G6b, a Novel Immunoglobulin Superfamily Member Encoded in the Human Major Histocompatibility Complex, Interacts with SHP-1 and SHP-2*

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The G6b gene, located in the class III region of the human major histocompatibility complex, has been suggested to encode a putative receptor of the immunoglobulin superfamily. Genomic sequence information was used as a starting point to clone the corresponding cDNA. Reverse transcriptase polymerase chain reaction showed that expression of the gene is restricted to certain hematopoietic cell lines including K562, Molt 4, and Jurkat. Several splice variants were detected, varying only in their C-terminal parts. One of the potential membrane-bound isoforms contained two immunoreceptor tyrosine-based inhibitory motifs in its cytoplasmic tail. Four of the isoforms were expressed as epitope-tagged proteins in the cell lines K562 and COS-7. The two splice isoforms lacking the hydrophobic transmembrane segment were secreted from the cell. Glycosidase treatment of the four recombinant proteins provided evidence for N- and O-glycosylation. Immunofluorescence studies indicated that the spliced isoforms having a transmembrane segment were directed to the cell membrane. The G6b isoform containing two immunoreceptor tyrosine-based inhibitory motifs in its cytoplasmic tail was found to be phosphorylated on tyrosine residues after peroxanadate treatment of cells and, subsequently, interacts with the SH2-containing protein-tyrosine phosphatases SHP-1 and SHP-2. Mutagenesis studies showed that phosphorylation of tyrosine 211 is critical for the interaction of G6b with SHP-1 and SHP-2.

The Ig superfamily receptors constitute a large group of cell surface proteins involved in the immune system and cellular recognition (1, 2). Members of this family are characterized by an extracellular part containing at least one immunoglobulin domain, a transmembrane segment, and a cytoplasmic tail. A subset of the Ig superfamily is the inhibitory receptor characterized by the presence of one or more immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tail.

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The consensus sequence of the ITIM is generally described as (L/V)I(S/T)X(Y)(L/V) (3). Following phosphorylation of the tyrosine residue within this motif, the SH2 domain containing protein-tyrosine phosphatases SHP-1 and/or SHP-2 can be recruited to the receptor, where they can dephosphorylate membrane-bound phosphoproteins, thus modulating the signaling cascade. SHP-1 is a non-transmembrane protein primarily expressed in hematopoietic cells and is considered to play a negative role in cell signaling (4). Identified substrates for SHP-1 are the linker of activated T-cells (5, 6) and the adapter protein Slp-76 (7). In contrast, SHP-2 has been considered to act primarily as a positive signal transducer (8). Possible substrates for this phosphatase are the platelet-derived growth factor beta receptor (9) and PZR (10). SHP-2 modulates the signal strength of receptor-protein-tyrosine kinases and is also involved in cytokine and antigen signaling not involving receptors with intrinsic kinase activity (11). Although the two phosphatases appear to have opposing roles, there are examples of ITIM-containing receptors that recruit both SHP-1 and SHP-2 (12, 13). Other receptors are reported to recruit primarily only one form (14, 15). Both phosphatases SHP-1 and SHP-2 contain two SH2 domains and a catalytic domain (16, 17).

The human major histocompatibility complex (MHC) is located on chromosome band 6p21.3 and spans ~3.6 megabases of DNA. The complete sequence of this region has been determined (18). The central MHC region is termed the class III region and comprises 0.8 megabases. Of the 58 genes in this region, 40% are known or predicted to have a role in the immune system or inflammation, such as tumor necrosis factor, lymphotixin-α, and lymphotixin-β. Susceptibility to a wide range of diseases has been linked to the MHC, including insulin-dependent diabetes mellitus (19), rheumatoid arthritis (20), and ankylosing spondylitis (21). Although disease susceptibility is often due to allelic differences in the class I and class II antigens, there is evidence that loci located in the class III region may also contribute (21, 22). For this reason the detailed characterization of genes located in the class III region with a potential role in the immune system is of great interest.

G6b is an uncharacterized gene located in the class III region of the MHC that encodes a putative cell surface receptor of the Ig superfamily. Its predicted gene product was found to contain a potential signal peptide, a variable type Ig domain, and a transmembrane segment (23). Interestingly, we have observed that the intracellular stretch also contains two tyrosine residues in ITIM consensus sequences.

In this study, the available genomic sequence information was used as a starting point to obtain the cDNA encoding the...
G6b protein. By RT-PCR, several alternative splice variants of the G6b mRNA were identified in the bone marrow-derived cell lines K562, Molt4, and Jurkat but not in other hematopoietic and fibroblast cell lines studied. These mRNAs encode proteins with different C termini, some of which lacked a transmembrane segment. Expression of four of the proteins as epitope-tagged fusion proteins in mammalian cells allowed their further characterization. An association of the splice isoform containing the ITIM motifs with SHP-1 and SHP-2 was shown following pervanadate-induced tyrosine phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Reverse Transcriptase-PCR**—RNA isolation from human cell lines was performed as described previously (24). cDNA synthesis was carried out using a Promega Reverse Transcription System and ~1 μg of poly(A)+ RNA according to the manufacturer’s instructions. Control PCR reactions with β-actin primers were performed on each cDNA reaction (forward 5'-CTTGGCGGCGGAGATGGC-3' and reverse 5'-TGTTGGTGAAGGCTGAGCC-3'). PCR primers for G6b were designed based on the genomic sequence (GenBankTM accession number AF129756). To obtain the complete open reading frame, nested PCR was performed on the cDNA samples. In the first round, forward primer 5'-AAAACACATTCCCTCAGAC-3' (nt position 23402–23421) was used. In the second round, forward 5'-CCTACTGATGCTGATTCGTC-3' (nt position 26676–26695) was used in combination with reverse primer 5'-GGGAGTTGGAAGATCGGCCC-3' (nt position 24972–24991). In each round, 25 cycles were performed using an annealing temperature of 60 °C. PCR fragments were cloned into the T7-epitope tag (Promega). Clones were checked by sequencing on an Applied Biosystems (Applied) 377 automated DNA sequencer using Big Dye terminators.

**Expression of Proteins in COS-7 Cells and K562 Cells**—For expression of epitope-tagged proteins in mammalian cells, the open reading frames of the different splice isoforms were cloned into the pcDNA3 vector (Invitrogen) fused to a T7-epitope tag (MASMTGGQQMGRDP). To express the G6b isoforms fused at the C terminus to the T7-epitope tag, PCR copies were made of the open reading frames removing the stop codons. The forward primer 5'-TTATAGCTATGCGGGTGTTCGTTGTTCGC-3' was used, creating a HindIII site (underlined). The reverse primer (either 5'-TACATTCCGCGGCCGACACGCAATTACACTGCACATGAGA-3' (for G6b splice isoforms A and B) or 5'-TACATGCCGGCGGCCGACACGCAATTACACTGCACATGAGA-3' (for C) and 5'-TACATGCGGCGGCCGACACGCAATTACACTGCACATGAGA-3' (for D)) was used. These two reverse primers obliterates the stop codons and introduced Nhel sites (underlined), allowing direct fusion to the T7 tag sequence in the T7.TagBlast vector (25). PCR fragments were cloned into this vector using HindIII-Nhel. Constructs were checked by DNA sequencing as described above. The inserts encoding the fusion proteins were cloned into the pcDNA3 vector using HindIII-NolI.

The splice isoforms with the T7-epitope tag fused to the N terminus, an expression vector was created containing the human CD3 signal peptide (26) instead of the G6b signal peptide followed by the T7-epitope tag in pcDNA3. First, a fusion between the CD3 signal peptide and the T7-epitope tag was constructed in pBlueScript. The CD3 leader-T7 tag fusion was amplified by PCR using primers 5'-GGTCTCAGAAATGACGCTCTGGTCGAATG-3' (introducing a NotI site (underlined) and 5'-GATCTATGGTATTCGGACGAC-3' containing a BamHI site (underlined). The resulting fragment was cloned into the pcDNA3 vector using NdeI and BamHI, yielding the plasmid CD33-T7-pcDNA3. The G6b splice isoforms were PCR-amplified with primer 5'-GTCTAATGATCCGAGGAACCAGCGGGGCGG-3', introducing a BamHI site (underlined) and removing the first 15 amino acids of the potential signal sequence, and 5'-AATATGCCGCCGGCTCTTCAAATACATCACACTGC-3', introducing a NotI site (underlined) and maintaining the stop codon. The resulting PCR fragments were digested with BamHI and NotI and cloned into CD33-T7-pcDNA3 digested with BamHI and NotI. The clones were checked by DNA sequencing. Mutations of tyrosine to phenylalanine in the cytoplasmic tail of the N-terminal-tagged G6b-B construct were made using the QuikChange® mutagenesis method (Stratagene) according to the manufacturer’s instructions.

Proteins were transiently expressed in COS-7 cells using the DEAE-dextran method as described elsewhere (25). Three days after transfection, cells and supernatants were harvested. K562 cells were transfected with Lipofectin (Life Technologies, Inc.) according to the manufacturer’s instructions. Three days after transfection, cells were maintained in the presence of 0.5 μg/ml G418. For direct analysis on SDS-polyacrylamide gels, cells were washed once with phosphate-buffered saline and lyzed in SDS-PAGE sample buffer (27). Supernatants were routinely cleared by centrifugation. SDS-PAGE was done according to Laemmli (27) on 12% polyacrylamide gels. Western blot immunostaining was performed with the anti-T7 tag monoclonal antibody (Oncogene). Immunoreactive proteins were detected with horseradish peroxidase-coupled secondary antibody followed by detection with ECL (PerkinElmer Life Sciences). Immunofluorescence localization studies were performed as described elsewhere (28), and staining was examined with a Nikon Eclipse E800 microscope linked to a MicroRadiance confocal imaging system (Bio-Rad).

**Pervanadate Treatment**—Pervanadate was prepared by mixing sodium orthovanadate (Sigma) and hydrogen peroxide (Sigma) in phosphate-buffered saline to final concentrations of 1 and 10 μM, respectively, and leaving the mixture for 15 min at room temperature. To remove excess hydrogen peroxide, catalase (Sigma) was added to a final concentration of 0.2 mg/ml. Cells were stimulated by a 10-fold dilution of pervanadate in pervanadate-buffered saline for 10 min at 37 °C. Cells were lysed in lysis buffer (10 mM Tris/HCl, pH 7.5, 1% Nonidet P-40, 50 mM NaCl, 0.02% sodium azide, and 1 mg/ml bovine serum albumin) supplemented with protease inhibitor mixture (200× diluted). Lysates were cleared by centrifugation at 4 °C, and immunoblotting was performed with the anti-T7 mAb and protein A-Sepharose (Sigma) at 4 °C. Proteins were eluted with SDS-PAGE sample buffer and analyzed on 12% polyacrylamide gels followed by Western blot immunostaining as described.

**Glycosidase Treatment**—Glycosidase treatment was performed using a Promega Reverse Transcription System and ~1 μg of poly(A)+ RNA derived from human natural killer cell lines NKL, NK92, and YT (data not shown).

**RESULTS**

**RT-PCR**—No human expressed sequence tag (EST) clones were found that contained any part of the coding region of G6b, although EST BE750421 was found to encode a putative bovine homologue. However, there were few human ESTs that corresponded to the genomic sequence ~1000–1600 nt downstream of the putative stop codon. Characterization of some of these clones (e.g. AA699838) indicated that they did not contain the complete open reading frame. Because one-step RT-PCR turned out not to be sensitive enough, nested RT-PCR was performed on cDNA preparations from poly(A)+ RNA derived from various human cell lines. The preparations from the bone marrow-derived cell lines K562 (erythroleukemia), Molt4, and Jurkat (T cell leukemias) yielded a number of differently sized PCR fragments (Fig. 1A, bands A–F) with the two closely migrating bands A and B (Fig. 1A) being of the expected size. PCR on the bone marrow-derived cell lines U937 (monocyte-like), Raji (B cell-like), HL60 (promyelocytic), and the fibroblast cell lines 3T3 and HeLa did not yield any of these products (Fig. 1). Similarly, in a separate experiment, we were not able to amplify by PCR G6b mRNA from human natural killer cell lines NKL, NK92, and YT (data not shown).

Sequence analysis of the generated RT-PCR products showed that the band with the lower molecular weight (band B) represents the form predicted by Genscan (23) and also anno-
Fig. 1. RT-PCR of G6b using cDNAs from various human cell lines as template. A, second round of the nested PCR reactions on human cDNA preparations using G6b-specific primers. The cell lines from which the cDNA samples were derived are denoted above the gel. Bands are designated A–G. Chr corresponds to the band derived from chromosomal contamination. B, β-actin control of the cDNA preparations. Molecular weight markers (in base pairs) are indicated on the right.

Expression of G6b Isoforms in COS-7 Cells—Four splice isoforms were selected for further characterization, two transmembrane segment-containing isoforms (G6b-A and G6b-B) and two soluble isoforms (G6b-D and G6b-E). The C terminus of G6b-A is identical to the C terminus of G6b-D, and the C terminus of G6b-B corresponds to the C terminus of G6b-E. The four splice isoforms were cloned into a pcDNA3 expression vector as fusions with a T7 epitope. The fusion proteins were transiently expressed in COS-7 cells. Multiple bands per lane were observed on SDS-polyacrylamide gel, which could be indicative of glycosylation, each band representing the protein in a different state of glycosylation (Fig. 3). As expected, only the isoforms lacking transmembrane segments (G6b-D and G6b-E) were present in the medium. The expected molecular sizes for the G6b splice variants without leader peptide are 24.3 (G6b-A), 23.2 (G6b-B), 19.5 (G6b-D), and 18.3 kDa (G6b-E). The observed molecular sizes of the proteins on SDS-polyacryl-
staining is virtually absent (Fig. 5C). This staining might be explained if the protein sticks to the cell after being secreted.

**Pervanadate Treatment and Interaction with SHP-1 and SHP-2**—Most inhibitory receptors, either of the Ig superfamily or lectin-like superfamily, contain at least two ITIMs with a typical spacing of 20–32 amino acids in the primary sequence (3) and are known to bind SHP-1 and/or SHP-2 after phosphorylation. G6b-B contains two tyrosine residues in consensus ITIM sequences in its cytoplasmic tail, and the spacing between these two ITIMs is 26 amino acids. To investigate whether this isoform was able to bind SHP-1 and/or SHP-2 after phosphorylation, COS-7 cells expressing the four N-terminal T7-tagged G6b isoforms were treated with pervanadate. In the case of the membrane-bound forms G6b-A and G6b-B, the N-terminal epitope tag (present outside the cell) is expected not to interfere with these interactions, which take place at the cytoplasmic tail. Analysis of total COS-7 lysates by Western blot with anti-phosphotyrosine antibodies showed that tyrosine phosphorylation in the cells was highly increased due to pervanadate treatment (data not shown). The different G6b isoforms were immunoprecipitated with the anti-T7 mAb from cells treated or untreated with pervanadate (Fig. 6). Induction of tyrosine phosphorylation on G6b was checked with a phosphotyrosine-specific antibody. Only the G6b-B isoform was found to be tyrosine-phosphorylated after pervanadate stimulation (Fig. 6). In the immunoprecipitate containing tyrosine-phosphorylated G6b-B, both SHP-1 and SHP-2 can be detected by Western blot immunostaining (Fig. 6). The presence of SHP-1 and SHP-2 is strictly dependent on both the presence of the ITIM-containing G6b-B isoform and on the induction of tyrosine phosphorylation by pervanadate on this molecule.

To investigate whether both cytoplasmic tyrosines of G6b-B or just one of them are phosphorylated, three mutant constructs were expressed in COS-7 cells, namely G6b-B(Y211F), G6b-B(Y237F), and G6b-B(Y211F/Y237F). Mutation of Tyr-211 to Phe resulted in a total loss of detectable tyrosine phosphorylation as well as a loss of interaction of G6b-B with SHP-1 and SHP-2 (Fig. 7). Mutation of Tyr-237 to Phe leads to a clearly detectable, although strongly reduced, level of tyrosine phosphorylation and SHP-1 and SHP-2 interaction. These results suggest that Tyr-211 is the only tyrosine residue to be phosphorylated by pervanadate.
phosphorylated and to be involved in SHP-1 and SHP-2 binding, even though Tyr-237 is in an ITIM consensus sequence.

To confirm the interaction of G6b-B with SHP-1 and SHP-2 in a human bone marrow-derived cell line, a similar experiment was performed using the human leukemic cell line K562, which expresses G6b at the RNA level (Fig. 1). The two membrane-bound forms of G6b (G6b-A and G6b-B) were expressed in this cell line. Only the G6b-B isoform was phosphorylated upon pervanadate treatment (Fig. 8). Both SHP-1 and SHP-2 can be detected in immunoprecipitates of tyrosine-phosphorylated G6b-B in transfected K562 cells, confirming the results obtained in COS-7 cells.

**DISCUSSION**

We have characterized the G6b gene located in the class III region of the human MHC, a region known to contain many genes with relevance in the immune system. RT-PCR on cDNA preparations from various human cell lines showed that the G6b gene is only expressed in a restricted set of hematopoietic cell lines, suggesting an immune-related function. Furthermore, it was observed that the RNA derived from this gene is alternatively spliced. One spliced form, which encodes a protein containing two ITIM sequences (G6b-B), appears to be less abundant at the RNA level than the form lacking these motifs (G6b-A). Besides these forms, other variants were detected encoding proteins lacking a transmembrane segment (G6b-D and G6b-E). When expressed in COS-7 cells, these latter forms were secreted, showing that the N-terminal hydrophobic segment indeed serves as a signal sequence.

Glycosidase treatment of the various G6b isoforms provided
tyrosine mAb, and polyclonal antibodies against SHP-1 and SHP-2.

Immunoprecipitations were performed using the anti-T7 mAb. Immunoprecipitates were analyzed by Western blot immunostaining with the anti-T7 mAb, anti-phosphotyrosine mAb, and polyclonal antibodies against SHP-1 and SHP-2. Molecular size markers (kDa) are indicated on the left of each blot.

**FIG. 7.** Pervanadate treatment of COS-7 cells transfected with the different G6b-B Tyr to Phe mutant cDNAs and co-immunoprecipitation with SHP-1 and SHP-2. Immunoprecipitations were performed with the anti-T7 mAb. Immunoprecipitates were analyzed by Western blot immunostaining with the anti-T7 mAb, anti-phosphotyrosine mAb, and polyclonal antibodies against SHP-1 and SHP-2. Molecular size markers (kDa) are indicated on the left of each blot.

**FIG. 8.** Pervanadate treatment of K562 cells transfected with G6b-A and G6b-B cDNAs and interaction with SHP-1 and SHP-2. K562 cells expressing T7-epitope-tagged G6b-A and G6-B were treated (+) or untreated (−) with pervanadate, and immunoprecipitations were performed using the anti-T7 mAb. Immunoprecipitates were analyzed by Western blot immunostaining with the anti-T7 mAb, anti-phosphotyrosine mAb, and polyclonal antibodies against SHP-1 and SHP-2. Molecular size markers (kDa) are indicated on the left of each blot.

evidence that the proteins are both N- as well as O-glycosylated. The extracellular part of the membrane-bound forms of G6b contains only one consensus N-glycosylation site. This site is located in the predicted B-strand of the V-type Ig domain, and the side chain of the asparagine residue (Asn-32) to be glycosylated is likely to be on the surface (see Ref. 29 for a detailed description of V-type Ig domains), making it a suitable residue for glycosylation. Secreted G6b-E contains a relatively high amount of O-glycosylation compared with the other isoforms. Because the difference between G6b-D and G6b-E lies in the C-terminal tail, it is likely that there is at least one extra O-glycosylation site in this part of G6b-E, which could explain the difference in glycosylation between these two forms. Although G6b-B contains a C terminus identical to the C termi-

nus of G6b-E, in the case of G6b-B, this tail is cytoplasmic and, therefore, not available for glycosylation.

Immunofluorescence with non-permeabilized cells using the N-terminal-tagged constructs showed that the T7 tag is outside the cells in the case of G6b-A and G6b-B. Although these constructs do not carry their own signal sequence, it confirms that the proteins are transported to the plasma membrane and not retained in the endoplasmic reticulum. These observations indicate that these proteins are able to fold correctly in these cells because proteins that are not able to fold correctly are retained in the endoplasmic reticulum and finally broken down (30). For the same reason, it can be assumed that G6b-D and G6b-E are able to correctly fold in COS-7 cells because these variants are secreted into the medium.

Of all the G6b isoforms analyzed, only G6b-B appears to be efficiently phosphorylated when COS-7 and K562 cells expressing this recombinant protein are treated with pervanadate. This is completely in line with expectations as G6b-A does not contain cytoplasmic tyrosine residues and the soluble isoforms do not have a part exposed to the cytoplasmic kinases even though G6b-E contains ITIM sequences identical to G6b-B. The interaction of tyrosine-phosphorylated G6b-B with SHP-1 and SHP-2 could be observed in two different cell lines, one of them (K562) expressing G6b mRNA endogenously. SHP-1 is known to be primarily expressed in hematopoietic cells (4), but we could clearly detect this protein in the monkey fibroblast cell line COS-7.

Although G6b-B contains two tyrosine residues in ITIM consensus sequences in its cytoplasmic tail (Tyr-211 and Tyr-237), site-directed mutagenesis suggested that only one of them (Tyr-211) gets phosphorylated upon pervanadate stimulation of COS-7 cells and that this tyrosine is responsible for SHP-1 and SHP-2 binding. This result was not anticipated because mutagenesis studies with similar receptors containing two tyrosine residues in ITIMs showed the involvement of both residues in SHP-1 or SHP-2 binding (15, 31). It is assumed that in these cases, the tandem SH2 domains of SHP-1 and SHP-2 bind the diposphorylated receptor with high affinity. However, the absence of detectable tyrosine phosphorylation of the Y211F mutant does not necessarily mean that Tyr-237 does not get phosphorylated in the wild type construct. It cannot be excluded that the phosphorylation of tyrosine 237 is dependent on the prior phosphorylation of tyrosine 211.

It has become clear that inhibitory receptors often have closely related activating homologues that are expressed on the same cell type and are believed to bind similar or identical extracellular ligands (32). Known examples are the killer inhibitory receptor family as well as the Ly49 family (32). In contrast to these cases, inhibitory and activating receptors are encoded by different genes. In contrast to the inhibitory proteins, the activating receptors generally have a short cytoplasmic tail lacking ITIM sequences. Furthermore, they possess a positively charged residue in the transmembrane segment involved in binding to signaling effector molecules such as CD3ζ, DAP10, and DAP12 (33, 34). The results obtained in this study with G6b resemble the studies with the mouse natural killer cell receptor 2B4 in which two splice variants exist that differ only in the cytoplasmic tail (35). The longer variant of 2B4 contains four consensus ITIM sequences, interacts with SHP-2 upon tyrosine phosphorylation, and inhibits NK cell-mediated lysis of tumor targets (36). In contrast, the shorter 2B4 isoform contains only one potential ITIM but does not get phosphorylated upon pervanadate stimulation and thus does not recruit SHP-2. This shorter variant has been shown to stimulate NK cell-mediated lysis of tumor targets (36). Analogous to these results, one can speculate that the G6b-A variant is the acti-
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vating counterpart of the inhibitory receptor G6b-B. However, both the short form of 2B4 as well as G6b-A lack a positively charged residue in the transmembrane segment, which is in contrast to the killer inhibitory receptor and Ly49 family of activating receptors.

In summary, we have shown that the G6b gene located in the class III region of the human MHC is expressed and processed in immune-related cells at the RNA level. Characterization of the different G6b isoforms expressed in mammalian cells is in line with G6b encoding a novel glycosylated cell surface receptor, although soluble variants are found as well. The interaction of G6b-B with SHP-1 and SHP-2 classifies this variant at least as a new member of the family of inhibitory receptors of the Ig superfamily and the first one found so far in the MHC. However, the G6b Ig domain does not contain any significant homology toward other Ig domain-containing proteins with known ligand. Identification of the extracellular ligand might be crucial in understanding the role of the G6b gene product in cellular signaling.

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