Identification of the CD45-associated 116-kDa and 80-kDa Proteins as the \( \alpha \)- and \( \beta \)-Subunits of \( \alpha \)-Glucosidase II *

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CD45 is an abundant, highly glycosylated transmembrane protein-tyrosine phosphatase expressed on hematopoietic cells. Herein we demonstrate that two proteins of 116 kDa and 80 kDa copurify with CD45 from mouse T cells. Microsequence analysis of the 116-kDa protein revealed high similarity to an incomplete human open reading frame that has been suggested to correspond to the catalytic \( \alpha \)-subunit of glucosidase II. We determined the nucleotide sequence of the mouse cDNA and observed that it encodes a protein product nearly identical to its human homologue and shares an active site consensus sequence with Family 31 glucosidases. Amino acid sequencing of the 80-kDa protein, followed by molecular cloning, revealed high homology to human and bovine cDNAs postulated to encode the \( \beta \)-subunit of glucosidase II. Antisera developed to the mouse \( \beta \)-subunit allowed us to demonstrate that the interaction between CD45 and glucosidase II can be reconstituted \textit{in vitro} in an endoglycosidase H-sensitive manner. The strong interaction between glucosidase II and CD45 may provide a paradigm for investigating novel aspects of the biology of these proteins.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U92793 and U92794.

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The abbreviations used are: PTP, protein-tyrosine phosphatase; DOC, sodium deoxycholate; Endo, endoglycosidase; ER, endoplasmic reticulum; GII, glucosidase II; kb, kilobase(s); H-LAG, human lysosomal \( \alpha \)-glucosidase; MHC, major histocompatibility complex; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase.

EXPONENTIAL PROCEDURES

Cell Lines and Monoclonal Antibodies—The mouse T-lymphoma cell lines SAKRTLS 12.1 (SAKRT) and BW5147 (BW), CD45-negative variants of these lines (SAKRT/CD45 + and BW/CD45 +), and a revertant of BW/CD45 expressing a truncated form of CD45 (BW/rev) (8) were obtained from Dr. R. Hyman (The Salk Institute, La Jolla, CA) and maintained as described previously (6). Monoclonal antibodies I3/2.3 (9) and M1/9.32 HL2 (American Type Culture Collection), recognizing pan-specific determinants in the CD45 ectodomain, and monoclonal antibodies M1/42.3.9.8 (ATCC), directed against class I major histocompatibility complex (MHC) molecules, were purified and directly coupled to

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cDNA using the primer pair 70–97 and 1681–1707. As a control, mock-treated SAKR cells were prepared as described above, passed through 0.45-μm filters (Costar), and applied overnight to an affinity column containing a 10-ml bed volume of Sepharose-I3/2 or -M1/42. The column was washed with 200–300 ml of high salt (0.5 M NaCl) wash buffer followed by approximately 150 ml of lysis buffer. Elution buffer (0.5 M DOC, 20 mM Tris, pH 7.6) was applied, and 1-ml fractions were collected, saving 40 μl from each for electrophoretic analysis.

Microsequence Analysis—The 13/2 immunoaffinity column fractions positive for 80-kDa and 116-kDa CD45-associated proteins were pooled and concentrated using M₃0,000 cut-off Ultrafree-MC Filters (Milipore), separated on preparative gels, transferred to Immobilon-P, and stained. Protein bands were excised for amino-terminal sequence analysis as described above, passed through 0.45-μm filters (Costar), and applied overnight to an affinity column containing a 10-ml bed volume of Sepharose-II/2 or -M1/42. The column was washed with 200–300 ml of high salt (0.5 M NaCl) wash buffer followed by approximately 150 ml of lysis buffer. Elution buffer (0.5 M DOC, 20 mM Tris, pH 7.6) was applied, and 1-ml fractions were collected, saving 40 μl from each for electrophoretic analysis.

Preparation of Cell Lysates and Immunoprecipitates—Post-nuclear extracts from 5 × 10⁶ viable SAKR or SAKR/CD45 cells were prepared as described above, passed through 0.45-μm filters (Costar), and applied overnight to an affinity column containing a 10-ml bed volume of Sepharose-II/2 or -M1/42. The column was washed with 200–300 ml of high salt (0.5 M NaCl) wash buffer followed by approximately 150 ml of lysis buffer. Elution buffer (0.5 M DOC, 20 mM Tris, pH 7.6) was applied, and 1-ml fractions were collected, saving 40 μl from each for electrophoretic analysis.

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Cloning of Glucosidase II α-Subunit—Based on microsequence analysis of the p116 protein, degenerate oligonucleotides were synthesized. These were synthesized for their ability to amplify DNA from random-primed, reverse-transcribed SAKR poly(A)⁺ RNA. Two primers, 5'-cttt cac aag cag gag ggc-3' (a sense oligonucleotide corresponding to an internal peptide sequence FEHQRA) and 5'-atg atg gag tgt cag tcct ctt ttt c-3' (an antisense oligonucleotide corresponding to internal peptide sequence MKDDPI), were found to amplify a 1.9-kb fragment. The PCR amplicon was cloned into the pCRII vector (Invitrogen), and partial sequencing confirmed the presence of nucleotide sequence in agreement with our amino acid microsequence data. The PCR amplicon was excised, labeled, and used to screen a Uni-ZAP XR oligo(dT)-primed mouse EL4 T-cell cDNA expression library (Stratagene). The longest clone was found to be incomplete after sequencing. To isolate the 5' end of the cDNA, two sequential rounds of PCR-RACE were performed with the Marathon cDNA Amplification kit (CLON-TECH) using SAKR cDNA as a template. The specific antisense PCR primers were designed to span nucleotides 1586–1609 (first round) and 231–260 (second round) and were used in conjunction with the adapter primer (AP1) supplied with the kit. Products of approximately 1.6 kb (first round) and 0.3 kb (second round) were TA cloned directly from the reaction mix. Positive clones containing sequences overlapping those of the incomplete cDNA were fully sequenced in both directions. Finally, the entire open reading frame of p116 was PCR-amplified using a primer pair spanning nucleotides 1–26 and 3065–3091, cloned into pCRII, and sequenced.

Cloning of Glucosidase II β-Subunit—The entire open reading frame of human homologue 80K-H (11) was cloned by RT-PCR from the A431 human epidermal carcinoma cell line using primers spanning nucleotides 129–149 and 1718–1739. This amplicon was radiolabeled and used to screen the EL4 cDNA library as described above. Five positive clones were rescued, each with inserts of different sizes corresponding to the 3' end of mouse glucosidase II β-subunit (GII β) mRNA, as revealed by partial sequencing. The longest of these, clone 80-5 (1.75 kb), was completely sequenced in both directions. The remaining 0.6-kb 5' end of the cDNA was cloned by PCR-RACE as outlined above, using a gene-specific primer spanning bases 603–636. The entire open reading frame was sequenced following RT-PCR amplification from SAKR genomic DNA using primers spanning bases 603–636. The entire open reading frame of p116 was PCR-amplified using a primer pair spanning nucleotides 129–149 and 1718–1739. This amplicon was radiolabeled and used to screen the EL4 cDNA library as described above. Five positive clones were rescued, each with inserts of different sizes corresponding to the 3' end of mouse glucosidase II β-subunit (GII β) mRNA, as revealed by partial sequencing. The longest of these, clone 80-5 (1.75 kb), was completely sequenced in both directions. The remaining 0.6-kb 5' end of the cDNA was cloned by PCR-RACE as outlined above, using a gene-specific primer spanning bases 603–636. The entire open reading frame was sequenced following RT-PCR amplification from SAKR genomic DNA using primers spanning bases 603–636. The entire open reading frame of p116 was PCR-amplified using a primer pair spanning nucleotides 129–149 and 1718–1739. This amplicon was radiolabeled and used to screen the EL4 cDNA library as described above. Five positive clones were rescued, each with inserts of different sizes corresponding to the 3' end of mouse glucosidase II β-subunit (GII β) mRNA, as revealed by partial sequencing. The longest of these, clone 80-5 (1.75 kb), was completely sequenced in both directions. The remaining 0.6-kb 5' end of the cDNA was cloned by PCR-RACE as outlined above, using a gene-specific primer spanning bases 603–636. The entire open reading frame was sequenced following RT-PCR amplification from SAKR genomic DNA using primers spanning bases 603–636. The entire open reading frame of p116 was PCR-amplified using a primer pair spanning nucleotides 129–149 and 1718–1739. This amplicon was radiolabeled and used to screen the EL4 cDNA library as described above. Five positive clones were rescued, each with inserts of different sizes corresponding to the 3' end of mouse glucosidase II β-subunit (GII β) mRNA, as revealed by partial sequencing. The longest of these, clone 80-5 (1.75 kb), was completely sequenced in both directions. The remaining 0.6-kb 5' end of the cDNA was cloned by PCR-RACE as outlined above, using a gene-specific primer spanning bases 603–636. The entire open reading frame was sequenced following RT-PCR amplification from SAKR genomic DNA using primers spanning bases 603–636. The entire open reading frame of p116 was PCR-amplified using a primer pair spanning nucleotides 129–149 and 1718–1739. This amplicon was radiolabeled and used to screen the EL4 cDNA library as described above. Five positive clones were rescued, each with inserts of different sizes corresponding to the 3' end of mouse glucosidase II β-subunit (GII β) mRNA, as revealed by partial sequencing. The longest of these, clone 80-5 (1.75 kb), was completely sequenced in both directions. The remaining 0.6-kb 5' end of the cDNA was cloned by PCR-RACE as outlined above, using a gene-specific primer spanning bases 603–636. The entire open reading frame was sequenced following RT-PCR amplification from SAKR genomic DNA using primers spanning bases 603–636.

Interaction of CD45 and Glucosidase II

RESULTS

Purification of CD45-associated Proteins—In our initial experiments to identify proteins that physically interact with CD45, we observed a protein with an apparent molecular mass of 116 kDa (p116) that was present in immunoprecipitates from CD45-positive T cells but absent from parallel immunoprecipitates from CD45-negative variants of these cells (6). Also visible on some of our gels, although frequently obscured by non-specific protein bands, was a protein with an apparent mass of approximately 80 kDa (p80, Fig. 1). Association of these pro-
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Blue-stained gels containing 40 proteins were eluted by addition of buffered 0.5% DOC. Shown are Coomassie Blue-stained gels containing 40 proteins.

SAKR mouse T-lymphoma cells were lysed in buffer containing 0.5% deoxycholate (DOC) (Fig. 1). Having failed to identify these proteins using antibodies to known molecules, we purified p116 and p80 by exploiting the differential detergent stability of these proteins using antibodies to known molecules, we purified p116 and p80. SDS-PAGE analysis of these fractions by total protein staining revealed two major components migrating at 116 and 80 kDa (Fig. 2A). As a further control, we confirmed that p80 and p116 could not be copurified using an isotype-matched anticlass I MHC molecule (M1/42) immunoprecipitation assays. After extensive washing in high salt (0.5 M NaCl) buffer, fractions were eluted in 0.5% DOC. SDS-PAGE analysis of these fractions by total protein staining revealed two major components migrating at 116 and 80 kDa and additional minor species (Fig. 2A). To control for nonspecific protein associations, lysates from an equivalent number of cells from a CD45-negative variant of SAKR were processed in an identical manner. Analysis of column eluates from this experiment revealed only faintly staining minor species, indicating that the 80-kDa and 116-kDa proteins are physically complexed with CD45 (Fig. 2B). As a further control, we confirmed that p80 and p116 could not be copurified using an isotype-matched anti-class I MHC molecule (M1/42) immunoprecipitation assay. After washing in buffer containing 0.5 M NaCl, proteins were eluted by addition of buffered 0.5% DOC. Shown are Coomassie Blue-stained gels containing 40 μl from each 1-ml column fraction. Arrows designate the locations of p116 and p80.

Fig. 2. Immunoaffinity purification of CD45-associated proteins. Nonidet P-40 lysates from 1.6 × 10^9 CD45^+ (A and C) or CD45^- (B) SAKR cells were loaded onto I3/2 (A and B) or M1/42 (C) immunoaffinity columns. After washing in buffer containing 0.5 M NaCl, proteins were eluted by addition of buffered 0.5% DOC. Shown are Coomassie Blue-stained gels containing 40 μl from each 1-ml column fraction. Arrows designate the locations of p116 and p80.

Teins with CD45 was stable in 0.5% Nonidet P-40 but not in 0.5% deoxycholate (DOC) (Fig. 1). Having failed to identify these proteins using antibodies to known molecules, we purified p116 and p80 by exploiting the differential detergent stability of the association. SAKR mouse T-lymphoma cells were lysed in buffer containing 0.5% Nonidet P-40 and passed through an immunoaffinity column containing the same pan-specific anti-CD45 antibody (I3/2) employed in our immunoprecipitation assays. After extensive washing in high salt (0.5 M NaCl) buffer, fractions were eluted in 0.5% DOC. SDS-PAGE analysis of these fractions by total protein staining revealed two major components migrating at 116 and 80 kDa and additional minor species (Fig. 2A). To control for nonspecific protein associations, lysates from an equivalent number of cells from a CD45-negative variant of SAKR were processed in an identical manner. Analysis of column eluates from this experiment revealed only faintly staining minor species, indicating that the 80-kDa and 116-kDa proteins are physically complexed with CD45 (Fig. 2B). As a further control, we confirmed that p80 and p116 could not be copurified using an isotype-matched anti-class I MHC molecule (M1/42) immunoprecipitation assay. After washing in buffer containing 0.5 M NaCl, proteins were eluted by addition of buffered 0.5% DOC. Shown are Coomassie Blue-stained gels containing 40 μl from each 1-ml column fraction. Arrows designate the locations of p116 and p80.

Microsequence Analysis and cDNA Cloning of p116 Identifies It as the Putative Catalytic Subunit of Glucosidase II—Having purified p116 and p80, we next subjected these proteins to amino-terminal microsequence analysis and utilized this sequence information to search the BLAST data base (12). Sequences from independently prepared samples of p116 revealed high homology to an incomplete human open reading frame (ORF), GenBank accession number D42041, that had been randomly isolated from the immature myeloid cell line KG1 (Table I, samples 1 and 2). This ORF predicts a putative signal sequence upstream of our amino-terminal sequence but is lacking an ATG start codon. Seeking further evidence that p116 is the mouse homologue of this molecule, we obtained internal amino acid sequence information from peptide fragments generated by cyanogen bromide digestion. This internal sequence data closely matched the protein sequence predicted by the human ORF (Table I, samples 3–7) with the exception of one peptide fragment (sample 4) that could only be partially aligned with the human protein. Intriguingly, the D42041 ORF recently has had a function ascribed to it as the catalytic (α) subunit of glucosidase II (GII), an α,1,3 ER luminal glucosidase, based on extensive microsequence analysis of a 110-kDa rat protein with glucosidase activity and deletion of a homologous gene in Saccharomyces cerevisiae (7).

Given that a full-length cDNA for GII had not been isolated in mammalian cells, and to confirm the identity of p116 as the mouse homologue of this molecule, we sought to clone and sequence the corresponding cDNA from mouse T cells. Several degenerate oligonucleotide primers were synthesized based on our peptide sequence data. Two of these amplified a 1.9-kb RT-PCR fragment from SAKR cDNA. Cloning and partial sequencing of this amplicon revealed high homology to D42041. Probing of an oligo(dT)-primed mouse T-cell cDNA library with this PCR product led to the isolation of two clones, each 2.9 kb in size, which appeared, from restriction analysis, to be derived from the same mRNA. One of these was sequenced in entirety and found to encode an ORF highly homologous to human D42021, containing a stop codon and downstream polyadenylation signal, but lacking over 1 kb of 5′ sequence. Two rounds of PCR-RACE led to the isolation of a cDNA fragment that contained 11 bases in the 5′-untranslated region. Juxtaposing the first ATG triplet is a motif that conforms well with the consensus sequence for initiation of translation in vertebrates (13). The predicted mass of the nascent product encoded by the longest ORF (109 kDa) and the fact that our amino acid sequence matches that predicted by the nucleotide sequence information matches that predicted by the nucleotide sequence at all but a single residue (Table I) strongly argue that the cDNA sequence we obtained corresponds to that of the p116 protein that we co-purified with CD45.

Shown in Fig. 3 in alignment with human ORF D42041, the cDNA product of the cDNA contains a long, hydrophobic leader peptide that appears to be cleaved at Ala-32, based on our amino-terminal sequence analysis (13). The predicted mass of the nascent product encoded by the longest ORF (109 kDa) and the fact that our amino acid sequence matches that predicted by the nucleotide sequence at all but a single residue (Table I) strongly argue that the cDNA sequence we obtained corresponds to that of the p116 protein that we co-purified with CD45.
of sources, does not appear to function as a transmembrane domain (7, 17, 18). Given that the protein product of our mouse cDNA shares 90% identity with human ORF D42041, which has recently been shown to have glucosidase II catalytic function (7), we conclude that the cDNA we have cloned encodes the α-subunit of mouse glucosidase II, a protein that stably associates with CD45 in T cells.

Glucosidase II α-Subunit Possesses Regions of Homology to Family 31 Glucosidases—That the large subunit of glucosidase II alone, accounts for the catalytic activity of the molecule is suggested by a number of studies (7, 16–18). Data base analysis of mouse and human GIIα protein sequences revealed regions of considerable homology to Family 31 glycosyl hydrolases. Contained within this family are a number of ancestrally related gene products, including the intestinal sucrase-isomaltase complex and lysosomal α-glucosidase (19, 20). Shown in Fig. 3 is a portion of the carboxyl-terminal sequence of human lysosomal α-glucosidase (H-LAG) (21) aligned with mouse and human GIIα. A significant degree of homology exists between the aligned regions of these molecules, with 36% sequence identity between H-LAG and mouse GIIα. In contrast to this, the remaining amino- and carboxyl-terminal portions of H-LAG (amino acids 1–477 and 777–952, respectively) cannot be aligned with GIIα sequences. Of particular interest is the

| No. | Mouse protein sequence | Human D42041 sequence |
|-----|-----------------------|-----------------------|
| 1   | vDRSNFKTFDESmFkRQ     | VDRSNFKTESSFCKRQ (32–49) |
| 2   | vDRSNFKTF              | VDRSNFKTE               |
| 3   | vDrSnFkTdx              | VDRSNFKTEDS (32–43)    |
| 4   | MAFEHQGRAPVFKDkVPCaLaGSV | LEFHFRAPRSQPKSPDAGGDA (175–198) |
| 5   | MtqagpaavVv            | IGAGPKAAVL (894–904)   |
| 6   | MkdqitlFvpsqgT         | MKDDPITFVALSFQSCTQG    |

*M. Samples 1 and 2 were subjected to NH2-terminal sequence analysis. Samples 3–7 are peptide fragments generated by cyanogen bromide digestion. Peptide 3 corresponds to the NH2-terminus of the 116-kDa protein.

**Sequences are denoted in standard single-letter code. Some sequencing cycles yielded multiple peaks; in these cases the major peak is indicated in lowercase type. An "x" has been placed at positions where amino acid identity could not be determined. The identity of the methionine at the first position of peptides 4–7 is inferred.

Regions of the human D42021 ORF (GenBank) homologous to those sequenced in the 116-kDa mouse protein are presented. The location of these residues in the human protein is indicated in parentheses. Boldface type identifies human sequences that do not match those in the mouse protein.

Residue underlined is the only site that does match the protein sequence predicted by the cDNA clone of the 116-kDa mouse protein, presented in Fig. 3.

**Fig. 3. Amino acid sequence alignment of mouse GIIα with its human homologue and a Family 31 glycosyl hydrolase.** The deduced amino acid sequence of mouse GIIα, is compared with the predicted sequence of human homologue D42041. Also aligned with these sequences is a portion (amino acids 478–776) of H-LAG. Dots identify residues identical with the mouse sequence; gaps are signified by hyphens. An arrow marks the amino terminus of the mature protein, and a hydrophobic stretch of amino acids is underlined. An arrowhead denotes the position of the single Asn-linked glycosylation consensus sequence. The region corresponding to the active site in H-LAG is presented in boldface type, and an asterisk indicates the catalytic nucleophile.
conservation of a number of residues in the region surrounding Asp-518 in H-LAG (Fig. 3, residues 514–521). Asp-518 has been identified by mutagenesis as the catalytic nucleophile of H-LAG, while Trp-516 has also been shown to be essential for catalytic function (22). These residues, together with flanking sequences, define the catalytic consensus sequence for Family 31 glycosyl hydrolases: (G/F)-(L/I/V/M)-W-(X)-L-V-A-G, while Trp-516 has also been shown to be essential for catalytic function (22). As can be seen from the alignments in Fig. 3, human GIH<sub>α</sub> possesses the entire catalytic consensus sequence of acid α-glucosidase, while the mouse sequence predicts a conservative substitution of Tyr for Phe at position 560. Although all of these enzymes are α-glucosidases, their specific activities are distinct. Lysosomal α-glucosidase is an acidic hydrolase that functions to cleave α-1,4- and α-1,6 linkages in glycogen, maltose, and isomaltose (27, 28), while glucosidase II has been shown to act upon α-1,3 glucosidic linkages present on immature ER glycoproteins, although it also possesses α-1,4 activity (29). Moreover, the pH optima of these enzymes appear to be mutually exclusive (15, 28). Of note, Family 31 surface and isomaltase possess substrate specificities and catalytic consensus sequences similar to those of lysosomal α-glucosidase (21, 23), but exhibit neutral pH optima (30). Taken together, these observations imply that the conserved catalytic consensus sequence in GIH<sub>α</sub>, shared among a number of apparently ancestrally related genes encoding functionally divergent α-glucosidases, represents the active site of the molecule, while other non-conserved regions may direct the specificity of the enzyme.

**Identification and cDNA Cloning of the 80-kDa CD45-associated Protein Corresponding to the Putative β-Subunit of Glucosidase II**—Microsequence information derived from the amino terminus of purified p80, in addition to limited sequence data from an internal cyanogen bromide-digested peptide fragment (Table II), revealed a close relationship to a human cDNA that was originally cloned as a potential PKC substrate, termed 80K-H (11). More recently, this protein has been proposed to function as a second (β) subunit of glucosidase II (GIH<sub>β</sub>), based primarily on the observation that it copurifies with the α-subunit from rat microsomes and is resistant to biochemical separation from this larger subunit (7). The high degree of amino acid conservation between the amino termini of mouse p80 and human 80K-H (Table II) led us to attempt to isolate the mouse cDNA by cross-species hybridization. A RT-PCR amplicon corresponding to the entire coding region of human 80K-H was used to screen a mouse T-cell cDNA library for homologous sequences. Five independent clones were rescued from the library, and their inserts were completely or partially sequenced. These sequences, define the catalytic consensus sequence for Family 3 representing the NH<sub>2</sub> terminus of the 80-kDa protein.

### Table II

| No. | Mouse protein sequence<sup>a</sup> | Human 80K-H sequence<sup>b</sup> |
|-----|----------------------------------|----------------------------------|
| 1   | VeVYKPrGVs1                     | VVXEKRPGVSL (15–25)             |
| 2   | VeVYKRP                        | VVXEKRPGVSL (15–20)             |
| 3   | VeVYKpGGSNHLHfyeESKFTCLGDGATIFPD | VVXEKRPGVSLNHNYTDKPTCGLDSATIFPD (15–49) |
| 4   | MyQE                            | MYQE (462–466)                  |

<sup>a</sup> Sample 1 was subjected to NH<sub>2</sub>-terminal sequence analysis. Samples 2–4 are cyanogen bromide-cleaved peptide fragments, with peptides 2 and 3 representing the NH<sub>2</sub> terminus of the 80-kDa protein.

<sup>b</sup> Sequences are presented as outlined in the footnote to Table I. The identity of the methionine at the first position of peptide 4 is inferred.

<sup>c</sup> Regions of the human 80K-H protein homologous to those sequenced in the 80-kDa mouse protein are presented as described in the footnote to Table I.

Data base analysis of the complete coding region revealed striking homology to the human 80K-H protein and an unpublished bovine sequence (GenBank accession number U49178), also shown in Fig. 4, but no additional significant primary sequence homologies to other known proteins. The mouse cDNA encodes a highly acidic 59-kDa nascent protein, which lacks a transmembrane domain but possesses a hydrophobic signal sequence that, based upon amino-terminal sequencing of the mature protein, is cleaved at position 14 in mouse (Table II) and at the corresponding site in other species (7, 11, 31, 32). The mouse protein shares 86% amino acid identity with its human homologue and 82% identity with the bovine protein, with conservation of a number of motifs of potential functional significance. Shared by all species is a carboxyl-terminal His-Asp-Glu-Leu (HDEL) sequence, which may serve as an ER-retention/retrieval signal by virtue of its ability to interact with a specialized membrane receptor (33–35). A region containing tandem glutamic acid repeats is also present in all species; such negatively charged domains are common among ER proteins and may function to allow these proteins to coordinate Ca<sup>2+</sup>, possibly as an additional ER retention mechanism (36–38). The unusual charge properties of this region may contribute to the anomalously slow migration of this molecule by SDS-PAGE, as has been observed for similar proteins (11). In addition to the possible Ca<sup>2+</sup> binding activity of the acidic stretch, two putative EF-hand motifs are present, which may confer high affinity Ca<sup>2+</sup> binding capacity (39). Finally, it is noteworthy that the distribution of cysteine residues, clustered near the amino- and carboxyl termini of the molecule, is entirely conserved between species, in agreement with evidence for the presence of intrachain disulfide linkages in the rat homologue (7). In summary, the second cDNA we have cloned encodes a product that is highly conserved on an evolutionary basis and possesses several hallmarks of an ER protein. Our ability to copurify this molecule with the catalytic subunit of glucosidase II and the major glycoprotein CD45 is in agreement with a study in the rat system proposing that it represents the strongly associated β-subunit of mouse glucosidase II, which may function, in part, to retain the catalytic subunit in proximity to the membrane of the endoplasmic reticulum (7).

*The Association between CD45 and Glucosidase II Can Be Reconstituted in Vitro*—Antisera to two distinct regions of GIH<sub>β</sub> were generated by injecting recombinantly expressed GST-fusion proteins into rabbits. Both sera (anti-80.1 and anti-80.2), but not preimmune sera from these rabbits, were capable of blotting and immunoprecipitating the 80-kDa protein from SAKR cells or 13/2 column fractions (Fig. 5A and data not shown). In addition, GIH<sub>α</sub> immunoprecipitated with one antibody could be immunoblotted with the other antibody, and vice versa (data not shown). As an additional control, we confirmed that immunoreactive protein is absent from immunoprecipitates prepared using M1/42 antibody (Fig. 5B). A second antibody to CD45 that is known to co-precipitate the 116-kDa...
(α-subunit) protein (6) was also found to co-precipitate GIIb (Fig. 5B). These results convincingly argue that the cDNA clone we obtained representing GIIb encodes the same molecule that we had purified by virtue of its ability to form a stable complex with the α-subunit and CD45.

We previously reported that the interaction between CD45 and the 116-kDa (α-subunit) protein could be reconstituted in vitro by mixing affinity purified CD45 that had been stripped of associated molecules with lysates from CD45-negative cells (6). Since the 80-kDa region of these protein-stained gels was obscured by nonspecific bands, we repeated these experiments and assayed for in vitro association of the β-subunit with CD45 using our specific antisera. As anticipated, we found that washing CD45 immunoprecipitates with 0.5% DOC led to release of the 80-kDa associated protein (Fig. 6, lane 3). By incubating CD45-positive immune complexes that had been washed with DOC with lysates from SAKR/CD45 cells, we were able to reconstitute the association between CD45 and GIIb (Fig. 6, lane 4). The association could not be reconstituted if CD45-negative beads were incubated with the cell lysates (Fig. 6, lane 5). In conjunction with our previous analysis of the 116-kDa protein, and consistent with the success of our purification strategy (Fig. 2A), these data indicate that the interaction between CD45 and glucosidase II is stable and of high affinity.

**Binding of Glucosidase II to CD45**

*Interaction of CD45 and Glucosidase II*

**Fig. 4. Comparison of mouse, human, and bovine proteins encoding the putative β-subunit of glucosidase II.** Predicted amino acid sequence of the 80-kDa mouse protein is shown together with homologous human and bovine (U94178) open reading frames. Human and bovine sequences matching those in mouse are indicated with dots; gaps (−) are marked. An arrow identifies the amino terminus of the processed protein. Two putative EF-hand domains are underlined, and a highly acidic stretch is presented in boldface type. The carboxy-terminal HDEL tetrapeptide is italicized. Cysteine residues, all of which are conserved among the three species, are noted with asterisks.

Moreover, we failed to copurify glucosidase II with another cell-surface glycoprotein, the class I MHC molecule (Fig. 2C). We thus sought to investigate the structural requirements mediating the association of these proteins. To first eliminate the possibility that the CD45 cytoplasmic domain, which is not exposed to the ER lumen, is required for these proteins to associate, we confirmed that the association between GIIb and CD45 can occur in a revertant of a CD45-negative cell (8) that expresses reduced levels of a truncated form of CD45 lacking most of the cytoplasmic domain (Fig. 7A). This result is in accordance with our previous observation that the 116-kDa α-subunit also associates with this mutant form of CD45 (6). We next asked whether immature N-linked oligosaccharides of the high mannose, Endo H-sensitive type are necessary for the interaction of these proteins. We incubated CD45 that had been washed with DOC to strip it of associated proteins with either Endo H, which releases high mannose type linkages, Endo F, which releases all N-linkages, or digestion buffer alone. We then assayed for the ability of GIIb from CD45-negative lysates to become associated with deglycosylated CD45. As is clear from the data presented in Fig. 7B, our ability to reconstitute the association between GIIb and CD45 was dependent upon the presence of N-linked carbohydrates, specifically those of the high mannose, Endo H-sensitive type. Based on the known specificity of glucosidase II, we speculate that terminal α-1,3 glucose linkages are specifically required. We cannot, however, formally exclude the possibility that other binding determinants on CD45 may interact with GIIa and GIIb.
Glucosidase II is a neutral exo-glucosidase that localizes primarily to the ER and transitional elements (40, 41) and is involved in the processing of N-linked triantennary core glycans following their co-translational transfer to nascent polypeptide chains. Glucosidase II acts in sequence after glucosidase I has cleaved the terminal, α-1,3-linked Glc from protein-conjugated Glc₆(mannose)₆(N-acetylglucosamine)₂ to hydrolyze the inner two α-1,3-linked Glc units (42).

The activity of glucosidase II in the ER is counteracted by that of a UDP-Glc-glycoprotein glucosyltransferase, which can add back a single Glc linkage to (mannose)₆(N-acetylglucosamine)₂ (7). The opposing activities of these two enzymes has been accounted for in a “quality-control” model of ER processing, whereby incompletely folded proteins must present a monoglycosyl moiety to be recognized by the folding chaperone calnexin (43–46). This scheme ascribes a dual function to glucosidase II. By removing the first α-1,3-linked Glc, glucosidase II allows nascent polypeptides to interact with calnexin, and by removing the innermost Glc, it regulates the dissociation of folded proteins from calnexin and allows for their egress from the ER.

The design of lengthy purification protocols allowed several groups to isolate glucosidase II and characterize its activity (15, 16, 18, 29, 47). However, the molecular identity of this enzyme was not revealed until recently, when Trombetta et al. reported the results of exhaustive microsequence analysis of two proteins that copurify with two non-overlapping portions of mouse glucosidase II. These authors expressed as recombinant GST-fusion proteins. A, extracts from CD45⁺ (+) and CD45⁻ (−) cells were subjected to immunoprecipitation using the anti-80 sera or preimmune sera from the same rabbits. The Western blot shown was probed with anti-80.1 serum. The side arrow marks the position of GII, B, immunoprecipitates were prepared from CD45⁺ and CD45⁻ SAKR cells using the antibodies indicated. Anti-80.1 serum was employed in Western blot analysis.

**DISCUSSION**

Glucosidase II is a neutral exo-glucosidase that localizes primarily to the ER and transitional elements (40, 41) and is involved in the processing of N-linked triantennary core glycans following their co-translational transfer to nascent polypeptide chains. Glucosidase II acts in sequence after glucosidase I has cleaved the terminal, α-1,3-linked Glc from protein-conjugated Glc₆(mannose)₆(N-acetylglucosamine)₂ to hydrolyze the inner two α-1,3-linked Glc units (42). The activity of glucosidase II in the ER is counteracted by that of a UDP-Glc-glycoprotein glucosyltransferase, which can add back a single Glc linkage to (mannose)₆(N-acetylglucosamine)₂ (7). The opposing activities of these two enzymes has been accounted for in a “quality-control” model of ER processing, whereby incompletely folded proteins must present a monoglycosyl moiety to be recognized by the folding chaperone calnexin (43–46). This scheme ascribes a dual function to glucosidase II. By removing the first α-1,3-linked Glc, glucosidase II allows nascent polypeptides to interact with calnexin, and by removing the innermost Glc, it regulates the dissociation of folded proteins from calnexin and allows for their egress from the ER.

The design of lengthy purification protocols allowed several groups to isolate glucosidase II and characterize its activity (15, 16, 18, 29, 47). However, the molecular identity of this enzyme was not revealed until recently, when Trombetta et al. reported the results of exhaustive microsequence analysis of two proteins that copurify with a heterooligomer with glucosidase II activity from rat liver microsomes (7). We now report the cloning of two homologous mouse cDNAs that encode proteins that copurify with the transmembrane PTP, CD45, in T cells.

The larger of these proteins (116 kDa in mouse, 110 kDa in rat) is a molecule that we partially characterized as a tyrosine-phosphorylated transmembrane glycoprotein that physically associates with CD45 in a wide variety of hematopoietic cell lines (6). Our present work reveals that the cDNA sequence encodes a nascent protein with a mass of 109 kDa that shares 90% sequence identity with the product of an uncharacterized gene encoding a PTP (GenBank accession number D42041) that lacks an initiation codon and 5'-flanking sequences. An internal region near the carboxyl termini of these mouse and human proteins contains considerable sequence identity to Family 31 glycosyl hydrolases, including a highly conserved catalytic consensus sequence. This observation, combined with the finding that disruption of a gene homologous to our cDNA in S. cerevisiae resulted in the complete loss of glucosidase II activity (7), suggests that the protein encoded by this cDNA is the catalytic unit of glucosidase II (GII). We have also been able to detect glucosidase II activity in CD45 and GII, but not in class I MHC immunoprecipitates (data not shown); however, in the absence of an antibody to the catalytic subunit, it is difficult to determine if the activity that we observe is significant.

The mouse cDNA encoding the smaller protein that we copurify with CD45 and that was copurified with glucosidase II activity by Trombetta et al. (80 kDa in mouse, 90 kDa in rat) shares high sequence identity with a previously cloned human protein, termed 80K-H (11), and an unpublished bovine se-quence (U49178). The human cDNA was originally isolated in an attempt to clone what appeared to be two major PKC substrates of 80 kDa, termed 80K-H (for “high”) and 80K-L (for “low”) based on their gel mobilities (48). The clone isolated was 80K-H, however, proved to be a poor substrate for this kinase (48), in contrast to the 80K-L protein, which was revealed to be the biochemically similar, but genetically distinct, myristylated alanine-rich protein kinase C substrate (48–50). More recently, the 80K-H protein was identified as one of several proteins in the 90-kDa molecular mass range to become tyrosine-phosphorylated in response to basic fibroblast growth factor (32). In conflict with these data implicating 80K-H in intracellular signal transduction cascades is information suggesting a role for this molecule as a cell-surface receptor or paracrine growth factor (33, 49–51). In support of this notion, 80K-H has been identified as a receptor for the growth factor midkine (52), as a substrate for a dual specificity phosphatase (53), and as a substrate for a protein tyrosine kinase (54). These and other data (55, 56) raise the possibility that 80K-H mediates growth factor-induced signal transduction pathways in a cell type- and context-dependent manner.

**Fig. 5. Reactivity of specific antisera generated against recombinant GII.** Polyclonal antisera (anti-80.1 and anti-80.2) were generated to two non-overlapping portions of mouse glucosidase II, expressed as recombinant GST-fusion proteins. A, extracts from CD45⁺ (+) and CD45⁻ (−) cells were subjected to immunoprecipitation using the anti-80 sera or preimmune sera from the same rabbits. The Western blot shown was probed with anti-80.1 serum. The side arrow marks the position of GII, B, immunoprecipitates were prepared from CD45⁺ and CD45⁻ SAKR cells using the antibodies indicated. Anti-80.1 serum was employed in Western blot analysis.

**Fig. 6. In vitro reconstitution of the association between CD45 and GII.** I3/2 immunoprecipitates were prepared from CD45⁺ (1) and CD45⁻ (2) SAKR cells. Parallel I3/2 immunoprecipitates from CD45⁺ (3 and 4) or CD45⁻ (5) cells were washed with 0.5% DOC to release CD45-associated proteins and re-incubated with lysis buffer (3) or with lysates from CD45⁻ cells (4 and 5). Proteins were resolved by SDS-PAGE, and GII was detected with anti-80.1 serum.

**Fig. 7. Structural requirements for GII binding to the CD45 ectodomain.** A, I3/2 immunoprecipitates prepared from BW, BW/CD45⁻, and BW/rev cell lysates were subjected to immunoblotting with anti-80 sera or preimmune sera from the same rabbits. The Western blot shown was probed with anti-80.1 serum. The side arrow marks the position of GII, B, immunoprecipitates were prepared from CD45⁺ (Mock) or CD45⁻ (Endo F, Endo H, or buffer alone) SAKR cells. Parallel I3/2 immunoprecipitates from CD45⁺ and CD45⁻ SAKR cells. Parallel I3/2 immunoprecipitates from CD45⁺ and CD45⁻ SAKR cells. Parallel I3/2 immunoprecipitates from CD45⁺ and CD45⁻ SAKR cells.
co-receptor on cells of the monocyte/macrophage lineage, conferring an ability to bind proteins modified by advanced glycation end products, a heterogeneous series of complex end-products resulting from the non-enzymatic reaction of free-amino groups of proteins with reducing sugars such as Glc (51). Added to this is the report from Trombetta et al. identifying 80K-H as the β-subunit of glucosidase II, based on the finding that GIβ and GIγ exist as a heterooligomer and are refractory to biochemical separation (7).

Although it is not yet possible to completely resolve these diverse observations, a number of clues to the localization of this molecule emerge from analysis of its cDNA sequence. Highly revealing is the fact that 80K-H possesses a hydrophobic signal sequence that is cleaved at the same position in the mouse protein as in the human and rat proteins identified in the studies described above (7, 11, 31, 32). Since the mature protein lacks a transmembrane domain, this implies that the protein initially enters the secretory pathway. Also present in 80K-H, and conserved among mouse, human, and bovine, is a carboxyl-terminal HDEL tetrapeptide. This sequence has been shown to interact with a membrane receptor that functions to target proteins containing specific carboxyl-terminal tetrapeptides (33). Moreover, mutation of the HDEL tetrapeptide found in ER proteins has been shown to favor the secretory pathway (34, 35). 80K-H has in common with other ER proteins a highly acidic region that may bind Ca²⁺ at low affinity, forming a “zipper” structure linking ER proteins in a loose matrix (36–38). 80K-H also possesses a pair of 12-amino acid motifs that may form two interacting helix-loop-helix EF-hand structures (39). Such motifs have been shown to coordinate Ca²⁺ in other ER proteins, and may allow for conformational regulation by Ca²⁺ levels or confer a buffering capacity on the protein (34, 35). Finally, 80K-H possesses a polar distribution of conserved Cys residues and possesses intrachain disulfide linkages (7). Taken together, these observations are consistent with 80K-H residing in the ER lumen.

Our biochemical data fit best with the suggestion by Trombetta et al. that 80K-H represents the β-subunit of glucosidase II. We have shown that the 80-kDa protein co-purifies with GIβ and a major cellular glycoprotein. Moreover, we have found that the 80-kDa protein can associate with glucosidase II activity in the absence of detectable CD45 (data not shown). Although GIβ does not appear to be necessary for GIβ catalytic function (7, 16–18), it may be capable of modulating the activity of the holoenzyme. Additionally, it may function to retain the α-subunit in association with the luminal face of the ER membrane. If correct, this could explain why an 80–90-kDa protein was consistently observed to be present in GIβ preparations after exhaustive purification steps (7, 16, 18) and also help reconcile the ability of GIβ to partition to the membrane fraction as a non-integral protein (6, 18). It is important to note, however, that GIβ (80K-H) has been purified in the apparent absence of the larger catalytic subunit (11, 31). Our data do not exclude the possibility that 80K-H may adhere to a cell-surface protein(s) in certain cell types, where it may contribute to the binding of proteins modified by advanced glycation end products. The basis for detecting an admittedly small subpopulation of tyrosine-phosphorylated 80K-H in a previous study, however, is unclear (32); we have been unable to detect tyrosine phosphorylation of this protein in response T-cell receptor-stimulation of CTL clone cells (data not shown).

An important question arising from our study is why are we able to copurify glucosidase II with CD45. Our evidence that the interaction between glucosidase II and CD45 is dependent on high mannose triantennary core oligosaccharide linkages supports the hypothesis that glucosidase II interacts with immature CD45 that presumably contains terminal α-1,3 Glc linkages. The simplest explanation for our results is that GIβ and GIγ interact with CD45 in the same way that they do with all immature glycoproteins; however, because CD45 is a highly expressed, multivalent ligand, we are able to detect the association. Nevertheless, our observations that we are unable to copurify glucosidase II with class I MHC molecules or detect the association of these ER proteins with class I molecules by blotting or enzymatic assay, suggest that there may be something special about the way in which glucosidase II interacts with CD45. Moreover, it is notable that we failed to copurify detectable levels of any other enzyme or receptor that recognizes oligosaccharides present on CD45, or any other ER chaperone molecule that interacts with CD45.

It is also possible that our data may relate to novel aspects of CD45 maturation or function. That maturation of CD45 is highly unconventional in some cell types is revealed by a report demonstrating the presence of high mannose triantennary oligosaccharide linkages on cell-surface CD45 derived from immature thymocytes (52). It has further been noted that in a resting T-cell line approximately 30% of total cellular CD45 is retained in the cis-Golgi (53). Within minutes of activating this cell with anti-Thy-1, this intracellular pool of CD45 was found to disperse, partitioning to an insoluble intracellular fraction. It is tempting to speculate that a novel function of glucosidase II may be to target and/or retain a portion of newly synthesized CD45, possibly in a transitional ER compartment. It is not clear whether the PTP activity of the cytoplasmic domain of CD45 may be required to perform a function in this particular subcellular microenvironment; however, it is interesting that the cytosolic phosphatase, PTP1B, has been shown to localize to the endoplasmic reticulum (54). In addition, tyrosine kinase and phosphatase inhibitors can block vesicular transport (55). Also intriguing, particularly in view of the fact that GIβ may bind Ca²⁺, is a report detailing defects in regulation of intracellular Ca²⁺ by cells deficient in CD45 (56) and another indicating that re-distribution of intracellular pools of CD45 correlates with changes in the ability of T cells to liberate Ca²⁺ from internal stores (57). Further investigation of the interactions between CD45 and glucosidase II may provide novel insights into the functions of these molecules.

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