Association of Tyrosine Phosphatases SHP-1 and SHP-2, Inositol 5-Phosphatase SHIP with gp49B1, and Chromosomal Assignment of the Gene*

We have analyzed the molecules participating in the inhibitory function of gp49B1, a murine type I transmembrane glycoprotein expressed on mast cells and natural killer cells, as well as the chromosomal location of its gene. As assessed by SDS-polyacrylamide gel electrophoresis and immunoblot analysis, tyrosine-phosphorylated, but not nonphosphorylated, synthetic peptides matching each of the two immunoreceptor tyrosine-based inhibitory motif (ITIM)-like sequences found in the cytoplasmic portion of gp49B1 associated with the ~65-kDa tyrosine phosphatase SHP-1 and ~70-kDa SHP-2 derived from RBL-2H3 cells. In addition, the phosphotyrosyl peptide matching the second ITIM-like sequence also bound the ~145-kDa inositol polyphosphate 5-phosphatase SHIP. Thus, it has been strongly suggested that the inhibitory nature of gp49B involves the recruitment of SHP-1, SHP-2, and SHIP for the delivery of inhibitory signal to the cell interior upon phosphorylation of tyrosine residues in their ITIMs. The gp49B gene has been found to be in the juxtaposition of its cognate gene, gp49A. The gene pair was shown to locate in the B4 band of mouse chromosome 19. In this region, no conserved linkage homology to human chromosome 19, where the genes for killer cell inhibitory receptors are found, has been identified.

The immunoreceptor tyrosine-based inhibitory motif (ITIM) is a set of amino acid sequences that deliver an inhibitory signal upon phosphorylation of the specific tyrosine residues. The motif is found in cytoplasmic portions of FcγRIIB (1–3), CD22 (4, 5), and inhibitory receptors expressed by human and mouse natural killer (NK) cells (6). The consensus sequence of ITIM is (V/I)-X-Y-X-(L/V) (7). In B cells, when F(ab′)2 fragments of anti-membrane immunoglobulin antibody induce cross-linkage of the surface B cell antigen receptors, a signal transduction cascade is elicited through the receptor that results in B cell proliferation and differentiation into antibody-producing cells. In contrast, stimulation with intact anti-membrane Ig antibody results in co-cross-linkage between the B cell antigen receptor and FcγRIIB, in attenuated B cell signal transduction due to the phosphorylation of ITIM of FcγRIIB, and binding of src homology 2 (SH2)-containing proteins, which inhibit calcium signaling pathway (2, 3). The importance of the inhibitory nature of FcγRIIB in the humoral immune response in vivo was demonstrated in the FcγRIIB-deficient mice generated by gene targeting (8). The proteins to be associated with the phosphorylated ITIM sequence of FcγRIIB was verified to be an SH2-containing tyrosine phosphatase, SHP-1 (9), and an SH2-containing inositol polyphosphate 5-phosphatase, SHIP, in B cells, whereas in mast cells SHIP was shown to be preferentially recruited (10).

On the other hand, human and mouse NK cells express two kinds of inhibitory molecules containing ITIMs. One of which, human killer cell inhibitory receptor (KIR), is a type I transmembrane glycoprotein belonging to Ig superfamily (6, 11, 12). In contrast, human CD94 and mouse Ly-49 molecules are type II transmembrane glycoprotein with C-type lectin structure (13, 14). Despite these quite different structures, both Ly-49 and KIR recognize allelic groups of the major histocompatibility complex class I molecules on target cells. Engagement of these inhibitory receptors results in a dominant negative signal that prevents lysis of the target cells. Tyrosine phosphorylation of their ITIMs and recruitment of SHP-1 was shown to be critical for the inhibitory function (7, 15).

Recently, it was shown that mouse NK cells as well as mast cells also express KIR-like molecules, gp49A (16) and gp49B1 (17), the latter of which contains two ITIM-like sequences in its cytoplasmic portion (17–21). Although the physiological function of gp49 molecules is unknown, it was demonstrated that the co-ligation of gp49B1 and a high affinity IgE receptor FcεRI on the surface of mast cells suppresses FcεRI-mediated exocytosis, suggesting that this molecule possibly functions as an inhibitory receptor (19). Moreover, transfection experiments have indicated that the cytoplasmic tail of gp49B1 inhibits lysis of major histocompatibility complex class I-negative cell line by mouse and human NK cell lines, showing the inhibitory nature of the gp49B1 molecule in NK cells (20). Thus, elucidation of the physiological ligand for gp49 as well as the biochemical characteristics of the inhibitory cascade of the molecule should be valuable for understanding the mode of action that regulates
activating and inhibitory signaling in cells of the immune system.

We report here that both of the phosphorylated ITIM-like sequences of gp49B bind SHP-1 and SHP-2, whereas the phosphotyrosyl second ITIM-like sequence associates with SHIP from RBL-2H3 cells. Moreover, we found that the gp49B gene and the cognate gp49A gene are co-localized on the B4 region of mouse chromosome 10, which is apparently not a syntenic position of human chromosome 19, where genes for the KIR family are present (6, 22).

EXPERIMENTAL PROCEDURES

Screening and Isolation of Mouse Genomic DNA Clones for gp49B—A 1.0-kilobase pair (kb) CDNA of gp49B was prepared by reverse transcription-polymerase chain reaction (PCR) of mRNA preparation from WEHI-3 cells using an oligo(dT) primer for a reverse transcription reaction, and the forward primer (5'-CGATAAGCTTGGCTGACTCACCATG-3') corresponding to nucleotides 7–24 (17) containing a HindIII restriction site, and the backward primer (5'-CGATGGATCCCTTGGTTTACCTGAGACTCACCATG-3') corresponding to the residues 1075–1095 of gp49B containing a BamHI restriction site for a PCR reaction. After the PCR amplification and digestion with HindIII and BamHI, the gp49B DNA was cloned into the HindIII/BamHI sites of pUC19. The cDNA insert was labeled with random primer labeling kit (Takara Shuzo Co., Otsu, Japan) and [γ-32P]dCTP (specific activity, ~3,000 Ci/mmol, Amersham Corp.), and was used to screen a 129/Sv mouse genomic library (Stratagene). 8.3 x 10⁸ plaques were screened under stringent conditions (23). The resulting two positive clones were plaque-purified, and they were subcloned into the plasmid pUC19 or pBluescript (Stratagene) and sequenced by the dideoxy chain termination method (24) using a Cy5 AutoRead sequencing kit and an ALFexpress DNA sequence (both from Pharmacia Biotech Inc.).

Cell Culture—Bone marrow-derived mast cells from C57Bl/6 or B10.A mice were prepared as described previously (25). RBL-2H3 cells (obtained from the Japanese Cancer Research Resources Bank, Tokyo) were maintained in RPMI 1640 plus 10% fetal calf serum.

Affinity Isolation of Cellular Proteins and Immunoblot Analysis—For the detection of cellular proteins that bind to two ITIM-like sequences found in the cytoplasmic portion of gp49B, four biotinylated peptides were synthesized: the sequences are DPPQGIVYAQQPK-amide (in one-letter code, designated as Y1, corresponding to amino acid residues 294–305, see Ref. 17), DPGGIP(Y3)AQVKK-amide (Y1), ETQD-VTQCELCL-amide (Y2, residues 316–327), and ETQDVTQ(Y3)AQVKK-amide (Y2), where each N-terminal residue is biotinylated, and (Y3) denotes a phosphotyrosine residue.

RBL-2H3 cells and bone marrow-derived mast cells were solubilized by adding extraction buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM phenylmethyl sulfonil fluoride, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM disodium EDTA, 1% glycerol) to the cell pellet. The extract was then incubated on ice for 10 min, and the insoluble material was removed by centrifugation at 15,000 x g for 15 min at 4 °C. The soluble extract was pre-cleared with 1.0 ml of avidin-Sepharose FF beads/107 cell eq (Pharmacia) in extraction buffer. The soluble extract was precleared with 1.0 ml of avidin-Sepharose FF beads/10⁷ cell eq (Pharmacia) in extraction buffer. The soluble extract was precleared with 1.0 ml of avidin-Sepharose FF beads/10⁷ cell eq (Pharmacia), followed by centrifugation. The pellet was then washed four times by centrifugation in extraction buffer containing 0.1% gelatin and devoid of aprotinin and leupeptin, resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromphenol blue, 10% glycerol, and 150 mM 2-mercaptoethanol), boiled for 1 min to liberate immunoprecipitated proteins, and centrifuged. The affinity-isolated materials or cell lysates were subjected to SDS-polyacrylamide gel electrophoresis separation using 7.5% gel and then resolved on 7.5% SDS-polyacrylamide gel electrophoresis and probed with rabbit anti-SHIP antiserum, anti-SHP-1 IgG, or anti-SHP-2 IgG. Mock, mock affinity isolation without any peptide but with avidin-Sepharose; Lysate, total cell lysate without any adsorption.

Association of SHP-1, SHP-2, and SHIP with ITIM of gp49B1

We report here that both of the phosphorylated ITIM-like sequences of gp49B bind SHP-1 and SHP-2, whereas the phosphotyrosyl second ITIM-like sequence associates with SHIP from RBL-2H3 cells. Moreover, we found that the gp49B gene and the cognate gp49A gene are co-localized on the B4 region of mouse chromosome 10, which is apparently not a syntenic position of human chromosome 19, where genes for the KIR family are present (6, 22).

FIG. 1. Association of SHP-1, SHP-2, and SHIP with phosphotyrosyl ITIM-like sequences of gp49B1. RBL-2H3 cell lysate was adsorbed with the indicated peptides. Affinity bound proteins were resolved on 7.5% SDS-polyacrylamide gel electrophoresis and probed with rabbit anti-SHIP antiserum, anti-SHP-1 IgG, or anti-SHP-2 IgG. Mock, mock affinity isolation without any peptide but with avidin-Sepharose; Lysate, total cell lysate without any adsorption.

mosomal assignment of the mouse gp49B gene. Preparation of R-banded chromosomes and fluorescence in situ hybridization were performed as described by Matsuda et al. (26) and Matsuda and Chapman (27). The chromosome slides were hardened at 65 °C for 2 h, denatured at 70 °C in 70% formamide in 2 × SSC, and dehydrated in cold ethanol (5 min each in 70 and 100%).

The mouse 6.7-kb genomic DNA fragment inserted in SacI site of pBluescript were labeled by nick translation with biotin 16-UTP (Boehringer Mannheim) following the manufacturer’s protocol. The labeled DNA fragment was ethanol-precipitated with a 10-fold excess of mouse Cot-1 DNA (Life Technologies, Inc.), sonicated salmon sperm DNA and E. coli tRNA, and then denatured at 75 °C for 10 min in 100% formamide. The denatured probe was mixed with an equal volume of hybridization solution to make final concentration of 50% formamide, 2 × SSC, 10% dextran sulfate, and 2 mg/ml bovine serum albumin (Sigma). The mixed probe was placed on the denatured chromosome slides and incubated overnight at 37 °C. The slides were washed in 2 × SSC, 0.1% formamide in 2 × SSC at 37 °C for 20 min and in 2 × SSC and 1 × SSC for 20 min each at room temperature. After rinsing in 4 × SSC, they were incubated under coverslips with fluoresceinated avidin (Vector Laboratories) at a 1:500 dilution in 1% bovine serum albumin, 4 × SSC for 1 h at 37 °C. They were washed sequentially with 4 × SSC, 0.1% Nonidet P-40 in 4 × SSC and 4 × SSC for 10 min each on a shaker, rinsed with 2 × SSC, and then denatured with 0.75 μg/ml propidium iodide (Sigma). Excitation at wavelength 450–490 nm (Nikon filter set B-2A) and near 955 nm (UV-2A) was used for observation. Kodak Ektachrome ASA 100 films were used for microphotography.

Linkage Mapping with Interspecific Backcross Mice—Recombinant mice used in this study were generated by mating male feral-derived mouse stocks, Mus spretus, with female C57BL/6J, and backcrossing the F1 female with male M. spretus (28). Genomic DNA derived from kidneys of each backcross mouse was digested with restriction endonucleases. The resulting fragments were separated on 0.8% agarose gels and transferred to a nylon membrane (Bio-Rad). The DNA was used to determine the genotype of each animal by Southern blot hybridization following the standard protocol. According to the result obtained by the cytogenetic mapping using fluorescence in situ hybridization, microsatellite DNA markers (Research Genetics, Huntsville, AL) for linkage analysis were chosen. All PCR reactions were performed in a total 15-μl reaction mixture containing 125 ng genomic DNA and 15 pmol oligonucleotide primers. Amplification conditions were 95 °C for 10 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 50 s, and 72 °C for 10 min. The PCR products were visualized under UV light with etidium bromide staining after separation on polycrylamide gel.
RESULTS AND DISCUSSION

Analysis of Cellular Components Associating with Cytoplasmic Domain of gp49B—
gp49B has two possible ITIM sequences in its 74-amino acid cytoplasmic domain (17–19). One is
IVYAQV, and the other is VTYAQL: they are separated by 18
amino acid residues. On the other hand, its cognate molecule
gp49A has a short cytoplasmic region comprised of 42 amino
acid residues, and has no such inhibitory sequences within this
region (16). Transfection experiments have shown that the
whole cytoplasmic portion of gp49B is sufficient for the inhib-
itory nature of the gp49B molecule in NK cells (20). A question
arises whether the possible ITIM sequences of gp49B actually
function as inhibitory motifs that interact with any cellular
component or not. To address this issue, we attempted to detect
cellular proteins associating with these sequences. Cellular
proteins of RBL-2H3 cells and bone marrow-derived mast cells
were lysed and then incubated with tyrosine-phosphorylated or
nonphosphorylated synthetic peptide matching each ITIM-like
sequence found in the cytoplasmic portion of gp49B. The bound
proteins were separated by SDS-polyacrylamide gel electro-
phoresis and subjected to immunoblot analysis. As shown in
Fig. 1, both anti-SHIP-1 and anti-SHIP-2 antibodies detected a
−65- and −70-kDa protein, respectively, bound to pY1 and pY2
but not to the corresponding nonphosphotyrosyl peptides. Un-
expectedly, we found that anti-SHIP antiserum clearly de-
tected a −145-kDa protein associating with pY2 but not with
pY1 and the corresponding nonphosphotyrosyl peptides. Detec-
tion of possible association of Syk kinase using anti-Syk anti-
serum was not successful (data not shown). Therefore, we con-
cluded that each phosphorylated ITIM-like sequence mainly
binds SHP-1 and SHP-2, whereas the second possible ITIM also
associates with SHIP from RBL-2H3 extract. Inhibition of cel-
ular activation signal by SHP-1 is associated with inhibition of
early tyrosine phosphorylation events, release of Ca2+
intracellular stores, and secondary influx of extracellular Ca2+
within the cells. Since the recruitment of SHP-1, SHP-2 and
SHIP is dependent on the presence of the phosphotyrosine
residue, the phosphorylation of specific tyrosine residues in
ITIM-like sequences of gp49B should be prerequisite for re-
cruitment of SHP-1 and SHIP in stimulated cells such as mast
cells. Since the gp49-specific antibody is not available at pres-
ent, we cannot verify that the cytoplasmic tail of gp49B is
tyrosine-phosphorylated within the cells. Possible candidates
that phosphorylate the tyrosine residues in the cytoplasmic
domain of gp49B are a src family kinase or a Syk/Zap-70 family
kinase such as Lyn or Syk, but this issue remains to be clari-
fied. A possibility remains that the preference of the associa-
tion with SHP-1 or SHIP could be dependent on the cell type.

Juxtaposition of gp49A and gp49B Genes—As mentioned
above, two related genes have been identified for mouse gp49,
gp49A and gp49B, by cloning of cDNA for each molecule (16,
17), cloning of genomic DNA for gp49B (17), and blot hybrid-
ization analysis of total genomic DNA (16). Recently, several
groups of genes with almost the same structural characteristics
but different in their cytoplasmic regions are revealed. One
example is a group of KIR that comprises inhibitory KIR mol-
ecules and the molecules with nearly the same extracellular
domains but with no inhibitory cytoplasmic domains such as
some of the NK-associated transcripts (for review, see Refs.
29–32). The genes for them have been found to be clustered in
human chromosome 19q13 (22). Therefore, it is interesting to
test a hypothesis that gp49 genes also locate in a limited area
of a mouse chromosome.

The first step toward this issue, we have isolated the genomic
clones for gp49B using a gp49B cDNA fragment as a probe. We
initially characterized the exon/intron structures of one of the
positive clones (clone 1, harboring a 16-kb insert and contain-
ing all of the exons for gp49B). Unexpectedly, we found that
this genomic clone 1 also contained a 3′ part of the gene for
gp49A as shown in Fig. 2. An overlapping clone, clone 2, was
also shown to contain all of the gp49A exons and a 5′ part of
the gene for gp49B by sequencing analysis. Thus, we found that
gp49A and gp49B genes are in the juxtaposition and in the
same orientation: they are apart by 4.4 kb (Fig. 2). In addition,
sequencing analysis of the gp49A and gp49B genes from 129/Sv
mouse revealed that there is no nucleotide substitution in the
exon sequences when compared with the respective genes from
BALB/c and C3H mice, respectively (data not shown), support-
Assignment of the mouse gp49B gene was performed by showing in Fig. 3, the signals of the gp49B gene were detected on species. Hence, the notion that gp49 genes are not polymorphic within a

**FIG. 4. Location of gp49 locus on mouse chromosome 10.** A, the segregation patterns of the mouse gp49B gene with flanking eight microsatellite DNA markers, D10Mit3, D10Mit38, D10Mit20, D10Mit31, and D10Mit7, in the backcross mice are shown. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6 × M. spretus)F1 parent. □ represent the presence of M. spretus allele, and ■ represent the presence of C57BL/6 allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. B, the partial chromosome 10 linkage map shows the location of mouse gp49B locus in relation to the flanking DNA markers. Recombination distances between loci are shown in centimorgan to the right of the chromosome.

**Chromosomal Mapping of the gp49B Gene**—The chromosomal assignment of the mouse gp49B gene was performed by direct R-banding fluorescence in situ hybridization using a mouse genomic DNA fragment as a biotinylated probe. As shown in Fig. 3, the signals of the gp49B gene were detected on the R-band-positive B4 band of mouse chromosome 10 (33).

For fine linkage mapping of the mouse gp49B gene, genomic DNAs of C57BL/6J, M. spretus and their F1, were digested with six different restriction endonucleases to find the restriction fragment length variants using Southern blot hybridization. The restriction fragment length variant with HindIII, 6.1 kb in C57BL/6J and 5.4 kb in M. spretus, was used to examine the concordance of segregation of the restriction fragment length variant with the segregation of microsatellite markers, D10Mit3, D10Mit38, D10Mit20, D10Mit31, and D10Mit7. Gene order was determined by minimizing the number of multiple recombinations among the loci on the same chromosome. Comparative pairwise loci analyses showed the gene order and recombination frequency for gp49B on mouse chromosome 10 (genetic distance in centimorgan ± S.E. and the number of

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