Biliary glycoprotein (Bgp, C-CAM, or CD66a) is an immunoglobulin-like cell adhesion molecule and functions as a tumor suppressor protein. We have previously shown that the Bgp1 isoform responsible for inhibition of colonic, liver, prostate, and breast tumor cell growth contains within its cytoplasmic domain two tyrosine residues positioned in immunoreceptor tyrosine-based inhibition motif (ITIM) consensus sequences. Moreover, we determined that these residues, upon phosphorylation, associate with the protein-tyrosine phosphatase SHP-1. In this report, we have further evaluated the structural bases of the association of Bgp1 with Tyr phosphatases. First, we demonstrate that Bgp1 also associates with the SHP-2 Tyr phosphatase, but not with an unrelated Tyr phosphatase, PTP-PEST. Association of Bgp1 and SHP-2 involves the Tyr residues within the Bgp1 ITIM sequences, Val at position +3 relative to the second Tyr (Tyr-515), and the SHP-2 N-terminal SH2 domain. In addition, our results indicate that residues +4, +5, and +6 relative to Tyr-515 in the Bgp1 cytoplasmic domain play a significant role in these interactions, as their deletion reduced Bgp1 Tyr phosphorylation and association with SHP-1 and SHP-2 by as much as 80%. Together, these results indicate that both SHP-1 and SHP-2 interact with the Bgp1 cytoplasmic domain via ITIM-like sequences. Furthermore, they reveal that the C-terminal amino acids of Bgp1 are critical for these interactions.

Biliary glycoprotein (Bgp),1 also known as C-CAM or CD66a, is a cell-surface immunoglobulin-like glycoprotein and a member of the carcinoembryonic antigen family (1, 2). Whereas one BGP gene has been identified in human, two very similar Bgp genes (Bgp1 and Bgp2) are expressed in the mouse (3, 4). The BGP genes in all species studied so far are subjected to very similar alternative splicing events (1, 3, 5). The most commonly encountered isoforms of the mouse Bgp1 gene exhibit four extracellular Ig domains and cytoplasmic domains, which include either a short 10-amino acid peptide (BgpS) or a longer version of 73 amino acids (BgpL) (3, 6). Bgp1 is expressed in epithelial cells of the gastrointestinal tract (7). In addition, the Bgp1 protein is expressed in epithelial cells of the reproductive system, where its expression is hormonally controlled (7). Bgp1 is also present at the surface of endothelial cells and in hematopoietic cells, in particular in B cells, macrophages, and interleukin-2-activated T cells (7–10).

Several functions have been attributed to the biliary glycoprotein. First, it functions as an intercellular adhesion molecule (6, 11, 12). This function requires the first Ig domain (13, 14) and is independent of the cytoplasmic region (15, 16). Second, mouse Bgp1 and Bgp2 behave as receptors for all strains of the mouse hepatitis viruses (4, 17), whereas human BGP binds to bacterial proteins from Escherichia coli, Salmonella typhimurium, or Neisseria gonorrhoeae (18–20). Furthermore, we have recently shown that Bgp1 acts as a negative regulator of colon tumor cell growth and that this role is dependent on the presence of the longer cytoplasmic domain of Bgp1 (21). Similar findings have been reported in human prostate and breast carcinoma models (22, 23).

Recent reports have also suggested that Bgp1 behaves as a signal transduction molecule. Several physiological events promote the Tyr phosphorylation of Bgp1 on one or two Tyr residues within its cytoplasmic domain (Tyr-488 and Tyr-515). BGP becomes Tyr-phosphorylated by Src-like Tyr kinases in activated neutrophils (24) and in human colon carcinoma cells (25) and is a Tyr phosphorylation substrate for the insulin receptor (26). Moreover, we (27) and others (28) have shown that Tyr-488 also undergoes phosphorylation when cellular phosphatases are inactivated by the pharmacological inhibitor vanadate. Whereas the physiological significance of Bgp1 Tyr phosphorylation remains largely to be defined, it has been reported that stimulation of Bgp1 (CD66a) in neutrophils leads to activation of Rac1, PAK, and Jun N-terminal kinase (29).

Interestingly, the amino acid sequences surrounding Tyr-488 and Tyr-515 of Bgp1 perfectly match that of an immunoreceptor tyrosine-based inhibition motif (ITIM). This includes a phosphorylated Tyr residue preceded by a Val, Ile, or Leu residue at position −2 and followed by a critical Leu or Val residue at position +3 (30, 31). In addition, as defined using mutated phosphopeptides, other residues such as those positioned at −4 as well as +1, +2, +4, and +5 relative to the Tyr

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1 The abbreviations used are: Bgp or BGP, biliary glycoprotein; ITIM, immunoreceptor tyrosine-based inhibition motif; PCR, polymerase chain reaction; GST, glutathione S-transferase.

### The Carboxyl-terminal Region of Biliary Glycoprotein Controls Its Tyrosine Phosphorylation and Association with Protein-tyrosine Phosphatases SHP-1 and SHP-2 in Epithelial Cells*

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residue within the ITIM might also contribute to the effective binding of SHP-1 with the natural killer cell inhibitory receptors (31). The ITIM consensus sequence is found in a number of hematopoietic cell-surface receptors such as Fc\γ receptor IIB, the B cell accessory receptor CD22, and the natural killer cell inhibitory receptors. These receptors associate with the cytosolic protein-Tyr phosphatase SHP-1, which possesses two N-terminal SH2 domains, leading to attenuation or inhibition of Tyr phosphorylation-elicted signaling in various cellular systems (30, 32, 33). Optimal SHP-1 association with Tyr-phosphorylated proteins is mediated by both of two tandem N-terminal SH2 domains (34). In most cases, once SHP-1 is bound to receptors, it catalyzes their dephosphorylation (35).

As our studies related to Bgp1-dependent tumor inhibition were being conducted in mouse colonic carcinoma cells (21), we questioned whether Bgp1 Tyr phosphorylation was conducive to any significant protein associations in this cellular background. We have recently shown that Tyr phosphorylation of the mouse Bgp1 cytoplasmic domain in CT51 mouse colonic carcinoma cells led to its binding to the protein-Tyr phosphatase SHP-1 and that this event required the presence of both Tyr-488 and Tyr-515 (27). In vitro binding assays confirmed that either one of the SHP-1 SH2 domains could bind to Bgp1 (27). We now report that, in addition to its interaction with SHP-1, Bgp1 physically associates with another ubiquitously expressed cytosolic protein-Tyr phosphatase, SHP-2 (36, 37). The association with SHP-2, like that with SHP-1, is dependent upon the expression of both Bgp1 cytoplasmic Tyr residues and the phosphorylation of at least one Tyr residue. Binding of SHP-2 requires a Val residue within the second ITIM sequence of the Bgp1 cytoplasmic domain. In addition, the N-terminal SH2 domain of SHP-2 interacts with Tyr-phosphorylated Bgp1. Furthermore, in reconstituting the association of Bgp1 deletion and point mutants with the Tyr phosphatases in CT51 mouse colonic epithelial cells and 293 human embryonic kidney cells, we found that the Bgp1 carboxyl-terminal region is critical for the regulation of its association with the protein-Tyr phosphatases SHP-1 and SHP-2. Residues +4, +5, and +6 relative to Tyr-515 within the second Bgp1 ITIM are necessary for maximal phosphatase association with Bgp1. These results represent the first report that residues located outside the classical ITIM consensus sequence influence the in vivo binding of the Tyr phosphatases SHP-1 and SHP-2. These three residues may consequently impede a particular conformation on the Bgp1 cytoplasmic domain or may be involved in anchoring other Bgp1 cytosolic partners to the cell surface.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Growth of CT51 mouse colon carcinoma cells, generously provided by Dr. Michael Brattain (Medical College of Ohio, Toledo, OH), and isolation of the Bgp1 cDNA constructs by retrovirus-mediated infection have been described previously (21). Cell clones were selected from G418-resistant populations (G418: 1.5 mg/ml), and Bgp1-positive clones were identified by fluorescence-activated cell sorter analyses and immunoblotting as reported previously (21). Experiments were performed with either cell populations or a minimum of two clones for each transfected cell line. Pervanadate treatment of cells was performed by incubating transfected CT51 cells in α-minimal essential medium for 10 min at 37 °C with a solution of 10 mM H2O2 and 100 μM sodium vanadate (38). Cells were collected from the dishes by scraping, followed by centrifugation and subsequent washes in the same vandate-containing medium. Human embryonic kidney cells (HEK293) were obtained from the American Type Culture Collection (ATCC CRL1573) and grown in Dulbecco’s modified Eagle’s medium containing 2 mM glutamine and 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) at 37 °C in a humidified atmosphere of 5% CO2. Calcium precipitates were removed after 18-h incubations, and the cells were cultured for an additional 24 h and then lysed.

**Cloning of the SHP-2 cDNA from a Mouse Thymocyte cDNA Library—**The mouse Syt cDNA (GenBank™ accession number L08663) was kindly provided by Dr. Gen-Sheng Feng (Indiana University School of Medicine, Indianapolis, IN). The C-terminal regions of mouse BALB/c 3T3 Syt and brain SHP-2 were differenced, and the mouse SHP-2 cDNA, the Syt cDNA was used as a probe on a C57B1 mouse thymocyte cDNA library, kindly provided by Dr. L. A. Matis (Yale University School of Medicine, New Haven, CT). Several cDNA clones were isolated and subjected to nucleotide sequencing (40). Clone 9 encoded a 4-kilobase pair cDNA encompassing the complete coding sequence of the Tyr base pairs identical to that found in a mouse SHP-2 cDNA (GenBank™ accession number D84372). Cloning of the mouse PTP-PEST cDNA has previously been reported (41).

**Site-directed Mutagenesis—**Mutations were introduced into the cytoplasmic domain of the Bgp1 cDNA by single-strand excision with M13K07 helper virus containing BlueScript SK® plasmid containing a 1.1-kilobase pair SacI-HindIII fragment of the Bgp1 cDNA (the HindIII site is from a BlueScript SK® vector containing the full-length Bgp1 cDNA). The mutations Y488F, Y515F, and Y488F,Y515F have been described previously (27). The S503A mutant was produced in the same way using the following primer, creating a new EcoI site: 5′-ACCAACCGCAGACTGACGCCCCTTTTCT-3′ (nucleotides in boldface represent the mutations). The Δ465 deletion mutant introduced a stop codon at amino acid 484 and a new SacI site (5′-TAAACGGTCTAGACGTGACAATCAG-3′), whereas the Δ495 mutant introduces a stop codon at amino acid 496 and a new BsiI site (5′-TCCTAATCTCTGAACACCCAGG-3′). The Δ510 deletion mutant has a stop codon at amino acid 511 and an added Styl site (5′-TTCTTCTCTCAGGAAACAGTTA-3′). The Δ518 deletion mutant has a stop codon inserted at amino acid 519 and a new Msel site (5′-TTATCGAAGTTAATGAAGTGCAGTAC-3′). Point mutations were also introduced in the C-terminal region of the Bgp1 cytoplasmic domain using an overlap PCR technique (42). Two PCR fragments were generated for each mutant using a combination of common and specific oligonucleotides. The common oligonucleotide KM4 (5′-ACCAACCGCAGACTGACGCCCCTTTTCT-3′) is located at nucleotides 1352–1375 within the Bgp1 cDNA and contains an endogenous StyI site, and a common T oligonucleotide (5′-TAAATGCACTACTATTAGGG-3′) of the Bluescript SK® vector was also used. Mutation of the three terminal Lys residues to Ala residues was accomplished using the forward primer 5′-TCAGAATGTCGACGCCCCTTTTCTGAC-3′ and the reverse primer 5′-TATATGCTCAGGCCCCTTTTCTGAC-3′. For mutation of the terminal Lys residues to Arg residues, the forward primer 5′-TATATGCTCAGGCCCCTTTTCTGAC-3′ and the reverse primer 5′-TAATTCAGAAGTAAAGTGTCGAC-3′ were used. The S503A deletion mutant intro-duced a new stop codon at amino acid 519 and removes an

**Phosphatase Reactivity Assays—**Western blots of cell lysates were performed by incubating Cys-453 with Ser (27). Phosphatase-inactive SHP-2 mutants were generated by converting Cys-453, present in the active site of the enzyme, to Ser. These mutations were created by the overlap PCR technique (42) using the following combination of primers: nucleotides 1353–1369 (5′-GCATTATGATTCAGGAAAGAG3′) and nucleotides 1555–1603 (5′-GTTCTACGAGCGCTTGGA3′), whereas the 483 deletion mutant introduces a new stop codon at amino acid 484 and removes an

**Antibodies—**An anti-mouse Bgp1 polyclonal antibody, specific to the extracytoplasmic Ig domains of Bgp1, has been previously described (6). Antibodies specific to the C-terminal regions of either SHP-1 or SHP-2 were raised in rabbits using TrpE fusion proteins. A fragment from the mouse SHP-2 cDNA corresponding to amino acids 553–597 was amplified by PCR and cloned into the pATH11 vector, and antibodies were generated against the induced purified fusion protein. Neither the SHP-1 nor the SHP-2 antibodies exhibit cross-reactivity. A polyclonal antibody was raised against the non-catalytic portion of mouse PTP-PEST has previously been described (41). This antibody does not cross-react with the human PTP-PEST phosphatase. A mouse monoclonal antibody specific to v-Src was purchased from Oncogene Research Products (Cambridge, MA), whereas an anti-phosphotyrosine (Tyr(P)) antibody (4G10) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The polyclonal antibody to the GST protein was a kind gift from...
Association of SHP-2 with Biliary Glycoprotein

Results

Association of Bgp1 and SHP-2 in CT51 Mouse Colonic Carcinoma Cells and HEK293 Cells—We have previously demonstrated that SHP-1 associates with Bgp1 in mouse colonic carcinoma cells (27). Another SH2-containing protein-Tyr phosphatase, SHP-2, is also recruited to the cell surface following activation of Tyr-phosphorylated receptors (36, 37). We therefore verified whether SHP-2 was expressed in CT51 cells and whether it also associated with Bgp1. None of the available antibodies directed to the mouse Bgp1 protein elicited Bgp1 Tyr phosphorylation. As no specific ligands for Bgp1 capable of provoking Tyr phosphorylation in colonic cells have yet been identified, we therefore made use of the pharmacological inhibitor pervanadate, which is known to inactivate endogenous protein phosphatases, thereby mimicking activation of protein-tyro sine kinases (35, 38). A Bgp1-expressing CT51 cell clone was subjected to pervanadate treatment; the cells were lysed with non-ionic detergent-containing buffers; and the Bgp1 protein was immunoprecipitated with anti-Bgp1 antibodies. SHP-2 was revealed by blotting with its specific antibody (Fig. 1A). SHP-2 was absent from immune complexes prepared from untreated cells (Fig. 1A, lane 1), but was found associated with Bgp1 immune complexes prepared from cells treated with pervanadate (lane 2). Expression of Bgp1 and SHP-2 in the CT51 cells was monitored by immunoblotting cell lysate proteins with their respective antibodies (Fig. 1A, lower panels). Therefore, upon Bgp1 Tyr phosphorylation, the SHP-2 Tyr phosphatase associates with Bgp1, presumably via its cytoplasmic domain.

To evaluate the potential association of Bgp1 with SHP-2 in a different cellular system, we also performed transfection experiments in HEK293 cells. HEK293 cells were therefore transiently transfected with the wild-type Bgp1 cDNA in combination with the SHP-1 and SHP-2 Tyr phosphatase constructs (Fig. 1B). As it has previously been shown that Src induces Tyr phosphorylation of Bgp1 (25), we included the constitutively activated mouse c-src Y529F cDNA in these transfection assays. Levels of Bgp1 Tyr phosphorylation and Bgp1 association with the various phosphatases were determined after lysis of the transfected cells, immunoprecipitation of Bgp1, and detection with either anti-Tyr(P) antibodies or the phosphatase-specific antibodies (Fig. 1B). HEK293 cells do not express the endogenous Bgp proteins as revealed by immunoprecipitation/immunoblotting experiments using Bgp-specific antibodies (Fig. 1B, third panel, lane 1). As revealed on the anti-Tyr(P) immunoblots (Fig. 1B, first panels, lanes 2 and 5), transfection of the Bgp1 and c-src Y529F cDNAs in these cells led to Bgp1 Tyr phosphorylation. HEK293 cells do not endogenously express the SHP-1 Tyr phosphatase, but SHP-2 was present in the lysates (Fig. 1B, fourth panels, lanes 1 and 2 versus lane 5). We then examined whether adding the wild-type SHP-1 or SHP-2 cDNA or their catalytically inactive versions (C453S or C459S, respectively) altered the Bgp1 Tyr phosphorylation status and consequent Tyr phosphatase association. The Cys-to-Ser mutations in the SHP-1 and SHP-2 Tyr phosphatases inactivate their phosphatase activity, but do not compromise their binding characteristics (35). The levels of Bgp1 expressed in the transfected cells were uneven, with this protein being particularly abundant in Fig. 1B (lane 3). However, this reinforced the fact that, despite the large amount of Bgp1 in this sample, its Tyr phosphorylation level was characteristically lower when the wild-type SHP-1 phosphatase was present compared with samples in which the catalytically inactive SHP-1 phosphatase was expressed (17-fold higher in this experiment) (Fig. 1B, lane 4 versus lane 3). The -fold values of Bgp1 Tyr phosphorylation were normalized to the expression of the Bgp1 protein present in each lane. Furthermore, when equal levels of Bgp1 were expressed as in Fig. 1B (lanes 6 and 7), the levels of Bgp1 Tyr phosphorylation were again lower when the wild-type SHP-2 phosphatase was included in the assay relative to those when the Cys-to-Ser SHP-2 mutant was used (7-fold higher) (Fig. 1B, lane 7 versus lane 6). This result suggested that the wild-type SHP-1 and SHP-2 phosphatases may be using Bgp1 as a substrate. Correspondingly, in both cases, the association of the SHP-1 or SHP-2 phosphatase with Bgp1 was concomitant with the Tyr phosphorylation levels of the latter protein. This was quantified as a 15-fold increase in Cys-to-Ser SHP-1 or SHP-2 binding relative to wild-type SHP-1 or SHP-2 phosphatase binding.

To investigate the specificity of the Bgp1 interaction with the SHP Tyr phosphatases, we considered whether other protein-Tyr phosphatases would also bind to this protein. PTP-PEST is a ubiquitous enzyme that contains an N-terminal phosphatase...
FIG. 1. Bgp1 associates with SHP-2 in mouse colonic carcinoma cells and HEK293 cells. A, a CT51 Bgp1-transfected cell line was treated or not with the phosphatase inhibitor pervanadate (PV). Cells were lysed with detergent-containing buffers, and 600 μg of cell lysate proteins were immunoprecipitated (IP) with anti-Bgp1 antibodies. Bound proteins were resolved by 8% SDS-polyacrylamide gel electrophoresis and immunobotted with an anti-SHP-2 antibody (first panel). Expression of the Bgp1 and SHP-2 proteins was monitored by immunoblotting 100 μg of cellular proteins with either anti-Bgp1 (second panel) or anti-SHP-2 (third panel) antibodies.

B, HEK293 cells were transiently transfected with either wild-type Bgp1 and constitutively activated mouse c-src Y529F together with either wild-type (WT) SHP-1 or SHP-2 or their catalytically inactive
Association of SHP-2 with Biliary Glycoprotein

Association of Bgp1 with SHP-2 Requires Both Bgp1 Tyr Residues—We have previously demonstrated that the presence of both Bgp1 Tyr residues and the phosphorylation of Tyr-488 was necessary for maximal association of the SHP-1 phosphatase (Fig. 3, first panel, lanes 1 and 2), although the PEST phosphatase and Bgp1 were adequately expressed (second and third panels) and phosphorylated in these cells (data not shown). Due to the specificity of the antibody used, the mouse PTP-PEST phosphatase, was not detected in untransfected HEK293 cells (Fig. 1, first panel, lanes 1–4) as well as recruitment of the SHP-2 Tyr phosphatase (second panel, lanes 6 and 8) as well as recruitment of the SHP-2 Tyr phosphatase (second panel, lanes 6 and 8). Mutation of either Tyr-488 or Tyr-515 within the Bgp1 cytoplasmic domain still gave rise to a Tyr-phosphorylated Bgp1 protein (Fig. 3, third panel, lanes 9–12). This result is at variance with our own previously reported data (27), where we had been unable to detect phosphorylation of Tyr-515 when Tyr-488 was mutated. In this report, we used a different anti-Tyr(P) antibody (4G10), which detected a Tyr-phosphorylated Bgp1 protein in the Y488F mutant clone. Note that the Bgp1 expression in this clone was lower than in the Y515F clone (Fig. 3, third panel, lanes 9–12), yet Bgp1 Tyr phosphorylation was still detectable (Fig. 3, first panel, lane 12). Mutation of either one of the Bgp1 Tyr residues led to abrogation of SHP-2 Tyr phosphatase association (Fig. 3, second panel, lanes 10 and 12). This result was further confirmed using Bgp1 deletion mutants in which Tyr-488 or Tyr-515 was removed from the cytoplasmic domain (see below) or in transient transfection assays in

cDNA constructs. Cells were lysed, and 600 μg of cell lysate proteins were immunoprecipitated with anti-Bgp1 antibodies. Phosphorylated proteins present in the immune complexes were detected with anti-Tyr(P) antibody 4G10 (first panels). Association of SHP-1 and SHP-2 with the Bgp1 protein was detected with their respective antibodies (second panels). Expression of Bgp1 (third panels), SHP-1 or SHP-2 (fourth panels), and c-Src (fifth panels) was monitored by immunoblotting 50 μg of cell lysate proteins with relevant antibodies. C, similar experiments as those described for B were performed with the PTP-PEST phosphatase construct. Association of the PTP-PEST phosphatase with Bgp1 was monitored using anti-PEST antibodies (first panel), whereas expression of Bgp1 (second panel), PTP-PEST (third panel), and c-Src (fourth panel) was monitored with their respective antibodies. Molecular mass standards are indicated on the left (in kilodaltons), and the positions of Bgp1, SHP-1, SHP-2, and PTP-PEST are shown on the right. PTP, protein-tyrosine phosphatase.
HEK293 cells (data not shown); in all these cases, loss of SHP-2 association was noticed when either one of the Tyr residues was mutated. As expected, mutation of both Tyr residues together revealed neither Bgp1 Tyr phosphorylation nor SHP-2 association (Fig. 3, first and second panels, lane 14). Therefore, both Bgp1 Tyr residues are necessary for maximal association of the SHP-1 (27) and SHP-2 Tyr phosphatases.

**Molecular Requirements for Maximal Bgp1 Tyr Phosphorylation and Association with the SHP-1 and SHP-2 Tyr Phosphatases**—To investigate whether other subregions or residues of the Bgp1 cytoplasmic domain were involved in binding to SHP-1 or SHP-2, a series of deletion mutants depicted in Fig. 2 was prepared. The Δ518 mutant was designed to remove the three carboxyl-terminal Lys residues, well conserved (KK(K/Q)) among the mouse, rat, and human BGP cytoplasmic domains. Furthermore, truncations removing the residues surrounding Tyr-515 (Δ510 mutant), those encompassing a conserved Ser phosphorylation site located at Ser-503 (Δ495 mutant) (43, 44), and finally those positioned close to Tyr-488 (Δ483 mutant) were also created. A Ser-503 point mutant converting this residue to a non-phosphorylatable Ala was also designed (S503A mutant). These Bgp1 mutants were stably expressed in CT51 cells, and cell populations or clones were derived and subjected to analyses for the Bgp1 Tyr phosphorylation status and its association with the SHP-1 and SHP-2 Tyr phosphatases. Inactivation of the endogenous phosphatase activity was effected by pervanadate treatment of the cells, and the Bgp1 protein was recovered by immunoprecipitation. Proteins associated with the immune complexes were subjected to immunoblotting with either anti-Tyr(P) or anti-SHP-2 antibodies (Fig. 4). As shown previously, the pervanadate treatment provoked enhanced Tyr phosphorylation of Bgp1 and its binding to SHP-2 (Fig. 4, first and second panels, lanes 1 and 2). However, removing a fragment of the cytoplasmic domain including both Tyr residues (Δ483 mutant) did not reveal any Tyr phosphorylation or association of the Bgp1 cytoplasmic domain with the Tyr phosphatase (Fig. 4, first and second panels, lanes 9 and 10). Although the expression of this Bgp1 mutant was less than that seen with the wild-type protein (Fig. 4, third panel, compare lanes 9 and 10 with lanes 1 and 2), longer exposures of the blots did not reveal any Bgp1 Tyr phosphorylation (data not shown). Interestingly, deleting the last three Bgp1 Lys residues reduced Bgp1 Tyr phosphorylation by at least 50% and almost completely abrogated binding of Bgp1 to SHP-2 (inhibition of 80 ± 14%) (Fig. 4, second panel, lanes 3 and 4). Further removal of residues at the C terminus eliminating amino acids surrounding Tyr-515 (Δ510) or Ser-503 (Δ495) completely abolished SHP-2 binding to Bgp1, which exhibited barely visible levels of Bgp1 Tyr phosphorylation, detectable only after long exposures of the blots (Fig. 4, first and second panels, lanes 5–8). In contrast, mutating Ser-503 to Ala did not produce significant differences when compared with results obtained with the wild-type Bgp1 protein (Fig. 4, third panel, compare lanes 1 and 2 with lanes 11 and 12, relative to the Bgp1 expression). SHP-1 association with these mutants was also examined and gave essentially similar results (data not shown). It should also be noted that comparable results were obtained by transiently transfecting HEK293 cells with the same Bgp1 deletion or point mutants together with c-src and catalytically inactive SHP-1 or SHP-2 phosphatase constructs (data not shown). Therefore, the Bgp1 C-terminal region is involved in controlling the levels of Bgp1 Tyr phosphorylation and, consequently, Bgp1 association with the Tyr phosphatases SHP-1 and SHP-2.

As the three C-terminal Lys residues appeared to be crucial in these interactions and since this represented the first observation that residues outside the ITIM consensus sequence affected the in vivo binding of the phosphatases to a cell-surface protein, we further investigated the possible mechanisms responsible for this reduction. We first focused on two issues: the
charge of these residues and their role within a potential protein kinase C consensus sequence, S<sup>V</sup>XXK<sub>1–3</sub> (45). Accordingly, additional Bgp1 mutants were generated in which the three Lys residues were converted to either Arg (3K→3R) or Ala (3K→3A) (Fig. 2) or alternatively, Ser-516 within the protein kinase C site was also mutated to Ala. As shown in Fig. 4, replacing the Lys residues with Arg or Ala residues did not hinder Bgp1 Tyr phosphorylation or reduce its association with the SHP-2 Tyr phosphatase whether tested in cell populations or clones (Fig. 4, first and second panels, lanes 15–20). Similarly, mutation of Ser-516 to Ala had no effect on either Bgp1 Tyr phosphorylation or the levels of SHP-2 binding (data not shown). Similar binding results were obtained with the SHP-1 phosphatase (data not shown). Hence, it appears that the presence of these Lys residues, more than their net charge or their role in a potential protein kinase C-mediated Ser-516 phosphorylation, is the determining factor in regulating association of Bgp1 with the Tyr phosphatases. In addition, the involvement of Val-518, located within the second ITIM consensus sequence site (pY<sup>518</sup>XXV), was also investigated by mutating it to Ala (V518A). This mutation completely eliminated SHP-2 and SHP-1 binding to Bgp1 (decrease of 97 ± 0.7%) (Fig. 4, second panel, lanes 13 and 14) (data not shown), although it did not significantly affect Bgp1 Tyr phosphorylation (first panel, lanes 13 and 14). Thus, the second ITIM consensus sequence is crucial for association of either SHP-1 or SHP-2 with Bgp1.

In Vitro Binding of SHP-2 to Bgp1—We have previously described that the two SH2 domains of SHP-1 bind equally well to Tyr-phosphorylated Bgp1 (27). To determine whether similar binding properties could be demonstrated for SHP-2, various bacterially expressed GST fusion domains of SHP-2 bound to glutathione-Sepharose beads were incubated with proteins from Bgp1-expressing CT51 cell lines. After several washes, bound proteins were eluted, resolved by electrophoresis, and detected by immunoblotting with anti-Bgp1 or anti-GST antibodies (Fig. 5). Equal molar amounts of the GST fusion proteins were adsorbed onto the glutathione-Sepharose beads, as revealed on an anti-GST immunoblot (Fig. 5B). In both unstimulated and pervanadate-treated cells, no Tyr-phosphorylated Bgp1 bound to the GST protein alone (Fig. 5A, lanes 9 and 10) or to the carboxyl-terminal SH2 domain (C-SH2) of SHP-2 (lanes 5 and 6), whereas a low but consistent amount of Bgp1 associated with the SHP-2 N-terminal SH2 domain (N-SH2) in each of four repeated experiments (lanes 7 and 8). Using fusion
proteins containing the two SHP-2 SH2 domains increased Bgp1 binding by a factor of 11 relative to binding with the N-terminal SH2 domain alone (Fig. 5A, compare lanes 3 and 7). Interestingly, under pervanadate-treated conditions, the binding of Bgp1 to full-length SHP-2 increased by 14–15-fold (Fig. 5A, lane 1) relative to the binding under untreated conditions (lane 2). Therefore, in contrast to the results obtained with SHP-1 in similar experiments (27), the SHP-2 N-terminal SH2 domain binds to the Bgp1 cytoplasmic domain. Moreover, full-length SHP-2 demonstrated a 40–45-fold increased binding relative to the N-terminal SH2 domain alone, suggesting that the presence of other domains or the overall conformation of the SHP-2 Tyr phosphatase may influence its binding to Bgp1. A low amount of Bgp1 from the untreated transfected CT51 cells was found to be associated with full-length SHP-2 (Fig. 5A, lane 2), demonstrating that a fraction of Bgp1 is endogenously Tyr-phosphorylated in these cells, as previously shown in Fig. 3.

**DISCUSSION**

In this work, we have demonstrated that the protein-Tyr phosphatase SHP-2 associates with the Tyr-phosphorylated form of Bgp1 in CT51 mouse colon carcinoma cells and in 293 human embryonic kidney cells. The association of Bgp1 with SHP-2 seems to parallel that observed previously with SHP-1 (27), except that only the N-terminal SH2 domain of SHP-2 appears capable of this interaction, whereas either SH2 domain of SHP-1 was competent in binding to the Bgp1 cytoplasmic domain. There may be, however, large differences in the relative affinities of the SH2 domains of SHP-1 or SHP-2 for binding to the cytoplasmic domain of Bgp1; this is currently being measured using surface plasmon resonance technology.

We have also investigated the molecular requirements leading to the association of Bgp1 with the Tyr phosphatases. Mutational analyses had previously shown that Tyr-488 was subject to Tyr phosphorylation events (27); we have now confirmed using point mutations that Tyr-515 is also capable of sustaining Tyr-phospho-transfer in vivo, albeit with less apparent efficiency than Tyr-488. Yet, association of both Tyr phosphatases requires the presence of both Bgp1 Tyr residues as well as their Tyr phosphorylation. Interestingly, deletion of the Lys residues located at the C-terminal end of Bgp1 significantly diminished SHP-2 binding, suggesting some direct or indirect interactions between the C-terminal residues and the Tyr residues for efficient phosphorylation of the cytoplasmic domain. Further deletions of a few residues within the Bgp1 cytoplasmic domain (Δ510 mutant) almost completely abrogated Bgp1 Tyr phosphorylation, and association with the phosphatases was undetectable. In addition, elimination of the protein kinase C phosphorylation site at Ser-503, previously shown to regulate both bile transport and internalization of the insulin receptor (43, 44), produced minimal differences in Bgp1 Tyr phosphorylation compared with that detected with wild-type Bgp1. However, conversion of Val-518 to Ala abolished SHP-2 binding and is therefore crucial for these protein interactions.

The terminal Lys residues of Bgp1 are located at positions +4, +5, and +6 relative to the second ITIM consensus sequence. Although they are positioned outside the classical ITIM consensus sequence, they appear to play a determining role in the association of either of the Tyr phosphatases. Several reports have suggested that residues +3, +4, and +5 relative to the ITIM sequences can dramatically affect either maximal SHP-1 or SHP-2 binding or levels of activity (31, 46, 47). Substitutions at positions +1, +2, +4, and +5 relative to the first ITIM of the natural killer cell receptor decreased phosphatase activation and presumably binding by approximately 50% (31). These results were obtained using synthetic peptides encompassing residues adjacent to the first Tyr residue within the cytoplasmic domain of the natural killer cell inhibitory receptor (31). Similarly, the crystal structure of the SHP-2 phosphatase complexed with peptides indicated that the side chains of phosphatase residues +3 and +5 relative to the phosphotyrosine interacted with the peptides (46). Furthermore, high affinity binding of the SHP-2 N-terminal SH2 domain requires contact with residues beyond position +3 of the ITIM (47). The results presented in this report thereby further reinforce these findings and provide additional evidence that residues +4, +5, and +6 downstream of an ITIM (TX51XXV) can dramatically influence the in vivo association and presumably the activity of the protein-Tyr phosphatases.

Several mechanisms have been explored to explain the decreased Bgp1 Tyr phosphorylation following the deletion of the three Lys residues located at the C-terminal end of Bgp1. These Bgp1 residues are likely to represent important hallmarks as they are conserved across species (KKK/K/Q). First and most obvious, we considered the effect of loss of net charge of the cytoplasmic domain. This could possibly lead to altered conformation of this domain, which would render Tyr-488 more or less accessible to Tyr kinases and phosphatases. However, replacement of the Lys residues with either uncharged Ala residues or charged Arg residues restored the binding of both SHP-1 and SHP-2 to Bgp1, indicating that the presence of three residues, irrespective of their identity, constitutes the most determinant factor. Furthermore, we had speculated that the three Lys residues might be part of a protein kinase C consensus sequence leading to the phosphorylation of Ser-516 and potentially to variations in the levels of Bgp1 Tyr phosphorylation. However, replacement of the three Lys residues with Ala residues or mutation of Ser-516 to Ala did not alter Bgp1 Tyr phosphorylation levels and consequent association of the Tyr phosphatases. Alternative mechanisms remain to be investigated; for instance, the proximity of the last Bgp1 ITIM sequence to the carboxyl-terminal end of the protein could potentially result in greater accessibility of the phosphatases to the Bgp1 phosphotyrosine residues, and this should be more closely examined. This is not a unique occurrence as SHPs or SIRP proteins also exhibit a similar ITIM sequence near their carboxyl-terminal ends (48, 49). In addition, other Ig-like proteins such as ILT1 and ILT2 also contain an ITIM sequence in this same position (50). Moreover, the CD22 adhesion molecule, shown to act as an accessory receptor to the B cell receptor complex and to associate with SHP-1 (51), contains six potential ITIM consensus sequences, the last one of which is at the extreme carboxyl-terminal end of the protein (52). In this particular case, it has been shown that competition with a peptide corresponding to the sixth ITIM sequence of CD22 can inhibit binding of SHP-1 to this glycoprotein (52).

Another mechanism to consider is that this Bgp1-conserved Lys sequence motif may also be involved in its own protein-protein interactions, and disruption of such associations through deletion of the Lys residues might induce dramatic changes in the local environment. We have recently identified Bgp1 cytosolic partners in a yeast two-hybrid screen, and the potential role of the Lys residues in regulating the association of these proteins with Bgp1 is currently being examined. Another mechanism involved might be the disruption of Bgp1 dimers. Hunter et al. (53) have reported that Bgp1 forms homodimers in equilibrium with monomers either in intact epithelial cells or as purified proteins. Conformation of the Bgp1 cytoplasmic domain may be governed as a complex dynamic...

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S. Sadekova and N. Beauchemin, manuscript in preparation.
process, as suggested by recent models proposed by Obrink (54). One of these models proposes that Ser and/or Tyr phosphorylation of the Bgp1 cytoplasmic domain could lead to dissociation of dimerized cytoplasmic domains, thereby rendering them capable of sustaining novel interactions such as those observed with the SHP-1 or SHP-2 Tyr phosphatase (54). In this context, modulation of the Tyr phosphorylation levels of Bgp1 dependent upon the presence and/or accessibility of its three C-terminal Lys residues would constitute a regulatory event.

It should be noted that our study represents the first report (to our knowledge) of either Tyr phosphatase associating with a cell-surface adhesion molecule in intestinal cells. A few reports have examined the roles played by SHP-1 or SHP-2 in association with cell-surface receptors in epithelial cells. For instance, both SHP-1 and SHP-2 are known to associate with the epidermal growth factor receptor in A431 human epidermoid carcinoma cells, where SHP-1 mediates the dephosphorylation of this receptor (55). In MCF-7 human breast carcinoma cells or in TRMP canine kidney epithelial cells, SHP-1 associates with the platelet-derived growth factor receptor and the p85 subunit of phosphatidylinositol 3-kinase and negatively regulates platelet-derived growth factor receptor-mediated activation of the c-fos promoter (56). This same receptor as well as the epidermal growth factor receptor were also shown to bind to SHP-2 in epithelial cells (57), where, in the latter case, SHP-2 acts as a positive mediator of epidermal growth factor signaling (58). Similarly, in primary mammary epithelial cells, the protein-Tyr phosphatases may act as regulatory targets via the extracellular matrix proteins in integrin-mediated milk signaling (58). Similarly, in primary mammary epithelial cells, SHP-2 acts as a positive mediator of epidermal growth factor signaling (58). One of these models proposes that Ser and/or Tyr phosphorylation of this receptor could lead to dissociation of association of the Tyr phosphatases with Bgp1 (48, 49). These proteins contain, within their cytoplasmic domain, four ITIM consensus sequences, one or several of which are involved in their association with the SHP-1 and SHP-2 Tyr phosphatases. These proteins also convey negative phosphorylation signals in response to growth factor stimulation or upon cell adhesion-induced signaling.

In addition, Bgp1 is an intercellular adhesion molecule whose homophilic abilities are likely to be controlled by intracytoplasmic associating partners. Recent studies on PECAM-1 (platelet endothelial cell adhesion molecule-1) give credence to this suggestion (62–64): PECAM-1 is a member of the Ig superfamily and is responsible for contacts between leukocytes or platelets with the surface of endothelial cells. Indeed, the last 10 amino acids of Bgp1, surrounding Tyr-515, demonstrate striking homology to a region of 18 amino acids within PECAM-1, particularly those centered around Tyr-686 (Bgp1, SSPTATVYSEVVKK, PECAM-1, LGTATVYSEERKVD). Recently, Jackson et al. (65, 66) have demonstrated that PECAM-1 becomes Tyr-phosphorylated on two Tyr residues during platelet aggregation (Tyr-663 and Tyr-686) and that, consequently, SHP-2 binds to activated PECAM-1. The maximal SHP-2-binding site in PECAM-1 is dependent upon the presence and phosphorylation of Tyr-686, which corresponds to the conserved sequence in Bgp1 (67). Furthermore, Famigletti et al. (68) have convincingly demonstrated that a point mutation of Tyr-686 is sufficient to convert heterophilic adhesion to homophilic binding. It is therefore tempting to speculate that SHP-1 and/or SHP-2 binding to Bgp1 may cause switches in its intercellular adhesion characteristics; this hypothesis is currently being studied.

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REFERENCES

3 L. Izzi and N. Beauchemin, manuscript in preparation.
The Carboxyl-terminal Region of Biliary Glycoprotein Controls Its Tyrosine Phosphorylation and Association with Protein-tyrosine Phosphatases SHP-1 and SHP-2 in Epithelial Cells
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