucleotides were tagged with restriction cleavage sites and the resulting PCR fragments, submitted to restriction cleavage and cloned into the pGEX2TK vector. The cloned PCR fragments were submitted to DNA sequencing (Sanger *et al.*, 1977). Fusion proteins were bound to glutathione-coupled Sepharose beads (approximately 4 µg of fusion protein per binding reaction) and incubated with 1.0-1.5 mg of pervanadate-treated or non-treated total cell lysate proteins for 3 h at 4°C in 1 X lysis buffer as described by Yi *et al.*, 1993b. After washing the beads four times with lysis buffer, adsorbed proteins were eluted, submitted to electrophoresis and analysed by immunoblotting with an anti-Bgp antibody or an anti-pTyr antibody.

Acknowledgments

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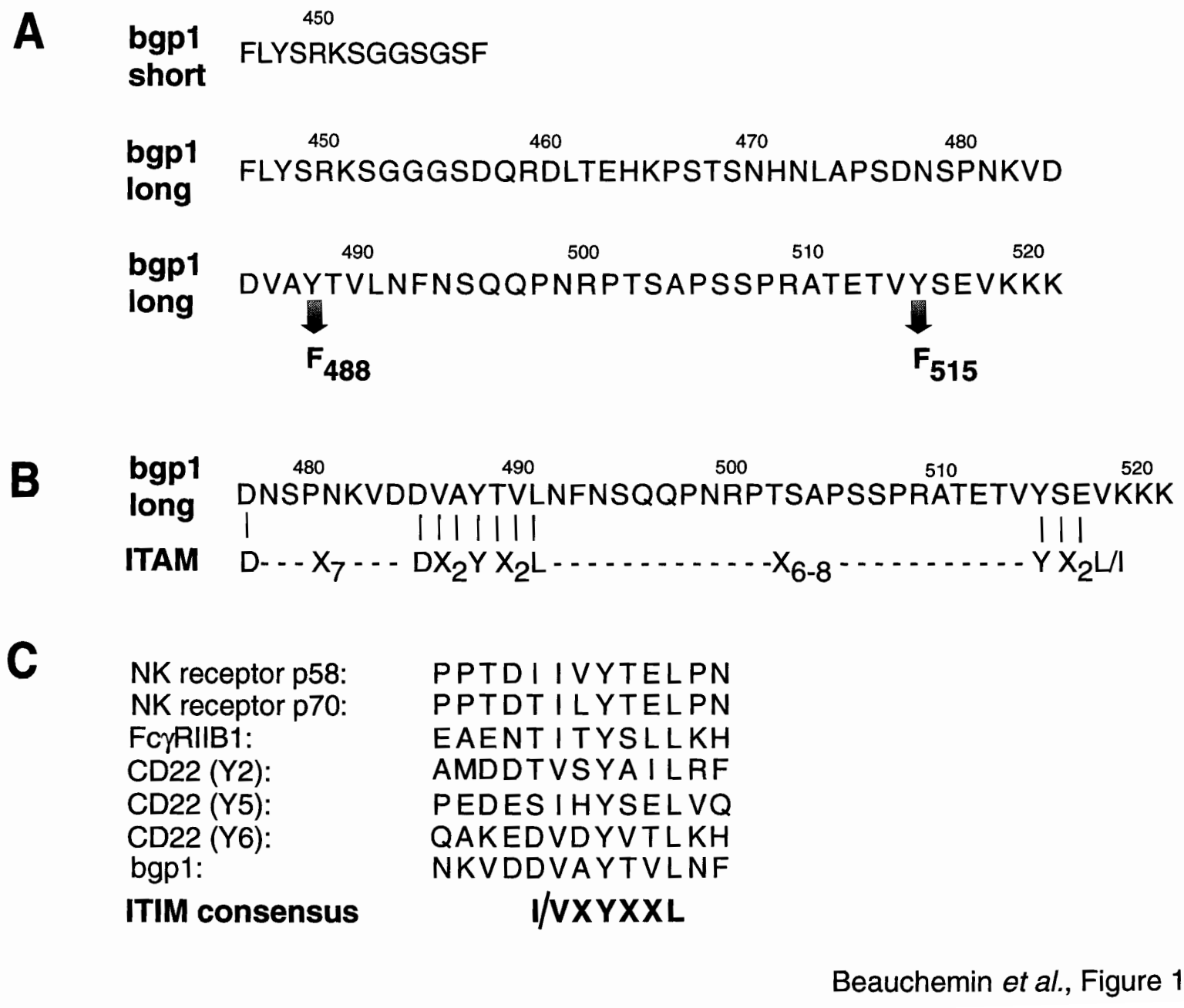
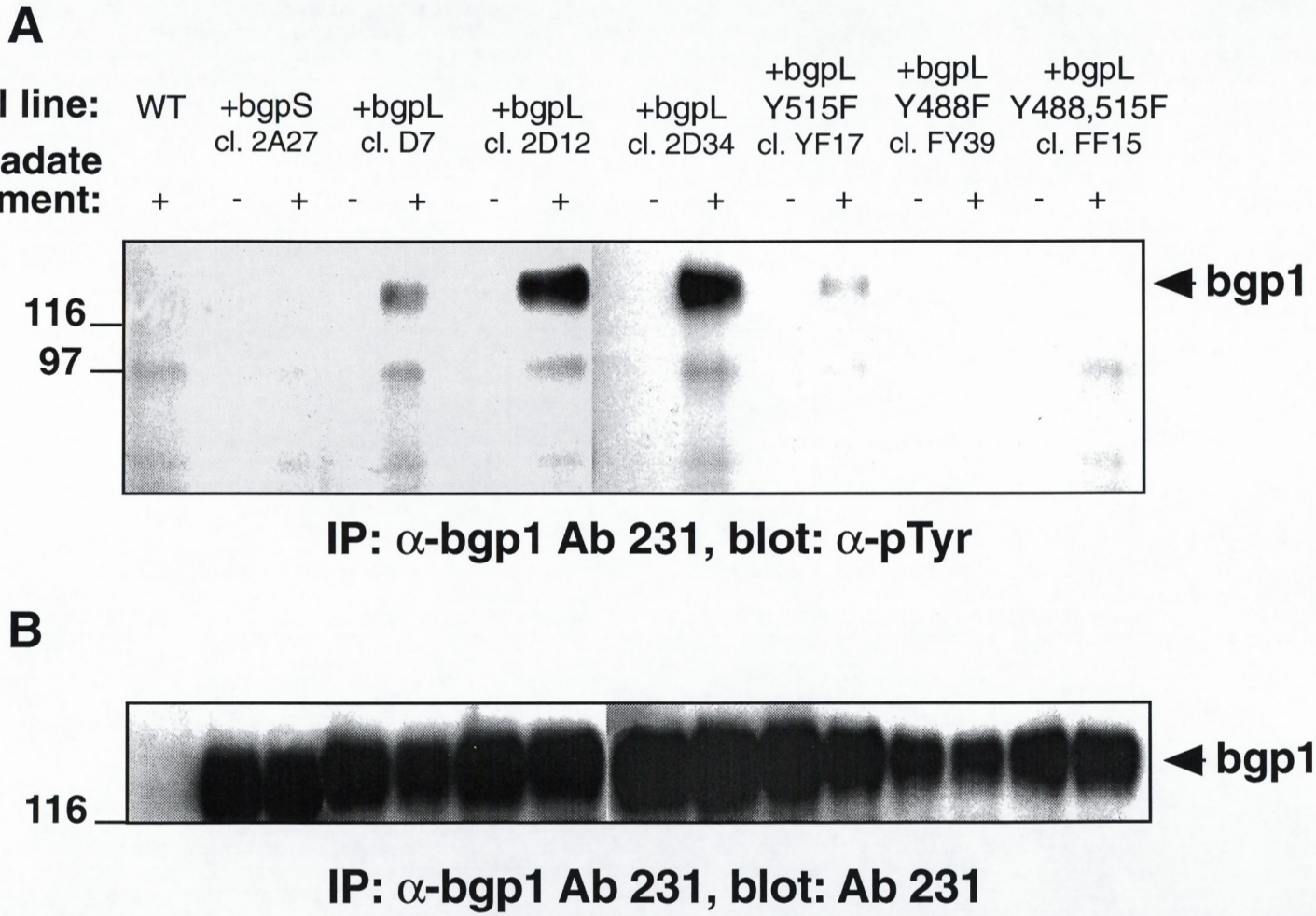
Figure 1 Consensus sites present on the Bgp cytoplasmic domains. (**A**) The amino acid sequence in single letter code is shown for the two alternatively spliced cytoplasmic domains of the Bgp isoforms. Numbers over the sequence correspond to the amino acid position in the full length sequence (McCuaig *et al.*, 1993). The large arrows below the Bgp sequence represent the positions of the Y→F mutations presented in this study. (**B**) Similarity of the Bgp cytoplasmic sequence and the consensus binding site for the Immunoreceptor Tyrosine-based Activation Motif. (**C**) Similarity of the Bgp cytoplasmic sequence and the consensus site for the Immunoreceptor Tyrosine-based Inhibition Motif present in various receptors (D'Ambrosio *et al.*, 1995; Doody *et al.*, 1995; Burshtyn *et al.*, 1996). The Y2, Y5 and Y6 residues represent the phosphotyrosines described in CD22 (Doody *et al.*, 1995).



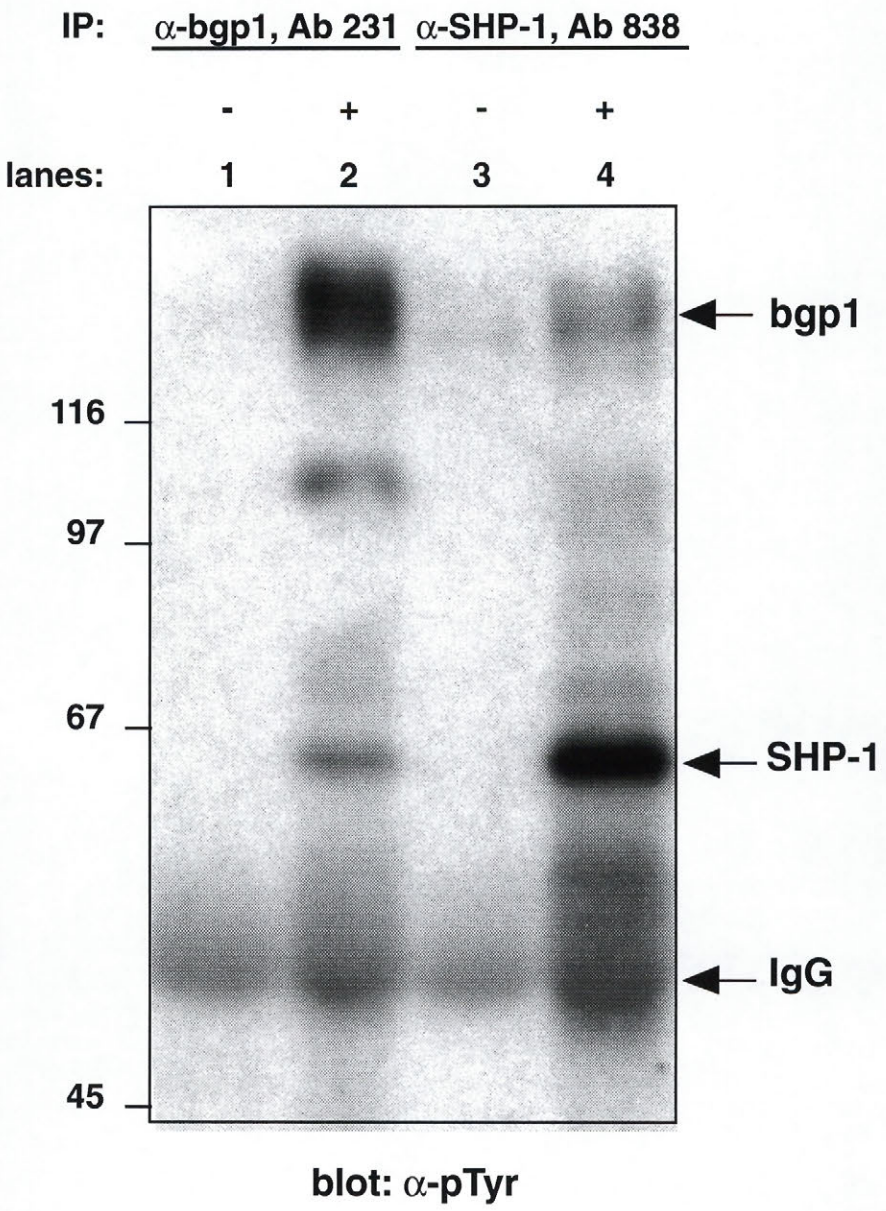
Beauchemin *et al.*, Figure 1

Figure 2 Tyrosine phosphorylation of the Bgp cytoplasmic domain. (**A**) Parental CT51 cells (WT) or CT51 cells retrovirally-infected with the *bgp*1 cDNAs expressing sequences of either a short (BgpS) or a longer (BgpL) cytoplasmic domain or Bgp mutated in either one (Y488F or Y515F) or both (Y488,515F) tyrosine residues were treated (+) or non-treated (-) with the phosphatase inhibitor pervanadate for 10 min at 37°C. The clone identifications are indicated beneath the cell lines. Total cell lysates were prepared and 1 mg of proteins were subjected to immunoprecipitation with anti-Bgp antibodies (Ab 231). Immune complexes adsorbed onto protein A Sepharose were subjected to electrophoresis and the proteins were immunoblotted with pY20 and pY72 anti-phosphotyrosine antibodies. (**B**) The same immunoblot was reprobed with anti-Bgp antibodies (Ab 231). The positions of the molecular weight markers (in kilodaltons) are indicated on the left and the position of Bgp on the right of the immunoblots.



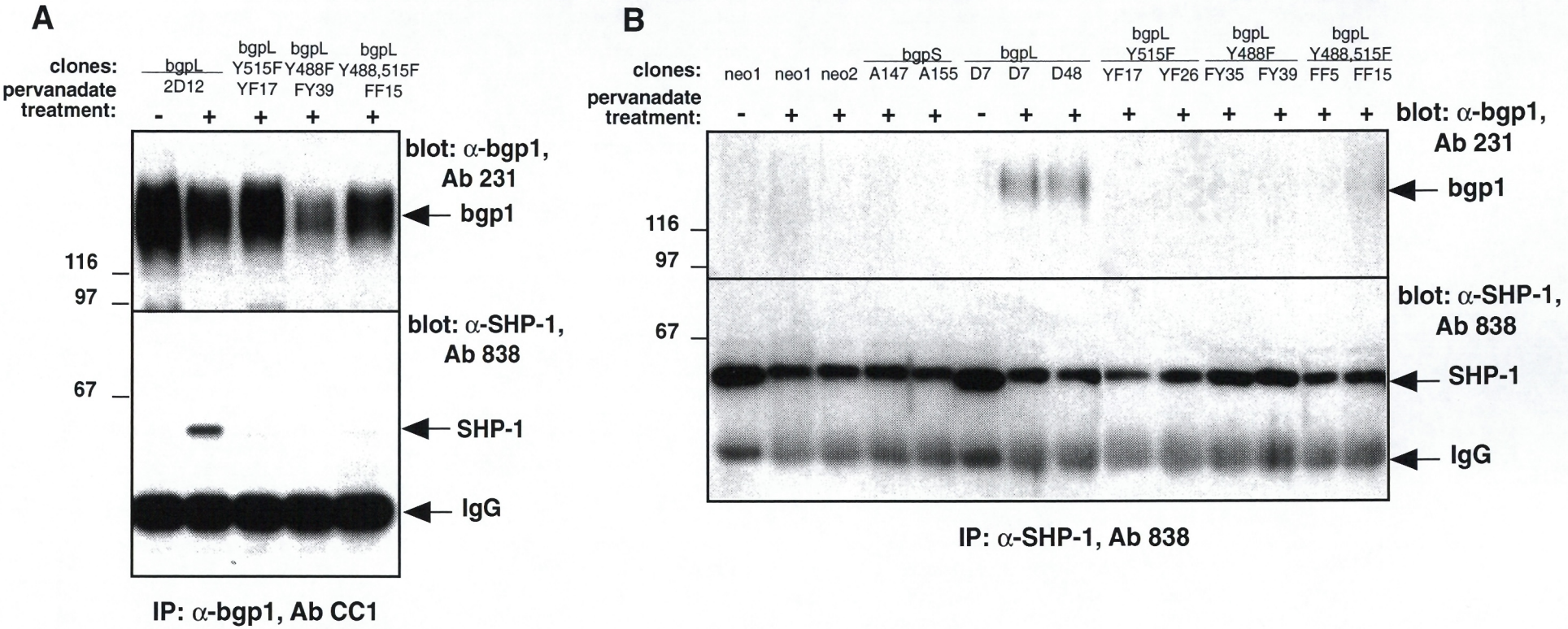
Beauchemin *et al.*, Figure 2

Figure 3 Association of a Tyr phosphorylated protein with Bgp. CT51 cells expressing the Bgp protein with a longer cytoplasmic domain (cl. 12) were treated (+) or not (-) with pervanadate for 10 min at 37°C and total cell lysates prepared. 1 mg of proteins were subjected to immunoprecipitation with either anti-Bgp (Ab 231) or anti-SHP-1 (Ab 838) antibodies. The immune complexes were separated by electrophoresis and the resulting proteins were immunoblotted with anti-phosphotyrosine antibody 4G10. The positions of the molecular weight markers (in kilodaltons) are indicated on the left and the position of Bgp, SHP-1 and Ig on the right of the immunoblots.



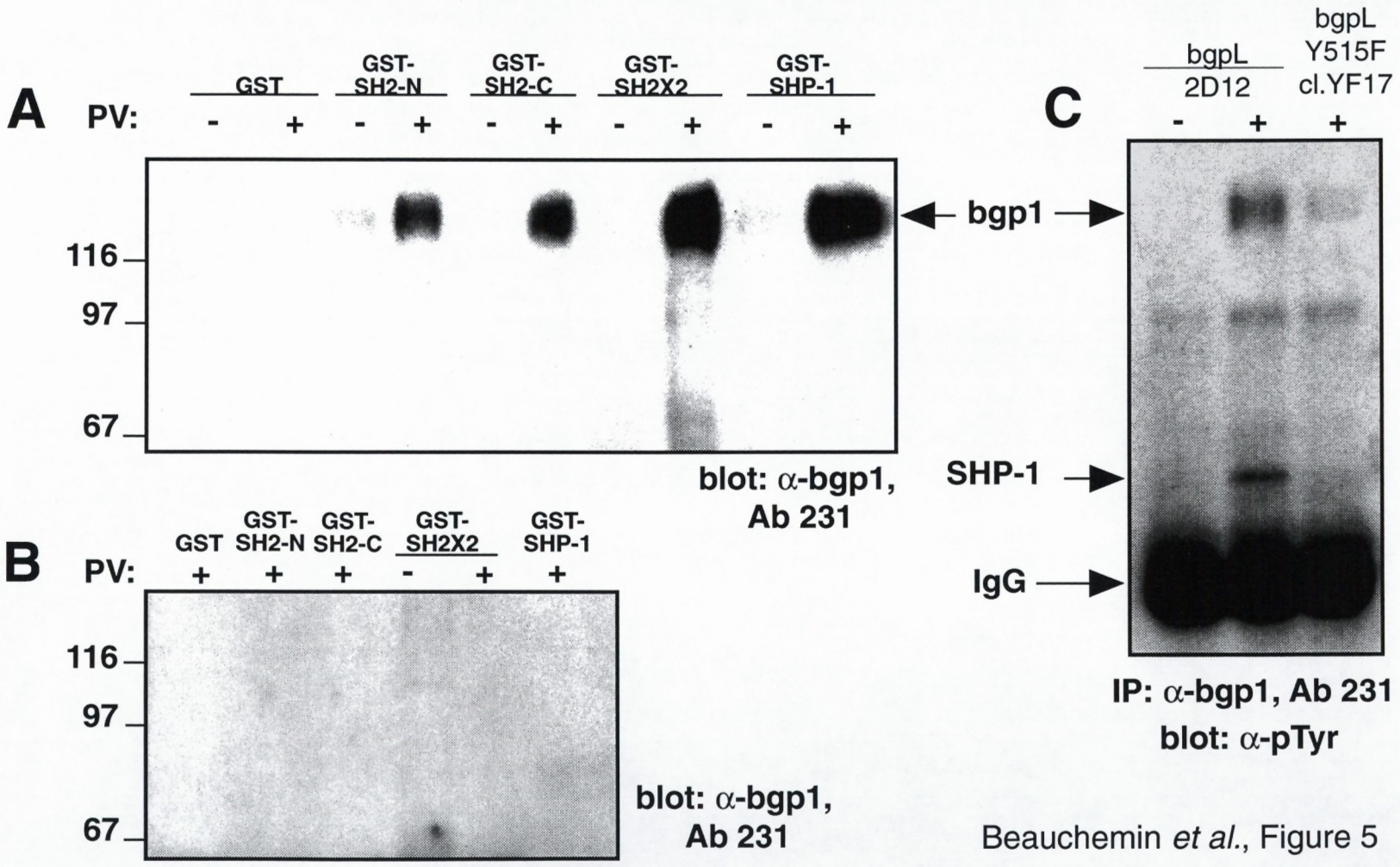
Beauchemin *et al.*, Figure 3

Figure 4 Association of the protein tyrosine phosphatase SHP-1 to Bgp. (**A**) CT51 cells transfected with the wild-type Bgp construct exhibiting a longer (BgpL) cytoplasmic domain, or cells transfected with Bgp mutated in either one (Y488F or Y515F) or both (Y488,515F) of its cytoplasmic tyrosine residues were treated (+) or not (-) with pervanadate for 10 min at 37°C. The clone numbers are indicated beneath the cell lines. Total cell lysates were prepared and 2 mg of total cell lysate proteins were subjected to immunoprecipitation with the anti-Bgp monoclonal antibody CC1, immune complexes were separated by electrophoresis and the resulting proteins were immunoblotted with either anti-Bgp (top, Ab 231) or anti-SHP-1 (bottom, Ab 838) antibodies. (**B**) The experiment was performed as above, but 1.0 mg of proteins were subjected to immunoprecipitation with anti-SHP-1 antibodies (Ab 838). The positions of the molecular weight markers (in kilodaltons) are indicated on the left and the position of Bgp, SHP-1 and Ig on the right of the immunoblots.



Beauchemin *et al.*, Figure 4

Figure 5 Binding of GST-SHP-1 fusion proteins to Bgp. (**A**) Cell lysate proteins prepared from CT51 cells expressing the BgpL construct exhibiting a longer cytoplasmic domain (cl. 12) treated (+) or non-treated (-) with pervanadate for 10 min at 37°C were incubated for 3 h at 4°C with various GST fusion proteins, comprising either none (GST) one (GST-SH2-N or SH2-C) or both (GST-SH2X2) SHP-1 SH2 domains, or the full-length SHP-1 phosphatase (GST-SHP-1), which had been immobilized on glutathione-Sepharose beads. The beads were washed, and the bound proteins resolved by electrophoresis and immunoblotted with anti-Bgp antibodies (Ab 231). The positions of the molecular weight markers (in kilodaltons) as well as the position of Bgp, SHP-1 and Ig are indicated. (**B**) The same experiment was repeated with the cell lysate proteins prepared from the CT51 cells expressing the Y515F mutant Bgp (cl. 17). (**C**) Total cell lysate proteins from the clones mentioned above were subjected to immunoprecipitations with antibodies to Bgp (Ab 231). The immune complexes were collected on protein A Sepharose beads, the proteins were denatured, resolved by SDS-PAGE electrophoresis and transferred to membranes which were immunoblotted with anti-phosphotyrosine antibodies pY20 and pY72.



Beauchemin *et al.*, Figure 5

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Chapter 4

Discussion

Discussion

The aberrant expression of several cell adhesion molecules (CAMs) have been implicated in the progression of many epithelial malignancies. For example, several members of the integrin family of CAMs were observed to be down-regulated in colorectal carcinomas (27), lung cancers (9), skin cancers (30), and breast carcinomas (43). However, the observed down-regulation of any particular molecule during carcinogenesis does not constitute proof of its involvement in the process. Functional studies must be performed to exclude the possibility that the observed aberrant expression is simply a consequence, and not a cause, of the cancer progression.

Introduction of α5ß1 integrin into transformed Chinese hamster ovary (CHO) cells resulted in the acquisition of a more normal phenotype (13). Transfected CHO cells exhibited a reduced saturation density in culture, a reduced ability to form colonies in soft agar, and most importantly, they were non-tumorigenic when injected subcutaneously into nude mice (13).

Like members of the integrin family, the cell adhesion molecule, BGP, was also observed to be down-regulated in several epithelial malignancies in three species (14, 26, 33, 17). To investigate the potential role of BGP in epithelial cancer progression, it was re-introduced into a mouse colon cancer cell line (CT51) that had completely lost BGP expression. As shown for the α5ß1-transfectant CHO cells, CT51 cells, individually transfected with two BGP isoforms, exhibited a reduced saturation density in culture and a reduced ability to grow in soft agar. However, a difference in the ability of the two

BGP isoforms to revert the transformed phenotype of CT51 was observed when transfected cells were subjected to an *in vivo* tumorigenicity assay. CT51 cells transfected with the BGP isoform possessing a short cytoplasmic tail (10 aa) was unable to inhibit tumor formation, while the BGP isoform with the long cytoplasmic domain (73 aa) reduced tumor formation by 80%. This result was intriguing, since both isoforms of BGP function as cell adhesion molecules *in vitro* (22, 36). In addition, the discrepancy between the results from the *in vitro* and *in vivo* tumorigenicity assays suggests different mechanisms may be operating in the respective assays.

The mechanism of tumor suppression by cell adhesion molecules is thought to be mediated by their adhesive properties. They have been postulated to play a role in the initial step of metastasis; the detachment of cells from the primary tumor. This is not to say that CAMs may not employ other mechanisms to suppress tumor formation, such as the transduction of anti-proliferative signals. In fact, integrins are well-known to be involved in signal transduction (16). However, several lines of experimental evidence do in fact attribute the adhesive properties of CAMs to their ability to suppress tumor formation. Qian *et al.* demonstrated that the expression of α4ß1 integrin on highly metastatic melanoma cells significantly reduced their invasive potential (32). α4ß1 integrin is a cell-cell and cell-matrix adhesion molecule (40, 5). The introduction of α4 cDNA into the $\alpha4^{-}ß1^{+}$ parental melanoma cell line, B16, promoted homotypic adhesion (32). Treatment with a monoclonal antibody against α4, not only inhibited homotypic adhesion *in vitro*, but also reverted the invasion suppression phenotype mediated by the α4ß1 integrin (32). This indicated that the effects elicited by α4ß1 were due to its

adhesive properties. In fact, it was demonstrated that α4ß1 only inhibited tumor cell invasion, but not cell growth. B16-α4 cells, injected subcutaneously, did not metastasize to the lung, while control transfectants (B16-neo) readily metastasized. However, when either B16-neo or B16-α4 cells were injected intravenously (bypassing the invasion step of metastasis), both cell lines were observed to metastasize to the lung (32). This brings to light an important distinction between a tumor suppressor protein and an invasion suppressor protein. Most CAMs, due their adhesive properties, are invasion suppressor proteins. They inhibit the initial steps of metastasis without having much effect on the growth rate of the primary tumor. E-cadherin (a Ca^{2+}-dependent CAM) was similarly shown to restrict the invasive nature of highly malignant epithelial cell lines without affecting their actual growth rates (7, 25). This classifies it as an invasion suppressor protein, rather than a tumor suppressor protein.

The fact that BGP transfectants grew at similar rates to the control transfectants at low cell densities, but exhibited a reduced saturation density at confluence, is more consistent with an invasion suppressor role than a tumor suppressor role. In concordance with the mechanism of invasion suppressors, the adhesion properties of BgpA (short-tail isoform) and BgpD (long-tail isoform) may be responsible for the inhibitory effects observed *in vitro*. However, the potential invasion suppressor properties of the BGP isoforms were not tested *in vivo*.

The parental cell line, CT51, is known to readily metastasize to the lung when injected subcutaneously (3), illustrating its invasive potential. Unfortunately, the ability of BGP transfectants of CT51 cells to metastasize has not yet been thoroughly

investigated. Since primary tumor growth was the only parameter monitored, the experiments in question addressed the *in vivo* tumor suppressor potential of the BGP isoforms, and not their invasion suppressor potential. As mentioned above, the long-tail isoform (BgpD) was able to inhibit primary tumor formation, while the short-tail isoform (BgpA) was not. This result indicated that BgpD elicited a true tumor suppressor phenotype, and this property was not due to adhesion, but rather to some element within its intracytoplasmic domain.

The similarity between the effects of BgpA and BgpD in the *in vitro* tumorigenicity assays may have been due to their adhesion properties, while the different phenotypes observed *in vivo* may have been due to the activation of a signal transduction cascade through the long cytoplasmic tail of BgpD. The *in vivo* environment may have provided the necessary ligands and/or counter-receptors required to elicit the anti-proliferative effects conferred by BgpD. The absence of such *in vivo* cues in the growth curve assays could explain the near wild-type growth rates at low cell densities. Taken together, these results suggest that BgpD is a tumor suppressor and also a potential invasion suppressor, while BgpA is simply a potential invasion suppressor. Further investigations of the metastatic potential of both BgpA and BgpD transfectants are required to confirm the invasion suppressor theory.

The tumor suppressive role of mouse BGP has been extended to the rat BGP homolog (C-CAM1) in a recent report by Hsieh *et al.* (15). They demonstrated that transfection of rat BGP into a human prostatic cancer cell line, PC-3, reduced its tumorigenicity *in vitro* and *in vivo* (15). Furthermore, transfection of an antisense BGP

construct into a rat non-tumorigenic prostatic epithelial cell line, NbE, resulted in a reduction of the endogenous BGP levels and a consequent increase in tumorigenicity *in vivo* (15). This report supports the observation that BGP acts as a tumor suppressor in epithelial malignancies by extending the findings to another species (rat) and to another tissue (prostate). The short-tail rat BGP isoform was not examined in this study and nor was the invasive potential of the control and transfected cells.

Determining the mechanisms of BGP-mediated tumor suppression would require investigating elements on the long-cytoplasmic tail and proteins that interact with it. The calcium-binding protein, annexin VI, has been demonstrated to potentially interact with rat BGP (21). Interestingly, annexin VI has been demonstrated to suppress the proliferation of epithelial vulval carcinoma (A431) cells in low serum concentrations (38). Annexin VI also exhibited tumor suppressor activity in A431 cells as determined by an *in vivo* tumorigenicity assay (37). It is tempting to speculate that BGP may mediate its tumor suppressor activity through annexin VI or *vice versa*, since they associate within the cell (21), and they have overlapping patterns of tissue expression (11). However, the simultaneous expression of both proteins in the cell lines exhibiting the tumor suppressor phenotype must first be demonstrated before such speculations can be seriously addressed.

In an attempt to systematically pin-point the important amino acid sequences on the long-cytoplasmic tail responsible for the tumor inhibition phenotype, a deletion mutagenesis strategy was devised. Six deletion mutants of the BgpD cytoplasmic domain were constructed (Figure 1, p.94). The mutants progressively shorten the long cytoplasmic domain of 73 aa to 13 aa. In addition, Ser^{503}, within the protein kinase C

Figure 1

Deletion Mutants of the BgpD Cytoplasmic Domain

BgpD SRKSGGGSDQRDLTEHKPSTSNHNLAPSDNSPNKVDDVA**Y**TVLNFNSQQP**NRPTS**APSSPRATETV**Y**SEVKKK

Δ2 SRKSGGGSDQRDLTEHKPSTSNHNLAPSDNSPNKVDDVA**Y**TVLNFNSQQP**NRPTS**APSSPRATETV**Y**SEV

Δ3 SRKSGGGSDQRDLTEHKPSTSNHNLAPSDNSPNKVDDVA**Y**TVLNFNSQQP**NRPTS**APSSPRA

Δ4 SRKSGGGSDQRDLTEHKPSTSNHNLAPSDNSPNKVDDVA**Y**TVLNFNS

Δ5 SRKSGGGSDQRDLTEHKPSTSNHNLAPSDNSPNKV

Δ6 SRKSGGGSDQRDLTEHKPSTSNHN

Δ7 SRKSGGGSDQRDL

BgpA SRKSGGSGSF

Figure 1: **Deletion mutagenesis statagey.** The six deletion mutants (Δ2 – Δ7) are shown from the shortest deletion to the longest deletion. The naturally-occuring cytoplasmic domains of two Bgp1 isoforms (BgpD and BgpA) are shown for comparison. The two tyrosines are indicated in a larger font and in bold. The PKC consensus sequence is indicated in bold. These mutants were generated by site-directed mutagenesis, as described (Chapter 3, p.71) and inserted into the pLXSN vector. These mutants were stably expressed in CT51 as described (Chapter 2, p.52).

(PKC) consensus sequence, was mutated to Ala^{503}. This mutation has already been observed to adversely affect bile acid transport (34) and receptor-mediated internalization of insulin (12). Transfection of these constructs into CT51 cells and subjecting them to tumorigenicity assays should reveal the elements necessary for tumor inhibition.

The intracytoplasmic tail of BGP has been shown to be phosphorylated on tyrosine residues by several groups (1, 35, 23). Since there is compelling evidence that tyrosine-phosphorylated proteins interact with Src homology 2 (SH2) domain-containing proteins (20), several candidate BGP-interacting proteins were screened. It was shown that tyrosine-phosphorylated BgpD associated with the SH2-containing cytoplasmic protein, SHP-1 (also known as PTP1C, HCP, and SH-PTP1). SHP-1 is a non-receptor protein tyrosine phosphatase (PTPase) that has two SH2 domains in its N-terminal region, followed by a phosphatase domain and a unique C-terminal domain of approximately 83 aa (24, 31). SHP-1 is predominantly expressed in hematopoietic and some epithelial cells (31). The role of SHP-1 in several cellular backgrounds has been investigated in detail. For example, SHP-1 was demonstrated to be specifically recruited to the erythropoietin receptor (EPO-R) in erythroid progenitor cells upon stimulation with the ligand, erythropoietin (EPO) (19). Upon ligand binding, EPO-R is known to transduce its proliferative signals by activating a constitutively-associated protein tyrosine kinase (PTK), Janus kinase 2 (JAK2) (41). Upon recruitment to EPO-R, SHP-1 was demonstrated to dephosphorylate JAK2 rendering it inactive, which consequently down-modulated the previously induced proliferative signal (19). The more N-terminal SH2 domain (N-SH2) was responsible for the recruitment to tyrosine-phosphorylated EPO-R,

while the second SH2 domain (C-SH2) appeared dispensable (19).

The inhibitory effects of SHP-1 has been dramatically illustrated by the phenotype of the *motheaten* (*me/me*) mouse; an established mouse strain deficient in SHP-1 expression (see review 2). Among other defects, these mice exhibit hyperproliferation and inappropriate activation of many cells of the myeloid lineage. In a recent report, Chen *et al.* demonstrated that SHP-1 negatively regulated signalling by the growth factor, colony-stimulating factor 1 (CSF-1) (6). The lack of SHP-1 in *me/me* mice resulted in the hyperphosphorylation of the CSF-1 receptor and a hyperproliferative response of *me/me* macrophages upon CSF-1 stimulation (6).

SHP-1 has also been implicated in the down-regulation of B cell receptor (BCR) signalling by two different pathways. Recruitment of SHP-1 to tyrosine-phosphorylated FcγRIIB1 or CD22 has been shown to negatively regulate BCR signalling (8, 10). Similarly, in natural killer (NK) cells, the inhibitory receptor, p58, mediated the inhibition of target cell lysis by the recruitment and activation of SHP-1 (4).

The above examples all point to an inhibitory role for SHP-1 in cellular physiology. Since SHP-1 also associated with tyrosine-phosphorylated BgpD, it was thought to be directly involved in the observed *in vivo* tumor suppressor phenotype. However, mutation of two critical BgpD tyrosine residues, necessary for SHP-1 association, did not revert the tumor suppressor effects of BgpD (28). This indicated that other elements within the long cytoplasmic domain were responsible for the tumor inhibition phenotype. As mentioned above, the deletion mutagenesis strategy should pin-point these elements (Figure 1, p.94).

Since SHP-1 does not appear to be involved in the *in vivo* anti-proliferative effects of BgpD, its association must regulate an alternative function. A candidate function worth investigating is the potential BGP-regulated internalization of insulin and its receptor (12). Uchida *et al.* demonstrated SHP-1 to be a substrate of the insulin receptor (IR) tyrosine kinase; phosphorylation of Tyr^{538} in the unique C-terminal region of SHP-1 resulted in a five-fold increase in phosphatase activity (39). However, they could not demonstrate SH2 domain-mediated association between SHP-1 and IR (39). The phosphorylation of BGP by the IR tyrosine kinase, and the subsequent recruitment of SHP-1 to the membrane (via BGP), could possibly bring the PTPase into the appropriate vicinity to become phosphorylated and activated by the insulin receptor. This indirect route of recruitment and activation of SHP-1 could provide the time-delay required for the insulin receptor to signal before it is presumably shut down by SHP-1 dephosphorylation. It must first be demonstrated that the insulin receptor is a suitable substrate for dephosphorylation by SHP-1 before the above hypothesis can be further investigated.

The mechanism of SHP-1 binding to BgpD appears to be entirely novel. Efficient SHP-1 association required the presence of two tyrosine residues within the cytoplasmic tail. Mutation of one tyrosine or the other abolished SHP-1 association *in vivo* and *in vitro*. Furthermore, either SH2 domain (N-SH2 or C-SH2) efficiently bound BGP *in vitro*. These results are in contrast to previously reported mechanisms of SHP-1 binding: a single particular phosphotyrosine residue is normally sufficient for SHP-1 binding (8, 19), and the association is mediated by either the N-SH2 domain (9, 42) or the C-SH2

domain (4, 8), but not both domains.

The two SH2 domains of SHP-1 have recently been demonstrated to have different functions in terms of regulating the PTPase domain (29). The N-SH2 domain exhibited an inhibitory effect on the PTPase domain unless it was occupied by a phosphopeptide, while the C-SH2 domain did not exhibit this property (29). These findings led Pei *et al.* to propose that the N-SH2 domain served, not only as a recruitment module, but also a regulatory domain, whereas the C-SH2 domain simply behaved in a recruiting capacity (29). The novel mechanism of SHP-1 association with BgpD indicates that the interaction of the N-SH2 and C-SH2 domains with each other and with the phosphoprotein in question may not be as simple and straight-forward as Pei *et al.* (29) suggest. Investigation of the phosphatase activity of SHP-1 in the presence of BGP-derived phosphopeptides will begin to unravel the novel mechanisms mediating this association.

Future Directions

The tumor suppressive properties of mouse and rat BGP in epithelial cancers may be exploited to treat human cancers of epithelial origin, such as colorectal and prostate carcinomas. Although it has been demonstrated that rat BGP can suppress the tumorigenicity of a human prostatic cancer cell line, PC-3 (15), the ability of human BGP to function in the same capacity has yet to be investigated. Despite this fact, recombinant adenovirus delivery of rat BGP to experimental *in vivo* human prostatic tumors has been investigated (18). Kleinerman *et al.* showed efficient and stable expression of rat BGP in PC-3 cells infected with a recombinant adenovirus containing the rat BGP cDNA (18). Furthermore, they demonstrated the ability of this recombinant adenovirus to suppress *in vivo* tumor growth for 2 to 3 weeks per dose (18). These preliminary results are encouraging with respect to the potential clinical utility of BGP gene therapy. Future investigations should address the potential tumor suppressive properties of human BGP isoforms possessing the longer cytoplasmic domain. Gene therapy with human cDNA constructs is preferable to using cDNA from another species, such as rat.

The elucidation of the mechanisms of BGP-mediated tumor inhibition will also aid in designing treatments for epithelial cancers. As mentioned above, the current deletion mutagenesis strategy (Figure 1, p.94) should initiate the solving of this puzzle. Before gene therapy with BGP can be realized, the normal function of this glycoprotein must first be determined. Without this knowledge, any adverse side-effects due to the introduction of BGP into cells cannot be properly assessed.

Although the association of SHP-1 to BGP does not appear to be involved in the tumor inhibition phenotype, it is still an interesting and important finding. The interaction of BGP with SHP-1 may shed some light on the normal functions of BGP *in vivo*. Future studies into the *in vivo* significance of this interaction can be carried out in the SHP-1-deficient *motheaten* (*me/me*) mouse (2). Hepatocytes from *me/me* livers can be analyzed for changes in bile acid transport activity and altered kinetics of receptor-mediated internalization of insulin (two activities known to be affected by the state of BGP phosphorylation). Any unusual phenotypes observed with respect to these assays can be further investigated by transfection experiments of primary cell cultures or established cell lines. The Bgp1-null mouse (currently being established) should also prove to be very useful in determining the *in vivo* functions of BGP and the significance of its association to SHP-1.

The possibility of BGP performing different roles in different cell types is quite likely. Although this makes the task of identifying these functions difficult and complex, the final results from the combined investigations will prove to be very interesting and exciting. The identification of BGP ligands and the mechanisms controlling the observed alternative splicing will provide insight into the *in vivo* functions of BGP in normal cellular physiology. They will also shed light on the importance of the long cytoplasmic domain of BGP and its role in tumor inhibition.

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