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PIG-B, a membrane protein of the endoplasmic reticulum with a large lumenal domain, is involved in transferring the third mannose of the GPI anchor

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Many eukaryotic cell surface proteins are bound to the membrane via the glycosylphosphatidylinositol (GPI) anchor that is covalently linked to their carboxy-terminus. The GPI anchor precursor is synthesized in the endoplasmic reticulum (ER) and post-translationally linked to protein. We cloned a human gene termed *PIG-B* (phosphatidylinositol glycan of complementation class B) that is involved in transferring the third mannose. *PIG-B* encodes a 554 amino acid, ER transmembrane protein with an amino-terminal portion of ~60 amino acids on the cytoplasmic side and a large carboxy-terminal portion of 470 amino acids within the ER lumen. A mutant PIG-B lacking the cytoplasmic portion remains active, indicating that the functional site of PIG-B resides on the lumenal side of the ER membrane. The *PIG-B* gene was localized to chromosome 15 at q21–q22. This autosomal location would explain why *PIG-B* is not involved in the defective GPI anchor synthesis in paroxysmal nocturnal hemoglobinuria, which is always caused by a somatic mutation of the X-linked *PIG-A* gene.

Keywords: GPI anchor/mannosyltransferase/PIG-B

Introduction

Many eukaryotic cell surface proteins are attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor that is covalently linked to the carboxy-termini. At least 50 mammalian proteins, such as cell surface hydrolytic enzymes, adhesion molecules, receptors and other antigenic molecules, are GPI anchored (Ferguson and Williams, 1988; Kinoshita *et al.*, 1995). The GPI anchor is used by many protozoa (Thomas *et al.*, 1990; McConville and Ferguson, 1993; Ferguson, 1994). A number of essential proteins, namely the variant surface glycoprotein of *Trypanosoma brucei* (Cross, 1990; Englund, 1993; McConville and Ferguson, 1993) and presumably also the circumsporozoite proteins of *Plasmodium* parasites (Moran and Caras, 1994; Reymond *et al.*, 1995) that cover almost the entire surface of these unicellular organisms are GPI anchored. Yeast *Saccharomyces cerevisiae* is also rich in GPI-anchored proteins (Herscovics and Orlean, 1993) and biosynthesis of GPI anchor is essential for growth (Leidich *et al.*, 1994).

The core backbone of the GPI anchor is conserved from yeast to mammals and it has the common structure, ethanolamine-P-6Manα1,2Manα1,6Manα1,4GlcNα1,6*myo*-inositol1-P-lipid (Ferguson *et al.*, 1988; Homans *et al.*, 1988; Fankhauser *et al.*, 1993; Treumann *et al.*, 1995). Among organisms and cell types, this core is modified variously by side groups and the type of lipid varies. The precursor of the GPI anchor is pre-assembled (Bangs *et al.*, 1985; Ferguson *et al.*, 1986) and post-translationally linked to peptides in the endoplasmic reticulum (ER) (Fasel *et al.*, 1989; Conzelmann *et al.*, 1990). The nascent peptides that are to be GPI anchored have a signal sequence at their carboxy-termini that direct GPI anchoring (Englund, 1993). This GPI signal peptide is cleaved and the new carboxy-terminus is linked to the ethanolamine of the GPI anchor precursor via an amide bond. This is presumably a transamidation reaction.

Synthesis of the GPI anchor precursor in the ER consists basically of the sequential addition of sugar components and ethanolamine phosphate (EtN-P) to phosphatidylinositol (PI). The first reaction is transfer of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to PI to form GlcNAc-PI (Masterson *et al.*, 1989). This is de-*N*-acetylated to form GlcN-PI (Hirose *et al.*, 1991). Three mannosyl residues (Man) are then transferred. To the third Man, EtN-P is transferred from phosphatidylethanolamine to complete the core backbone (Menon and Stevens, 1992). The GPI anchor precursor is added to proteins on the lumenal side of the ER, whereas the first two intermediates, GlcNAc-PI and GlcN-PI, face the cytoplasm (Vidugiriene and Menon, 1993, 1994), indicating that biosynthesis of the GPI anchor precursor begins on the cytoplasmic side. Therefore, either an intermediate or the mature precursor should flip into the ER lumen.

Many mutant mammalian cell lines that are deficient in GPI anchor synthesis or its transfer to peptides have been established (Hyman, 1988; Hirose *et al.*, 1992a; Mohney *et al.*, 1994). They have been grouped into several complementation classes by somatic cell fusion analysis. Mutants of classes A, C and H are defective in the first step, indicating that at least three genes are involved in this step (Stevens and Raetz, 1991; Sugiyama *et al.*, 1991). Class J (Mohney *et al.*, 1994) and other (Stevens *et al.*, 1996) mutants are defective in deacetylation of GlcNAc-PI. Class E is a mutant of Dol-P-Man synthase (Chapman *et al.*, 1980), being defective in GPI anchor as well as *N*-glycan synthesis. The class B mutant is defective in the third mannosylation (Puoti *et al.*, 1991; Sugiyama *et al.*, 1991) and class F is defective in EtN-P transfer to the third Man residue (Sugiyama *et al.*, 1991; Puoti and Conzelmann, 1993). Mature GPI anchor precursors are formed but not transferred to proteins in class K (Mohney *et al.*, 1994). Using class A and H cells as recipients for cDNA libraries, *PIG-A* and *PIG-H* (or *GPI-H*) genes,

respectively have been cloned by means of expression cloning (Kamitani *et al.*, 1993; Miyata *et al.*, 1993). Using class F cells, the *PIG-F* gene, a putative ethanolamine phosphotransferase, has been cloned (Inoue *et al.*, 1993).

Little is known about three GPI mannosyltransferases, termed GPI mannosyl transferases I–III (GPIMT-I –III). All Man residues are donated by Dol-P-Man (Menon *et al.*, 1990), and Dol-P-Man synthesis is essential for GPI anchoring as shown in class E mutants. For the first Man, polyprenol phosphate-Man is also used at lower efficiency (DeLuca *et al.*, 1994). One report states that GPI anchor intermediates bearing two or more Man residues face the cytoplasmic side on the microsomes of *T.brucei*, indicating that Man residues are added on the cytoplasmic side of the ER membrane (Vidugiriene and Menon, 1994). This is in contrast to mannosylation in *N*-glycosylation intermediates in which Man residues donated from Dol-P-Man are added on the lumenal side of the ER membrane (Hirschberg and Snider, 1987). Dol-P-Man is also used in the ER lumen for the *O*-glycosylation of yeast proteins (Orlean, 1990). To obtain insight into this potentially different topological usage of Dol-P-Man, we cloned *PIG-B*, a putative GPIMT-III, using class B mutant cells.

Results

Expression cloning of PIG-B cDNA

The S1A-b cell line is a GPI anchor-deficient murine thymoma mutant of complementation class B (Hyman, 1988). We used expression cloning to obtain a cDNA that complements the deficiency of S1A-b cells. To ensure the recovery of plasmids from transfected and sorted cells, we used the polyoma replication system and established an S1A-b/C4 clone that stably expresses high levels of polyoma large T antigen (PyT). A human cDNA library constructed in a vector bearing a polyoma origin of replication was screened for clones that complement Thy-1 expression on the S1A-b/C4 cells. We obtained the plasmid p42G302 that contained an insert of 1837 nucleotides. Since the complementation of Thy-1 expression by this cDNA was partial (see below), we thought it a partial cDNA and cloned the upstream portion using the 5′-RACE system. Of nine clones analyzed, clone 07 had the longest insert containing the upstream 92 nucleotides. Others contained 54–74 nucleotides of essentially the same sequences, except for one position at which two nucleotides appeared, suggesting a genetic polymorphism. Clone 07 was ligated to the original cDNA to obtain a full-length cDNA. The full-length and partial cDNAs were transiently expressed in S1A-b cells. The partial cDNA induced surface Thy-1 expression on only 16% of cells at a subnormal level (Figure 1a). In contrast, the full-length cDNA restored it on 40% of cells to the wild-type level (Figure 1b).

To confirm that cloned cDNAs complement the deficient transfer of the third Man residue, S1A-b cells stably expressing either the full-length or the partial cDNA were established, metabolically labeled with [^{3}H]mannose and mannolipids were analyzed. As shown in Figure 2, the mature GPI anchor precursors (H7 and C/H8) were synthesized in the wild-type cell line, TIMI (lane 3), but not in S1A-b cells (lane 2). Instead, an intermediate, which was

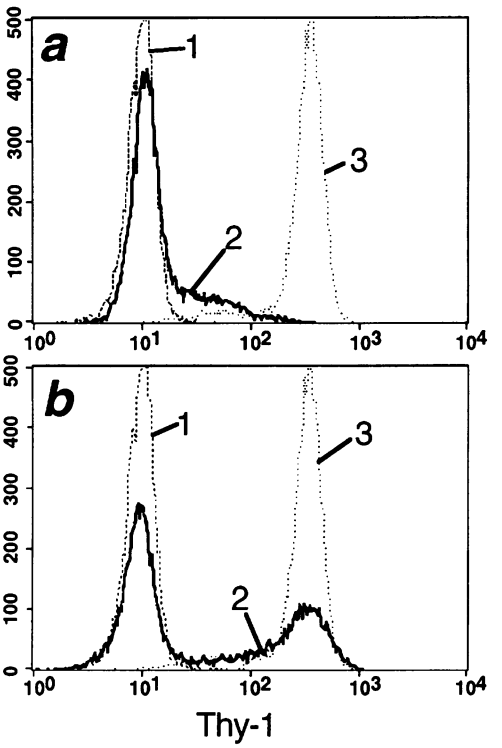

Fig. 1. Restoration of the surface expression of Thy-1 on class B mutant S1A-b cells with partial and full-length *PIG-B* cDNAs. S1A-b cells transiently transfected with each *PIG-B* cDNAs were stained for Thy-1 and analyzed in a FACScan. (**a**) S1A-b (line 1), S1A-b cells transfected with the partial *PIG-B* cDNA (line 2) and wild-type TIMI cells (line 3). (**b**) S1A-b cells (line 1), S1A-b cells transfected with the full-length *PIG-B* cDNA (line 2) and wild-type TIMI cells (line 3).

thought to bear two Man residues, accumulated in S1A-b cells (lane 2) as described (Puoti *et al.*, 1991; Sugiyama *et al.*, 1991). A transfectant expressing the full-length cDNA synthesized mature type GPI anchor precursors similarly to the wild-type TIMI cells, and the accumulation of the intermediate disappeared (lane 1). Therefore, transfer of the third Man was restored by the expression of the cloned cDNA. A transfectant expressing the partial cDNA also synthesized mature GPI anchor precursors, but the intermediate still accumulated (lane 4), indicating the partial restoration of the transfer of the third Man residue.

We thus cloned the target cDNA. We termed the gene *PIG-B* for phosphatidylinositol glycan of complementation class B. The *PIG-B* gene product may be GPI mannosyltransferase III.

Characteristics of PIG-B cDNA

The *PIG-B* cDNA consists of 1929 bp and the longest open reading frame, spanning nt 45–1706, encoded a predicted protein of 554 amino acid residues (Figure 3). The sequence around the initiation codon at nt 45–47 agrees well with the Kozak consensus sequence (Kozak, 1987). The partial cDNA that starts from nt 96 was partially active as described above. Thus, either a peptide presumably translated from the second methionine codon located at nt 333–335 had partial activity or a read-through peptide generated at low levels using a translation initiation site within the vector had activity. To determine which was true, full-length and partial cDNAs as well as a cDNA starting from the second methionine site were cloned into

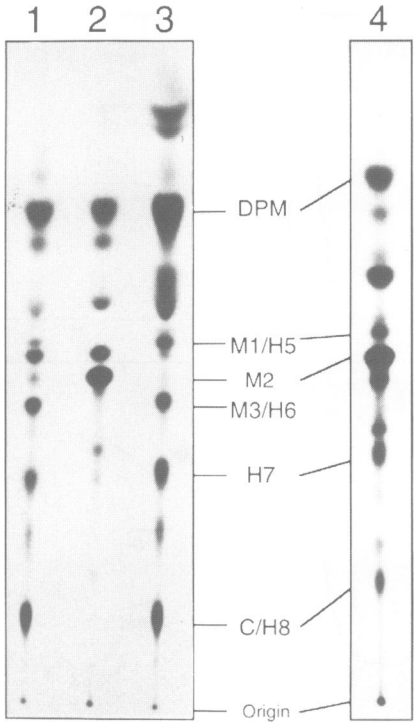

Fig. 2. Restoration of biosynthesis of the mature GPI anchor precursors with *PIG-B* cDNAs. A stable transfectant with the full-length *PIG-B* cDNA (lane 1), S1A-b cells (lane 2), wild-type TIMI cells (lane 3) and a stable transfectant with the partial *PIG-B* cDNA (lane 4) were labeled with D-[^{3}H]mannose in the presence of tunicamycin. Radiolabeled lipids were analyzed by thin layer chromatography. C/H8 and H7, the mature type GPI anchor precursors; M1–M3, intermediates containing one to three mannose residues, respectively, with acylation on PI and ethanolamine phosphorylation on the first mannose; H5 and H6, identical to M1 and M3, respectively (Hirose *et al.*, 1992b); DPM, dolichol phosphate-mannose.

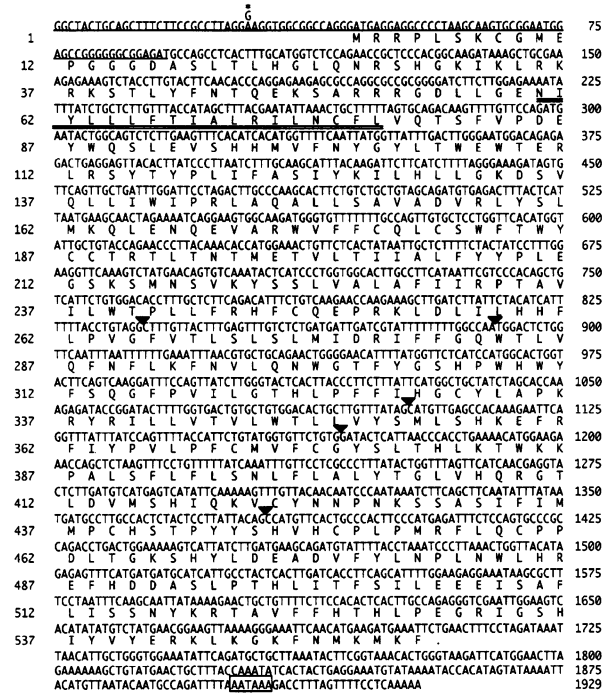

Fig. 3. The nucleotide sequence of *PIG-B* cDNA and its deduced amino acid sequence. The predicted amino acid sequence of the longest open reading frame is shown in single-letter codes below the nucleotide sequence. The region of 92 nucleotides at the 5′ terminus obtained by means of 5′-RACE is underlined. The putative transmembrane domain is doubly underlined. A polyadenylation signal sequence is boxed. The polymorphic position 29 is marked by an asterisk. The arrowheads indicate some of the exon boundaries determined by analysis of a partial *PIG-B* genomic clone of 17 kb. The nucleotide sequence data reported here will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the accession No. D42138.

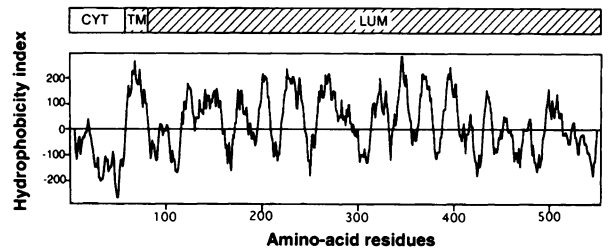

Fig. 4. A hydrophobicity profile of the predicted PIG-B protein. The hydrophobicity profile is drawn using the algorithm of Kyte and Doolittle (1982) with a window setting of 11. A schematic representation of domain organization is shown above the hydropathy profile. CYT, TM and LUM indicate cytoplasmic, transmembrane and lumenal domains, respectively.

an expression vector so that they all used the same consensus initiation sequence, and they were then transfected into class B mutant cells. The two former cDNAs complemented the surface Thy-1 expression to the wild-type level, whereas the latter did not, indicating that the read-through product was active (data not shown).

There was no typical amino-terminal hydrophobic sequence of the signal peptide and there were several hydrophobic segments in the deduced PIG-B protein sequence (Figures 3 and 4). There was no overall homology with known glycosyltransferases.

The size of *PIG-B* mRNA was assessed by Northern blotting. Poly(A)$^+$ RNAs extracted from the B lymphoblastoid cell line JY-25 and its GPI anchor-deficient class A mutant JY-5 (Hollander *et al.*, 1988; Miyata *et al.*, 1993) were hybridized with a *PIG-B* cDNA probe. Both cell lines expressed a single *PIG-B* mRNA species of 2.0 kb (Figure 5), suggesting that the full-length *PIG-B* cDNA corresponds to this mRNA.

We next tested whether *PIG-B* is the gene mutated in the class B mutant. Since detection of mouse transcripts on Northern blot analysis with human cDNA probe was not successful (data not shown), we cloned the mouse counterpart of *PIG-B*, termed *Pig-b*. The *Pig-b* cDNA consists of 2196 bp and encodes a protein of 542 amino acids which has 77% amino acid identity with human PIG-B (DDBJ/EMBL/GenBank accession No. D84436).

The cloned *Pig-b* cDNA restored the surface expression of Thy-1 on S1A-b cells after transfection, confirming its function (data not shown).

Levels of *Pig-b* transcripts in parental S1A and mutant S1A-b were assessed by RT–PCR (Figure 5B). The class B mutant had only 10% of the wild-type level. The amplified cDNAs were cloned and transfected into S1A-b cells to determine their functional activity. Three of the five clones derived from wild-type S1A were active, whereas none of seven clones from S1A-b were active (data not shown), indicating that the *Pig-b* transcripts found in S1A-b cells are non-functional as well as quantitatively

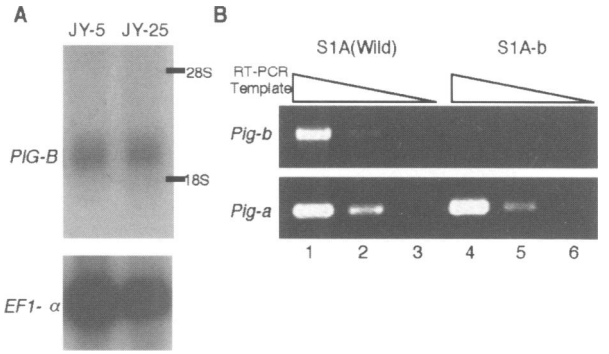

Fig. 5. Analyses of *PIG-B* transcripts in human and mouse cell lines. (**A**) Northern blots of *PIG-B* transcripts in human cell lines. Poly(A)$^+$ RNAs from the human B lymphoblastoid cell line JY-25, which expresses GPI-anchored proteins normally, and JY-5, which does not express GPI-anchored proteins due to having an abnormal *PIG-A* gene, were hybridized with the *PIG-B* cDNA probe (upper panel) and rehybridized with elongation factor 1α (EF1-α) gene cDNA (a gift from Dr S.Nagata) to evaluate the amounts of RNA applied (lower panel). (**B**) RT–PCR analysis of mouse *Pig-b* transcripts in S1A-b and S1A cells. *Pig-b* and *Pig-a* transcripts were reverse transcribed with an oligo(dT) primer from total RNA of S1A-b and S1A, and the entire coding region of *Pig-b* (1770 bp) and a part of *Pig-a* coding region (330 bp) were amplified by PCR, with 4-fold dilutions of the cDNAs (lanes 1–3, S1A; lanes 4–6, S1A-b).

abnormal. We concluded, therefore, that *Pig-b*/*PIG-B* is the class B gene.

ER localization and orientation of PIG-B protein

To localize the intracellular expression site of PIG-B, its amino-terminus was tagged by glutathione-*S*-transferase (GST) and the fusion construct, GST–PIG-B, was expressed in a class B thymoma mutant. The fusion protein was active as shown by complementation of the surface expression of Thy-1 (data not shown). The transfectant cells were disrupted and fractionated into cytoplasm, nuclei and membranes. The nuclei were separated further into non-ionic detergent-soluble and high salt-soluble fractions. The membranes were solubilized by non-ionic detergent. The PIG-B fusion proteins collected from these fractions by beads bearing glutathione were assessed by Western blotting against anti-GST antibodies. GST–PIG-B was detected in detergent-soluble membranes and nuclei but not in the cytoplasm or the salt extract of the nuclei (Figure 6). Therefore, PIG-B is membrane bound. The molecular mass of the GST–PIG-B fusion protein was estimated to be 90 kDa, being similar to a predicted size of 92 kDa.

To localize the exact site of expression further, membranes were fractionated into ER, Golgi and plasma membranes. GST–PIG-B was detected mainly in the fraction containing the ER (Figure 7), indicating that PIG-B is an ER membrane protein.

We then investigated whether PIG-B is a transmembrane protein in the ER and, if so, how it is oriented. For this, we also tagged the carboxy-terminus with GST to test whether the carboxy-terminus is within the ER lumen or in the cytoplasm. This fusion (termed PIG-B–GST) was also active, as determined by restoration of the surface Thy-1 expression on class B mutant cells upon transfection (data not shown). Cells expressing PIG-B–GST and those expressing GST–PIG-B were disrupted to obtain membrane vesicles bearing PIG-B–GST or GST–PIG-B. These

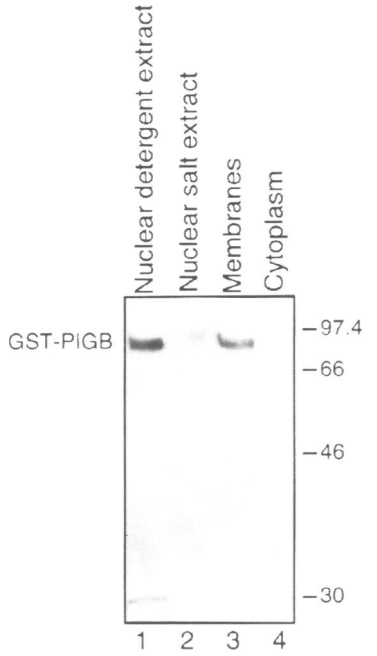

Fig. 6. Membrane localization of PIG-B protein. S1A-b cells expressing the fusion protein GST–PIG-B were disrupted by passage through a 22 gauge needle, and nuclei were precipitated by centrifugation at 1000 *g* for 10 min. The supernatant was fractionated into the cytoplasm (supernatant, lane 4) and membranes (pellet, lane 3) by centrifugation at 10^5 *g*. The nuclei were extracted with a buffer containing 0.5 M NaCl and separated by centrifugation at 10^5 *g*. The supernatant and pellet fractions were designated as Nuclear salt (lane 2) and Nuclear detergent extracts (lane 1), respectively. NP-40 was added to these fractions at a final concentration of 1% to solubilize the membranes, then beads bearing glutathione were added. The captured GST fusion proteins eluted from the beads were analyzed by Western blotting with anti-GST antibodies.

vesicles were digested with proteinase K followed by Western blotting against anti-GST antibodies. As shown in Figure 8A lane 5, a fragment of ~80 kDa was protected when GST was located at the carboxy-terminus. The protected fragment was not seen when the vesicles were made leaky by 1% NP-40 (lane 4). There was no protection when the amino-terminus was tagged by GST (lane 2). These results indicated that PIG-B is a transmembrane protein, of which 85% at the carboxy-terminal is within the ER lumen, and that the hydrophobic segment close to the amino-terminus is a transmembrane domain. To confirm that these vesicles were intact, we simultaneously analyzed the proteinase K susceptibility of protein disulfide-isomerase (PDI), a lumenal, reticulum-associated protein (Lambert and Freedman, 1985). As shown in Figure 8B, PDI was resistant to proteinase K in both vesicles (lanes 2 and 5). When the vesicles were made leaky by detergent, a cleavage product appeared (lanes 3 and 6), showing that the vesicles were intact.

Localization of functional sites of PIG-B by deletion mutagenesis

To determine whether the cytoplasmic small domain is essential for PIG-B function, we prepared a deletion mutant of PIG-B in which the first 209 nucleotides are missing, tagged it with GST at the carboxy-terminus and transfected it into class B mutant cells (Figure 8C). The mutant lacking the first 209 nucleotides, and therefore

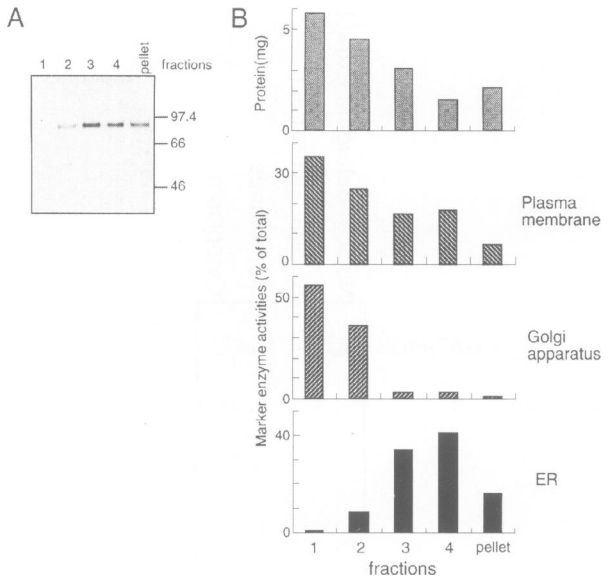

Fig. 7. ER localization of PIG-B protein. S1A-b cells expressing GST–PIG-B were disrupted by nitrogen cavitation and centrifuged at 10 000 *g* for 15 min. The supernatant was fractionated further by sucrose gradient centrifugation (see Materials and methods). The fractions and pellet were adjusted to 1% NP-40, 150 mM NaCl and 1 mM EDTA. GST–PIG-B fusion protein was detected as described in the legend to Figure 6 (**A**). Each fraction was characterized by assaying for protein content and organelle-specific marker enzyme activities, alkaline phosphodiesterase I for plasma membrane, α-mannosidase II for Golgi apparatus, and dolichol phosphate-mannose synthase for ER (**B**).

the entire amino-terminal hydrophilic portion (designated Δ209PIGB-GST), co-migrated with the fragment derived from PIG-B–GST that was protected from proteinase K (lanes 2 and 3), indicating that this amino-terminal hydrophilic portion corresponds to the cytoplasmic domain. This mutant was functional in restoring the surface Thy-1 expression (Figure 9), indicating that the cytoplasmic domain is not necessary for function.

Partial characterization of the genomic structure of PIG-B and its chromosomal localization

Using *PIG-B* cDNA as a probe, we isolated a *PIG-B* genomic clone of 17 kb. Analysis of this clone revealed four exons within it; nt 839–890, 891–1102, 1103–1167 and 1168–1381 (for cDNA nucleotide number, see Figure 3). Therefore, the *PIG-B* gene has at least six exons. A mixture of the 3.6 kb *Sal*I and *Eco*RI fragment and the 3.6 kb *Eco*RI fragment derived from this genomic clone was used as the probe for fluorescence *in situ* hybridization (FISH). *PIG-B* was localized to chromosome 15 at 15q21–q22 (Figure 10).

Discussion

The GPI anchor precursor is synthesized in the ER and post-translationally linked to protein. All GPI anchors from various eukaryotes contain three Man residues in their core backbone. Little is known about the molecular mechanism of transfer of these residues. Here we cloned the human *PIG-B* gene, which is involved in transfer of the third Man residue. A murine class B Thy-1-deficient mutant cell accumulates an intermediate of the GPI anchor

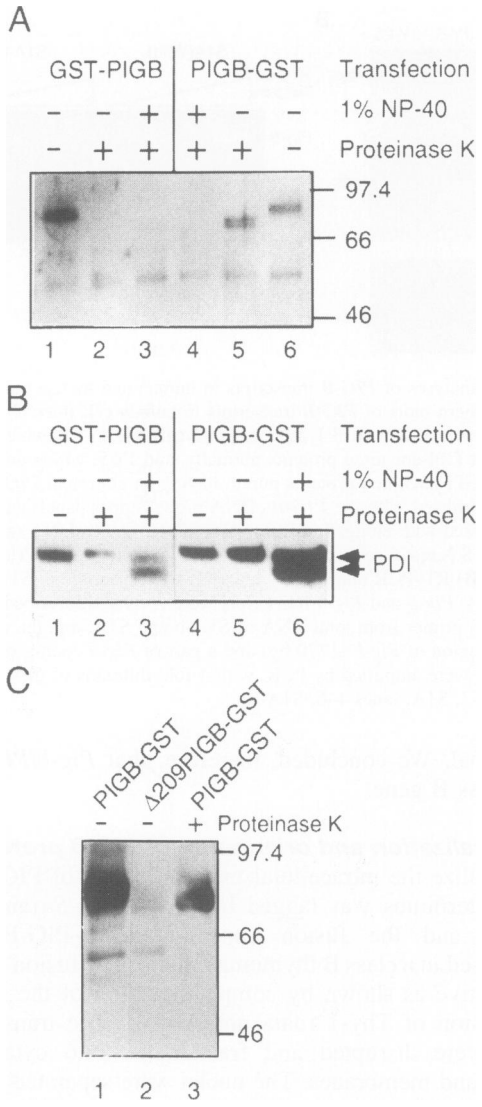

Fig. 8. Orientation of PIG-B protein. (**A**) Proteinase K protection assay. Transfectants expressing GST–PIG-B or PIG-B–GST were disrupted by passage through a 22 gauge needle. The vesicles were incubated with buffer alone (lanes 1 and 6), or proteinase K in the absence (lanes 2 and 5) or presence (lanes 3 and 4) of 1% NP-40. The reaction was terminated with PMSF. GST fusion proteins were collected with glutathione–Sepharose after solubilization with detergent and Western blotted against anti-GST antibodies. (**B**) Vesicle intactness was assessed by Western blotting of PDI in the samples described in (A) after removing the GST fusion proteins. (**C**) Comparison of GST fusion proteins in lysates of transfectants expressing PIG-B–GST (lane 1) or Δ209PIGB–GST (lane 2) with the fragment of PIG-B–GST protected from proteinase K (lane 3).

precursor bearing two Man residues, indicating the deficient transfer of the third Man residue (Puoti *et al.*, 1991; Sugiyama *et al.*, 1991). Upon transfection into class B cells, the *PIG-B* cDNA restored transfer of the third Man residue, leading to the generation of mature GPI anchor precursors and subsequent surface expression of GPI-anchored Thy-1 proteins. *PIG-B* cDNA encodes a new ER transmembrane protein of 554 amino acids. Although there is no apparent homology between PIG-B and known glycosyltransferases, and the enzyme activity of PIG-B protein has not been shown, the ER localization of PIG-B supports the notion that PIG-B is GPI mannosyltransferase III itself or its subunit.

PIG-B protein consists of a small amino-terminal cytoplasmic domain, a transmembrane domain and a large carboxy-terminal lumenal domain. The cytoplasmic domain was not necessary for function because a deletion mutant of PIG-B lacking the entire cytoplasmic domain was active. Therefore, the functional site of PIG-B resides on the lumenal side of the ER membrane.

All three Man residues in the GPI anchor are transferred from Dol-P-Man (Menon *et al.*, 1990). Dol-P-Man is synthesized by Dol-P-Man synthase, an ER membrane protein, from GDP-Man and Dol-P. Since GDP-Man does not translocate across the ER membrane (Hirschberg and Snider, 1987) and since Dol-P is available on the cytoplasmic side of the ER, it is thought that Man is transferred to Dol-P on the cytoplasmic side of the ER. Dol-P-Man is used on the lumenal side of the ER membrane for the mannosylation of *N*-glycan precursor intermediates. It is also used in the ER lumen for *O*-glycosylation of yeast proteins (Orlean, 1990). Therefore, Dol-P-Man should flip into the lumen after its synthesis.

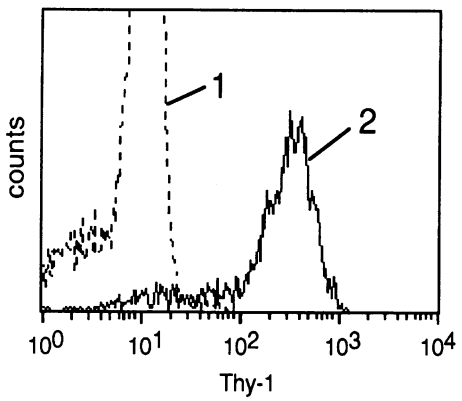

Fig. 9. Functional activity of a PIG-B mutant lacking the cytoplasmic domain. S1A-b (line 1) and its transfectant expressing Δ209PIG-B–GST (line 2) were analyzed for the surface expression of Thy-1.

The Lec35 mutant of Chinese hamster ovary (CHO) cells synthesizes Dol-P-Man but cannot use it as a Man donor for *N*-glycan (Lehrman and Zeng, 1989; Zeng and Lehrman, 1990), raising the possibility that Lec35 mutant is defective in Dol-P-Man flipping. Significantly, the Lec35 mutant accumulates a GPI anchor intermediate glucosaminyl-acylPI (Camp *et al.*, 1993), indicating that it cannot use Dol-P-Man for a GPI anchor either. This is consistent with the notion that Dol-P-Man is also used on the lumenal side for a GPI anchor synthesis.

The orientation of the functional domains of PIG-B suggests the lumenal usage of Dol-P-Man for the third mannosylation of GPI anchor precursor. This is, however, inconsistent with the reported orientation of the intermediate GPI anchor precursors of *T.brucei* (Vidugiriene and Menon, 1994) and of intermediate protein GPI anchor precursors of *Leishmania major* (Mensa Wilmot *et al.*, 1994). One explanation for this may be that the topology of GPI mannosylation is different in mammalian and protozoan cells. In this context, cloning protozoan homologs of PIG-B and analysis of their membrane topology would be interesting. Another, but less likely, possibility is that PIG-B acts as a mannosyltransferase subunit that associates with another as yet unknown catalytic subunit bearing a cytoplasmic catalytic site; hence, mammalian GPI mannosylations also occur on the cytoplasmic side.

Paroxysmal nocturnal hemoglobinuria (PNH) is a somatically acquired genetic disease characterized by defective GPI anchor biosynthesis in affected clonal hematopoietic cells. In all patients characterized to date, the GPI anchor deficiency is caused by a somatic mutation of the *PIG-A* gene (Takeda *et al.*, 1993). This gene, which participates in the first step in GPI anchor synthesis (Miyata *et al.*, 1993), is X-linked (Takeda *et al.*, 1993). Since only one allele of *PIG-A* is active in female as well as male cells due to the X-inactivation phenomenon, one inactivating mutation in *PIG-A* would result in defective GPI anchor synthesis. For autosomal GPI synthesis genes,

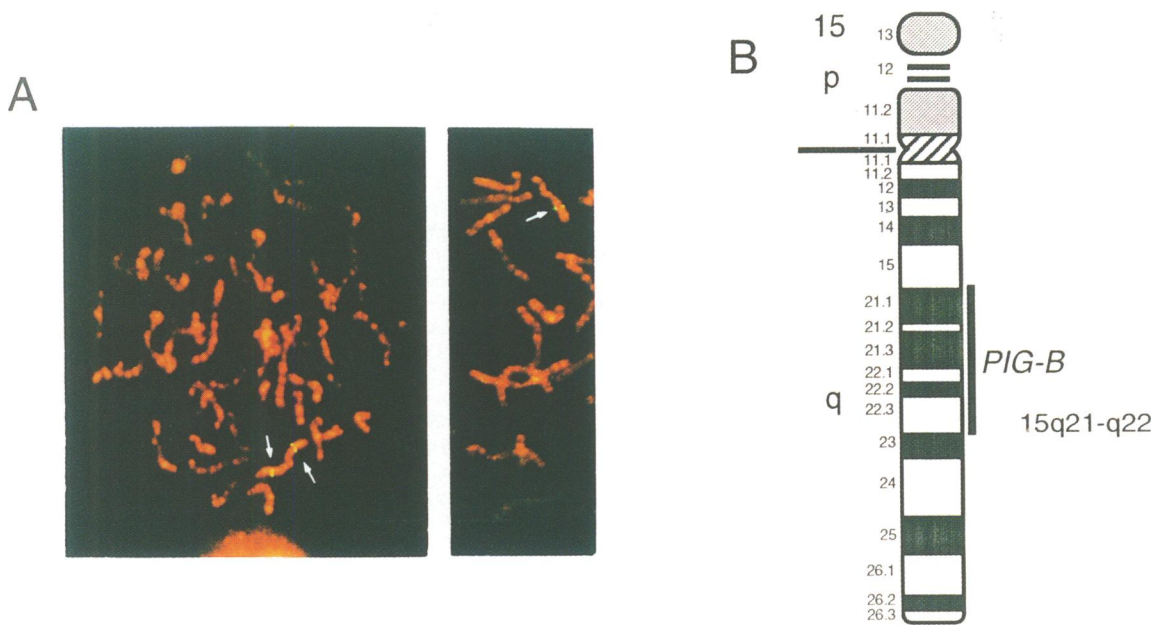

Fig. 10. Chromosomal localization of the *PIG-B* gene determined by fluorescence *in situ* hybridization with a genomic *PIG-B* probe. Arrows indicate positive signals in 15q21–q22 (**A**). An ideogram of chromosome 15 and the location of the *PIG-B* gene are shown in (**B**).

two inactivating mutations would be required. The autosomal location of *PIG-B* would, therefore, account for the absence of patients with PNH caused by a *PIG-B* mutation.

Materials and methods

Cells and plasmids

The murine Thy-1-negative thymoma mutant S1A-b (Hyman, 1988) and wild-type thymoma TIMI were provided by R.Hyman (Salk Institute). Parental, wild-type S1A was obtained from the American Type Culture Collection (Rockville, MD). We used TIMI as a control wild-type thymoma in some of the experiments because parental S1A was not available to us in the initial stages of this study.

A human cDNA library prepared from the human myelocytic leukemia cell line P39/TSU (Matsumoto and Seya, 1993) in the eukaryotic expression vector, pCEV4 (Itoh *et al.*, 1990), was a gift from Drs M.Matsumoto and T.Seya. A fragment containing PyT but lacking the polyoma origin of replication was obtained from pdl3027 (Jat *et al.*, 1982) by digestion with *Bam*HI and *Hin*cII. The fragment was blunt-ended and ligated to *Eco*RI- and *Nru*I-digested and blunt-ended pRc/CMV, a mammalian expression vector bearing the cytomegalovirus promoter for expression (Invitrogen, San Diego, CA). The plasmid obtained was termed pS7neo. A 4.9 kb fragment containing luciferase cDNA was derived from PGV-CS (Toyo Ink, Japan) by digestion with *Bam*HI and *Xho*I and inserted into a 3.9 kb vector fragment (*Eco*RI and *Xho*I) of pME18Sf(+) to obtain pMEluc. Thereafter, the luciferase fragment obtained from pMEluc by cutting with *Bam*HI and *Pst*I was inserted into pCEV4 to obtain pCEVluc, which bears the origin of replication of the polyoma virus. The expression vector pME18Sf (+) was a gift from Dr K.Maruyama (Tokyo Medical and Dental University, Tokyo).

Plasmids were transfected into $0.5–1\times10^7$ cells suspended in 0.8 ml of HEPES-buffered saline (Chu *et al.*, 1987) by electroporation at 300 V and 500 μF using a Gene-pulser (Bio-Rad, Richmond, CA).

Cloning of PIG-B cDNA

To establish a PyT-expressing cell line from S1A-b cells, we transfected them with pS7neo and selected transfectants with G418. To identify the clone with the highest level of PyT activity, we transfected the cells with pCEVluc. One of the transfectants, the S1A-b/C4 clone, which had the highest luciferase activity was selected as a host cell line for expression cloning.

A sample of 200 μg of the cDNA library plasmids was transfected into S1A-b/C4 cells (total 10^8 cells) by electroporation. Two days later, transfected cells were stained with biotinylated anti-Thy-1 monoclonal antibody G7 (a gift from Dr T.Tadakuma, National Defense Medical College, Japan) followed by phycoerythrin-conjugated streptavidin (Biomeda, Foster City, CA) and sorted using a FACStar (Beckton Dickinson, Mountain View, CA). From 127 Thy-1-positive cells sorted, 1.5×10^4 independent colonies were recovered as described by Hirt (1967) and by transformation into *Escherichia coli* MC1061. Pooled plasmids obtained from these transformants were transfected again into S1A-b/C4 cells (200 μg of DNA into 10^8 cells). A total of 327 Thy-1-positive cells were obtained from the second sorting performed 2 days later. Plasmids obtained from the sorted cells containing ~5000 independent colonies were separated into five 96-well plates. Ten miniprep plasmid pools each derived from 48 wells were transfected into S1A-b/C4 cells, and the transfected cells were analyzed for Thy-1 expression. One pool that gave Thy-1-positive signals was divided further into smaller pools, which were screened again. Finally, one positive well contained plasmid 42G302 that restored the expression of Thy-1 on the cell surface.

To obtain a full-length cDNA of *PIG-B*, we amplified the 5′ end of *PIG-B* cDNA from human placental poly(A)$^+$ RNAs using a 5′-AmpliFINDER RACE kit (Clontech, Palo Alto, CA). In brief, the 5′ end of cDNA was first synthesized from the poly(A)$^+$ RNAs with the antisense *PIG-B*-specific primer (P1: 5′-GCCACCAGGGATGAGTAT-TTGACAC-3′). After AmpliFINDER anchor ligation to the 3′ end of the *PIG-B* cDNA, we amplified a 5′ portion of *PIG-B* by PCR using an AmpliFINDER anchor primer and a nested *PIG-B*-specific primer (P2: 5′-AAACACCCATCTTGCCACTTCCTGA-3′).

Cloning of mouse Pig-b cDNA

To amplify a fragment of mouse *Pig-b* cDNA by PCR from a mouse cDNA library, we synthesized degenerated primers each corresponding to nucleotides 1146–1171 and complementary to nucleotides 1353–1378 of *PIG-B* cDNA. Using these primers, a specific band of predicted size (233 bp) was amplified from an F9 embryonic carcinoma cell cDNA library. This DNA fragment has a sequence homology with *PIG-B*. Based on this sequence, we synthesized nested primers to amplify 5′ and 3′ ends using a Marathon kit (Clontech, Palo Alto, CA). For amplification of the 5′ end, *Pig-b* mRNA was reverse transcribed from total RNA of S1A cells using a *Pig-b*-specific primer (5′-GAA-GCTGGGTCAGGACCTCG) and a 5′ fragment of 1.3 kbp was amplified using nested *Pig-b* primers (5′-ATCCAGGGTGCCTCTCTGATGAACT and 5′-CTGATGAACTAGGCCAGTGTAGAAT) and the linker primer supplied with the kit. For amplification of the 3′ end, RNA was reverse transcribed using the first strand primer supplied with the kit and a 3′ fragment of 900 bp was amplified using nested *Pig-b* primers (5′-CTTCCTGCTCTTGTCAAATGTGCCA and 5′-AGTTCATCAGAGA-GGCACCCTGGAT) and the first strand primer. The full-length *Pig-b* cDNA was constructed from these fragments.

To amplify the entire coding region of *Pig-b* by RT–PCR, cDNAs reverse transcribed with oligo(dT) primer from S1A and S1A-b RNA were used as templates and two oligonucleotides, 5′-AGTGGCATATCG-ATGCCTACAAGAAAGTGA and 5′-TGGCTTCCTCTCATTTCTAGA-TCACCTACA were used as primers. To assess the amounts of total cDNA, a 330 bp fragment of *Pig-a* was amplified with primer set 5′-GCTGATGTCAGCTCGGTGCTTAC and 5′-GATTCTCTTTGGTCCC-TCTCCTC by PCR (Kawagoe *et al.*, 1994).

In vivo labeling of the cells with [^{3}H]mannose

Cells (4×10^6) were labeled with 100 μCi of D-[2-^{3}H]mannose (American Radiolabeled Chemicals, MO) for 45 min, then the lipid fraction was extracted and separated with chloroform/methanol/water (10:10:3) on DC-Alufolien Kieselgel 60 (MERCK, Germany) (Hirose *et al.*, 1992b). The plate was analyzed by fluorography.

Mutagenesis of PIG-B cDNA

Deletion mutants of *PIG-B* cDNA that lack various lengths of the 5′ portion but which have the same initiation sequence for translation were constructed as follows. A fragment containing the coding region of the *OCH1* gene, a yeast mannosyltransferase, was prepared by digestion with *Sac*I and *Kpn*I of pSP-OCH1 (Nakayama *et al.*, 1992) (a gift from Dr Y.Jigami). This was ligated into *Sac*I- and-*Kpn*I-digested pBS II KS+ and the plasmid was termed pBS-OCH1. This plasmid was prepared to use the 5′-non-coding sequence. An *Sph*I site which contains an artificial translation initiation codon was introduced into the 5′ ends of various lengths of the 5′ coding region of PIG-B by PCR. The upstream primers were 5′-GCGCCGCATGCATCTTCTTGGAGAAAATAT-3′ to delete 209 nucleotides and 5′-TGAAGTTTCACATCGCATGCTTTT-CAA-3′ to delete 338 nucleotides (*Sph*I sites underlined). The common downstream primer was 5′-TCCACCCAGCAATGTTAAGATATCTAG-3′, which introduced an *Eco*RV site (underlined) at the 3′ end of the PCR products. The PCR products were digested by *Sph*I and *Eco*RV, and ligated into *Sph*I- and *Eco*RV-digested pBS-OCH1. Fragments that contained various lengths of the 5′ portion of *PIG-B* coding region plus the same 5′-non-coding region and initiation codon were obtained from these plasmids by digestion with *Sal*I and *Eco*RV and inserted into the mammalian expression vector pME18Sf(+).

Expression and analysis of PIG-B, its fusion proteins with GST and their mutants

A cDNA of the coding region of *Schistosoma japonicum* GST was obtained from pGEX4T (Pharmacia Biotech, Uppsala, Sweden) and fused to either the 5′ or 3′ end of a full-length *PIG-B* cDNA, or to the 3′ end of mutant *PIG-B* cDNAs. Full-length and partial *PIG-B* cDNAs, the PIG-B–GST fusion cDNAs and their mutants were inserted into pME18Sf(+) and transfected into S1A-b cells which were used either 2 days later as transient transfectants or selected with G418 to establish stable transfectants. Fusion proteins of PIG-B and GST extracted from cells with 1% NP-40 were collected by glutathione–Sepharose beads and Western blotted against goat anti-GST and peroxidase-conjugated anti-goat IgG antibodies, followed by development using RENAISSANCE Kit (Du Pont, Boston, MA). PDI was detected by Western blotting with rabbit anti-PDI antibodies (a gift from Drs R.Masaki and A.Yamamoto, Kansai Medical University, Osaka, Japan).

Isolation of subcellular fractions

S1A-b cells (2×10^8), stably expressing GST–PIG-B fusion proteins were resuspended in a buffer containing 0.25 M sucrose, 10 mM HEPES–NaOH pH 7.5, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl

fluoride (PMSF), 1 μg/ml leupeptin, 10 μg/ml aprotinin and 0.1 mM TLCK, and disrupted by means of nitrogen cavitation (400 p.s.i. N_2 pressure for 30 min) and a tight pestle Dounce homogenizer. Nuclei and cell debris were removed by centrifugation at 1000 *g* for 10 min and 10 000 *g* for 15 min, respectively at 4°C. The supernatant (PNS) was layered on the top of a discontinuous sucrose gradient consisting of 3.6 ml of 38% sucrose, 1.8 ml of 30% sucrose and 1.8 ml of 20% sucrose made in the same buffer and centrifuged at 25 000 r.p.m. for 3 h at 4°C in an SW41Ti rotor (Beckman Instruments Inc., Fullerton, CA) (Vidugiriene and Menon, 1993). The gradient was collected in fractions and membranes were dissolved by adding NP-40 to a final concentration of 1%. Plasma, Golgi and ER membranes were located by measuring levels of their membrane enzymes, alkaline phosphodiesterase I, α-mannosidase II and dolichol phosphate-mannose synthase, respectively (Vidugiriene and Menon, 1993).

Proteinase K protection assay

Transfectants ($2–5\times10^8$ cells) expressing GST fusion proteins were lysed by 10 passages through a 22 gauge needle in 5 ml of 0.25 M sucrose buffer containing 10 mM HEPES–NaOH pH 7.5, 1 mM DTT and 0.5 mM PMSF. Ten μl of 10 mg/ml proteinase K and 2 μl of 0.5 M EDTA were added to 1 ml of the lysates and these mixtures were incubated at 4°C for 3–5 h. Of the remaining lysates, 1 ml was incubated with proteinase K in the presence of 1% NP-40. The reaction was stopped by adding 20 μl of 0.1 M PMSF followed by centrifugation at 100 000 *g* for 1 h at 4°C. The pellets were solubilized by 1 ml of TNE buffer (10 mM Tris–HCl, pH 7.8, 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 2 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin and 0.1 mM TLCK). Insoluble debris was removed by centrifugation at 1000 *g* for 10 min. GST fusion proteins were collected from the supernatants using glutathione–Sepharose and Western blotted.

FISH

FISH proceeded using the *PIG-B* genomic DNA probe as described (Takeda *et al.*, 1993).

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