Erythrocyte Spectrin Is an E2 Ubiquitin Conjugating Enzyme[†]

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ABSTRACT: The involvement of red blood cell spectrin in the ubiquitination process was studied. Spectrin was found to form two ubiquitin-associated derivatives, a DTT-sensitive ubiquitin adduct and a DTT-insensitive conjugate, characteristic intermediate and final products of the ubiquitination reaction cascade. In addition to spectrin and ubiquitin, ubiquitin-activating enzyme (E1) and ATP were necessary and sufficient to form both the spectrin−ubiquitin adduct and conjugate. No exogenous ubiquitin-conjugating (E2) or ligase (E3) activities were required, suggesting that erythrocyte spectrin is an E2 ubiquitin-conjugating enzyme able to target itself. Both ubiquitin adduct and conjugate were linked to the α subunit of spectrin, suggesting that the ubiquitin-conjugating (UBC) domain and its target regions reside on the same subunit.

Spectrin is a major structural component of the erythrocyte membrane skeleton. The spectrin membrane skeleton is a two-dimensional meshwork of proteins that covers the cytoplasmic surface of the erythrocyte membrane and is responsible for its biconcave shape and properties of elasticity and flexibility essential for its circulatory travel (*1*). The membrane skeleton viewed by negative staining and electron microscopy is primarily a hexagonal lattice (*2*) with actin protofilaments at the center and six corners of the hexagons, interconnected by spectrin tetramers. Spectrin is composed of two large subunits: 280 kDa α and 246 kDa β subunits (*3*, *4*). The simplest form of spectrin is an antiparallel $\alpha\beta$ heterodimer. Within the skeleton, however, it is an $(\alpha\beta)_2$ tetramer, a flexible rod formed by head-to-head linkage of two heterodimers (*5*, *6*). Spectrin tetramers bind actin protofilaments at its tail regions, thereby cross-linking F-actin (*7*−*10*). The spectrin−actin interaction is strengthened by the binding of protein 4.1 to the tails of spectrin (*11*−*13*) and adducin to both spectrin and F-actin (*90*, *91*). The membrane skeleton is attached to the membrane bilayer in two ways. Protein 4.1 binds to the transmembrane protein glycophorin C (*1*, *14*, *15*), and ankyrin, bound to β spectrin, also binds to the transmembrane protein band 3 (*16*−*20*).

Lux and colleagues have demonstrated that erythrocyte membrane skeletons derived from irreversibly sickled cells (ISCs)[1] retain the sickled shape (*21*). The core components of the membrane skeleton, which are minimally required to maintain shape, are spectrin, F-actin and protein 4.1 (*22*, *23*). Defects in these proteins can cause structural rearrangements in the membrane skeleton resulting in abnormal red cell shape and stability. Studies conducted in our laboratory revealed a posttranslational modification in ISC β actin (*24*−*26*), affecting the actin−actin interaction and, as a result, the ability of the membrane skeleton to disassemble and reassemble (*27*). The studies also suggested a functional difference between ISC and normal spectrins in the ability of the spectrin−4.1−actin ternary complex to disassemble at 37 °C (*24*). A structural difference between normal and ISC spectrin was directly demonstrated with a rabbit autoantibody (*28*): a modified form of spectrin recognized by the antibody was found in normal human erythrocytes. The modified spectrin was present in sickle cells to a much lower extent than in normal erythrocytes (*28*). The nature of the spectrin structural modification was not determined at that time. It was shown, however, that the modification was associated with the α subunit of spectrin and was sensitive to reducing agent (*28*). Here we provide evidence that the modified α′ spectrin is an ubiquitin adduct.

Ubiquitin is an 8.6 kDa highly conserved polypeptide found both free and covalently conjugated to other proteins in all eucaryotic cells (*29*, *30*). Protein ubiquitination is a posttranslational modification of target proteins, a dynamic process involving ubiquitin conjugation enzymes and de-ubiquitination enzymes (*31*). Ubiquitination is involved in cell growth regulation and controls such diverse processes

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[1] Abbreviations: E1, E2, and E3, ubiquitin activating, conjugating, and ligating enzymes, respectively; GSH, reduced glutathione; GSSG, oxidized glutathione; ISC, irreversibly sickled cell; LC, liquid chromatography; MS, mass spectrometry; PBS, phosphate-buffered saline; PBST, PBS with 0.05% Tween 20; TFA, trifluoroacetic acid; UBC, ubiquitin conjugating.

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as cell cycle progression (*32*, *33*), transcriptional activation (*34*, *35*), growth factor mediated signal transduction (*36*, *37*), and apoptosis (*38*−*40*). The ubiquitin family includes a growing number of ubiquitin-like proteins with diverse regulatory functions (*30*, *41*). For most known substrates, ubiquitination leads to protein degradation by the 26S proteosomal complex (*31*, *42*) and, for some plasma membrane proteins, through an endocytic lysosomal/vacuolar pathway (*43*). Recent studies have shown that the ubiquitination can also regulate enzymatic activity (*44*) and protein associations.

The conjugation of ubiquitin to proteins is a multistep process. A cascade of reactions catalyzed by several classes of enzymes is required to form an isopeptide bond between the C-terminal glycine of ubiquitin and a lysine ϵ-amino group of the target acceptor protein (*43*). Ubiquitin-activating enzyme (E1), utilizing ATP, forms a high-energy thioester bond between its active site cysteine and the C-terminal glycine of ubiquitin via formation of ubiquitin−adenylate, an activated ubiquitin intermediate. The activated ubiquitin is next transferred from E1 to an active site cysteine of the ubiquitin-conjugating enzyme (E2). The final step, formation of isopeptide linkage between ubiquitin and the target protein, is sometimes catalyzed by a ubiquitin−protein ligase (E3). It is believed that the E3 binds the E2−ubiquitin complex and initiates direct transfer of ubiquitin from E2 to the target (for review, see ref *45*). In other cases, such as the HECT family of E3s (*46*), an additional thioester intermediate between ubiquitin and E3 is formed (*43*, *47*). Recently, an additional conjugation factor, named E4, was shown to be required for multiubiquitination (*48*).

Previous studies have shown that erythrocyte spectrin (*49*), as well as hippocampal erythroid and nonerythroid spectrins (*50*), is a target for the ubiquitination system. Proteins of the spectrin family are ubiquitous components of eucaryotic cells (*51*). While spectrin is an extremely well studied structural protein component of the membrane skeleton, no enzymatic activity has ever been reported for this protein. In this study, we demonstrate that erythrocyte spectrin is not only a target for the ubiquitination system but also an ubiquitin-conjugating (UBC) E2 enzyme able to ubiquitinate itself.

EXPERIMENTAL PROCEDURES

Antibodies. Spectrin antisera, recognizing both α and β spectrin were prepared as described (*51*). Antisera, recognizing only α spectrin (or β spectrin), were prepared from the spectrin antisera diluted 1:16 in PBS (10 mM $NaPO_4$, 150 mM NaCl, pH 7.6) by incubation with electroblotted β spectrin (or α spectrin) at 4 °C overnight to bind corresponding IgGs. Spectrin antisera free of IgGs against α spectrin or β spectrin were used as anti-β spectrin or anti-α spectrin, respectively. Final dilutions are given from original undiluted spectrin antisera.

Red Blood Cell Membranes and Spectrin Extract. Human red blood cell membranes and spectrin extract were prepared as described (*10*) with the following exceptions. White ghost membranes were extracted with 1.5 volume of 0.1 mM EDTA, pH 8.0, for 30 min at 37 °C. The extract (spectrin extract) was concentrated by Centriprep YM 50 centrifugal filter (50K molecular weight cutoff) devices (Millipore).

SDS−PAGE and Western Blotting. SDS−PAGE was performed according to Laemmli (*52*). Unless otherwise indicated, samples were solubilized in loading buffer (1% SDS, 1 mM EDTA, 10% sucrose, 0.4 mg/mL pyronin Y, 10 mM Tris-HCl, pH 8.0) for 20 min at 37 °C in the absence or presence of reducing agent (32 mM DTT). Staining was with silver (Bio-Rad) according to the manufacturer's protocol or with 0.05% Coomassie blue R-250 in 45% methanol containing 9% acetic acid (gels were destained in 25% ethanol containing 8% acetic acid). Separated proteins were electroblotted onto nitrocellulose membrane in transfer buffer: 25 mM Tris-HCl, 192 mM glycine, pH 8.3, 0.01% SDS, and 20% methanol. The membrane was blocked in PBST (10 mM $NaPO_4$, pH 7.6, 150 mM NaCl, 0.05% Tween 20) containing 5% milk. Primary antibodies were used at 1:15 000 (anti-α spectrin), 1:2000 (anti-β spectrin), and 1:500 (anti-ubiquitin, Biømeda) in PBST for 2 h at room temperature. Where indicated, anti-ubiquitin was premixed with free ubiquitin (Sigma): 25 μg of ubiquitin/μL of undiluted antibody. The membrane was washed five times in PBST. Visualization was by ^{125}I-protein A (35 Ci/g, ICN) at 0.1 mCi/L in PBST for 2 h at room temperature. The membrane was washed, dried, and autoradiographed.

Immunodot Assay. Samples (7−200 μL) were applied onto nitrocellulose membrane using a Minifold I filtration manifold (Schleicher and Schuell). The membranes were washed, blocked, and incubated with primary antibody and ^{125}I-protein A as described in the procedure for Western blotting. The dots were cut out and assayed for radioactivity in a Packard 500 γ counter.

Immunoprecipitation. Immunoprecipitation reactions were carried out in PBS containing 0.1% SDS, 1% Nonidet P-40, and 0.5% sodium deoxycholate at 4 °C. One milligram of spectrin extract proteins was incubated with 50 μL of anti-α spectrin (corresponding to 3.3 μL of undiluted antisera), with 5 μL of anti-ubiquitin, or in the absence of the antibodies for 1 h in a final volume of 1 mL. Where indicated, 1.5 mg of ubiquitin was premixed with spectrin extract. A protein A-coated agarose suspension (50 μL) (Santa Cruz) was added to each mixture, and the incubation was continued overnight. The agarose-bound immune complex was collected by centrifugation (1000*g*, 5 min), washed five times with 1 mL of incubation buffer, and solubilized in 60 μL of loading buffer with 32 mM DTT for 5 min at 100 °C, and 25 μL aliquots were analyzed by SDS−PAGE on a 7% polyacrylamide gel followed by silver staining and Western blotting. The blots were probed with anti-α spectrin as described in the procedure for Western blotting.

Separation of the Spectrin Heterodimer, First Gel Filtration. The spectrin heterodimer was separated from the spectrin extract by gel filtration. The spectrin extract (∼10−12 mg/mL) was chromatographed on a Sepharose CL-4B column (1.5 cm × 152 cm) equilibrated with gel filtration buffer: 5 mM $NaPO_4$, 50 mM NaCl, and 0.1 mM EDTA, pH 7.5. Collected fractions (3.06 mL) were analyzed by immunodot assay. The spectrin heterodimer ($V_e = 1.6V_0$) peak fractions were combined and concentrated by Centriprep YM 50. This procedure leads to homogeneous spectrin as indicated by SDS−PAGE (*6*).

DTT Treatment of the Spectrin Heterodimer, Second Gel Filtration. Spectrin heterodimer (8 mg/mL) was incubated with 32 mM DTT in gel filtration buffer for 30 min at 37

°C and chromatographed on a Sepharose CL-4B column (1.6 cm × 60 cm) equilibrated with gel filtration buffer containing 32 mM DTT. Collected fractions (2.14 mL) were analyzed by immunodot assay with anti-α spectrin and anti-ubiquitin. Low molecular weight fractions showing reactivity only to anti-ubiquitin were combined. The combined fraction, referred to as "Ubl", was concentrated under vacuum in a SpeedVac (Savant) to ~0.5 mL, dialyzed (3.5 kDa molecular mass cutoff) against diluted (1: 10) gel filtration buffer, and further concentrated to 20−250 μL. The Ubl fraction was analyzed by SDS−PAGE (see legend to Figure 3) and Western blotting (see Sequencing by Edman Degradation).

Sequencing of the 8.5 kDa Protein by Edman Degradation. The Ubl fraction derived from ~30 mg of the DTT-treated spectrin heterodimer was solubilized in the presence of DTT and separated by SDS−PAGE on a 15% polyacrylamide (mini gel). The gel was soaked for 5 min in transfer buffer: 10 mM cyclohexylaminopropanesulfonic acid (CAPS), pH 11.0, and 10% methanol. The proteins were transferred to the SequiBlot PVDF membrane (Bio-Rad) and stained with 0.06% Coomassie blue R-250 in 50% methanol. A 1.5 mm strip of the membrane was probed with anti-ubiquitin as described in the procedure for Western blotting. An 8.5 kDa band, recognized by the ubiquitin antibody, was sequenced. Automated Edman degradation was performed on a Procise-cLC sequencer (Applied Biosystems) according to the manufacturer's instruction at the Protein Sequencing Center at the State University of New York Health Science Center at Brooklyn (Brooklyn, NY).

8.5 kDa Protein Sequence Analysis by Mass Spectrometry. To purify the 8.5 kDa protein, recognized by anti-ubiquitin, the Ubl fraction (0.25 mL) derived from ~100 mg of DTT-treated spectrin was chromatographed by reverse-phase HPLC (System Gold, Beckman) on a ODS C18 column (0.46 × 15 cm) with precolumn. Water and acetonitrile (CH_3CN) with 0.1% trifluoroacetic acid (TFA) were used as solvent A and solvent B, respectively. Fractions were eluted by a gradient of 5−95% solvent B over 40 min at 1 mL/min flow rate. Fractions, eluted between 9.6−10.6, 10.6−11.9, 11.9−13.6, 13.6−15.6, 15.6−16.3, 16.3−17.3, 17.3−18.3, 18.3-21.0, and 21.0−22.8 min, were collected, dried under vacuum in a SpeedVac, redissolved in 50 μL of 25 mM Tris-HCl, pH 8.5, and analyzed by immunodot assay. Two microliters of the 15.6−16.3 min fraction, showing the highest reactivity to anti-ubiquitin, was analyzed by SDS−PAGE on a 15% polyacrylamide (mini gel). An 8.5 kDa protein, contained in 32 μL of the 15.6−16.3 min fraction, was digested with 8 μg/mL endoproteinase Lys-C (Boehringer-Mannheim) for 48 h at 35 °C in digestion buffer (25 mM Tris-HCl, 1 mM EDTA, pH 8.5, 0.01% SDS). At 24 h of the incubation, fresh enzyme (4 μg/mL) was added. Commercial ubiquitin was digested at 0.2 mg/mL under the same conditions. Both digests were analyzed by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS).

A Magic 2002 HPLC system (Microchrom BioResources, Auburn, CA) connected to a LCQ DECA ion trap mass spectrometer with electrospray source (Finnigan Corp., San Jose, CA) was used for the LC/MS/MS experiments. A C18 reverse-phase column (1 × 150 mm) served for the separation of the peptides in the enzymatic digest. Two microliters of the concentrated digest containing about 0.5 μg of peptides was injected. Solvent A was an aqueous solution of 0.05% TFA and 2% acetonitrile, and solvent B was acetonitrile containing 0.05% TFA and 2% water. A linear gradient was applied from 5% solvent B to 65% solvent B in the first 20 min after injection, and the flow rate was 50 μL/min. The electrospray voltage was set to 4.0 kV and the temperature of the heated capillary to 210 °C.

During the chromatographic separation, the LCQ DECA performed the "triple play" experiment to identify the protein from which the peptides were originated. The experiment produces three kinds of mass spectra in rapid sequences (about 25 sequences/min). The first spectrum in the sequence is a conventional mass spectrum recorded in a fast and wide scan range (*m*/*z* 300−2000). The second is a high-resolution spectrum recorded in a narrow, slower scan across the most intense peak, presumably the molecular ion peak found in the conventional spectrum. This high-resolution spectrum provides the precise mass/charge (*m*/*z*) ratio for the ^{12}C monoisotopic molecular ion and its ^{13}C satellites, which allows the determination of the ion's charge state (the value of *z*). The third kind of spectrum is the tandem mass spectrum (also called MS/MS or daughter ion or product ion spectrum) obtained by the decomposition of the isolated molecular ion in an MS/MS process characteristic to the ion trap. The MS/MS spectra compiled during the chromatographic process are submitted to a database search by use of the software tool SEQUEST (*53*, *54*) to identify the protein of origin. SEQUEST correlates uninterpreted tandem mass spectra with amino acid sequences from protein and nucleotide databases. With large databases such as nr.fasta that contains over 400 000 proteins, the delta correlation coefficient between a confident first hit and a second hit is larger than 0.1.

Isolation of Spectrin Subunits. The purified spectrin heterodimer was chromatographed by reverse-phase HPLC (System Gold, Beckman) on a Protein C4 (Vydac) column (0.46 × 25 cm) to separate spectrin subunits (D. G. Kakhniashvili and S. R. Goodman, manuscript submitted). Water and acetonitrile with 0.1% TFA were used as solvent A and solvent B, respectively. Four hundred micrograms of the spectrin heterodimer was injected into the column equilibrated with 45.0% solvent B. The spectrin subunits were eluted by a gradient of 45.0−48.5% solvent B over 7 min at 1.0 mL/min flow rate; elution was continued with 48.5% solvent B for 7 min followed by a gradient of 48.5−58.5% solvent B over 20 min. The pure α spectrin (retention time 13.31 min) and β spectrin (retention time 20.40 min) were dialyzed against 20 mM Tris-HC1, pH 7.5.

Assay for Ubiquitin−Spectrin and Conjugate Formation. The incubation buffer consisted of 75 mM Tris-HC1, pH 7.5, 5 mM $MgC1_2$, 3 mM ATP, 10 mM creatine phosphate, 10 units/mL creatine phosphokinase, 1 μM leupeptin, 1 μM pepstatin, and 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF). Human erythrocyte lysate fraction, containing all three ubiquitinating enzymes (fraction II), was prepared according to Hershko et al. (*55*). In the first set of experiments (Figure 7, panels A and B) 20 μg of erythrocyte membrane protein was incubated with 3 μM (10 Ci/mmol) ^{125}I-ubiquitin (Amersham-Pharmacia) and 23 μg of fraction II at 37 °C in a final volume of 30 μL. Incubation was at 37 °C. At indicated times, the incubation mixture was centrifuged for 15 min at 16000*g* at 4 °C. Membrane pellets and supernatant were solubilized in the absence of reducing agents (37 °C, 20 min) or in the presence of 100 mM DTT

for 5 min at 100 °C. The solubilized proteins were analyzed by SDS−PAGE on a 5% polyacrylamide gel (14 × 16 cm), followed by Coomassie blue staining and autoradiography.

In the second set of experiments (Figure 7, panels C−F), 20 μg of erythrocyte membrane protein and/or 5 μg of purified spectrin heterodimer were (was) incubated with 1 μM (30 Ci/mmol) ^{125}I-ubiquitin (Amersham-Pharmacia) and 0.1 μM E1 enzyme (Calbiochem) at 37 °C in a final volume of 30 μL. Samples were processed as described above. For samples without membranes, only supernatant fractions were analyzed.

In the third set of experiments (Figure 9), 10 μg of purified spectrin heterodimer, 10 μg of DTT-treated spectrin heterodimer, 5 μg of α spectrin, or 8 μg of β spectrin was incubated with 0.6 μM (67 Ci/mmol) ^{125}I-ubiquitin and 0.1 μM E1 enzyme for 120 min at 37 °C in a final volume of 60 μL. The DTT-treated spectrin heterodimer (see Experimental Procedures) was separated from low molecular weight components with a Centriprep YM-100 centrifugal filter (100K molecular weight cutoff) device (calculated dilution of the low molecular weight components 1:1000) and dialyzed against 20 mM Tris-HC1, pH 7.5, to remove DTT. The solubilized proteins were analyzed by SDS−PAGE ± DTT on a 5% polyacrylamide gel followed by Coomassie blue staining and autoradiography.

RESULTS

As mentioned in the introduction the α subunit of normal human red blood cell spectrin contains a modification (*28*). The modified α spectrin, referred to as α′ spectrin, migrates slightly slower than α spectrin in SDS−PAGE. In addition, α′ spectrin appeared to be sensitive to the reducing agent DTT which converts it back to α spectrin. The nature of the spectrin modification remained unknown. To determine the nature of the modification, two features of α′ spectrin, sensitivity to DTT and recognition by the rabbit autoantibody, were employed. The antibody would allow us to trace the modification once separated from spectrin by DTT treatment. During the course of our studies, however, we found that α′ spectrin was also recognized by ubiquitin polyclonal antibodies. Anti-ubiquitin was used for detection in all subsequent experiments.

Ubiquitin Antibody Interacts with α′ Spectrin. Figure 1 is a SDS−PAGE and Western blot analysis of red blood cell membrane proteins separated in the absence and presence of DTT. Both the Coomassie blue staining (Figure 1, panel A, lane b) and the Western blot with anti-α spectrin (Figure 1, panel B) revealed a band corresponding to α′ spectrin, as well as α spectrin, when the proteins were separated in the absence of DTT. Figure 1, panel B, demonstrates that a band corresponding to α′ spectrin was also recognized by anti-ubiquitin. Free ubiquitin (25 μg/μL of undiluted antibody), as a competitor, prevented the reaction. α′ spectrin was not detected by either Coomassie blue staining (Figure 1, panel A, lane c) or Western blotting (Figure 1, panel C) when the proteins were separated in the presence of DTT. The only protein detected by anti-α spectrin in the presence of DTT was α spectrin (Figure 1, panel C). No protein was detected by anti-ubiquitin (Figure 1, panel C) in the presence of DTT. Anti-β spectrin did not react with α′ spectrin (Figure 1, panel

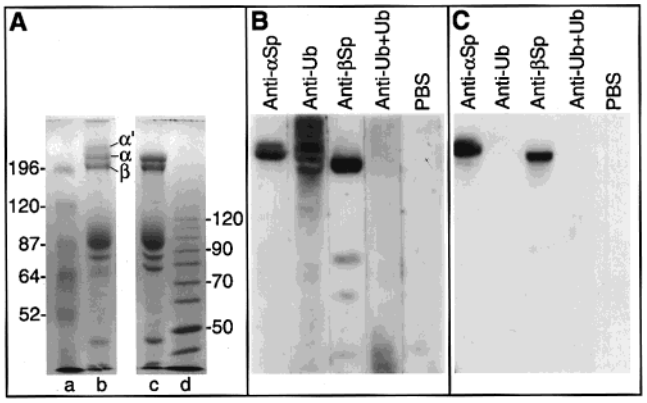

FIGURE 1: Recognition of α′ spectrin by anti-ubiquitin. Erythrocyte membrane proteins (80 μg/lane) were analyzed by SDS−PAGE on a 7% polyacrylamide gel followed by Coomassie blue staining (panel A) and Western blotting (panels B and C). The proteins had been solubilized in the absence (panel A, lane b; panel B) or presence of 32 mM DTT (panel A, lane c; panel C). The bands corresponding to α′, α, and β spectrins are indicated by corresponding Greek letters (panel A, lane b); the molecular mass standards (Gibco BRL) are given in kilodaltons (panel A, lanes a and d). The blots (panels B and C) were probed with anti-α spectrin (1:15 000), anti-β spectrin (1:2000), anti-ubiquitin (1:500) in the absence and presence of free ubiquitin (25 μg/μL of undiluted antibody), and PBS (no antibody), as indicated. Visualization was with ^{125}I-protein A.

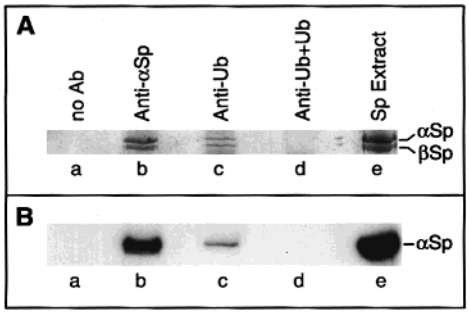

FIGURE 2: Immunoprecipitation of spectrin by anti-ubiquitin. Spectrin extract proteins were immunoprecipitated with anti-α spectrin (lane b) or with anti-ubiquitin in the absence (lane c) and presence (lane d) of free ubiquitin (1.5 mg/mL). The antibodies were omitted in the control mixture (lane a). Immunoprecipitates, as well as 3 μg of spectrin extract (lane e), were solubilized in loading buffer with 32 mM DTT for 5 min at 100 °C. The proteins were analyzed by SDS−PAGE on a 7% polyacrylamide gel followed by silver staining (panel A) and Western blotting (panel B). The blots were probed with anti-α spectrin (1:15 000) and visualized with ^{125}I-protein A.

B). This experiment showed that the DTT-sensitive modification in erythrocyte membrane α spectrin carried epitopes recognized by anti-ubiquitin polyclonal antibody. In addition to α′ spectrin, anti-ubiquitin revealed two bands with lower apparent molecular masses (Figure 1, panel B). These lower bands were not studied further.

The interaction between ubiquitin and spectrin was also demonstrated by immunoprecipitation. Spectrin extract proteins (extracted from erythrocyte membranes with low ionic strength buffer) immunoprecipitated by anti-ubiquitin or anti-α spectrin under stringent conditions were solubilized in the presence of DTT and analyzed by SDS−PAGE and Western blotting using anti-α spectrin as a primary antibody. Both Coomassie staining (Figure 2 , panel A, lane c) and Western blot (Figure 2, panel B, lane c) showed that anti-ubiquitin immunoprecipitated spectrin. Excess free ubiquitin

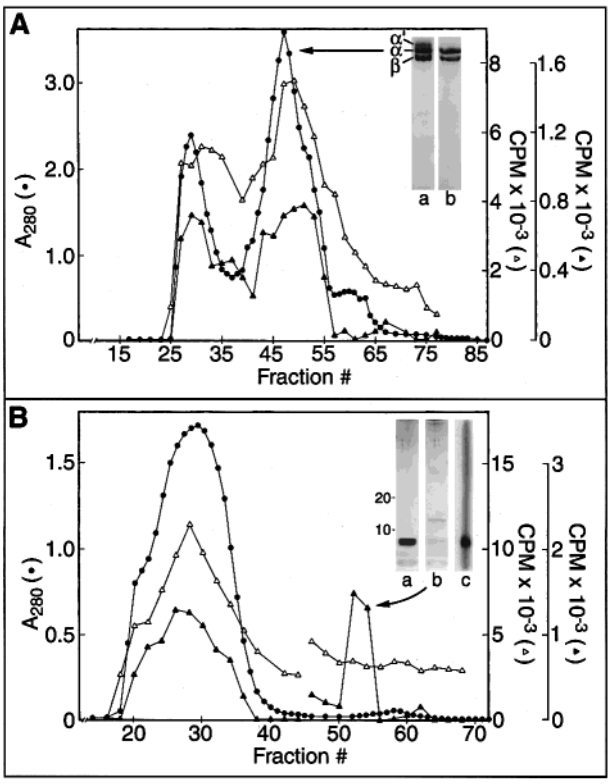

FIGURE 3: Separation of spectrin and a 8.5 kDa protein carrying ubiquitin epitopes. (A) 16 mL of spectrin extract (~190 mg) was chromatographed by gel filtration on a Sepharose CL-4B column (1.5 × 152 cm). 3.06 mL fractions were collected, and 7 μL aliquots were analyzed by immunodot assay. The dots were probed with (△) anti-α spectrin (1:15 000) or (▲) anti-ubiquitin (1:500) followed by ^{125}I-protein A and assayed for radioactivity in a γ counter. Spectrin heterodimer peak fractions (44−50) were combined. The insert demonstrates purified spectrin (fractions 44−50) separated by SDS−PAGE in the absence (a) or presence (b) of DTT. (B) 6 mL of the purified spectrin heterodimer (48 mg) was incubated with 32 mM DTT for 30 min at 37 °C and rechromatographed by gel filtration on a Sepharose CL-4B column (1.6 × 60 cm). 2.14 mL fractions were collected, and aliquots (15 μL for fractions 16−44, 150 μL for fractions 46−68) were analyzed by immunodot assay as in (A). Low molecular mass fractions 51−54 were combined (Ubl). Insert: 0.1 μg of commercial ubiquitin (a) and the Ubl fraction derived from ~1.5 mg (b) and ~35 mg (c) of DTT-treated spectrin were solubilized in the presence of DTT and separated by SDS−PAGE on a 15% polyacrylamide gel. The separated proteins were stained with silver (a, b) or electroblotted onto PVDF membrane (c). A 1.5 mm strip of the blotted membrane was probed with anti-ubiquitin (1:500) and vizualized with ^{125}I-protein A (c).

(1.5 mg/mL) prevented the reaction (Figure 2, lanes d). This experiment clearly demonstrates a covalent complex between α spectrin and the ubiquitin reactive protein.

Separation of Spectrin and Its Modification Carrying the Ubiquitin Epitope. Next we created a method to separate spectrin from this modification. Spectrin extract was chromatographed by gel filtration on a Sepharose CL-4B column to purify spectrin heterodimer. Fractions were analyzed by immunodot assay using anti-α spectrin and anti-ubiquitin as primary antibodies. Absorbance and immunostaining with spectrin antibody demonstrated (Figure 3, panel A) a typical distribution of spectrin between its oligomeric (fractions ~25−35) and heterodimeric (fractions ~43−55) fractions. As shown in Figure 3, panel A, immunostaining profiles with spectrin and ubiquitin antibodies are similar, indicating that spectrin and ubiquitin epitopes are bound to each other.

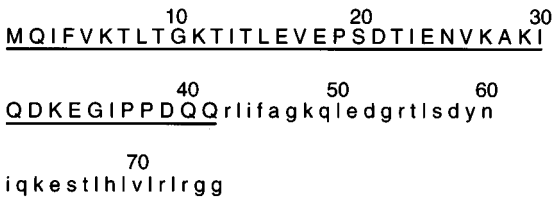

FIGURE 4: N-Terminal sequence of 8.5 kDa protein. The N-terminal sequence (41 residues) of 8.5 kDa protein, determined by Edman degradation, is compared with the complete (76 residue) sequence of ubiquitin (*29*) determined by mass spectrometry. Capital letters denote identical residues determined by automated sequencing.

Purified spectrin heterodimer (fractions 44−50, Figure 3, panel A inset), displaying reactivity to both spectrin and ubiquitin antibodies, was treated with DTT and rechromatographed by gel filtration on a smaller Sepharose CL-4B column. Fractions were analyzed by immunodot assay using spectrin and ubiquitin antibodies and Figure 3 panel B shows that spectrin and ubiquitin epitopes were separated. Ubiquitin antibody, but not α spectrin antibody, revealed a low molecular mass peak separated from the major spectrin peak. A significant portion of the reactivity to ubiquitin antibody remained bound to spectrin. It should be noted that in this experiment, unlike that presented in Figure 1, DTT treatment of spectrin was carried out without denaturing agents.

The combined low molecular mass immunoreactive peak (fractions 51−54, Figure 3, panel B) was analyzed by SDS−PAGE and Western blotting. Silver staining revealed two bands with apparent molecular masses of ~8.5 and ~13 kDa (Figure 3, panel B inset, lane b), and only the ~8.5 kDa band was recognized by ubiquitin antibody (Figure 3, panel B inset, lane c). Thus, the ~8.5 kDa protein carrying ubiquitin epitopes was separated from spectrin by gel filtration, following DTT treatment. N-Terminal sequencing of the 13 kDa peptide by Edman degradation yielded the following sequence: VIRVYIASSV. No matches to this sequence were found in the Swiss Protein Database. Therefore, the 13 kDa protein has not been previously studied and does not match to any known E1, E2, or E3 enzyme.

Identification of the ~8.5 kDa Protein. The ~8.5 kDa protein was identified as ubiquitin by two different methods: Edman degragation and mass spectrometry (MS). The protein, separated and transferred to the PVDF membrane, was sequenced by automated Edman degradation obtaining up to 41 residues from the N-terminus. The N-terminal sequence determined was identical to the published (*29*) ubiquitin sequence (Figure 4).

We obtained the remaining sequence for the ~8.5 kDa protein by LC/MS/MS experiments. For mass spectrometry the ~8.5 kDa protein was purified by reverse-phase HPLC (Figure 5 , panels A−C), and both the purified protein and the commercial ubiquitin (Figure 5, panel D) were digested with endoproteinase Lys-C (Figure 5, panel E). Both digests were analyzed by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). Typical results from the LC/MS/MS analysis are depicted in Figure 6. Panel A is the base peak (most abundant ion) trace. This chromatogram was constructed by plotting the base peak intensities in the conventional spectra versus elution time. The mass spectrum recorded at retention time 10.15 min as marked is shown in panel B as an example. The dominant ion in this spectrum

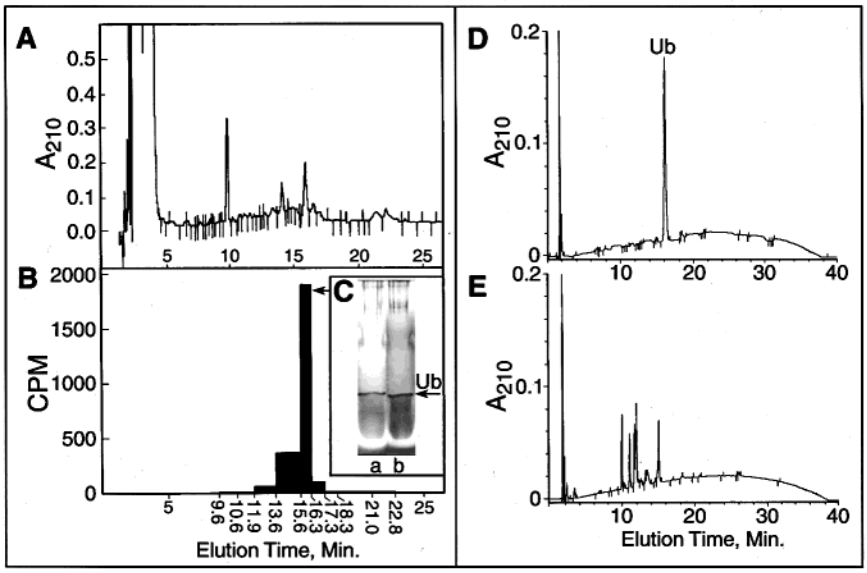

FIGURE 5: Isolation of 8.5 kDa protein. (A) The Ubl fraction (250 μL) derived from ~100 mg of DTT-treated spectrin was chromatographed by reverse-phase HPLC. Collected fractions, eluted as indicated between 9.6 and 22.8 min, were dried under vacuum, redissolved in 50 μL of 25 mM Tris-HCl, pH 8.5, and analyzed by immunodot assay (B) and SDS−PAGE (C). (B) Immunodot assay. 2 μL of the HPLC fraction, eluted between 15.6 and 16.3 min, and 5 μL of the other fractions were analyzed. The dots were probed with anti-ubiquitin (1:500) followed by ^{125}I-protein A and assayed for radioactivity in a γ counter. (C) SDS−PAGE. 2 μL of the HPLC fraction, eluted between 15.6 and 16.3 min (a), and 0.2 μg of commercial ubiquitin (b) were electrophoresed on a 15% polyacrylamide gel and stained with silver. (D, E) Reverse-phase HPLC of commercial ubiquitin (10 μg) before (D) and after (E) digestion with endoproteinase Lys-C for 48 h at 35 °C.

appears at *m*/*z* 895.2, which was more precisely measured by the slow/high-resolution scan resulting in the Zoom Scan spectrum in panel C. This spectrum was generated at 10.18 min. The precise mass/charge values of the three peaks in the center of this spectrum differ by half mass units (894.7 − 894.2 = 0.5 and 895.2 − 894.7 = 0.5), indicating that the ion is doubly charged. Thus, the mass of the ^{12}C monoisotopic ion is 2 × 894.2 = 1788.4, and the peptide's MW = 1786.4. The tandem mass spectrum in panel D was generated at 10.21 min by the dissociation of the doubly charged molecular ion at *m*/*z* 895.2. It exhibits extensive fragmentation and intense fragment ions that help identification. The first three hits of the automatic SEQUEST (*53*, *54*) search identified the peptide as the hexadecapeptide TITLEVEPSDTIENVK, a component of the protein ubiquitin from three different sources (*Homo sapiens*, *Quinqueloculina seminulum*, and *Drosophila melanogaster*).

Panels E−G present three other examples of tandem MS spectra obtained from peptide molecular ions *m*/*z* 835.1 (retention time 9.10 min), 890.7 (6.23 min), and 726.2 (10.87 min), respectively. Each of these peptides was declared by SEQUEST as a ubiquitin-specific peptide: ^{34}EGIPPDQQRLIFAGK^{48}, ^{49}QLEDGRTLSDYNIQK^{63}, and the C-terminal ^{64}ESTLHLVLRLRGG^{76}, respectively. The three peptides cover the whole amino acid chain from position 34 to the terminal 76 without any gap. Thus, the LC/MS/MS analysis provided an uninterrupted sequence for all of the 43 C-terminal residues, overlapping with the results of the Edman sequencing (34−41). These combined analytical techniques proved the identity of the 8.5 kDa protein as ubiquitin (Figure 4). It should be noted that the separation of intact ubiquitin from spectrin during DTT treatment excludes the possibility that the separation was due to contaminating protease activities.

Formation of Spectrin−Ubiquitin Adduct and Conjugate. We had analyzed erythrocyte α′ spectrin, a DTT-sensitive derivative of α spectrin synthesized in vivo, down to its constituent α spectrin and ubiquitin moieties. Ubiquitin does not contain any cysteine residue (see Figure 4). We hypothesized that the DTT-sensitive linkage between spectrin and ubiquitin might well be a thioester bond between ubiquitin's C-terminal glycine and an α spectrin cysteine residue(s). The formation of thioesters involving ubiquitin C-terminal glycine is mediated by ubiquitinating enzymes. Therefore, if our suggestion was correct, one could expect the ubiquitinating enzymes to mediate the formation of the characteristic thioester bond between ubiquitin and spectrin (spectrin−ubiquitin adduct). In the experiments described below we monitored the formation of the spectrin−ubiquitin adduct and/or conjugates (sensitive and insensitive to DTT, respectively) mediated by the ubiquitinating enzymes. As a source of ubiquitinating activities, erythrocyte lysate fraction II, containing ubiquitin-activating (E1) enzyme, conjugating (E2) enzyme, and ligase (E3), was added to the mixture containing spectrin and ^{125}I-ubiquitin. Washed erythrocyte membranes were used as a source of spectrin as well as other potential target proteins. After various times of incubation membranes were separated from soluble material by centrifugation. Reaction products associated with the membranes, and present in the supernatant, were analyzed separately by autoradiography following SDS−PAGE in the absence and presence of DTT. Time-dependent formation of two ubiquitinated bands corresponding to α′ and α spectrins was observed (between 10 and 60 min of incubation) when the reaction products were analyzed in the absence of DTT (Figure 7, panel B, lanes 1−6). In the presence of DTT (Figure 7, panel B, lanes 8−13), only the ubiquitinated band corresponding to α spectrin was observed, indicating that α′ and α spectrin bands represent ubiquitin adduct and conjugate, respectively. Both α spectrin derivatives were associated with the membranes (lanes 1−3 and 8−10) and were not found in the supernatant (lanes 4−6 and 11−13).

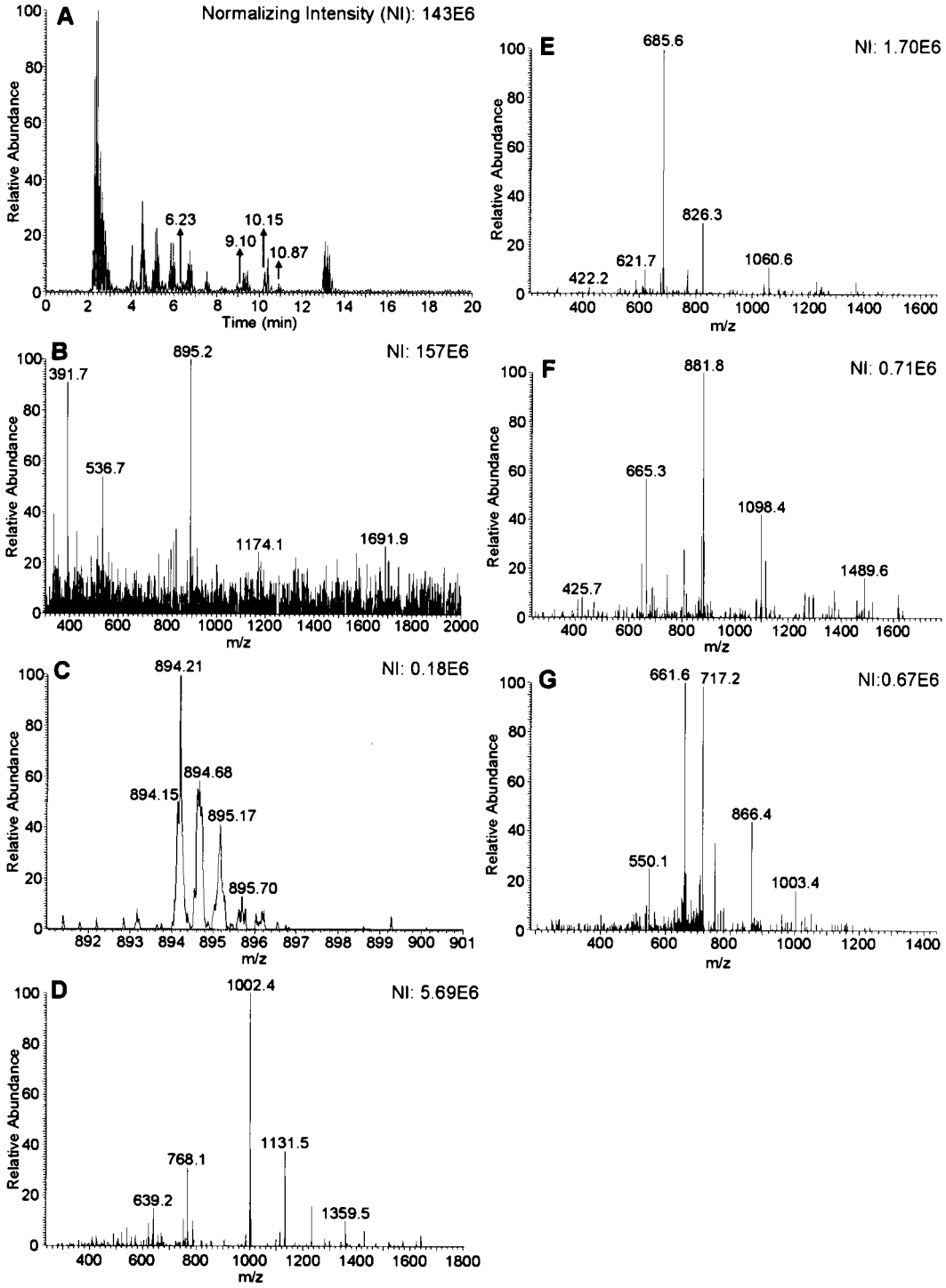

FIGURE 6: Base peak trace and sample mass spectra obtained in a triple play experiment on the digested 8.5 kDa protein. (A) Base peak trace: the intensities of the dominant ions in spectra versus retention time. (B) Full scan mass spectrum of the peptide eluting at 10.15 min. (C) Zoom scan across the dominant ion peak at *m*/*z* 895.2. (D) MS/MS spectrum of *m*/*z* 895.2. (E−G) MS/MS spectra of peptides with *m*/*z* 835.1 (retention time 9.10 min), 890.7 (6.23 min), and 726.1 (10.87 min), respectively.

No ubiquitin adduct or conjugate was observed in the absence of ATP (data not shown). This experiment demonstrated that erythrocyte spectrin can form both the ubiquitin conjugate and the DTT-sensitive adduct in the presence of fraction II and ATP.

Ubiquitin adducts, sensitive to reducing agents, are key intermediates of the ubiquitination process formed by E1, E2, and some E3 enzymes (*31*, *42*, *56*). The ability of spectrin to form an ubiquitin adduct raised the possibility that spectrin itself contained one or more of the ubiquitination enzymatic activities. In the experiments described below we determined whether spectrin was involved in the cascade of reactions conjugating ubiquitin to target proteins. The experiments, similar to those described above, were done with the following essential changes: the purified spectrin heterodimer, as well as erythrocyte membranes, was used as a spectrin source, and fraction II was replaced with purified (95% pure) commercial E1 enzyme. Analysis of reaction products in the absence of DTT (Figure 7, panel D) showed that ubiquitinated bands corresponding to α′ and α spectrins

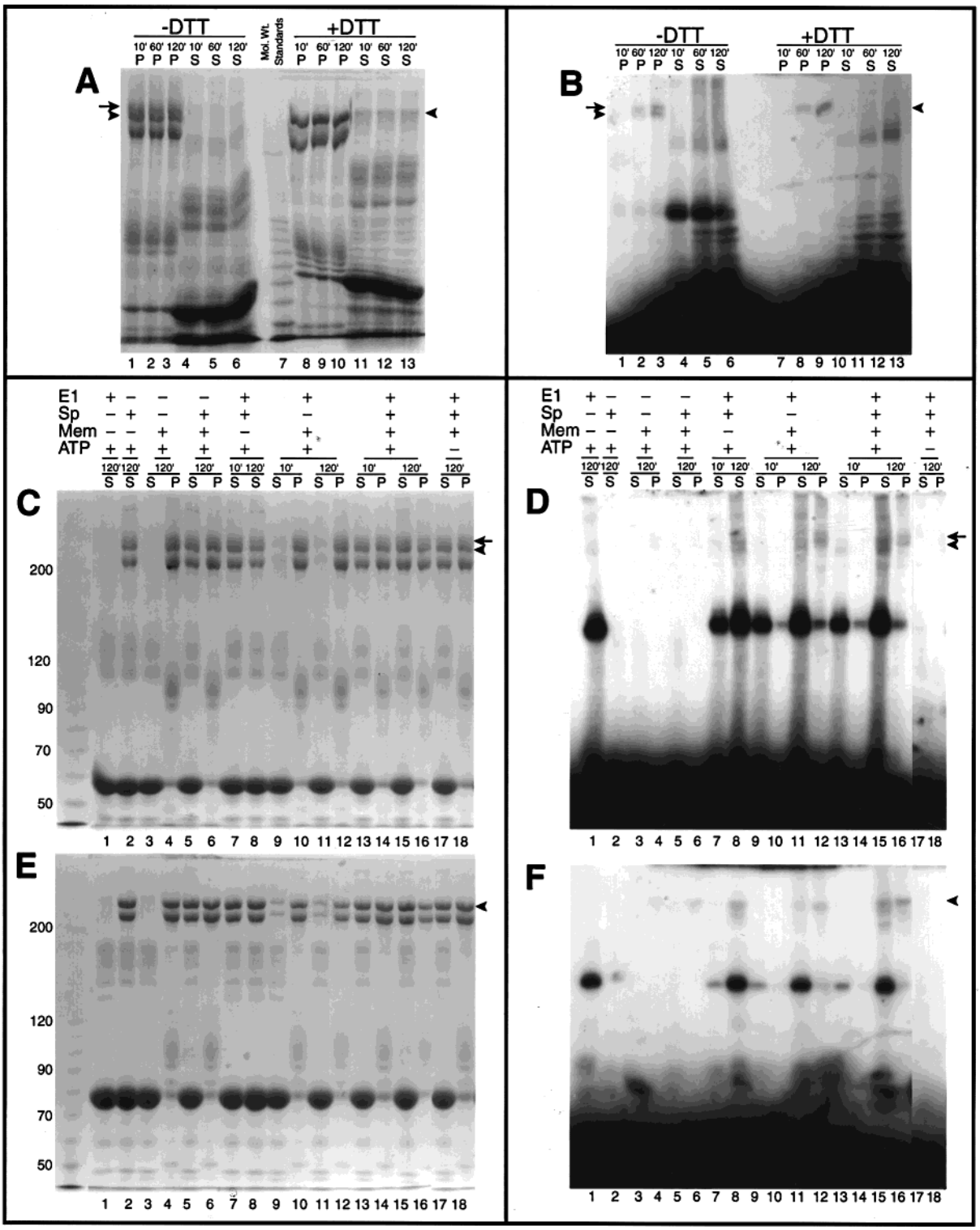

FIGURE 7: Formation of spectrin−ubiquitin derivatives. (A, B) Erythrocyte membranes (20 μg of protein) and 23 μg of fraction II were incubated with 3 μM ^{125}I-ubiquitin in a final volume of 30 μL at 37 °C (see Experimental Procedures). At the indicated time (10, 60, 120 min) the mixture was centrifuged at 16000*g* for 15 min at 4 °C, and the membrane pellets (P) and supernatant (S) were analyzed. The proteins were separated by SDS−PAGE on a 5% polyacrylamide gel (14 × 16 cm) in the absence (lanes 1−6) or presence (lanes 8−13) of DTT and stained with Coomassie blue R-250 (panel A). The gel was dried and autoradiographed (panel B). The Coomassie blue stained dry gel and autoradiograph were superimposed to identify radiolabeled bands. Lane 7 contains molecular mass standard proteins. (C−F) Erythrocyte membranes (20 μg of protein) and/or 5 μg of spectrin heterodimer, 0.1 μM E1 enzyme, and/or 3 mM ATP were incubated with ^{125}I-ubiquitin and samples processed as described in (A) and (B). For samples without membranes, only supernatant fractions (S) were analyzed. Proteins were electrophoresed in the absence (panels C and D) and presence (panels E and F) of DTT, stained with Coomassie blue (panels C and E), and autoradiographed (panels D and F). The arrow and arrowhead denote α' and α spectrins, respectively. Molecular masses of standard proteins are given in kilodaltons (panels C and E). The ubiquitinated band at ~130 kDa is the ubiquitinated form of the commercially available E1 (molecular mass ~120 kDa). Note its reduction in intensity in the presence of DTT.

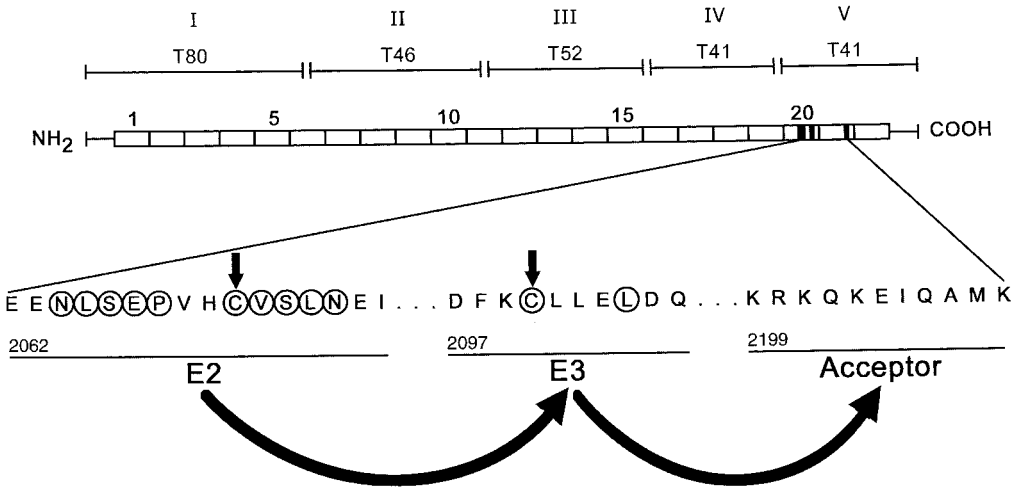

FIGURE 8: Working model of α spectrin's potential E2, E3, and acceptor sites. This model is based on our comparisons of the α spectrin sequence with known active site consensus sequences for E2 and E3 enzymes. All aspects of the model remain to be tested.

were formed (compare 10 and 120 min points) in the mixture containing E1 enzyme and spectrin, regardless of whether spectrin was added in the presence or absence of red blood cell membranes (lanes 7−16). In the presence of DTT (Figure 7, panel F), only the ubiquitinated band corresponding to α spectrin was observed (lanes 7−16), indicating that it represents a ubiquitin conjugate. α′ spectrin proved to be a DTT-sensitive ubiquitin adduct. Formation of both the ubiquitin adduct and conjugates was dependent on E1 enzyme and ATP (Figure 7, panels D and F). They were not observed in the absence of ATP (lanes 17 and 18) or E1 (lanes 2). The trace level of ubiquitination, observed in the mixtures lacking E1 but containing membranes as the spectrin source (Figure 7, panel F, lanes 4 and 6), was probably due to an E1 activity associated with the membrane. This E1 activity was not associated with spectrin itself, because no ubiquitination products were observed when E1 was replaced with the purified spectrin heterodimer (Figure 7, panel F, lane 2) in the absence of membranes.

Thus, E1 enzyme and ATP are both necessary and sufficient to form a DTT-sensitive spectrin−ubiquitin adduct, suggesting that spectrin possesses E2 activity. Consistent with this suggestion is our analysis comparing the α spectrin primary sequence to known E2 enzymes. α spectrin contains 20 cysteine residues (*3*) which might be capable of E2 or E3 activity. Inspection of sequences surrounding these residues for conservation of sequence and/or structure with known E2 (*45*, *69*) and E3 (*65*, *70*) enzymes revealed two segments of interest. Using computer programs COMPARE and PEPTIDE STRUCTURE of the Wisconsin GCG package (Version 10, 1999) and a structural prediction program, PROPSEARCH (*71*), to analyze the α-spectrin sequence indicated that a segment within spectrin repeat 20 would be a likely candidate for E2 enzymatic activity (see our structural analysis in Figure 8). Cysteine residue 2071 is surrounded by a sequence which conforms ∼70% (11 out of 16 residues) to the active site consenses sequence critical for E2 activities (*45*). Moreover, there is a cluster of aromatic residues within α spectrin positioned appropriately for the bipartite E2 motif (2118-YTWLT-2122) known to be key components of the E2 enzymatic activity. Although not as well conserved at the primary sequence level, a second region of interest surrounds cysteine residue 2100 which conforms to the cleft

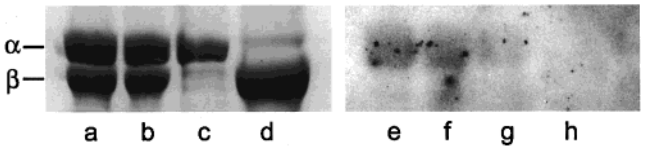

FIGURE 9: Ubiquitin of α spectrin and DTT-treated spectrin heterodimer. 10 μg of the spectrin heterodimer (lanes a and e), 10 μg of the DTT-treated spectrin heterodimer (lanes b and f), 5 μg of α spectrin (lanes c and g), or 8 μg of β spectrin (d and h) was incubated with 0.1 μM E1 enzyme and 0.6 μM ^{125}I-ubiquitin at 37 °C for 120 min in a final volume of 60 μL. Proteins were electrophoresed in the presence of DTT, stained with Coomassie blue (lanes a−d), and autoradiographed (lanes e−h).

structure surrounding the active site residues of E3 HECT domain enzymes (*46*, *70*). Finally, there is a cluster of lysine residues within spectrin repeat 21 (2199-KRKQKEIQAMK-2209) that could potentially contain an acceptor site for the ubiquitin molecule.

Therefore, α spectrin contains a 16 amino acid stretch in the α 20 repeat with 70% identity to the active site consensus sequence of known E2 ubiquitin-conjugating enzymes. Furthermore, in the presence of pure E1, ATP, and ubiquitin, α spectrin forms both a DTT-sensitive and DTT-insensitive linkage with ubiquitin. The most reasonable explanation for these results is that α spectrin possesses an E2 site at cysteine 2071 that is able to transfer ubiquitin (possibly via an E3 site) to an intramolecular target site (possibly in α spectrin repeat 21).

A less likely possibility is that our purified spectrin contains an attached small E2 conjugating enzyme which is becoming ubiquitinated in a DTT-sensitive manner and then transferring the ubiquitin to an α spectrin target site. This suggestion does not take into account the 70% identity between α spectrin residues 2062 and 2077 and the consensus active site sequence of known E2 enzymes (Figure 8), but we have tested it nonetheless.

In Figure 9 we demonstrate utilizing our cell-free ubiquitination assay that, in the presence of pure E1, ATP, and ^{125}I-ubiquitin, purified spectrin heterodimer (lanes a and e) and spectrin heterodimer stripped with DTT and repurified by gel filtration (lanes b and f) both form the ubiquitin−spectrin conjugate on SDS−PAGE plus DTT. As would be expected, the upper α′ spectrin band was present upon SDS−

PAGE minus DTT (data not shown). We conclude that any linkage between a hypothetical small E2 enzyme and spectrin would have to be DTT insensitive. Next we isolated α and β spectrin subunits by reverse-phase HPLC on a C4 column (see Experimental Procedures; Kakhniashvili and Goodman, manuscript submitted). Homogeneous α spectrin monomer in the presence of pure E1, ATP, and ^{125}I-ubiquitin formed the ubiquitin−spectrin conjugate (lanes c and g) on SDS−PAGE plus DTT. Once again, the upper α′ spectrin complex was present on SDS−PAGE minus DTT (data not shown). Finally, as would be expected from previous work, purified β spectrin does not become ubiquitinated (*28*, *49*, *66*) in our cell-free system (lanes d and h). Therefore, any small E2 attached to purified spectrin would have to be attached by a DTT-insensitive linkage (Figure 9), be present in stoichiometric amounts with spectrin (Figure 1), copurify through gel filtration chromatography and reverse-phase HPLC (Figure 9), be associated strictly with the α subunit (Figure 9), and be present in addition to α spectrin's endogenous E2 sequence (Figure 8). This possibility is remote. The most plausible explanation consistent with all of the data is that α spectrin contains its own E2 ubiquitin-conjugating activity.

DISCUSSION

In this study we demonstrate that erythrocyte spectrin is able to (a) accept activated ubiquitin from an E1 enzyme and form a DTT-sensitive spectrin−ubiquitin adduct and (b) transform the adduct into the spectrin−ubiquitin conjugate insensitive to reducing agents (conjugate ubiquitin to itself). Spectrin's E2 activity may also be able to conjugate ubiquitin to other yet unidentified proteins.

It is asumed that the target specificity of the ubiquitination system is determined at least in part by the E2 and E3 enzyme classes. The E2s, ubiquitin carrier/conjugating enzymes, represent a superfamily of related proteins usually of 15−35 kDa molecular mass. Members of the class were functionally defined by their ability to directly accept activated ubiquitin (thioester) from an E1 enzyme to form the corresponding E2−ubiquitin thioester.The criterion of direct thioester acceptor from E1 also identified an unusually large E2 (57), a 230 kDa protein (E2−230K) required for remodeling of the erythroid cell during terminal differentiation (*58*), and a giant E2, a 528 kDa endomembrane associated protein named BRUCE (*59*). By this criterion, we now identify spectrin as an E2 enzyme.

Structurally, E2s are related proteins comprising a highly conserved ∼150 residue domain, the UBC domain with a specific "active site" cysteine residue. Some E2s consist of the UBC domain only (class I); others possess C-terminal (class II), N-terminal (class III), or both C- and N-terminal (class IV) extensions (*59*−*61*). E2-like proteins with noncanonical UBC domains were also reported (*56*, *62*). These E2s, lacking active site cysteine, are unable to accept ubiquitin and directly conjugate it to substrate. The ability of spectrin to accept ubiquitin from E1 indicates that it contains a normal UBC domain.

In most cases, the interaction between E2 and the target protein is mediated by an E3 enzyme (*31*, *42*, *56*). It appears that distinct E3s interact with a subset of E2s, suggesting that specific E2/E3 tandems might contribute to the specificity of protein ubiquitination. In general, E3 binds to both E2 and the target protein, bringing them into close proximity. Ubiquitin is then transferred from E2 to the substrate either directly or via a E3−thioester intermediate. E3 enzymes, members of the family of the HECT domain proteins (*46*), can form thioester intermediates with ubiquitin and conjugate it to substrate (*47*). The HECT domain proteins have a modular structure consisting of the C-terminal HECT domain and different N-terminal extensions. The HECT domain is a conserved ∼350-residue catalytic domain with active site cysteine and by its function is similar to the UBC domain of E2s. Interestingly, the overall organization of BRUCE, a giant E2 with a large N-terminal extension and a C-terminal ubiquitin-accepting domain, more resembles an E3 enzyme with a HECT domain, suggesting that BRUCE may combine E2- with E3-like properties (*59*). Another enzyme with suggested E2- and E3-like functions is E2−230K (*57*). This large E2 contains two active site cysteines, suggesting that ubiquitin conjugation might be mediated by an intramolecular thioester cascade. We do not know, as of yet, whether spectrin can transfer ubiquitin directly from its E2 site to its target site or, alternatively, also contains an E3 site cysteine. Sequencing of the E2−230K enzyme did not demonstrate identity with any known protein (including α spectrin) (*58*).

The ability of spectrin to ubiquinate itself is not unique. Under some conditions, the catalytic intermediates formed by some ubiquitinating enzymes are able to transfer ubiquitin from an active site thiol to a nearby lysine (*63*, *64*). A function for these reactions, including spectrin autoubiquitination, is not yet established, though it was shown that Mdm2 protein, a so-called RING finger-dependent E3 enzyme, mediates its own ubiquitination in vitro and proteasome-dependent degradation in cells (65).

Corsi et al. (*66*) reported ubiquitination of erythrocyte spectrin dependent on an $E2_{14kDa}$ conjugating enzyme. Two sites located on protease-resistant domains III and V of α spectrin (*67*) were reported to be targets for ubiquitination (*68*), placing them at a considerable distance (ca. ∼1000 amino acids) from each other. It is not clear if these two sites are targets for two different ubiquitin-conjugating enzymes. Our data support the hypothesis that at least one of the ubiquitination sites within α-spectrin is modified by an intramolecular reaction. This would require the molecule to possess an E2 enzymatic activity, which transfers ubiquitin, via a thioester intermediate on α spectrin cysteine residues, from an activated E1 complex to an ϵ-amino linkage with lysine residue(s) within domain III and/or V. Our structural analysis of α spectrin indicates a likely E2 site at cysteine 2071, a possible E3 site at cysteine 2100, and a possible target site within residues 2199−2209 (Figure 8). These sequence motifs are in close proximity (∼150 residues) to one another and, importantly, are located in repeats 20 and 21 within domain V of the α spectrin molecule, one of the positions known to be ubiquitinated (*68*). Interestingly, utilizing recombinant peptides, Galuzzi et al. (*92*, *93*) have recently demonstrated two ubiquitination sites within α spectrin: one in α spectrin repeat 17 and the other in α spectrin repeats 20/21 (consistent with our computer prediction). While the lysine involved in the α spectrin repeat 17 linkage has been identified (*93*), the residue(s) involved in ubiquitin linkage to α spectrin repeats 20 and 21 remain(s) to be established. Thus, a reasonable possibility is that the ubiquitination of the erythrocyte α-spectrin is accomplished

by an intramolecular mechanism with cysteines 2071 and 2100 representing DTT-sensitive thioester linkages of E2 and E3 conjugating and ligating activities and a lysine cluster within repeat 21 as an acceptor site. We hasten to say that we do not know (1) whether spectrin requires an E3 ligating enzyme site to target itself (or other proteins) or (2) whether our computer search has identified the biologically appropriate cysteines and lysine. But this hypothetical model can be appropriately tested in the future by mutating these cysteine and lysine residues.

Recently, we reported that a DTT-sensitive modification of spectrin was present in high- and low-density sickle cells to a much lower extent than in normal erythrocytes (*28*, *72*). Now we know that the modification is a spectrin−ubiquitin adduct, an E2−ubiquitin intermediate indicating that sickle cells and normal erythrocytes differ in ubiquitin-conjugating activity associated with spectrin. This result, in hindsight, could be predicted from the following two sets of published data. First, sickle cells are under extreme oxidative stress. They have an abnormally high level of oxygen radicals (*73*, *74*) and a diminished level of reduced glutathione (GSH), an intracellular protector against oxidative stress (*75*, *76*). Upon oxidative stress the cells show a concomitant increase in the ratio of oxidized glutathione (GSSG) to GSH and cell density, with maximum GSSG/GSH values in ISCs (*77*) where failure to protect membrane proteins is particularly noticeable (*78*). Second, cellular redox status, increasing GSSG/GSH ratio, was shown to modulate protein ubiquitination in intact retina and retinal pigment epithelial (RPE) cells via S-thiolation of E1 and E2 enzymes, presumably by glutathiolation (*79*). Thiolated E1 and E2 enzymes are unable to form ubiquitin intermediates resulting in decreased levels of the ubiquitin intermediates and protein−ubiquitin conjugates. Thus, the increased GSSG/GSH ratio in sickle cells would be predicted to decrease the activity of ubiquitination system, and it does (*28*).

The ubiquitination activity of spectrin could play a profound role in erythropoiesis. Terminal differentiation of erythroid progenitor cells (defined as the point at which hemoglobin begins to be synthesized) includes the restructuring of the plasma membrane with the biogenesis of the erythroid membrane skeleton. Development of the membrane is not governed solely by regulation of expression of the constituent proteins followed by simple self-assembly. Rather it is a dynamic process with asynchronous synthesis and degradation of constituent proteins. Prior to terminal differentiation, the proteins of the membrane skeleton assemble only transiently into a membrane skeleton that is then degraded (*80*). During terminal differentiation some of these proteins are synthesized in excess, with one fraction assembling into a membrane skeleton but the other fraction being targeted for degradation (*81*, *82*). Turnover of spectrin subunits in developing erythroblasts is specific: β spectrin is degraded extremely rapidly ($t_{1/2} \approx 15$ min), whereas α spectrin is turned over more slowly ($t_{1/2} \approx 2$ h) (*82*), though it is synthesized in excess to β spectrin (*83*−*85*). α spectrin could play the role of the E2 (and maybe E3) enzyme and be a target of the ubiquitination system, thereby regulating turnover of membrane skeleton proteins during erythropoiesis. Deficiency in such spectrin ubiquitinating activity, as in sickle cells, could lead to excessive accumulation of oxidatively damaged proteins causing abnormal development of the membrane skeleton. This mechanism may lead to early removal of erythropoietic cells where the oxidative damage is beyond repair.

Mature erythrocytes have no ubiquitin- and ATP-dependent protein degradation but maintain significant levels of ubiquitin conjugates (*86*−*88*). In these cells spectrin-mediated ubiquitination activity could have an essential impact upon protein−protein interactions. Therefore, it is interesting to compare the ubiquitination sites within α spectrin repeats 20/21 to known functional regions. Spectrin heterodimer assembly, from α and β subunits, is initiated by a nucleation site involving α spectrin repeats 18−21 and β spectrin repeats 1−4 (*94*). The initial nucleation event begins by the α 21 repeat interacting with β1, then α20 with β2, and so on in a zippering mechanism (*94*). In other words, the α spectrin ubiquitination sites in repeats 20/21 fall directly within the heterodimer nucleation sites of α spectrin and may regulate heterodimer assembly.

The protein 4.1 binding domain (residues 207−445) (*95*) and adducin binding domain (residues 1−528) (*96*) contain all or part of the nonhelical N-terminal region of β spectrin plus all of β1 and part (or all) of the β2 repeat. This would put the 4.1 and adducin binding domains (β1 and β2 repeats) in direct contact with the α spectrin ubiquitination sites in α repeats 20/21. Therefore, α spectrin ubiquitination may regulate formation of the spectrin−4.1−actin and/or spectrin−adducin−actin ternary complex. This could offer a partial explanation for the difference in the ability of ternary complexes to disassemble when formed with spectrin isolated from normal or irreversibly sickled red blood cells (*24*).

No enzymatic activity was previously reported for erythrocyte spectrin or any member of the spectrin family. Is ubiquitin-conjugating activity a specific property of erythrocyte spectrin? Our initial study in hippocampus indicates that neuronal α SpI and α SpII are ubiquitinated in a DTT-insensitive covalent complex (*50*), but we do not yet know whether brain spectrins can serve as E2 enzymes in other nonerythroid cell types. As spectrin makes up 2−3% of the total protein in nonerythroid cells (*1*), it could potentially be a primary E2 enzyme for many ubiquitinated target proteins.

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