

A Deletion in the Gene Encoding the CD45 Antigen in a Patient with SCID

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SCID is a heterogeneous group of hereditary diseases. Mutations in the common γ -chain (γ^c) of cytokine receptors, including those for IL-2, IL-4, IL-7, IL-9, and IL-15, are responsible for an X-linked form of the disease, while mutations of several other genes, including Janus-associated kinase-3, may cause autosomal recessive forms of SCID. We investigated the first SCID patient to be described with minimal cell surface expression of the leukocyte common (CD45) Ag. CD45 is an abundant transmembrane tyrosine phosphatase, expressed on all leukocytes, and is required for efficient lymphocyte signaling. CD45-deficient mice are severely immunodeficient and have very few peripheral T lymphocytes. We report here that a homozygous 6-bp deletion in the gene encoding CD45 (PTPRC, gene map locus 1q31–32), which results in a loss of glutamic acid 339 and tyrosine 340 in the first fibronectin type III module of the extracellular domain of CD45, is associated with failure of surface expression of CD45 and SCID. Molecular modeling suggests that tyrosine 340 is crucial for the structural integrity of CD45 protein. This is the second description of a clinically relevant CD45 mutation, provides direct evidence for the importance of CD45 in immune function in humans, and suggests that abnormalities in CD45 expression are a possible cause of SCID in humans. *The Journal of Immunology*, 2001, 166: 1308–1313.

Severe combined immunodeficiency is a heterogeneous group of hereditary disorders of T and B lymphocyte function. Causative abnormalities are found in genes with intracellular (ADA, JAK3, ZAP70, RAG, and MHC) and extracellular expression (IL2RG, CD3E, and CD3G) (1–8). Affected genes include those involved in cytokine pathways (2, 3, 6), Ag presentation (5), purine salvage (1), and Ag receptor rearrangement and signal transduction (7). The leukocyte common Ag CD45 is an abundant hemopoietic-specific transmembrane protein tyrosine phosphatase (9, 10). The phosphatase activity of CD45 Ag is crucial for efficient lymphocyte Ag receptor signal transduction, as has been shown in CD45-negative cell lines and CD45-deficient mice (11–14). CD45-knockout mice are severely immunodeficient. Thymic development is blocked, so that positive selection does not proceed, resulting in very few T cells in the periphery. Although the development of B cells appears normal, their proliferative responses are significantly reduced (13, 14).

CD45 Ag can potentially exist in at least eight different isoforms generated by alternative splicing in the N-terminal extracellular domain of the molecule (15, 16). The expression of different CD45 isoforms depends on the state of activation and differentiation of hemopoietic cells, and in humans has been used to distinguish naive and memory T lymphocytes (17–21). Variations in CD45 isoform expression exist as normal variants (22), but can also be associated with some human disorders of immune activation (23–25). However, the mechanisms linking the expression of different CD45 isoforms to T cell function and the regulation of CD45 activity remain unclear.

Cale et al. (26) described an SCID patient from consanguineous parents with minimal cell surface expression of CD45. Recently, a second SCID patient lacking surface CD45 expression has been investigated, and the genetic lesions in the CD45 gene identified (27). Here we show that the patient described by Cale et al. (26) has a homozygous 6-bp deletion in the CD45 gene, which results in a loss of glutamic acid 339 and tyrosine 340 in the first fibronectin type III module of the extracellular domain of CD45 and is responsible for the lack of cell surface expression of CD45. These findings confirm that CD45 is essential for lymphocyte development and signaling and that abnormalities in CD45 expression can be a cause of SCID in humans.

Materials and Methods

Patient material

Fresh blood and frozen PBMC were obtained from the G. family from the Department of Immunology and Infectious Diseases, Great Ormond Street Hospital National Health Service Trust (London, U.K.) (26). EBV-transformed lymphoblastoid lines were derived from the parents. Fresh material from the SCID patient was very limited, and it proved impossible to derive an EBV line. Genomic DNA samples from European populations were provided by W. F. Bodmer (Institute of Molecular Medicine, University of Oxford, Oxford, U.K.). African pygmy samples were provided by L. L. Cavalli-Sforza (Department of Genetics, Stanford University School of Medicine, Stanford, CA). Samples from the Caucasus, Iran, and Central Asia were obtained as part of the anthropological expedition EurAsia '98 (see <http://popgen.well.ox.ac.uk/eurasia> for more details).

Immunofluorescence

Liquid nitrogen-stored PBMC from members of family G, Chinese hamster ovary (CHO),² and EL-4 CD45 transfectants were surface stained with the following mAbs against human isoforms CD45R0-PE, CD45RB-FITC, and CD45RA-PE (Dako, Carpinteria, CA) or with pan-CD45-PE (PharMingen, San Diego, CA) or pan-CD45 hybridoma supernatant (2D1, Imperial Cancer Research Fund, London, U.K.) with second layer F(ab')₂ sheep anti-mouse Ig-FITC (Sigma, St. Louis, MO). Isotype-matched mAbs were used as controls. Ten thousand events were collected on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and analyzed using CellQuest software.

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² Abbreviations used in this paper: CHO, Chinese hamster ovary; BMT, bone marrow transplantation.

Amplification and sequencing of CD45 cDNA

Total RNA was extracted from frozen PBLs using Tri-Reagent (Sigma). First-strand cDNA synthesis on 4 μ g of total RNA was performed using random hexadeoxynucleotide primers and the First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden). The CD45 cDNA was amplified using four pairs of primers, each pair amplifying ~1- to 1.3-kb overlapping parts of the CD45 cDNA: pair 1, 5' untranslated region forward (5'-CCTCGTCTGATAAGACAACAG-3') and ex10 reverse (5'-CATATTACCACACTGAAATC-3'); pair 2, ex10 forward (5'-CAGTTA CATGATTGTACAC-3') and ex17 reverse (5'-CAAGTCTCTGCTGTT CATC-3'); pair 3, ex16 forward (5'-GGCACTGATAGCATTCTGGC3') and ex28 reverse (5'-TCATCAGAGGATTCATCTGA-3'); and pair 4, ex27 forward (5'-GAGGACACAGCACATTGGAA-3') and 3' untranslated region reverse (5'-GTCTCTCACACAAACACACACC-3'). The PCR conditions for amplification of CD45 cDNA included 4-min incubation at 94°C followed by 30 reaction cycles (1 min at 94°C, 1 min at 55°C, 4 min at 72°C) and final 16-min extension at 72°C. The purified PCR products were subjected to direct automated sequencing using gene-specific primers.

PCR analysis for detection of the 1168-1173del

One hundred nanograms of genomic DNA was amplified by PCR using forward (5'-AATTAGAAAACCTTGAACCCG-3') and reverse (5'-ACT TGCGTTAGTAACTTGTGG-3') primers. The primers were in exon 11, on either side of the 1168-1173del and amplified fragments of 77 bp (mutant) or 83 bp (wild type). PCR conditions were as described above. The PCR products were analyzed on a 6% nondenaturing polyacrylamide gel or VisiGel Separation Matrix (Stratagene, La Jolla, CA). For amplification refractory mutation system-PCR analysis (28) the following primers were used: forward primers, CD45FDEL (5'-CTTGAACCCGAACATAAGTG 3') and CD45F (5'-CTTGAACCCGAACATGAGTA-3'); and reverse primer, CD45R (5'-AGCAATTTTACATATAAATGCAAG-3'). CD45-FDEL is specific for 1168-1173del, while CD45F is specific for the unmutated sequence. Typing reactions were performed in a volume of 10 μ l with the following buffer conditions: 67 mM Tris-HCl, 16.6 mM ammonium sulfate, 0.1% Tween 20, 177 μ M of each dNTP, 2.3 mM magnesium chloride, 0.4 U of AmpliTaq Gold polymerase (Perkin-Elmer, Norwalk, CT), 70 ng of template DNA, and 10 pmol of each primer. PCR was performed on a Hybaid MBS multiblock PCR machine (Middlesex, U.K.), with a touchdown protocol (an initial 10-min denaturation at 95°C, then 10 cycles of 25 s at 95°C, 25 s at 65°C dropping by 0.5°C/cycle, 30 s at 72°C, followed by 25 cycles of 25 s at 95°C, 25 s at 60°C, 30 s at 72°C, and a final 5-min extension at 72°C); products were resolved on a 1.6% agarose gel.

Generation of 1168-1173del CD45 mutant cell lines

The retroviral expression vector pZipNeoSV-LCA.6 containing the full-length cDNA for the human high m.w. CD45RABC isoform was provided by Dr. M. Streuli (Dana-Farber Cancer Institute, Boston, MA) (29). The 1168-1173del was introduced into the pZIPNeoSV-LCA6 construct according to the QuickChange Site-Directed Mutagenesis Kit (Stratagene) using forward (5'-GAAAACCTTGAACCCGAACATAAGTGTGACTC AGAAATACTC-3') and reverse (5'-GAGTATTTCTGAGTCACTATATGTTTCGGGTTCAAGTTTTC-3') primers. Clones containing the 6-bp deletion were identified by PCR as described above and sequenced to confirm that the correct mutation had been introduced. Exponentially growing CHO cells or EL-4 mouse thymoma cells (1×10^7) were mixed with 20 μ g of wild or mutated pZIPNeoSV(X)-LCA6 cDNA constructs in 0.8 ml of PBS, then subjected to electroporation (Bio-Rad Gene Pulser, Bio-Rad, Hercules, CA) at 950 μ F and 250 V. After 48-h recovery in medium, cells were selected for neomycin resistance in medium containing G-418 (1 mg/ml). Surviving clones were analyzed by immunofluorescence using PE-conjugated pan-human CD45 mAbs and Western blotting.

Western blot analysis

CHO cell transfectants (4×10^6) were lysed in 500 μ l of RIPA buffer for 30 min on ice. The supernatant was clarified by centrifugation at 10,000 \times g for 10 min at 4°C. An equal amount of protein from each of the cell lysates was loaded and separated by 10% SDS-PAGE. The gel was transferred onto nitrocellulose membrane, and the membrane was blocked in PBS, 0.1% Tween 20, and 5% nonfat dry milk overnight at 4°C. CD45 protein was detected using mouse anti-human CD45 mAb (3 μ g/ml; clone H130, PharMingen) followed by anti-mouse IgG-HRP conjugate (1:25,000; Pierce & Warriner, Cheshire, U.K.), followed by reaction with SuperSignal WestPico Chemiluminescent Substrate (Pierce & Warriner) for 5 min.

Results

Clinical features and lack of surface CD45 expression

We obtained material from an infant with SCID who exhibited minimal expression of CD45 Ag (26). The patient was the first child of consanguineous Kurdish parents. She was presented aged 2 mo with a rash, pyrexia, hepatosplenomegaly, lymphadenopathy, pneumonitis, and pancytopenia. CMV was detected in buffy coats, a liver biopsy specimen, nasopharyngeal aspirates, and urine. On the basis of immunologic investigations (see below) and clinical phenotype, a diagnosis of SCID with postnatally acquired CMV was made. She responded well to anti-CMV treatment and at 8 mo underwent bone marrow transplantation (BMT) from a matched unrelated donor. T cell engraftment was demonstrated 3 wk after BMT. Despite continuous anti-CMV treatment her CMV reactivated, and she died 55 days after BMT. Analysis of PBLs showed absolute lymphopenia, low T cell number ($0.26 \times 10^9/L$), with markedly low CD4⁺ ($0.07 \times 10^9/L$) and low CD8⁺ ($0.36 \times 10^9/L$) and normal B cell numbers ($1.2 \times 10^9/L$). Ig production was impaired, with low concentrations of IgM and IgA compared with age-matched normal controls. Because of the very limited availability of blood samples from the patient, further phenotypic and functional analysis was not possible.

The most striking feature was the markedly reduced expression of CD45 Ag on all leukocytes. Fig. 1 shows the expression of different CD45 isoforms on PBMC from the parents and the SCID patient. Minimal CD45 expression was detected with pan-CD45 and CD45RB Abs, and no expression was detected with CD45RA and CD45R0 Abs in the SCID patient, while both parents showed normal CD45 expression patterns.

Identification of CD45 mutation

To identify the molecular basis of the abnormal CD45 expression in the patient we performed RT-PCR and sequenced the CD45 cDNA. We found one silent and two conservative substitutions in the cytoplasmic domain and a 6-bp deletion in exon 11 in the extracellular domain of the coding sequence of CD45. The 6-bp deletion is at nucleotide position 1168 of the CD45 cDNA, 1168-1173del (accession no. NM 002838, huCD45RABC isoform). This does not produce a frame shift, but causes a deletion of two amino acids, glutamic acid 339 and tyrosine 340, Δ E339 Δ Y340 (Fig. 2a). The 1168-1173del was also confirmed in genomic DNA from the patient by amplification and sequencing of exon 11.

To identify mutation carriers we performed PCR analysis using primers amplifying the region with mutation generating 77-bp (mutant) or 83-bp (wild type) fragments, respectively. PCR analysis of genomic DNA of the patient's family revealed that both parents are heterozygous for the 1168-1173del, while a sibling is homozygous for the normal allele (Fig. 2b). The latter also shows normal CD45 expression, as determined by FACS analysis (data not shown). These results indicate that it is unlikely that the T cells in the SCID patient are of maternal origin, as there was no evidence in the PCR products for the presence of the normal allele. Other members of the family were not available for analysis.

The 1168-1173del is responsible for the lack of surface CD45 expression

To confirm directly that the 1168-1173del mutation is responsible for the observed abnormal CD45 surface expression in the patient, we introduced the 1168-1173del into a normal full-length CD45 cDNA in a retroviral expression vector and transfected CHO cells. FACS analysis showed lack of surface CD45 expression on the CD45 mutant CHO transfectant (Fig. 3a). By contrast, CHO transfectant with a wild-type human CD45 cDNA construct expressed

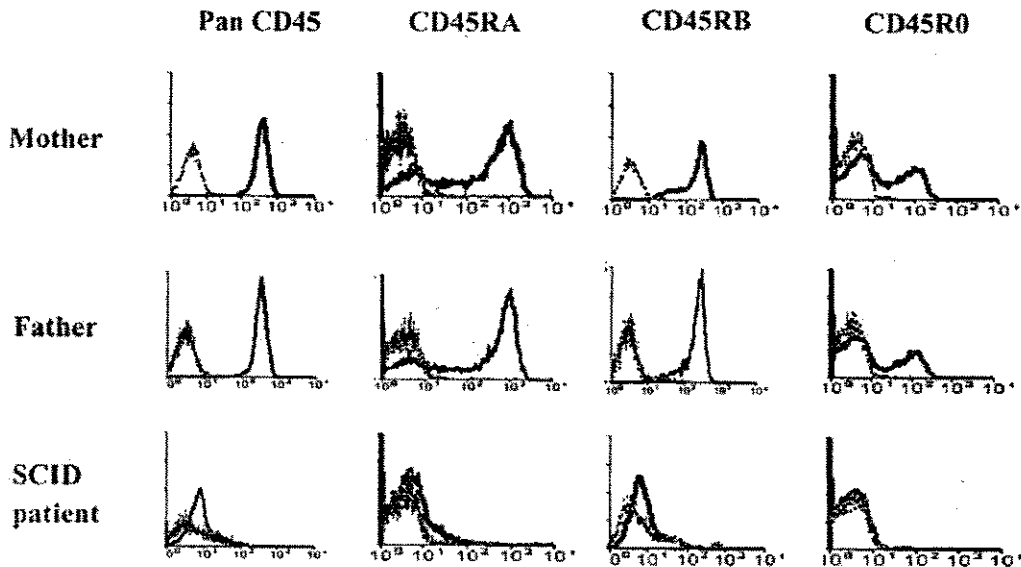


FIGURE 1. Expression of CD45 isoforms in the patient's family. Flow cytometric analysis was performed of PBMC from the SCID patient and her parents stained with isoform-specific CD45 mAbs (solid lines). Minimal CD45 expression was detected with pan-CD45 and CD45RB Abs in the SCID patient compared with that using relevant isotype control Abs (dotted lines). No detectable staining was demonstrated with CD45RA and CD45R0 Abs in the patient. Both parents show normal patterns of CD45 expression.

high levels of CD45 Ag, providing direct evidence that the 1168–1173del is responsible for abnormal CD45 expression in leukocytes of the SCID patient. The same results were obtained when the EL-4 mouse thymoma cell line was transfected with these constructs (data not shown).

The mechanism underlying the lack of CD45 cell surface expression is unknown. The 1168–1173 bp deletion was identified by RT-PCR from the patient's cells, suggesting that mRNA is produced. To see whether the 6-bp deletion affected CD45 expression at the protein level we performed Western blot analysis. The Western blot analysis detected CD45 protein with a M_r of 220 kDa in mutant CD45 CHO cell transfectants, although at a reduced level compared with the wild-type CHO transfectant (Fig. 3b). These results suggest that the 1168–1173del may affect the proper folding, stability, and correct cellular localization of the mutant CD45 protein.

Tyrosine 340 is crucial for the structural integrity of CD45

The deleted glutamic acid 339 and tyrosine 340 are part of the first of three fibronectin type III modules believed to form part of the extracellular domain of CD45 (30, 31). Tyrosine 340 is conserved in all CD45 sequences (human, rat, mouse, chicken, shark, and puffer fish), and the equivalent tyrosine is also highly conserved in known fibronectin sequences (Fig. 4a). Although the crystal structure of the extracellular domain of CD45 has yet to be solved, a molecular model of the first fibronectin module could be constructed using COMPOSER (32) based on known fibronectin structures (Fig. 4b). The model suggests that this tyrosine makes important contributions to maintenance of the packed core of the fibronectin structure (33). Deletion of this residue is likely to seriously destabilize the fibronectin module, leading to unfolding and intracellular degradation.

1168–1173del is not a common genetic polymorphism

To rule out the possibility that the 1168–1173del mutation in the SCID patient represented a common genetic polymorphism, we analyzed its frequency in the general population. Using ampli-

fication refractory mutation system-PCR analysis (28) the 1168–1173del was not detected in 518 unrelated healthy controls from varied ethnic backgrounds, including Europe, Africa, and East and Central Asia. This analysis included samples from 39 Kurds as well as 176 individuals from related ethnic groups (Iranians, Armenians, Azeris, and Turkmen); all were negative for 1168–1173del. Therefore, the deletion does not appear to be a common genetic polymorphism and has only been detected in the patient and members of her family.

Discussion

CD45 Ag has long been postulated to be crucial for immune function. Mice lacking the CD45 gene are severely immunodeficient and have a very similar phenotype to the SCID patient described in this study, with low T cell and normal B cell numbers. Here we have provided evidence for the existence of at least one naturally occurring mutant form of human CD45 that is associated with SCID. We have identified a 6-bp deletion in the gene encoding CD45 that results in the loss of glutamic acid 339 and tyrosine 340 in the first fibronectin type III module of the extracellular domain of CD45, identifying a region of the molecule important for CD45 structural integrity and lack of surface CD45 expression, which is almost certainly responsible for the immune deficiency in this patient.

In addition, our study provides direct evidence, through the transfection of murine and hamster cells with wild-type and mutant alleles, that the 6-bp deletion is responsible for the failure of CD45 surface expression on the patient's cells. The exact mechanism responsible for the lack of CD45 cell surface expression is unknown. RT-PCR analysis of CD45 cDNA in the patient's family revealed comparable amounts of wild-type and mutant CD45 transcripts in the patient, her parents, and the healthy sibling, suggesting that the 1168–1173del may not affect CD45 expression at the transcriptional level. Furthermore Western blot analysis of the cell transfectants showed that CD45 protein is produced, although not expressed, at the cell surface. While it is difficult to extrapolate to the patient from cell culture studies, this suggests that the 1168–

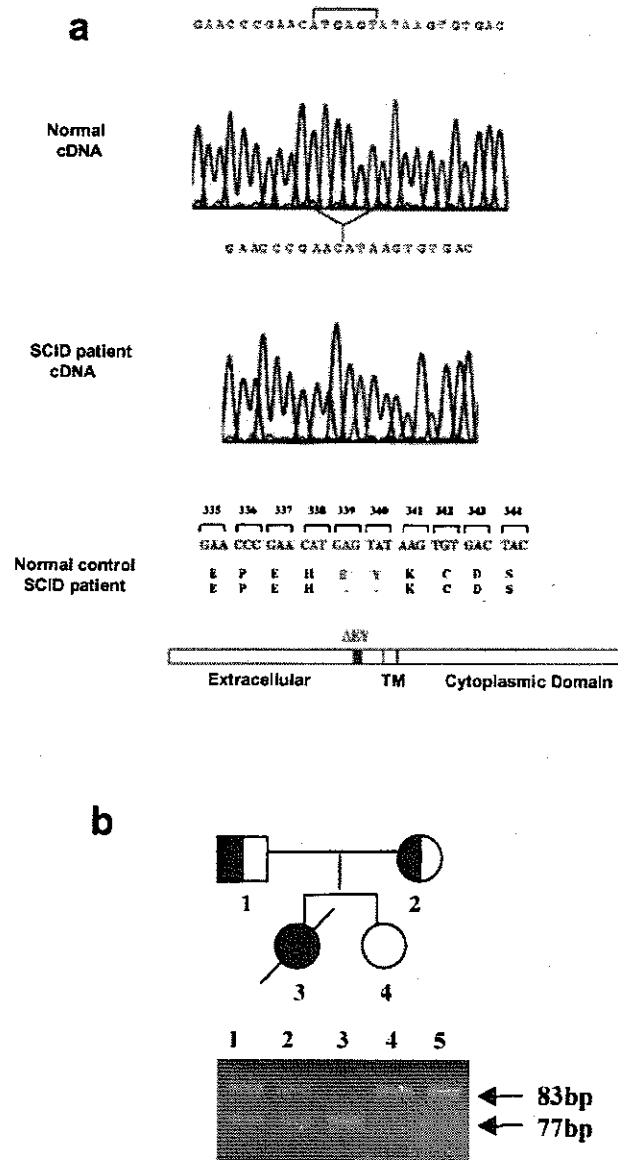


FIGURE 2. Identification of 1168–1173del mutation. *a*, At nucleotide position 1168 a 6-bp homozygous deletion was observed in CD45 cDNA from the SCID child. Wild-type (*top*) and mutant (*bottom*) sequences are shown, with the deleted bases annotated above the electropherogram. The predicted mutant CD45 protein lacks glutamic acid 339 and tyrosine 340. The deleted sequences are shown in red in the wild-type control. At the *bottom* a schematic representation of the relative position of the 2-aa deletion in the CD45 molecule is shown as a red box (not to scale). *b*, Pedigree of the patient's family. PCR analysis for detection of 1168–1173del was performed on genomic DNA with primers on either side of the mutation amplifying 77-bp (mutant) and 83-bp (wild-type) fragments visualized on 6% nondenaturing polyacrylamide gel. A homozygous mutant 77-bp band can be seen in *lane 3* (SCID patient), heterozygous patterns of two bands (77 and 83 bp) in *lanes 1* and *2* (patient's mother and father), and the normal homozygous pattern in *lane 4* (patient's sibling) and *lane 5* (normal control).

1173del may affect the proper folding, stability, and correct cellular localization of the mutant CD45 protein. This is in accord with the molecular modeling, which shows that tyrosine 340 has a crucial role in maintaining structural integrity of the first fibronectin type III of the extracellular domain of CD45. Tyrosine 340 is highly conserved topohydrophobic residue (33) responsible for

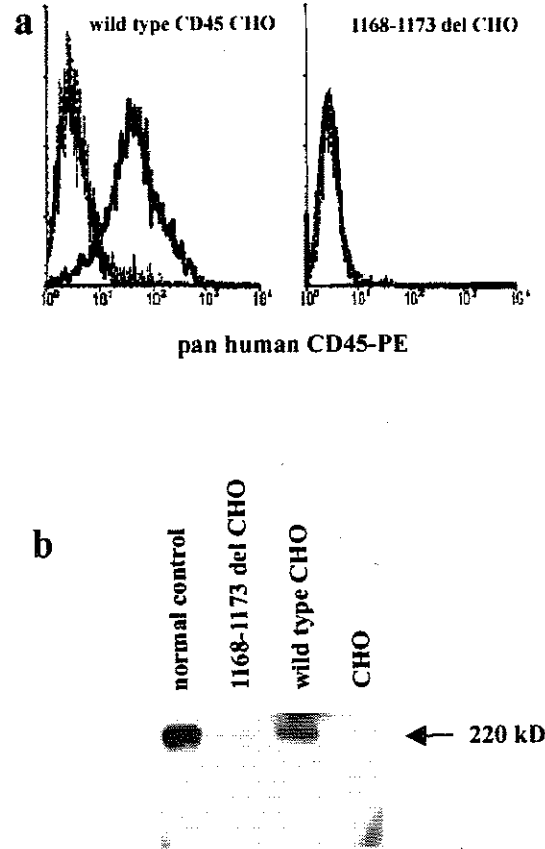


FIGURE 3. Analysis of 1168–1173del CHO cell transfectants. *a*, Wild-type and mutant CHO cells transfectants were stained with pan-human CD45-PE (solid lines) compared with isotype-matched control (dotted lines). Mutant CD45 CHO cell transfectants show lack of surface CD45 expression consistent with the pattern of CD45 expression in the SCID patient. Wild-type CD45 CHO cell transfectants express CD45. *b*, Lysates from CD45 CHO cell transfectants and EBV-transformed cells from a normal control were immunoblotted with anti-human CD45 mouse mAb. A specific band of 220 kDa was detected in wild-type, mutant CD45 CHO cell transfectants and the normal control.

key nonpolar interactions in the packed, hydrophobic core of the fibronectin module. Deletion of this residue would result in dramatic alterations in the thermodynamic stability of this module. This may lead to an inability of the fibronectin module to fold into a stable tertiary conformation, leading, through the exposure of otherwise buried, proteolytic cleavage sites in the unfolded protein, to a concomitant degradation by intracellular proteases (34). Further studies will be required to determine the precise mechanism for the lack of surface CD45 expression in the patient. For example, pulse-chase experiments could be used to follow the intracellular fate of the mutant molecules.

We have also examined the frequency of the mutant allele in 518 DNA samples, including 215 from ethnic groups related to the Kurdish patient. No examples of the 6-bp deletion were detected, suggesting that 1168–1173del is not a common genetic polymorphism. Very recently, another SCID patient lacking CD45 expression has been reported (27). This patient, similar to the case described here, was also presented at 2 mo of age and had greatly reduced numbers of peripheral T cells. The latter were unresponsive to mitogen, and despite normal B cell numbers, serum Ig levels decreased with age. Two separate genetic abnormalities, a large deletion on one allele and a point mutation at the other, distinct from that reported here, were shown to be associated with

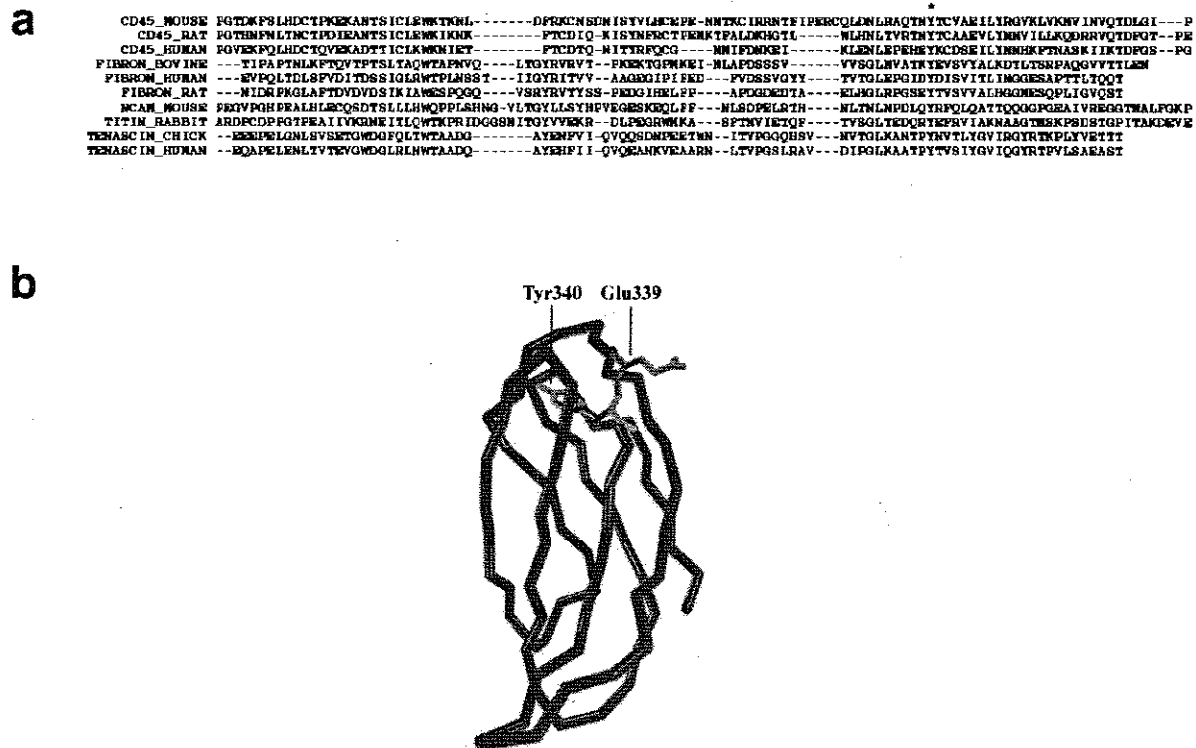


FIGURE 4. *a*, Sequence alignment of vertebrate CD45 proteins and a number of representative fibronectin type III module sequences. An asterisk marks the deleted tyrosine 340 identified in the SCID patient. *b*, The positions of the deleted residues, glutamic acid 339 and tyrosine 340, are shown on the C α trace of the modeled fibronectin type III module.

lack of CD45 expression on the patient's cells. The fact that now two SCID patients with different genetic lesions in CD45 have been identified suggests that although disease-associated CD45 polymorphisms may be rare, they should be considered in SCID patients without any other known cause.

In addition to the association of SCID with the lack of CD45 surface expression, abnormalities of CD45 splicing have been recognized in humans. We and other have demonstrated that a point mutation in the fourth (A) exon of CD45 prevents normal splicing of the N-terminal region of the gene, so that activated lymphocytes of these individuals express both high and low m.w. CD45 isoforms, in contrast to the normal pattern of low m.w. isoform expression (22). While these individuals are apparently normal, no homozygotes have yet been identified. In contrast, families with a similar defect in CD45 splicing associated with hemophagocytic lymphohistiocytosis or erythrocytic hemophagocytosis have been described (23, 24). Recently, linkage of abnormal CD45 splicing with a 32-bp deletion in CCR5 (CCR5del32), which confers resistance to infection with HIV isolates that use CCR5 as a coreceptor, has been reported (25). Although in all these cases no abnormality in the CD45 gene has yet been identified, this is made difficult by the large size of CD45, which has been estimated to be >120 kb (35).

This study provides evidence for the crucial role of CD45 in immune functions in humans, identifies abnormalities in CD45 expression as a possible cause for SCID, and suggests that CD45 screening should be included in the investigation of SCID patients and other patients with unexplained immunodeficiency.

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