Cloning a cDNA for human IgGFc binding protein (FcγBP) from human colonic epithelial cells reveals an mRNA and coding region of 17 and 16.2 kilobases, respectively. The predicted amino acid sequence contains 12 occurrences of a 400-amino acid cysteine-rich unit resembling that found in mucin. A motif (CGLCGN) in the N-terminal 450-amino acid sequences is necessary and sufficient to confer IgG Fc binding activity. FcγBP mRNA is expressed only in placenta and colonic epithelial cells. These results suggest that FcγBP may play an important role in immune protection and inflammation in the intestines of primates.  

Each antibody isotype has specific biological activities that are dependent on the Fc receptor (FcR) it binds (1, 2). Notably, the mucin-like protein we characterize here has the biological activity of IgG binding, suggesting that it is an important component of mucosal immune system and inflammation.  

**MATERIALS AND METHODS**

Isolation and DNA Sequence Analysis of cDNA Clones—Human colonic or ileal epithelial cells were isolated as described previously (13). The epithelial cells were pelleted and resuspended in PBS (pH 7.4) and centrifuged at 4 °C for 10 min. Total RNA was prepared with the method of Chomczynski and Sacci (20). Poly(A) + RNA for the cDNA library construction was prepared by affinity purification with oligo(dT)-latex beads (digoxigen-dT30, Takara Shuzo Co.). cDNAs were synthesized from the poly(A) + RNA using random primers and reverse transcriptase, as described by the manufacturer (cDNA kit, Amersham Life Science, Inc.). Then, EcoRI-NorI-BamHI linkers (Takara Shuzo Co.) were ligated with the cDNAs. A random primed cDNA library for screening with monoclonal antibodies was fractionated in size greater than 500 bp, with an average of about 1.5 kb, and constructed into the EcoRI site of pgt11 vector (Stratagene). The cDNA library was screened by standard procedures (21) with monoclonal antibody K9 or K17 directed against colonic FcγBP. Restriction endonuclease fragments (q and a/b as shown in Fig. 1B) from the isolated cDNA clones were used as probes to isolate longer cDNA clones from the cDNA library constructed as follows. Poly(A) + RNA prepared from human colonic epithelial cells was size-fractionated by agarose gels (>3 kb) and used to construct cgt10 libraries for the use of these probes.
screening with DNA probes. Subsequent screening was carried out with the amplified library. 2 × 10^6 phage clones were plated on Luria-Bertani agar dish (10 × 13 cm), and the plaques were transferred onto Biodyne A nylon filter (Nippon Genetics Co.). The filters were hybridized with probe q and probe a/b end-labeled with [32P]dCTP and [33P]dCTP, respectively to nucleotides 33 to 54 of the 908-bp primer-labeled 908-bp fragment derived from clone NZC as described in the manufacturer instruction manual. DNA sequencing of double-stranded plasmid DNAs and single-stranded DNAs rescued with VCSM13 helper phage was performed with standard dye primer-labeled cycle-sequencing techniques using an Applied Biosystems 373A DNA sequencer, and, in some experiments, internal sequencing primers were synthesized with a DNA synthesizer Model 394 (Applied Biosystems) for sequencing. Gene Works 2.3 (IntelliGenetics, Inc.) Macintosh software was used for the DNA sequencing analysis.

**Northern Blot Analysis**—20 µg of total RNA prepared from colonic epithelial cells or from the human HT-29–18-N2 (designated as N2) cells was subjected to denaturing formaldehyde-agarose gel electrophoresis, transferred, and fixed onto a nylon membrane. The blots were prehybridized in the same solution as described in the screening method. Hybridization was performed with probe a, q, or y in the same solution at 42 °C overnight. The blots were washed three times with 0.2 × SSC containing 0.2% SDS at 65 °C for 40 min and exposed to x-ray film at −80 °C overnight. For DNA sequencing, a nest of induction fractionally deleted from each end was generated by exonuclease III and mung bean nuclelease digests (Kilo sequence deletion kit, Takara Shuzo Co.) as described in the manufacturer's notice. DNA sequencing of double-stranded plasmid DNAs and single-stranded DNAs rescued with VCSM13 helper phage was performed with standard dye primer-labeled cycle-sequencing techniques using an Applied Biosystems 373A DNA sequencer, and, in some experiments, internal sequencing primers were synthesized with a DNA synthesizer Model 394 (Applied Biosystems) for sequencing. Gene Works 2.3 (IntelliGenetics, Inc.) Macintosh software was used for the DNA sequencing analysis.

**Zoo Blot Analysis**—High-molecular-weight DNA was isolated from human colon epithelial cells by the methods of Nelson and Krawetz (22). Animal genomic DNAs were purchased from CLONTECH. Each DNA (5 µg) was digested with restriction enzyme EcoRI, electrophoresed in 0.7% agarose gel, and transferred to nylon membrane under alkaline conditions. The filter was hybridized with a 32P-labeled 700-bp fragment of probe a.

**Screening of 5′-Flanking Region of Human Genomic DNA for Fc-βP—EMBL3 SP6/T7 human leukocyte genomic library (CLONTECH) was screened by plaque hybridization using a 32P-labeled random primer-labeled 909-bp BamHI fragment of clone NZ4 as a hybridization probe. Hybridization was performed at 42 °C in a hybridization solution containing 5 × SSPE, 50 (for pNZ4 probe) or 20% (for RPS1 probe) formamide, 2.5 × Denhardt's solution, 0.1% SDS, and 100 µg/ml heat-denatured salmon sperm DNA. The filter was washed 3 times with 0.2 × SSC and 0.2% SDS at 65 °C and exposed to x-ray film.

**Construction of Fc-βP Expression Plasmids—**Plasmids to express half of full-length cDNA were constructed as follows. Five cDNA fragments (see Fig. 1) were used for construction of expression cDNA. The fragment of nucleotide 1 (5′ end) to nucleotide 313 (3′ end) was derived from clone NZ4, nucleotide 314 (3′ end) to nucleotide 1244 (3′ end) was from clone NZC, nucleotide 1665 (3′ end) to nucleotide 1664 (3′ end) was from clone NZ1, and nucleotide 4443 (3′ end) was from clone N2. The fragments were ligated to each other, and the ligated 7.9-kb cDNA fragment was inserted at the cloning site of pcDL-SR0296, which was under control of the SV40 promoter, to yield pSR-NV11.

**Fc-βP Gene Deletion Derivatives and Their Expression—**The deletion constructs were prepared as follows. (i) The 5′-end BglII fragment of NZ4 clone was ligated to the BglII site of pUC72 clone to produce N2. (ii) NZC was ligated to the ~420-bp fragment of Y11 clone at the BstXI site to yield NZC. (iii) The XhoI cloning vector site on a vector-BstXI fragment from NZC was ligated to the BstXI-HincII fragment from Y11, and the resultant 1.7-kb fragment was inserted in the HincII-digested X1 clone to produce derivatives. (iv) NV11 was digested with SalI, washed, and the 7.9-kb fragment was ligated to both ends with NotI and inserted in pUC119, of which the HincII site changed to NotI site to generate pUC-NV11. pUC-NV11 was digested with HincII, BssHII, Thh1111, or SpI and SpeI, respectively, and each digest containing vector sequences was self-ligated to produce derHinc, derBssH, derThh, or derSpI, respectively. (v) derThh, derNX, and derHinc were digested with BssHII, and then each digest containing vector sequences was self-ligated to generate derBssHThh, derBssH, and derHincBssH. (vi) derNotI-digested insert of V11 was blunted by Klenow fragment and was inserted into the blunted SpeI site of NZC to produce NZCV11.

For expression, the original initiation site of each construct was alive except for X1. To express clone X1, an oligonucleotide adapter containing the initiation site sequences originated from Fc-βP cDNA (or NV11), intervened between HincIII- and EcoRI-site sequences, was designed as follows (5′-AAAGTCTCTGACGCGCATGCCGGGATCC-3′). The oligonucleotide was inserted into HincIII-EcoRI site of pBLS-STOP to make transcription start followed by cDNA sequences. The insertion sequences of clone X1 digested with EcoRI were ligated into EcoRI site of pBLS-STOP to produce X1 clone. Stop codon sequences were also designed as follows. The two adapter sequences were synthesized (upper strand 5′-CTAGTTAGTTAGTTAGTTAGGACC-3′ and lower strand 5′-GGCGGGTACCTTACTACTAA-3′). This double strand provided stop codon in all frames. It was inserted between SpeI and NotI site of pBLS-STOP plasmid and oriented in the correct reading frame. Then each vector sequence was ligated to itself, and the two adapter sequences were ligated to each other to yield derBssHThh, derBssH, and derHincBssH. The expression vectors containing these deletion constructs were transfected transiently in COS7 cells, and produced proteins were stained with Fc fragment of human IgG or monoclonal antibodies K9 and K17.

**Transfection**—For transient expression, COS7 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 10,000 bovine serum albumin, 1.5 mM MgCl2, 0.5 units Taq polymerase (Perkin-Elmer Cetus), and 20 pmol of each primer set in a total volume of 50 µl. The following oligonucleotide primers for PCR were synthesized: BC1 (forward 5′-ACCACTCTTGCACTGCGC-3′, 30 µM) reverse 5′-GTGCGGACGGACGG-3′, GS1 (forward 5′-ACCAGCGCCACGACGACGG-3′), and GS2 (reverse 5′-ACAGCAGGTTGTCGCCG-3′). Reverse-transcription PCR (RT-PCR) was also carried out. Total RNA (0.6 µg) from N2 cells was used as starting material. The first strand synthesis was performed at 60 °C for 30 min using avian myeloblastosis virus reverse transcriptase with primer GS1 or GS2 followed by inactivation of the enzyme at 95 °C for 5 min, and then each forward primer was added to the mixture. The thermal cycle profile for both PCR and RT-PCR was as follows: 95 °C for 1 min, 60 °C for 1.5 min, 72 °C for 2.5 min for 30 cycles. Each 7 ml of the amplification product was digested with 50 units of SmaI in a total volume of 10 µl at 30 °C for at least 12 h. The digested-amplification products were electrophoretically separated on 2% agarose gels and visualized by ethidium bromide staining under UV-light. Quantitative determination of products was carried out using scan analysis software with photography of the gels.

**Plasmids for cDNA Expression—**pcDL-SR0296 vector (23) was kindly provided by Dr. Takebe of the National Institute of Health (Japan). pMSXND vector (24) contains metallothionein promoter for foreign cDNA expression and dhfr gene for selection of clones and gene amplification.
IU/liter penicillin, and 100 mg/ml streptomycin. COS 7 cells were plated at 1 x 10^5 cells/35-mm tissue culture dish a day before transfection. Then the cells were transfected with pSHIV1 by lipofection procedure using 10 μg of DNA and 5 μl of Transfectam (Promega) per 500 μl of RPMI 1640 medium. After 6 h, medium was changed and cultured for a further 48 h. For permanent expression, CHO cells were transfected with a pMSXND vector bearing Fc gBP cDNA fragment, NV11, or the deleted cDNA by lipofection. Cells were passaged the day before transfections, and the subconfluent 2 x 10^6 cells were treated with CaCl₂-purified plasmids in the same way as COS cells. The cell medium was replaced with F-12 medium supplemented with nucleotides and 10% FBS and incubated at 37 °C for 2 days. Then the cells were selected in α-minimum Eagle’s medium without nucleotides containing 1 mg/ml G418, 10% FBS, and antibiotics. After culturing under these conditions for 14 days, the clonal cell lines were propagated with increasing concentrations of methotrexate, starting with 0.02–6.4 μM. The gene-amplified cells were confirmed by a higher amount of expression of Fc gBP, and clonal cells were obtained by limiting dilution.

RESULTS

Cloning and Sequencing of Full-length cDNA for Human Fc gBP—Random primed cDNA libraries (>600 bp) were constructed from human colonic epithelial cells using agt11 as a vector. The agt11 human epithelial cell library was first screened with two independent monoclonal antibodies against Fc gBP, K9, and K17 that block the binding of IgG Fc fragment (14). The screening of 1 x 10^5 recombinants with monoclonal antibody K9 led to the isolation of a cDNA clone 618 bp long (Fig. 1B) in the region defined by nucleotide position 13788–14405 (probe q). Furthermore, the screening of 6 x 10^5 recombinants with monoclonal antibody K17 led to the isolation of 7 clones, one of which was about 1300 bp long and could be digested with BamHI into 2 fragments (a and b). These adjacent fragment probes (a/b) were corresponding to nucleotide positions 7368–8045/8046–8697 or 10970–11649/11650–12390, respectively. The nucleotide sequences of probes a and ab revealed that they are independent cDNA clones. However, both probes hydridize to a single band of >16 kb on a Northern blot (described below). These results suggest that the two clones are derived from the same mRNA for Fc gBP. Three probes, a, b, and c, were used to determine the full-length cDNA sequence. Out of more than 70 clones isolated, 10 clones (T5, A43, A8, A31, A40, A53, V11, X1, Y1, and C72) were shown to cover most of full-length cDNA for Fc gBP except for 5’-terminal sequence (Fig. 1C).

Sequence analysis of these clones showed that the Fc gBP cDNA is composed of three homologous units that are tandemly repeated (see Fig. 1B). Probe q hybridizes with 3 regions, as does probe a/b. Each unit shares more than 95% homology with one another. Although a cDNA clone representing the 3’-terminal region, T5, was isolated from oligo(dT)-primed cDNA library, no 5’-terminal clones (i.e., extending from C72) were isolated from human colonic libraries. Thus, we constructed a cDNA library of human N2 cell line, which can differentiate into goblet cells and express Fc gBP (25, 26). Using a 5’-terminal probe derived from the C72 clone, we obtained a clone, NZ4, containing the 5’-terminal ATG. Sequences flanking the first ATG (GCC(A/G)CCATGG) in NZ4 clone are consistent with those described by Kozak (27) for an initiation codon. The complete nucleotide sequence data has been submitted to the DDBJ, EBI, and GenBankTM Data Banks; a map of the major restriction enzyme sites is shown in Fig. 1A.

Predicted Amino Acid Sequence and Sequence Homology of Fc gBP with MUC2 and von Willebrand Factor—A full-length sequence of the predicted amino acids is shown in Fig. 2A. A dot matrix plot of the entire predicted amino acid sequence against itself revealed 12 repeated domains flanked by unique N-terminal (450 amino acids) and C-terminal (160 amino acids) domains (data not shown). These 12 repeated domains (r1-r12 in Fig. 2B) were classified into 5 types that shared 30–40% homology. The R1 unit (r3-r5) corresponds to the first 5’-terminal A-B-C-Q region in Fig. 1. The repeated domains r1-r12 are each composed of about 400 amino acids.

As some mucin-related proteins have been reported, the predicted amino acid sequence of human Fc gBP was compared with those amino acid sequences, and a broader search for similarities to other protein sequences was carried out using Gene Works (IntelliGenetics, Inc.) loading GenBankTM Release 8.7. These analyses demonstrate that the Fc gBP sequence has significant similarity to portions of MUC2 and prepro-von Willebrand factor (vWF) but that it does not have homology to either Fc receptors or IgG-like domain. A close examination reveals that each repeated domain of Fc gBP was homologous with four MUC2 D domains and to four prepro-vWF D2 domains (about 30% homology). Interestingly, as shown in Fig. 3, Fc gBP, vWF, and MUC2 all have the conserved amino acid...
motif CGLCGN. These sequences are also characteristic of thioredoxin (28) and protein disulfide isomerase (29). Another feature of this protein is that it is high in cysteine content. As shown in Fig. 2B, 8.1% of the amino acid residues are cysteines (cf. 5.7% for human serum albumin (hSA)). A total content of the predicted serine/threonine residues for O-linked glycosylation is 12.3% (Fig. 2B, cf. 9.3% for hSA). Further, the content of hydrophobic and neutral amino acids is 78.6% (cf. 63.8% for hSA).

**Size Determination of Native mRNA for Human FcγBP—**
Northern blot analysis was carried out to detect the expressed mRNA for FcγBP from colonic epithelial cells. Probes q, a, or y (an 800-bp probe derived from the 5’-terminal region of clone Y1) were used. A single band of larger than 15 kb with a smeared front was detected with each probe, as shown in Fig. 4A. Probe y gave the same Northern blot pattern as with probes q and a, indicating that these three probes hybridize to the same mRNA. The same result was obtained using a part of clone NZ4 as a probe (data not shown).

As described above, however, mRNA size is critical for determining the number of 4.5-kb units (A-B-C-Q). To determine more precisely the size of the FcγBP mRNA, we compared it
with mRNAs for ryanodine receptor (15.2 kb) and for dystrophin (14 kb), which are among the longest mRNAs reported. We prepared the respective probe for them by PCR method, using poly(A)^+ RNA from human cytoskeletal muscle. Since FcγBP has been shown to be present in human N2 cells by immunohistochemical staining with monoclonal antibodies K9 and K17 (26), we carefully prepared the mRNA from the N2 cells, except for additional faint bands. Clusters of multiple start sites are common for genes lacking a TATA box (30).

**Transcription Start Sites**—To confirm that the 5'-terminal cDNA sequence of human FcγBP is identical to genomic DNA, approximately 2 × 10^6 recombinant phages from a human leukocyte genomic library were screened using the 5'-terminal fragment of clone NZ4 as a probe. One independent clone, GHFc1, and two overlapping clones, GHFc2 and GHFc3, were identified. A 1908-bp SacI/EcoRI fragment at the 5'-flanking region from GHFc1 as well as the exon 2/intron boundary regions from GHFc2 and GHFc3 were subcloned into pBluescript and sequenced using exonuclease III-generated deletion templates.

Fig. 5 shows the sequence of the first and second exons of genomic DNA, which completely coincides with the corresponding region of cDNA. The exon/intron splice sites were confirmed to be GT-AG border element consensus sequences. The first exon contains the putative ATG initiation codon suitable for Kozak's rule (27), as described above. Examination of the 5'-untranslated region reveals a TGA in-frame stop codon at −78 position of the gene. However, no TATA, CAAT, or other promoter/enhancer motifs were detected within 2 kb further upstream (data not shown).

**Distribution of SmaI RFLP**—To detect sequence variants and to study whether those variants are allele-specific or repeated region-specific in each allele, RFLP analysis with SmaI was investigated for 10 individuals and cultured N2 cells. We designed two distinct primer sets, as described under “Materials and Methods.” The PCR-amplified products are 186 bp (primers BC1 and GS4R) as shown in Fig. 7 and 107 bp (primers GS1 and GS3R) (data not shown), and the complete digestion of these products with SmaI results in fragmentation into 113 and 73 from 186 bp and into 72 and 35 from 107 bp, respectively. In the case of sample 1, only 113- and 73-bp SmaI-digested bands were observed, but 9 other cases showed the additional band (186 or 107 bp) non-digested with SmaI. Using primers GS1 and GS3R, consistent results were obtained by detecting a set of 72- and 35-bp fragments after SmaI digestion.

Reflecting the sensitivity to SmaI digestion, we determined two alternative sequences. One was present in clones of A53, A8, and others (ACT-GGC-TGC-CCC-GGG-GGT), which are sensitive to SmaI, and another sequence (ACT-GGC-TGC-CTG-GGG-GGT) present in clones of V11 and others, which are resistant to SmaI. This difference in sequence results in chang-
ing one amino acid residue of proline to leucine. As FcγBP gene has three large repeats, six large repeats are involved in a diploid genome. Although theoretical ratios of Smal-digested bands to undigested bands are 6:0, 5:1, 4:2, 3:3, 2:4, 1:5, or 0:6, no case in which all 6 repeated regions are Smal-resistant has yet been observed. RT-PCR of mRNA from N2 cells reveals the message to be all Smal-sensitive (Fig. 7, sample RT).

Expression Pattern in Human Tissues and Other Species—Human FcγBP mRNA expression in various human tissues was examined. 17-kb mRNA is expressed in the placenta and colonic epithelial cells, but no expression was detected in heart, brain, lung, liver, skeletal muscle, or kidney (Fig. 8). Since IgGFc binding activity has not yet been detected in non-human mammals such as mice and rabbits (13), we investigated the species specificity of FcγBP gene. The gene for FcγBP was detected in human and monkey but not in mouse, rabbit, rat, dog, bovine, or porcine by zoo blot analysis (data not shown).

Expression of Active Recombinant Molecules—The large size of the intact cDNA for human FcγBP precluded expression in a diploid genome. Although theoretical ratios of Smal-digested bands to undigested bands are 6:0, 5:1, 4:2, 3:3, 2:4, 1:5, or 0:6, no case in which all 6 repeated regions are Smal-resistant has yet been observed. RT-PCR of mRNA from N2 cells reveals the message to be all Smal-sensitive (Fig. 7, sample RT).

Inhibition of IgG Binding by Heat-aggregated IgG Using FcγBPf-Expressed CHO Cells—We isolated a CHO stable transfectant clone expressing FcγBPf. More than 90% of cells produced the protein product. Therefore, we determined the binding activity quantitatively using HRP-conjugated human IgG. Fig. 10 shows the comparative inhibition of HRP-labeled IgG binding in the presence of the various concentrations of human monomeric IgG or heat-aggregated IgG. Monomeric IgG can specifically inhibit the binding of HRP-conjugated IgG, and heat-aggregated IgG shows 10 times stronger inhibition than monomeric IgG. These results suggest that polymeric IgG, like the heat-aggregated form, has a higher affinity to FcγBP than monomeric IgG.

Functional Analysis of FcγBPf cDNA—Subsequently, we prepared nested deletions of NV11 cDNA to identify regions of cDNA essential for biological activity (Fig. 11). Monoclonal antibody reactivity and IgG binding activity of the protein fragment produced in the transfecants are also summarized in Fig. 11. Notably, clones deleted within the H region (ΔHinc, ΔHinc/BissH, or X1) can express proteins with monoclonal antibody reactivity but no IgG binding activity. These results suggest that the subregion from r1 through r5 (Fig. 11) is responsible for IgG binding and that the H region is essential for expression of functional molecules. Although about half the r5 domain is deleted in clone ΔSpl, its reactivity with monoclonal antibody K9 is retained. In clones ΔThH and ΔBshH/Tth, K17 reactivity is attributable to a part of the r6 domain, which is highly homologous to the r5 domain.

Prediction of IgG Binding Sites—Domains r1–r5 show 30–40% amino acid sequence homology with one another. We tried to determine whether each domain is responsible for IgG binding. COS cells transfected with the deleted cDNAs were treated with HRP-IgG for IgG binding assay and also incubated with the HRP-IgG in the presence of excess unlabeled competitors, monoclonal antibodies K9 and/or K17. When some r domains were deleted (e.g. clones NX and NZCY in Fig. 11), the strength of IgG binding was proportional to the number of intact r domains; a clone completely lacking r domains (such as NZC) showed no IgG binding activity. Since monoclonal antibodies (designated as FcγBPf) that can bind IgG Fc but not IgG F(ab')2.
K9 and K17 interact with the r3 and r5 domains, respectively, K9 and K17 competitively inhibited the IgG binding activity of clones expressing FcγBP containing r3 and/or r5 domains (Fig. 11). These findings suggest that at least r1, r3, and r5 domains are involved in the IgG binding capacity of FcγBP.

DISCUSSION

Predicted Characteristics of FcγBP—Our cDNA sequence reveals that full-length FcγBP comprises 5405 amino acid residues (Fig. 2). Based on its structural features, this protein can be divided into three major domains. The largest central domain is composed of 12 tandem repeats of about 400 amino acids each (Fig. 2B). Each repeat contains about 8% cysteine residues. Repeats are homologous (about 30–40%) to each other. The lability of IgG binding activity in periodic acid and hydrogen peroxide treatments (13) and broad bands observed on SDS-PAGE gels suggested that FcγBP is a highly glycosylated protein. The presence in the predicted amino acid sequences of many N- and O-linked glycosylation sites (15.5%) is consistent with this. The structure of FcγBP is related to the mucin-like protein MUC2. Taking together its intracellular transport and localization in goblet cells (14), this fact indicates that FcγBP may be a component of mucus.

Gel-forming mucin is thought to be a giant molecule formed by several to tens of molecules bound by inter-cystine bonds (31). The tandem repeat domain, for example, composed of repeats of 17-amino acid units for MUC3 (32), 16 for MUC4 (33), and 169 for MUC6 (34) is characteristic of conventional mucin. However, no such domain containing short repeat units was detected in the molecule of FcγBP. Nevertheless, FcγBP should be classified as one of the mucins because of the fact that (i) it has a high molecular weight (>200 kDa) with S-S linkages (13), (ii) it is secreted with mucus from goblet cells into the intestinal tract (14), (iii) it may be glycosylated (13), and (iv) it contains several cysteine-rich domains (Figs. 2 and 3).

Thus, FcγBP appears to be a mucin-like protein and to be involved in the maintenance of the mucosal structure as a gel-like component of the mucosa.

Gene Polymorphism—We analyzed cDNA libraries prepared from human tissue and the N2 cell line, and detected several different types of FcγBP cDNA, as assayed by SmaI sensitivity. Since a difference was specifically seen in the SmaI site of highly homologous 3.6-kb DNA repeats, we focused on the analysis of polymorphisms of this site. This analysis revealed several particular digestion patterns dependent on the number of SmaI sites, including polymorphisms of each allele in the FcγBP gene (Fig. 7). This finding also suggested the expression of FcγBP gene in different alleles in individuals. No major variations in the FcγBP gene, such as variations in the number of homologous repeats or in splicing, have been detected to date (Fig. 4). While the amino acid residue of the SmaI site was changed from proline to leucine or reverse, it is unknown whether polymorphism of the FcγBP open reading frame caused by point mutation alters the Fc binding activity of FcγBP related to colorectal diseases, as is the case for familial intestinal polyposis and polymorphisms of its causative gene (adenomatous polyposis coli gene) (35). The physiological function of gene polymorphisms in FcγBP remains to be elucidated.

Role of Subdomain—The protein FcγBP produced by the expression of an approximately 8-kb fragment including the 5' terminus of FcγBP cDNA (NV11 clone) contained an H domain and 6 r domains (Fig. 11). To identify the domain possessing Fc binding activity, we assessed activity using a series of deletions in cDNA. When some of the r domains were deleted, IgG binding tended to become weaker in proportion to the length of the

FIG. 6. Determination of mRNA initiation site for FcγBP transcript by primer extension analysis and S1 mapping analysis. A, the antisense 50-mer (5'-GCTGATAGTTCTGCAGGAAGGCTGTGAG-GAATTCTCCTGCGCCAGTGTC-3') oligonucleotide complementary to nucleotides +95–144 was synthesized and purified. The 50-mer oligonucleotide was used in primer extension after labeling the 5'-end. Universal primers were used for the sequence ladders with the single-stranded M13mp18 DNA template as size marker (lanes T, C, G, and A). The numbers from the primer and of the transcription start site are indicated on the left side and in parentheses, respectively. Lanes HCE and N2 indicate the cDNA reverse-transcribed from RNAs from human colon epithelium cells and N2 cells, respectively. Band locations are indicated on the A, and S1 indicates on the B. The arrow (+1) denotes the transcription start site indicated in Fig. 5.

FIG. 7. PCR/RFLP analysis for FcγBP gene. The PCR-amplified products (lane -S) that contained 186 bp (primer BC1/GS4R) were completely digested with SmaI (lane +S). Sample 1–4 DNAs were from the normal part of colon epithelium from the patients bearing colon cancer, and sample 5–10 DNAs were from the normal blood lymphocytes of independent volunteers. Lane RT denoted the cDNA for reverse transcript from RNA of N2 cells. The ratios of the whole numbers under the photos indicate the relative amounts of SmaI-digested and -undigested products obtained by image scan analysis.
remaining r domains. The deletion experiments also showed that r5 domain is critical for IgG binding. Competition experiments using inhibitory monoclonal antibodies suggest r3 and r5 domains are involved in the binding of Fc although the r1 domain of clone N2CY still shows significant binding activity. Staining with monoclonal antibodies revealed that K9 recognizes the r5 domain, as well as r11, and that K17 recognizes the r3 domain, as well as r6 (Fig. 11). Our results further suggest that both the r3 and r5 domains possess independent Fc binding sites as well as r1 domain. Therefore, we speculate that at least three IgG molecules can bind to a single FcγBP protein molecule. Furthermore, the inhibition of IgG binding by heat-aggregated form implies that the polymeric form such as immune complexes may be a better ligand for FcγBP.

Considering the lower cysteine content of H domain and its unique amino acid sequences, it is likely that it may differ in nature from r domains. Deletion of the H domain results in loss of Fc binding activity (Fig. 11). This suggests that the H domain plays an important role in maturation of biologically active protein products. We speculate that H domains are involved either in the processing of the FcγBP polypeptide into an active form with Fc binding activity or in the intracellular localization for suitable protein processing, such as Golgi apparatus or mucus granules. These speculations seem to be reasonable because the following facts were observed. Although the predicted molecular weight of the whole FcγBP (about 5,000 amino acids) is more than 500 kDa, Kobayashi et al. detected a more than 200 kDa band by non-reduced electrophoresis and 70–80 kDa bands under reduced conditions. Thus, the FcγBP may be processed by protease after translation. So far, our FcγBP expressed in COS and CHO cells is also recovered as broad bands of 50–80 kDa under reduced conditions by Western blot analysis.

**Physiological Functions of FcγBP**—Our previous study using monoclonal antibodies showed the presence of FcγBP in the mucosa of the large and small intestines (13, 14). In the present study, FcγBP mRNA is detected not only in the colorectal epithelial cells but also in the placenta (Fig. 8). These findings imply that FcγBP may be distributed in the systemic mucus, as are the secreted mucins MUC2, MUC3, and MUC4. FcγBP was detected only in humans and monkeys by Southern blotting. Therefore, FcγBP may play a role in the mucosal immune system of primates. IgA and IgM are transported within epithelial cells via polymeric Ig receptors, and they eliminate toxic antigens from the lamina propria. Analysis of the amino acid sequences of FcγBP, however, reveals no membrane-penetrating domain or signal peptide sequences (Fig. 2). Immunohistochemical studies reveal that FcγBP is transported from Golgi apparatus to the mucus follicles (14) although it is unknown whether protein localization is dependent on binding to IgG. It therefore seems unlikely that FcγBP serves as a transporter-like poly-Ig receptor or incorporates IgG-like FcRn. However, similar to poly-Ig receptor, a portion of which serves as a secretory component and contributes to multivalent IgA formation and its stabilization, it is likely that FcγBP enhances antigen trapping through its promotion of multivalent IgG.

### References

1. N. Harada, S. Iijima, K. Kobayashi, T. Yoshida, W. R. Brown, T. Hibi, A. Oshima, and M. Morikawa, unpublished data.

### Figures

**Fig. 8. Expression of FcγBP mRNA in human tissues.** The blots of poly(A)⁺ RNA from indicated human tissues were hybridized with 32P-labeled probe y and control β-actin probe.

**Fig. 9. Expression of protein product (FcγBPf) from FcγBP cDNA fragment in COS cells (a–c) and demonstration of IgG binding activity (d–f).** cDNA fragment, NV11, was constructed using clones N24, C72, Y1, X1, and V11 as described under “Materials and Methods.” The NV11 fragment was inserted into mammalian expression vector pcDL-SRα 296. The expression vector was transfected transiently into COS7 cells and stained with the indicated antibodies followed by HRP-conjugated anti-mouse IgG F(ab')₂ fragment to detect protein product. a, monoclonal antibody K9; b, monoclonal antibody K17; and c, without antibody. The IgG binding activity was detected by the combination of the indicated immunoglobulin and the second HRP-conjugated anti-human IgG F(ab')₂. d, human whole IgG; e, IgG Fc fragment; f, IgG F(ab')₂ fragment.

**Fig. 10. Competitive inhibition of IgG binding to FcγBPf by monomeric IgG and heat-aggregated IgG.** The CHO cells stably transformed by NV11 expression vector were cultured and fixed to 96-well micro test plate. HRP-labeled human IgG (5 μg/ml) was added to the cells with indicated concentrations of monomeric IgG (open circle) or heat-aggregated IgG (closed circle). After washing, the amount of bound labeled IgG was determined under the standard assay conditions, and the supernatant reaction mixtures were transferred for colorimetric determination at 495 nm with a plate reader.
Formation or protects IgG from degradation by bacterial proteases. Further, secreted into the mucus, native FcγBP may prevent the invasion of antigens into the mucosa by efficiently trapping antigen-IgG complexes through its binding to more than nine of the aggregated IgG complexes. Further studies are being conducted to reveal where FcγBP is able to bind IgG molecules during protein processing.

FcγBPf expressed from an 8-kb cDNA are cleaved into several polypeptides cross-linked by disulfide bonds. Intra- and inter-disulfide bonds are also suggested by the conserved motif (CGLCGN) in all the repeated domains (Fig. 3). Interestingly, such vicinal cysteines are conserved in all members of thioredoxins, as well as vWF and MUC2. Thioredoxins are involved in a wide variety of biochemical systems, and the vicinal cysteines (CGPC for thioredoxins) are essential for redox functions in *E. coli*. In mammalian cells, thioredoxin functions as an endogenous glucocorticoid receptor-activation factor (36). Moreover, thioredoxin-like domains have been found in several proteins of higher molecular weight, such as protein disulfide isomerase (29) and phosphoinositide-specific phospholipase C (37). Another interesting function of eukaryotic thioredoxin is its stimulation of interleukin 2 receptor expression in human T-cell leukemia virus (HTLV)-1 transformed T cells, an activity originally described as adult T-cell leukemia-derived factor (ADF) (38, 39). Recently, recombinant ADF prevented the cytotoxicity caused by H2O2 (40). These results suggest that high cysteine content, as well as the conserved sequences in human FcγBP molecules, serves as an antioxidant in mucus. This speculation led to protective elimination of antigen-immune complex from the intestinal tract.

Regarding the vicinal cysteines, another potentially interesting parallel is the possibility that FcγBP, like pro-vWF (41), may have the ability to catalyze its own oligomerization. Pro-vWF autocatalysis occurs in vitro at low pH and is dependent upon the integrity of two sets of vicinal cysteine residues, both with the sequence CGLC (42). These tetrapeptides apparently conduct thiol oxidation through a mechanism involving the formation of a strained 14-member ring joined at the sulfur atoms of the two cysteine residues. These sequences are conserved in each subdomain of FcγBP. These vicinal cysteines may be important in processing to an active form as well as H domain, and in formation of structural networks by mucin proteins.

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