Purification and Characterization of the Human Erythrocyte Band 3 Protein C-Terminal Domain†

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ABSTRACT: To clarify the function of the hydrophilic carboxyl-terminal tail of human erythrocyte membrane band 3 protein (HEM-B3), we purified two peptides, C1 (Ala893−Val911) and KS4 (Gly647−Arg656), from human erythrocyte band 3 protein preparations. Purified C1 peptides at concentrations from 5 to 80 μM were incubated with fresh human erythrocyte white ghosts. The C1 peptide demonstrated a novel protease activity, which cleaved glycophorin A (GPA) at Leu118−Ser119 in a dose-dependent manner. This activity was eliminated by trypsin. In a control experiment, the KS4 peptide did not cleave GPA under the same conditions. To help substantiate that the band 3 C-terminal tail peptide (C1) alone possesses the protease activity, two experiments were performed. First, the plasmids pGBKT$_7$-GPA-Ct and pGADT$_7$-AE1-Ct were cotransformed into the yeast strain AH109. The pGBKT$_7$-GPA-Ct plasmid contains the cDNA of the 33 amino acid residue section of GPA (Tyr93−Asn125) fused with the pGBKT$_7$ vector. The plasmid pGADT$_7$-AE1-Ct contains the cDNA of the C-terminal 33 amino acid residues of HEM-B3 fused with the GAL4 DNA-binding domain in the pGADT$_7$ vector. The results of the cotransformation experiment indicated that the C-terminal 33 amino acid residues of HEM-B3 interacted directly with the GPA C-terminal segment defined above. Second, we used a mammalian two-hybrid analysis to confirm the interaction relationship between the band 3 C-terminal segment and the GPA C-terminus. The C-terminus of GPA and the C-terminal 33 amino acid residues of HEM-B3 were subcloned into the DNA-binding domain and transcription activation domain vectors of the two-hybrid system, respectively. They were then cotransfected along with a chloramphenicol acetyltransferse (CAT) reporter vector into HeLa cells. The CAT activity measured in this experiment also indicated that there was interaction between the C-terminal 33 amino acid residues of HEM-B3 and the C-terminus of GPA.

Human erythrocyte membrane band 3 (HEM-B3;[1] anion exchanger 1, AE1) is a major integral membrane anion-exchange glycoprotein. It has a molecular mass of 95 kDa and composes about 25 wt % of the total membrane protein of the erythrocyte (*1*−*3*). HEM-B3 consists of three separate domains: the N-terminal (∼403 residues) 43 kDa cytoplasmic domain, the C-terminal membrane-spanning domain, and the cytoplasmic carboxyl-terminal tail domain (*4*−*6*). The N-terminal 43 kDa domain, which is water soluble, anchors to the erythrocyte skeleton by association with ankyrin and protein 4.1. It also binds an array of other proteins including protein 4.2. It binds the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, alderase, and phosphofructokinase as well as tyrosine kinase p72^{syk} and deoxyhemoglobin (*7*).

The C-terminal membrane-spanning domain, which transverses the cell membrane 14 times, is a highly conserved domain. It catalyzes Cl^-/HCO_3^- anion transport across the phospholipid bilayer (*8*). It also serves as the major antigen responsible for immune-mediated removal of senescent and abnormal erythrocytes (*9*). The topology of this domain has been reported, and the amino acid residues important for anion transport have been identified (*10*). There is, however, little detailed information of the acidic carboxyl-terminal tail of HEM-B3 (from Asn880 to Val911). This portion of the HEM-B3 molecule contains many acidic amino acids and thus has a net negative charge. It has been shown to be inaccessible to trypsin even though it is highly hydrophilic (*11*), indicating that the binding sites must be hidden, perhaps by interaction with other proteins.

Over the past few years, several studies have been focused on the acidic C-terminal tail of the band 3 protein. It contains a binding site for carbonic anhydrase (CA) at Asp887−

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[1] Abbreviations: ABTS, 2,2′-azinodi(3-ethylbenzothiazolinesulfonate) diammonium salt crystals; AE1, anion exchanger 1; Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; C1, Ala893−Val911 of band 3; CA, carbonic anhydrase; CAT, chloramphenicol acetyltransferse; ELISA, enzyme-linked immunosorbent assay; FRAP, fluorescence recovery after photobleaching; Gly, glycine; GPA, glycophorin A; HEM-B3, human erythrocyte membrane band 3 protein; KS4, Gly647−Arg656 of band 3; Leu, leucine; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; Ser, serine; Tyr, tyrosine; Val, valine.

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Asp890, and the CA activity is required for full anion transport activity of the band 3 protein (*12*, *13*). Although deletion of the 11 C-terminal amino acids does not affect the overall structure or anion transport activity of the band 3 protein, it does affect the surface membrane trafficking of the protein (*14*, *15*). In the present paper, we purified and collected the two HEM-B3 peptides (C1 peptide, Ala893−Val911, and KS4, Gly647−Arg656) from human erythrocyte membranes and incubated them with fresh human erythrocyte white ghosts to investigate the function of the C1 peptide and to search for a protein, which may interact with the hydrophilic carboxyl-terminal tail of HEM-B3. In our experiments we found that the band 3 C1 peptide demonstrated a novel protease activity. It was found to cleave GPA, another transmembrane protein of the human erythrocyte, at the Leu118−Ser119 bond. The two-hybrid analysis showed that there is a direct interaction between band 3 Asn880−Val911 and GPA Tyr93−Asn125.

EXPERIMENTAL PROCEDURES

Preparation of Human Erythrocyte White Ghosts. Human erythrocyte white ghosts were prepared as previously described by Dongkang et al. (*11*). Briefly, human blood was washed four times in phosphate-buffered saline (PBS) to remove the white cells from the blood and then suspended in PBS containing 0.2 mg/mL trypsin at 37 °C for 1 h to digest glycophorins. The preparations were then washed with PBS containing PMSF (2.5 μg/mL) three times to remove the trypsin. The pellets were lysed by adding more than 20 volumes of 5 mM $NaHCO_3$ with stirring on ice for 5 min. The solution was centrifuged at 24000*g* for 30 min at 4 °C and washed with the same buffer four times at 4 °C. The packed ghosts were stored at −80 °C until they were used. White ghosts (1 mg/mL) in 5 mM $NaHCO_3$ containing 0.15 M NaCl were digested with 15 μg/mL trypsin at 4 °C for 1 h with stirring (i.e., trypsin treatment with high-salt concentration). The cytosolic 40 kDa domain of HEM-B3 was thereby cleaved. After addition of antipain (10 μg/mL), the treated membranes were separated by centrifugation at 45600*g* for 20 min at 4 °C and then washed extensively with more of the same buffer. Peripheral membrane proteins in the washed membranes were stripped with 5 volumes of 100 mM NaOH at 4 °C. The alkali-stripped membranes were washed with 5 mM $NaHCO_3$ three times and resuspended in 5 mM $NaHCO_3$ at a protein concentration of 1.5 mg/mL. The membrane suspension was redigested with 15 μg/mL trypsin at 37 °C overnight (i.e., trypsin treatment with low-salt concentration). The peptides released to the supernatant were collected by centrifugation at 27200*g* for 30 min at 4 °C.

Purification of C1 and KS4 Peptides. The Cl and KS4 peptides were obtained from the supernatant described above by using HPLC. The HPLC system was equipped with a reverse-phase column (Cosmosil C-18, 4.6 × 250 mm) using a gradient of acetonitrile containing 0.1% trifluoroacetic acid. It was run for 150 min at a flow rate 0.8 mL/min. An amino acid sequencer was used to determine the purity and concentration of the C1 and KS4 peptide-containing fractions.

Preparation of Fresh White Ghosts for Incubation Experiments. Human blood was washed with PBS four times, lysed in more than 20 volumes of 5 mM $NaHCO_3$, and stirred on ice for 5 min. The lysate was centrifuged at 24000*g* for 30 min at 4 °C. The pellets were washed twice with 5 mM $NaHCO_3$ and once with 5 mM $NaHCO_3$ containing 0.15 mM NaCl. The fresh white ghosts were diluted to 1 mg/mL for incubation experiments.

Incubation Experiments. The C1 peptides collected from the HPLC fraction were quantitated by the amino acid sequencer and then were added to experimental tubes at concentrations of 5, 10, 30, and 80 μM. The peptides were evaporated by speed vacuum, and then 300 μL of fresh white ghosts was added to the tubes. The samples were mixed well and incubated at 37 °C for 1 h. The incubated solution was centrifuged at 25000*g* for 30 min. The supernatant was removed for HPLC analysis.

Yeast Two-Hybrid Assay. A cDNA fragment encoding the C-terminal 33 amino acid residues of HEM-B3 was fused in frame with the GAL4 DNA-binding domain in the $pGADT_7$ vector to create the hybrid bait protein, while the 33 amino acid residues of glycophorin A were fused to the $pGBKT_7$ vector. Both of the reconstructed plasmids were introduced into a yeast reporter strain by the lithium acetate transformation procedure of Gietz et al. Transformants were allowed to grow at 30 °C for 2−4 days until colonies were large enough to assay for β-galactosidase activity. Transformant cells were then plated directly onto sterile Whatman no. 1 filters that had been layered onto sterile growth media. After colonies had grown, the filters were assayed for β-galactosidase activity. The cells were disrupted by a cycle of freezing the filter paper with the yeast colonies on it in liquid nitrogen and thawing to room temperature. Each filter was then soaked with 2 mL of Z buffer (CLONTECH Matchmaker protocol manual) containing 5-bromo-4-chloro-3-indolyl β-D-galactoside. The filters were then placed in a covered plastic container at room temperature and checked at 30 min intervals. The filters were then dried and photographed to record the data.

Mammalian Two-Hybrid Assay. The cDNA fragment of the AE1 C-terminus and the C-terminus of GPA were subcloned into the transcription activity domain vector pVP16 and DNA-binding domain pM, respectively. One day before transfection, HeLa cells were seeded at 5×10^5/plate in six 10 cm plates. Using the calcium phosphate transfection method, 2.0 μg of both the pM-based plasmid and pVP16-based plasmid, as well as 1.0 μg of reporter plasmid pG5CAT, was cotransfected into the HeLa cells. The detailed transfection protocol was provided with the kit purchased from CLONTECH. The cells were incubated at 37 °C in 3% CO_2 overnight and then carefully washed twice with 5 mL of PBS. Then 10 mL of complete medium with 10% fetal bovine serum was added. The cells were incubated for 24−48 h, harvested and lysed, and assayed for CAT activity using the CAT enzyme-linked immunosorbant assay kit (Roche Molecular Biochemicals). The cell extracts (containing CAT enzyme) were added to the wells of a microtiter plate that was precoated with a polyclonal antibody to CAT. All of the CAT in the cell extracts was bound to the CAT antibodies. Digoxigenin-labeled antibodies to CAT were then added to bind to CAT, and an antibody to digoxigenin conjugated to peroxidase was added to bind to the digoxigenin. Finally, the peroxidase substrate ABTS was added. The peroxidase enzyme catalyzed the cleavage of the substrate producing a color reaction. The absorbance of the

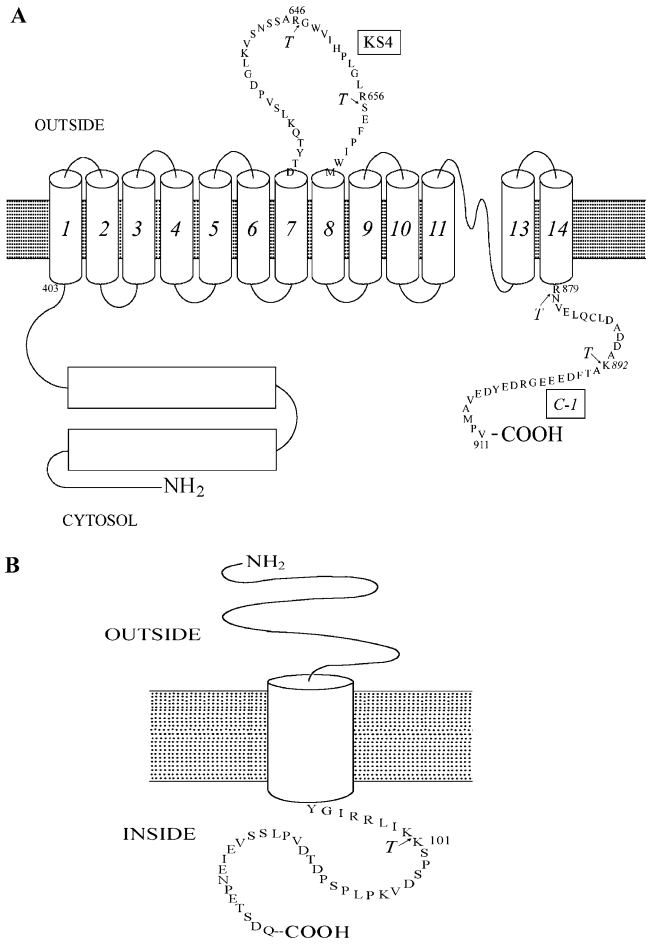

FIGURE 1: Topology model of band 3 and glycophorin A: (A) band 3; (B) glycophorin A.

sample was determined by an enzyme-linked immunosorbent assay reader and was directly correlated to the level of CAT present in the cell extracts.

RESULTS

Analysis of Peptides Released from the Erythrocyte Membrane. After removal of the cytosolic N-terminal domain of HEM-B3 and the peripheral membrane proteins, the transmembrane 55 kDa domain of HEM-B3 remained. The C1 and KS4 peptides were located at the intracellular and extracellular sides, respectively, of the erythrocyte membrane (Figure 1A, band 3; Figure 1B, GPA). The treated membranes were further digested with trypsin at 37 °C for 1 h, and the peptides released were analyzed by the standard HPLC procedure. Figure 2 shows the elution profile of one HPLC analysis. All major peaks were collected and rechromatographed with the same HPLC system. The purified peptides were sequenced by a gas-phase amino acid sequencer. The C1 and KS4 peptides were thus identified.

Analysis of Peptides Released from Fresh White Ghosts Which Were Incubated with C1 and KS4 Peptides. The purified C1 peptides were incubated with fresh white ghosts at 37 °C for 1 h at concentrations of 0−80 μM and then centrifuged at 15000*g*. The supernatant was analyzed by standard HPLC. Figure 3 shows the pattern obtained by HPLC analysis of the supernatant. The profile has a major peak at concentrations of 5 and 10 μM C1 peptide (panels A and B). We sequenced the fraction materials of the major peak. The sequencer data from the major peak fraction indicated the presence of the C1 peptide and the GPA Lys101−Asp130 sequence. However, when the C1 peptide concentration was increased up to 30 and 80 μM, the HPLC pattern produced by the supernatant changed from the one major peak produced at the lower concentrations to three peaks (panels C and D). The sequencer data identified the sequences of the material contained in each of the three peaks as follows: GPA Lys101−Leu118 (peak 1), GPA Ser119−Asp130 (peak 2), and C1 peptides (peak 3).

A Novel Protease Inactivated by Trypsin. To further investigate the properties of the C1 peptide of HEM-B3, six incubation experimental groups were performed. Group 1 had white ghosts only, group 2 had white ghosts + trypsin (15 μg/mL), group 3 had white ghosts + C1 peptides (40 μM), group 4 had white ghosts + C1 peptides + trypsin, group 5 had white ghosts + KS4 peptides (40 μM), and group 6 had white ghosts + KS4 peptides (40 μM) + trypsin. All of the incubated supernatants were analyzed by HPLC (Figure 4). In groups 1 and 2, no major peaks appeared in their HPLC profile (panels A and A′). Groups 5 and 6 showed one major peak that corresponded to the KS4 peptide sequence that was added to the incubation tube (panels C and C′). In group 3, the HPLC profile showed three major peaks: (peak 1) GPA Lys101−Leu118, (peak 2) GPA Ser119−Asp130, and (peak 3) C1, which are shown in panel B. However, in group 4, the three major peaks returned to a single peak (panel B′). The sequence data on this peak show the GPA Lys101−Asp130 and the C1 peptide sequence again. These results suggest that the preparation of purified C1 peptide has an unidentified protease activity, that it cleaves the amino acid bond of GPA at Leu118−Ser119, and that this protease activity was inactivated by trypsin. The amino acid sequencer profile indicates that the preparation contains no peaks corresponding to known protease sequences. Also, no peaks are seen which represent peptides as large as known proteases. In the control experiments using KS4, the peaks corresponding to GPA Lys101−Leu118 and GPA Ser119−Asp130 were not found, and no change occurred with the addition of trypsin to the incubation tubes. The presence of these results suggests that the HEM-B3 C-terminal tail might interact with the GPA C-terminal end and have a specific protease activity.

Yeast Two-Hybrid System Analysis of the AE1 C-Terminal End and C-Terminus of GPA. To test whether the AE1 C-terminal end Ala893−Val911 interacts with GPA Lys101−Asp130 directly, the cDNA coding for the entire 33 amino acid region of GPA was fused in frame to the GAL4 DNA-binding domain (in the vector pGBKT$_7$). Also, a cDNA coding for a 33 amino acid fragment of the AE1 C-terminus was fused to the GAL4 DNA-activating domain (in the vector pGADT$_7$). The two-hybrid plasmids were cointroduced into *Saccharomyces cerevisiae* AH109 cells, which contain the lacZ, his3, and ade reporter genes under different promoters with upstream GAL4 binding sites. Interaction of the two fusion proteins is necessary to juxtapose the GAL4 DNA-binding and activation domains, which then activate transcription of the reporter genes. We cultured the transformants on trp$^-$/leu$^-$/his$^-$/ade$^-$ medium in four separate groups, and then β-galactosidase activity was checked. Results of these experiments are shown in Table 1. Only the transformant, which was infected with pGBKT7-GPA-Ct and pGADT7-

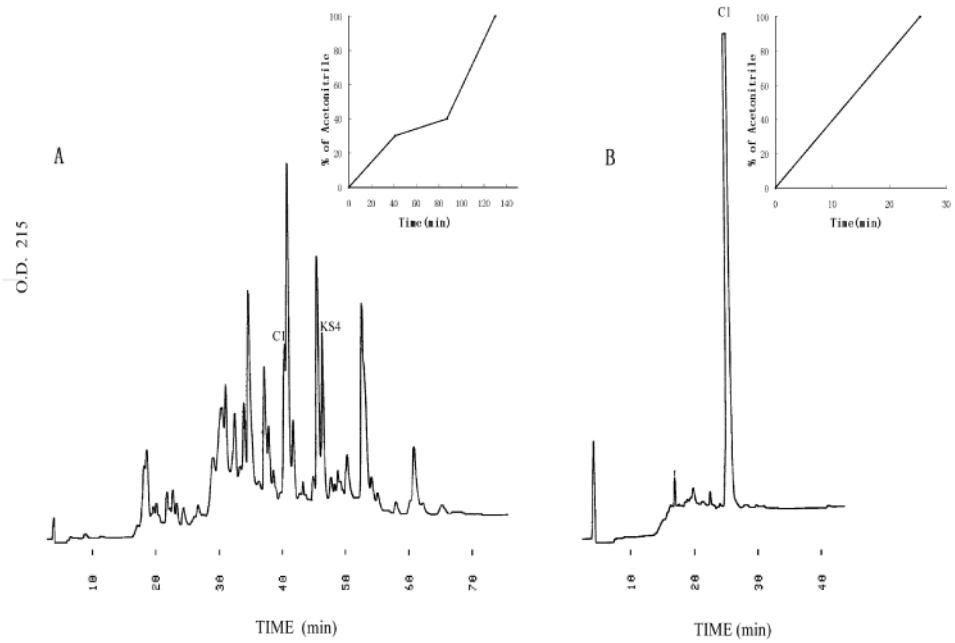

FIGURE 2: Purification of C1 and KS4 peptides from human erythrocyte membrane band 3 using reversed-phase Cosmosil C-18 columns. Panel A: Membranes were isolated from human erythrocytes, and the peripheral membrane proteins were removed with NaOH at 4 °C. After that, the cytosolic 40000 Da domain of band 3 was also removed by treating white ghosts with trypsin at 4 °C. Then the treated membranes were redigested with trypsin at 37 °C for 1 h. Peptides released into the supernatant were collected and analyzed using a gradient of acetonitrile containing 0.1% trifluoroacetic acid. Panel B: The collected C1 fraction was purified, and the single peak and amino acid sequencer data show the C1 peptide to be of high purity.

AE1-Ct, could grow on the $trp^-/leu^-/his^-/ade^-$ plate and possessed a β-galactosidase activity.

Mammalian Two-Hybrid Analysis. Figure 5 shows the interaction of the band 3 C-terminus and GPA C-terminus assessed by a mammalian two-hybrid system. HeLa cells were transfected with a DNA-binding-based vector, an activating-based vector, and a CAT reporter vector simultaneously. After treatment with lysozyme, cell extracts containing CAT were used immediately for CAT ELISA. The absorbance of the samples was measured at 405 nm using a microtiter plate reader. The absorbance of the cell extracts which were transfected with the pM-GPA-Ct and pVP16-AE1-Ct (group 1) was significantly higher than the negative control (groups 2, 3, 4, and 6). These results suggest that the C-terminus of band 3 interacts with the C-terminus of GPA.

DISCUSSION

Although the C-terminal 33 amino acid residue section of the band 3 protein contains two trypsin cleaving sites and is similar to the N-terminal domain in that it is very hydrophilic, the N-terminal cytoplasmic domain can be removed by treatment of the erythrocyte membrane with trypsin alone, whereas the C-terminal tail requires pretreatment with 100 mM NaOH to strip the erythrocyte membrane before trypsin will cleave it. This property led us to consider that the band 3 C-terminal 33 amino acid residues might interact with some other protein by ionic attraction. So, in the original experiment, we purified and collected band 3 C-terminal C1 peptides and incubated the C1 peptides with fresh white ghosts, expecting to draw off a protein from the erythrocyte membrane which would show a new band upon SDS−PAGE analysis of the supernatant. However, we were disappointed as no new interesting band was dyed when the incubated supernatant was run on SDS−PAGE (figure not shown). The supernatant was also analyzed by HPLC equipped with a reverse-phase column (Cosmosil, 4.6 × 250 mm). This method demonstrated that different major peptides were contained in the supernatant at different concentrations of C1 peptide. When the C1 peptide concentration reached 30 μM, the preparation of C1 peptide began to show a novel protease activity that cleaved the GPA C-terminus at Leu118−Ser119 and was inactivated by trypsin. The high purity of the C1 peptide preparation and the absence of peaks that represent peptides as large as known proteases in it indicate that the enzyme activity is closely related to the C1 peptide. The yeast two-hybrid and mammalian two-hybrid analyses showed that the C-terminal 33 amino acid residues of band 3 directly interact with the GPA C-terminus, supporting the idea that an enzyme−substrate relationship exists between them.

Several previous studies have suggested an interaction between HEM-B3 and GPA at an early stage in the biosynthesis and intracellular processing of the two proteins. It was initially observed that the glycosylation of HEM-B3 was altered in GPA-deficient En (a^-) red blood cells, as well as in other GPA mutant red blood cells (*16*, *17*). In addition, the fact that anti-GPA antibodies can reduce the rotational diffusion of HEM-B3 indicates that the two proteins may associate in the red blood cell membrane (*18*). This view is further strengthened by the observation that the binding of anti-GPA antibodies to its extracellular domain rigidifies the red blood cell membrane (*19*) and leads to the immobilization of both GPA and HEM-B3 as measured by the in situ FRAP (fluorescence recovery after photobleaching) technique (*20*). Furthermore, reconstitution of purified GPA and HEM-B3 in nonionic detergents indicates that GPA may directly associate with HEM-B3 (*21*). Immunological studies also

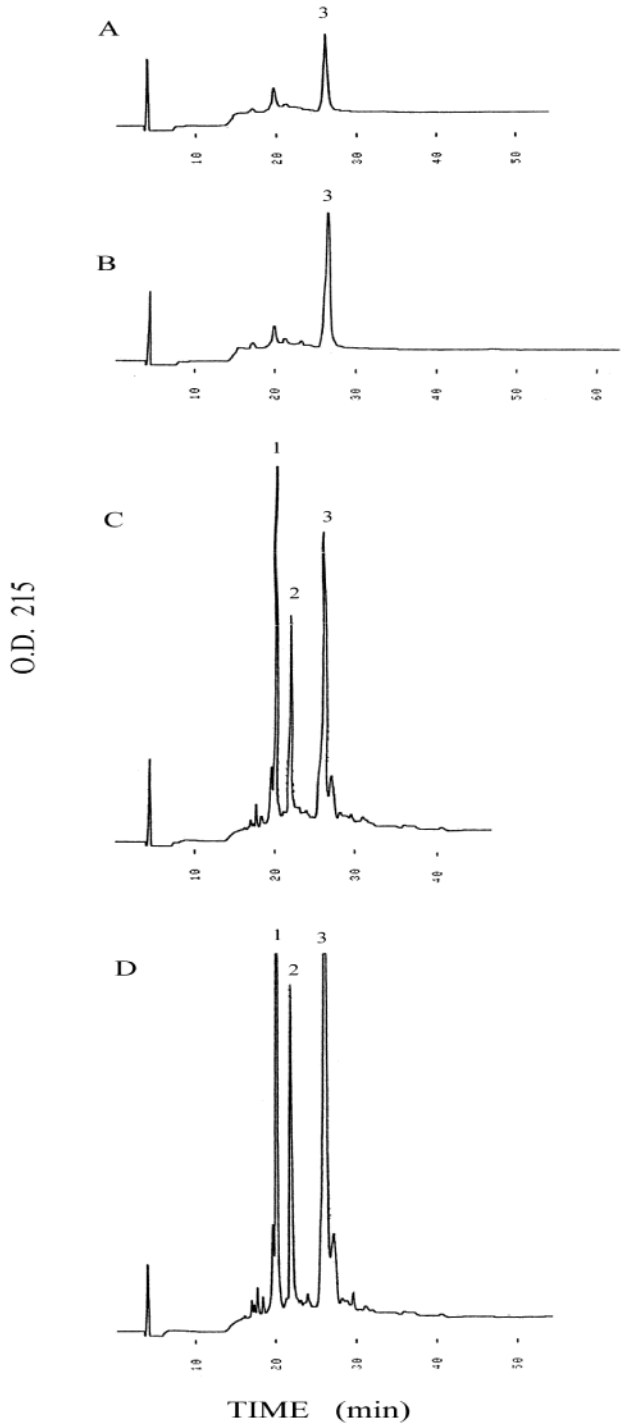

FIGURE 3: Effect of C1 peptide on fresh white ghosts. C1 peptides were purified from erythrocyte membranes, and 5, 10, 30, and 80 μM C1 peptide, respectively, was added to incubation tubes. After evaporation by speed vacuum, 1 mL of fresh white ghosts (1 mg/mL protein) was added to the tubes, and the mixture was incubated at 37 °C for 1 h. The supernatant was obtained by centrifugation at 15000*g* for 20 min. Analysis of peptides in the supernatant was carried out with a reversed-phase Cosmosil C-18 column. The major peaks were sequenced.

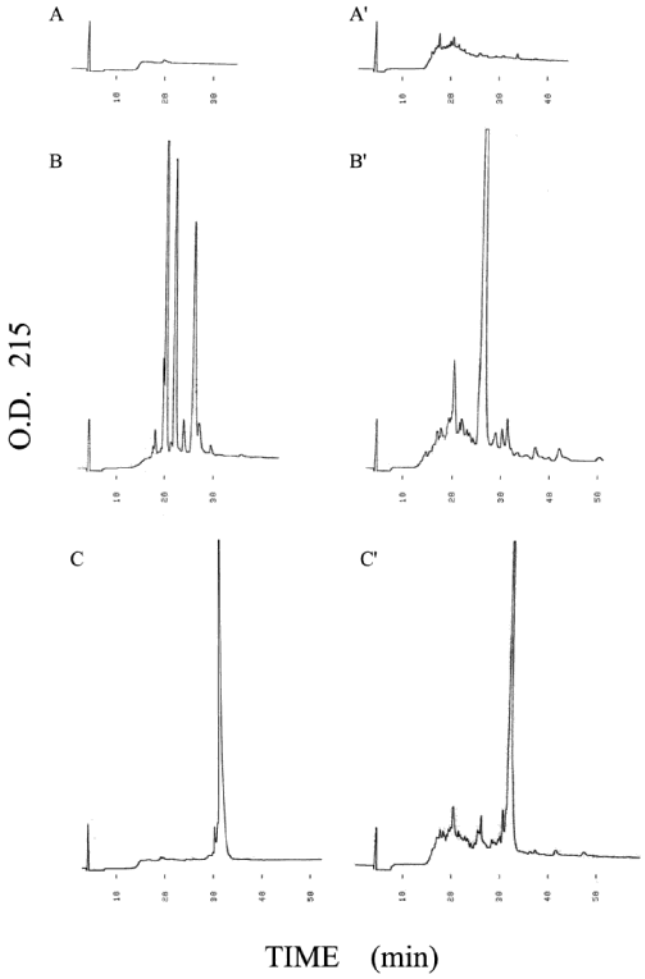

FIGURE 4: Specific analysis of C1 activity: (A) fresh white ghosts; (A′) fresh white ghosts + trypsin; (B) fresh white ghosts + C1 peptides (40 μM); (B′) fresh white ghosts + C1 peptides (40 μM) + trypsin (15 μg/mL); (C) white ghosts + KS4 peptides (40 μM) + trypsin (15 μg/mL); (C′) white ghosts + KS4 peptides (40 μM) + trypsin (15 μg/mL).

Table 1: β-Galacosidase Activity Analysis[a]

DNA-binding domain	activating domain	β-galacosidase activity
$pGBKT_7$	$pGADT_7$	−
$PGBKT_7$-GPA-Ct	$pGADT_7$	−
$pGBKT_7$	$pGADT_7$-AE1-Ct	−
$PGBKT_7$-GPA-Ct	$pGADT_7$-AE1-Ct	+

[a] Yeast two-hybrid assay of the cytoplasmic C-terminus of band 3 and GPA. AH109 cells were cotransformed with pGADT7-AE1-Ct and pGBKT7-GPA-Ct, pGADT7 and pGBKT7, pGADT7 and pGBKT7-GPA-Ct, and pGADT7-AE1-Ct and pGBKT7, respectively, and then the β-galacosidase activity was measured. β-Galacosidase activity is shown as the average of five independent transformants ± SD. Only the transformants which cotransformed with the band 3 C-terminus and GPA C-terminus exhibited β-galacosidase activity.

support a close GPA−HEM-B3 interaction: a monoclonal antibody raised against HEM-B3 was shown to coprecipitate GPA from red blood cell membranes (*22*−*24*). Finally, the expression of the antithetical antigens Wr^a and Wr^b, which represent alternative polymorphisms of HEM-B3, requires an interaction between HEM-B3 and GPA involving a region of the transmembrane domain of GPA (*25*). The presence of additional blood group epitopes, which are dependent on the interaction of GPA with HEM-B3, was also suggested (*26*, *27*), and the existence of one such epitope was recently documented (*28*).

Recently, evidence is accumulating that GPA has a role in facilitating the movement of band 3 to the cell surface. Coexpression of wild-type band 3 cRNAs with GPA induces an increased level of band 3 mediated chloride influx in *Xenopus* oocytes by facilitating the translocation of band 3

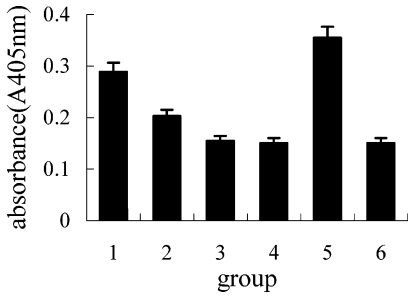

FIGURE 5: CAT activity values were measured by ELISA. The absorbance values are normalized by the CAT enzyme under standard dilutions, and the average value is calculated from three separate experiments (±SD). The absorbance of the experimental group increased remarkably when compared with the control groups ($p < 0.01$). Key: group 1, pM-GPA-Ct and pVP16-AE1-Ct; group 2, pM-GPA-Ct and pVP16; group 3, pM and pVP16-AE1-Ct; group 4, pM and pVP16; group 5, positive control pM3-VP; group 6, HeLa cells without transfection.

to the cell surface and potentially also by enhancing the anion transport function of HBM-B3 (*29*). Also, there is a close relationship between the hydrophilic carboxyl-terminal tail of band 3 and the cytoplasmic C-terminus of GPA. The cytoplasmic C-terminus of GPA is involved in the enhancement of band 3 protein movement to the cell surface. Deletion of the cytoplasmic C-terminus of GPA led to the loss of the ability to enhance band 3 specific chloride transport. The single point mutation GPA R97M (located on the cytoplasmic side of the transmembrane domain) also exhibited an impaired ability to increase the surface presentation of band 3 but efficiently enhances band 3 anion transport activity (*30*). These results imply that the cytoplasmic C-terminus of GPA is responsible for enhanced trafficking of the band 3 protein to the cell surface. The GPA-mediated enhancement of the band 3 surface presentation could be achieved either by direct interaction of the GPA C-terminus with band 3 or by interaction of the GPA C-terminus with other proteins which may facilitate band 3 trafficking. Taken together, these observations strongly suggest that GPA and HEM-B3 interact directly at the red blood cell membrane and that these interactions are associated with the movement of band 3 to the cell surface (*31*). Although recent studies have provided more clear information on the structure and function of HEM-B3 and its transmembrane and cytoplasmic domains, the direct interaction between GPA and band 3 has not yet been documented (*32*). In this paper, we focused on providing the evidence of the interaction between HEM-B3 Ala893−Val911 and GPA Tyr93−Asn125. Our experiments proved that the C-terminus of HEM-B3 interacts directly with GPA, and the interaction site was located in 32 amino acids of HEM-B3 and 33 amino acids of GPA.

The major new finding described here is that the segment of Asp893−Val911 of HEM-B3 interacts with GPA Lys101−Asn130 and is able to cleave it at the Leu118−Ser119 bond. At the present time, we do not know the natural tertiary structure that will demonstrate the enzyme activity. Also, the physiological meaning of the band 3 C-terminus protease activity and whether it has a function in any pathologic conditions are still uncertain. We believe that our work lays a good foundation for further studies of this peptide and provides clues for continuing research.

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