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Identification of α-Spectrin Domains Susceptible to Ubiquitination*

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Previously, we demonstrated that α-spectrin is a substrate for the ubiquitin system and that this conjugation is a dynamic process (Corsi, D., Galluzzi, L., Crinelli, R., and Magnani, M. (1995) *J. Biol. Chem.* 270, 8928–8935). In this study, we mapped the sites of ubiquitination on erythrocyte α-spectrin. A peptide map of digested α-spectrin, previously submitted to *in vitro* ^{125}I-ubiquitin conjugation, revealed the presence of four distinct labeled bands with M_r 40,000, 36,000, 29,000, and 25,500. Western blotting experiments using antibodies against each α-spectrin domain revealed that only IgG anti-αIII domain recognized the ^{125}I-labeled ubiquitin peptide of 29 kDa, whereas the IgG anti-αV domain recognized the M_r 40,000 ^{125}I-ubiquitin-labeled peptide. The other two labeled bands of M_r 36,000 and M_r 25,500 were identified as tetra and tri multiubiquitin chains. Ubiquitination of the αIII and αV domains was further confirmed by anti-α-spectrin domain immunoaffinity chromatography. Endoprotease Lys C-digested spectrin conjugated previously to ^{125}I-ubiquitin was incubated with antibodies against each trypsin-resistant domain of α-spectrin. Gamma counting of the radiolabeled antigen-antibody complexes purified by protein A chromatography showed labeling in the IgG anti-αIII and anti-αV complexes alone. Domain αIII is not associated with any known function, whereas domain αV contains the nucleation site for the association of the α and β chains. Ubiquitination of the latter domain suggests a role for ubiquitin in the modulation of the stability, deformability, and viscoelastic properties of the erythrocyte membrane.

Ubiquitin (Ub),[1] a 76-amino acid protein, has been found both free and covalently bound to target proteins via an isopeptide linkage between the carboxyl group of the terminal glycine moiety of Ub and free ε amino groups on the target protein (1). Rabbit reticulocyte fraction II (2) (protein adsorbed to DEAE 52-cellulose and eluted with 0.5 M KCl) contains the enzymatic system (E1, E2 s, E3 s) that is involved in ubiquitin conjugation. Usually, a protein can have one or more sites for ubiquitin, in which one ubiquitin or multiubiquitin chains can be linked. In the latter case, one Ub is linked to the lysine 48 of another ubiquitin bound to the substrate (3). Ub-conjugated proteins can either be degraded to small peptides by a large 26 S ATP-dependent protease complex, or the Ub moiety can be removed by Ub isopeptidases, releasing ubiquitin and the intact protein (4). Protein ubiquitination is a posttranslational process involved not only in protein degradation but also in other cellular functions. In fact, many studies have reported the *in vivo* presence of several stable Ub-protein conjugates that are not subject to degradation (5, 6). Moreover, another linkage in which ubiquitin is linked to a previously bound Ub involves lysine 63 of Ub, and these chains serve nonproteolytic functions (7). In general, ubiquitin is involved in different cellular processes such as transcriptional regulation (8), cell cycle regulation (9), stress responses (10), and modulation of the immune response (11). In particular, in the last decade Ub has been found to be bound to many specific substrates such as lysozyme (12), phytochrome (13), actin (14), histone (15), calmodulin (16), cAMP-dependent protein kinase (17), p53 (18), ABC-transporter Ste 6 protein (19), c-jun (20), transducin (21), T-cell antigen receptor (22), nuclear factor-κB1 precursor (23), inhibitor of κB-α (24), platelet-derived growth factor-β, and α receptors, and epidermal growth factor, colony-stimulating factor-I, fibroblast growth factor (25), cystic fibrosis transmembrane conductance regulator (26), and estrogen receptors (27), as well as others.

Recently, we demonstrated that red blood cell α-spectrin is also a specific substrate for ubiquitination (28). α- and β-spectrin are the major protein constituents of the red blood cell membrane skeleton and contribute to about 25% of total red cell membrane proteins. This membrane skeleton provides support for the overlying lipid bilayer and contributes to the viscoelastic properties and deformability of the membrane (29).

The spectrin molecule is composed of two subunits with apparent molecular masses of 240 kDa (α-spectrin) and 220 kDa (β-spectrin), intertwined side-to-side to form a heterodimer. The two α and β subunits of spectrin are different not only in molecular mass but also in constitution. In fact, the α-spectrin chain consists of series of 22 repeats of 106 residues, whereas the β chain consists of 17 repeats of 106 residues (30). The tryptic digestion of spectrin gave evidence of a linear disposition of trypsin-resistant domains (31). Five domains were defined on the α chain (αI to αV from the N terminus) and four on β chain (βI to βIV from the C terminus). The heterodimer α-β assembly requires a specific nucleation site located in the αV and βIV domains (32). A self-association between the N-terminal region of the α chain and the C-terminal region of the β chain is involved in the formation of a tetramer, which is the predominant form in the erythrocyte (33).

Moreover, spectrin forms noncovalent associations with other proteins of the cytoskeleton, such as band 2.1 (34), band 4.1, and actin (35). Other proteins such as adducin, tropomyosin, tropomodulin, and dematin function as accessory proteins of spectrin-actin junctions and are probably involved in the stabilization of spectrin-actin complexes (36). Furthermore,

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[1] The abbreviations used are: Ub, ubiquitin; E1, Ub activating enzyme; E2, Ub carrier protein; E3, Ub-protein isopeptide ligase; meUb, methylated Ub; PAGE, polyacrylamide gel electrophoresis; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; PMSF, phenylmethylsulfonyl fluoride.

spectrin and protein 4.1 interact through phosphatidylserine with the inner leaflet of the lipid bilayer (37).

In an attempt to gain insight into the potential biological role of α-spectrin ubiquitination, we searched for the site(s) of ubiquitination present on α-spectrin. The data reported in this study show that ubiquitination occurs on the αIII and αV domains of α-spectrin, suggesting that at least ubiquitination of the αV domain can play a role in cytoskeleton stability mediated by the α-β-spectrin nucleation site.

EXPERIMENTAL PROCEDURES

Materials—Ubiquitin, chloramine T, and many biochemical reagents were obtained from Sigma. Reticulocyte fractions were prepared as reported previously (28). Immobilized protein A was obtained from Pierce. The ECL Western blotting detection reagents, Hybond N nitrocellulose and carrier-free $Na^{125}I$, were from Amersham Corp. Endoprotease Lys C was from Boehringer Mannheim.

Ubiquitin Labeling—Reductive methylation of ubiquitin was carried out as described by Hershko and Heller (38). Native ubiquitin and methylated ubiquitin (meUb) were radiolabeled with carrier-free $Na^{125}I$ (Amersham Corp.) by the chloramine-T method (39). The specific activity obtained was 9400 cpm/pmol of ubiquitin for ^{125}I-Ub and 9000 cpm/pmol of ubiquitin for ^{125}I-meUb.

Electrophoresis and Western Blotting—SDS-polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli (40) as reported previously by Corsi *et al.* (28).The molecular mass standards used were 94, 66, 45, 31, 21, and 14 kDa (Pharmacia Biotech Inc.). Thirty-five μg of sample protein were loaded for each lane, unless otherwise indicated.

The gels were electroblotted according to Towbin *et al.* (41) using Hybond N nitrocellulose. Blots involving ^{125}I-Ub spectrin peptides were first dried and then exposed to obtain an autoradiographic film of the nitrocellulose. After membrane rehydration, the different lanes were cut and incubated with different polyclonal IgG against each α-spectrin domain (42).

Goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) was used at a 1:3000 dilution as a second antibody. Enhanced chemiluminescence (ECL; Amersham Corp.) was used as the detection system.

Assay of Ubiquitin Conjugation—Human red blood cell membranes were prepared from healthy volunteers according to Corsi *et al.* (28). The conjugation of ^{125}I-Ub to red cell membrane proteins was assayed as described previously (28) using 5 μM ^{125}I-Ub or ^{125}I-meUb (final concentration) for each incubation mixture and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) as antiproteolytic agent. The conjugation of ^{125}I-Ub to brain fodrin was assayed in a similar way.

Crude Spectrin Extraction—After 120 min of incubation at 37 °C to permit ^{125}I-Ub conjugation, the reaction mixture was centrifuged in an Eppendorf microcentrifuge at 16,000 × *g* for 15 min at 4 °C. The supernatant was removed, and the pelleted membranes were washed twice with phosphate-buffered saline, pH 7.4, containing 1 mM PMSF. The membranes were then washed an additional two times with a low ionic strength buffer containing 0.3 mM Na_2HPO_4, pH 8.5, 0.1 mM EDTA, 1 mM PMSF, and 0.2 mM AEBSF (extraction buffer) (43). The pellet thus obtained was resuspended in a small volume of the extraction buffer (1 ml/5 mg of membrane protein) and incubated at 37 °C for 30 min. The samples were then centrifuged at 200,000 × *g* in a SW 65 rotor for 30 min at 4 °C, and the supernatant was collected. This fraction, primarily ^{125}I-Ub-α-spectrin, α-spectrin, and β-spectrin chains, actin, and traces of band 4.1, was referred to as "crude spectrin." Total protein was adjusted to 1 mg/ml with extraction buffer. Before protein determination, crude spectrin was stored on ice at 4 °C overnight in 50 mM NaCl and 5 mM Na_2HPO_4, pH 8.0 (final concentration).

Isolation of Ubiquitinated α-Spectrin Peptides—Crude spectrin extracted as above was dialyzed overnight at 4 °C using dialysis tubing with a molecular weight cut-off of M_r 3500 against 2000 volumes of 10 mM Tris-HCl, pH 8.5, and 1 mM EDTA to eliminate PMSF and AEBSF. Five μg of endoprotease Lys C were resuspended in 50 μl of H_2O and added 1:10 (μl/μg of protein sample) to crude spectrin. The digestion was for 1.5 or 3 h at 28 °C and was stopped by adding 0.2 mM AEBSF and sample buffer 1:1 (v/v), after which the sample was boiled for 5 min. Thirty-five μg of protein sample were used for each lane of SDS-PAGE, and the relative autoradiogram of the gel was obtained. Quantitative determinations of radioiodinated proteins were performed by direct counting of the excised bands using a Beckman 5500 gamma counter. One hundred and seventy-five μg of spectrin digestion products (35 μg for five lanes) were used for Western blotting analysis. The nitrocellulose was used first to obtain the autoradiograms and subsequently was cut in five lanes and probed with IgG specific for each of the five different domains of α-spectrin.

Immunoprecipitation of ^{125}I-Ub-α-Spectrin Peptides with Different IgG Anti-α-Spectrin Domains—^{125}I-Ub was conjugated to human red blood cell membranes for 2 h at 37 °C in the presence of rabbit reticulocyte fraction II as described by Corsi *et al.* (28). Spectrin extraction and digestion were performed as described above. The digestion was stopped with 0.2 mM AEBSF, 1% (w/v) SDS, and boiling for 10 min. The sample was diluted 100-fold with 10 mM Tris-HCl, pH 7.5, divided into 5 aliquots, and then separately incubated with different antibodies against each domain of α-spectrin (αI to αV) previously buffered in 10 mM Tris-HCl, pH 7.5 (final concentration). A 5.5-fold excess of specific anti-α-spectrin-domain IgG (mol/mol) was used to form the antigen-antibody complex. The antigen-antibody mixtures were left for 2 h at room temperature and then overnight at 4 °C with gentle agitation. Five ml of immobilized protein A were equilibrated with 10 volumes of 10 mM Tris-HCl, pH 7.5, then with 3 volumes of the same buffer plus 1 mg/ml bovine serum albumin, and finally with 10 volumes of 10 mM Tris-HCl, pH 7.5. The protein A suspension was divided into 1-ml aliquots, each of which was incubated overnight at 4 °C with the different mixtures of antigen-antibody complexes formed as above. The 5 aliquots of protein A suspension with bound antigen-IgG complexes were packed in five different columns and washed with 10 volumes of 10 mM Tris-HCl, pH 7.5, until protein determination at 280 nm was zero. Five different eluates were obtained from the columns with 0.2 M glycine, pH 2, and then counted in a gamma counter.

Non-Erythroid Spectrin-like Protein Preparation—α- and β-fodrin from bovine brain were purified as reported by Davis and Bennet (44) and kindly provided by Dr. B. Geny (INSERM U 322, Paris, France). The purified fodrin was left at 0 °C in 1 M NaBr, 10 mM Tris-HCl, pH 8.2, 1 mM EGTA, 15 mM $Na_4P_2O_7$, 1 mM NaN_3, 1 mM dithiothrietol, and dialyzed against 1000 volumes of 50 mM Tris-HCl, pH 7.5, 0.2 mM AEBSF at 4 °C for 5 h immediately prior to use.

Other Determinations—Protein concentrations were determined by the method of Bradford (45) using bovine serum albumin as standard or spectrophotometrically at 280 nm.

RESULTS

Isolation of Ubiquitinated α-Spectrin Peptides—α-Spectrin is one of the most abundant membrane proteins (12.5%) in the erythrocyte membrane (29). It is known that it can be digested with trypsin into five trypsin-resistant domains of different molecular weights known as αI, αII, αIII, αIV, and αV (31). In an attempt to identify the site(s) of ubiquitination on membrane α-spectrin, we first performed an *in vitro* ubiquitination assay using human erythrocyte membrane fraction II as a source of ubiquitin-conjugating enzymes and ^{125}I-Ub. After two washing steps, crude spectrin was extracted as described under "Experimental Procedures." In a first approach, extracted crude spectrin was digested with trypsin at 1 mg/ml, 1:50 (μl/μg of crude spectrin). Unfortunately, trypsin digestion of ^{125}I-ubiquitinated spectrin produces many bands with low labeling radioactivity. Furthermore, trypsin was also found to be able to digest ^{125}I-Ub itself (46) and to cut polyubiquitin chains (data not shown). Thus, spectrin peptide patterns were produced using endoprotease Lys C instead of trypsin, and all of the data reported hereafter in this report were obtained with this proteolytic enzyme. The production of digested peptides was time-dependent. Endoprotease Lys C did not cut ubiquitin and produced four highly ubiquitinated bands of low molecular weight (M_r 40,000, 36,000, 29,000, and 25,500) as detected by autoradiography (Fig. 1*B, lanes 1* and *2*). Quantitative determinations obtained by gamma counting of excised radiolabeled bands showed that the M_r 36,000 and 25,500 peptides had an associated radioactivity four and three times higher than the M_r 40,000 and 29,000 bands (Fig. 1*C*).

Identification of α-Spectrin Ubiquitin Binding Sites—To identify the site(s) of ubiquitination on erythrocyte α-spectrin, we performed the experiment represented in Fig. 2*A*. The conjugation of ^{125}I-Ub to α-spectrin was obtained in a cell-free system using fraction II as a source of ubiquitin-conjugating

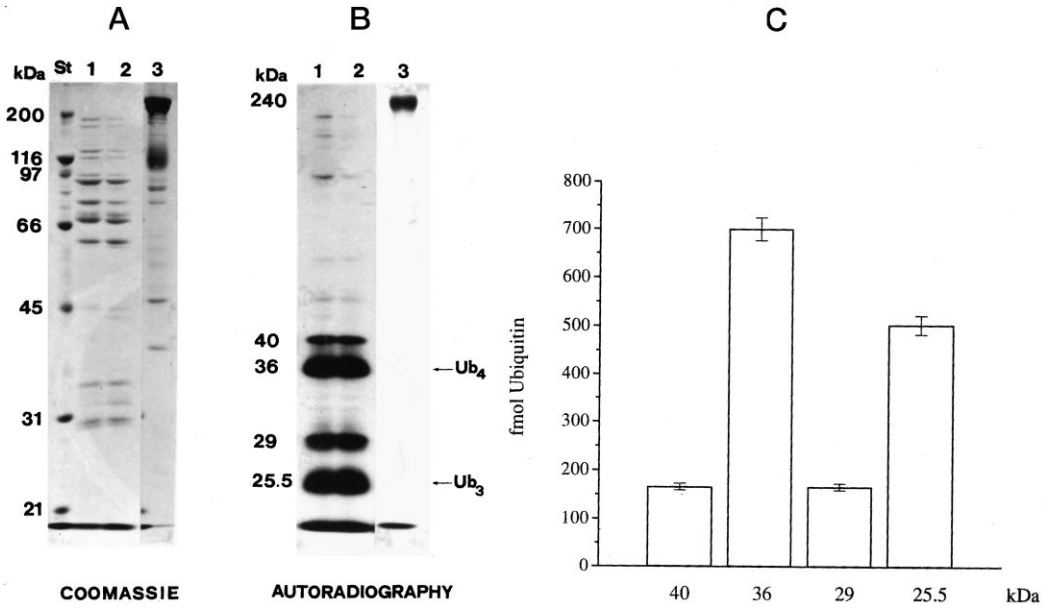


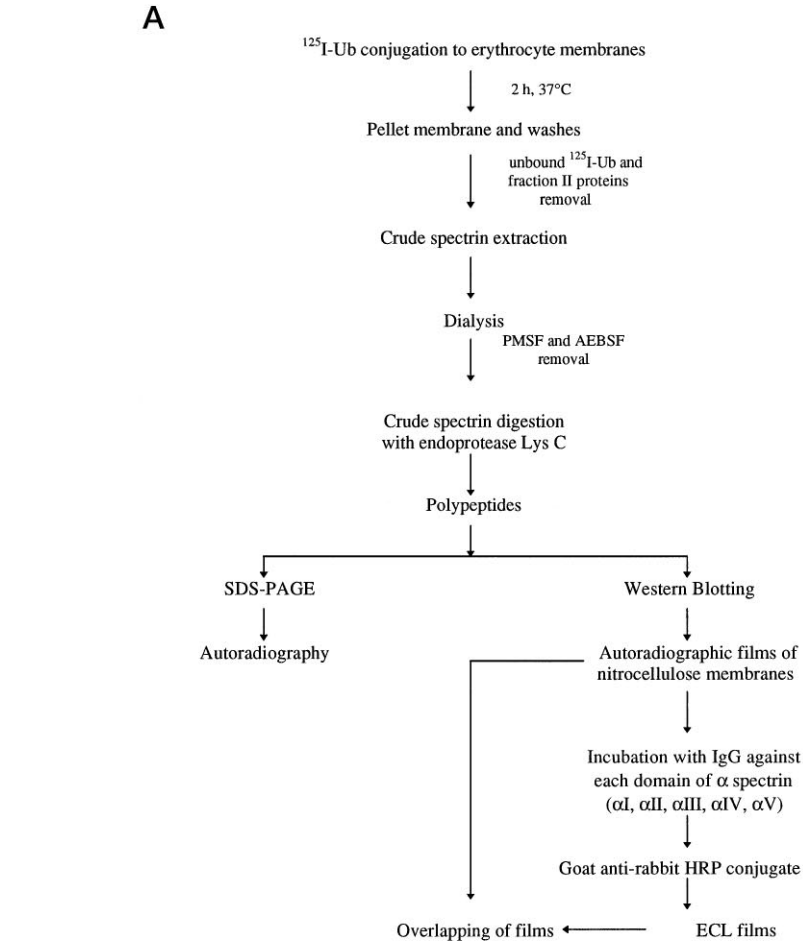
FIG. 1. **Monodimensional peptide mapping of ubiquitinated α-spectrin.** ^{125}I-Ub was conjugated to human erythrocyte membranes in an *in vitro* assay. Extracted crude spectrin was digested with endoprotease Lys C for 1.5 h (*lane 1*) or 3 h (*lane 2*). The digestion products were separated by SDS-PAGE in an 11% polyacrylamide gel and stained with Coomassie Blue R-250 (*A*). The radiolabeled polypeptides were detected by autoradiography of the dried gel (*B*). The bands corresponding to the radiolabeled peptides obtained using endoprotease Lys C were excised from the gel and counted in a gamma counter (*C*). The histogram shows the amount of absolute ubiquitin present in each band of different molecular weights (*left*). The values are the mean of five experiments ± S.D. Molecular size standards (*St*) are indicated in thousands (*kDa*). SDS-PAGE and relative autoradiogram of erythrocyte membranes (50 μg), in which α spectrin is ^{125}I-ubiquitinated, are shown in *lane 3*.

enzymes and spectrin extraction from the membrane was performed as described above. ^{125}I-Ub-α-spectrin was submitted to endoprotease Lys C digestion. One fraction of the sample (35 μg) was analyzed by SDS-PAGE and autoradiographed, whereas another fraction of the sample was divided into five aliquots (each of 35 μg), processed for SDS-PAGE on five different lanes, and Western blotted. The nitrocellulose membrane was dried and autoradiographed (Fig. 2*B, odd numbers*). The five lanes were then cut and probed separately using five different antibodies against each domain of α-spectrin (Fig. 2*B, even numbers*). The autoradiograms of the nitrocellulose membranes and the films obtained by ECL were then overlapped to observe the relative positions of the antibody-recognized peptides compared to those of the radiolabeled peptides. The autoradiogram of the gel (Fig. 2*B, lane B*) shows four different radioiodinated bands with molecular weights of M_r 40,000, 36,000, 29,000, and 25,500, as shown previously in Fig. 1*B, lanes 1* and *2*. The radioiodinated peptide of M_r 40,000 (Fig. 2*B, a*) was specifically recognized by IgG anti-αV (Fig. 2*B, lanes 9* and *10*), whereas the second radioiodinated peptide of M_r 29,000 (Fig. 2*B, c*) was recognized by IgG anti-αIII (Fig. 2*B, lanes 5* and *6*). The two strongly radioiodinated peptides of M_r 36,000 and 25,500 present in the autoradiograms were not recognized by any IgG. According to the minimal stoichiometry, one ubiquitin molecule is bound to each of the two bands of M_r 40,000 and 29,000; therefore, the molecular weights of the unconjugated peptides recognized by IgG anti-αIII and anti-αV are most likely M_r 31,000 and 20,000, respectively.

Presence of Polyubiquitin Chains in α-Spectrin Ubiquitination—An experiment identical to that described above (Fig. 2*A*) was performed using ^{125}I-meUb instead of ^{125}I-Ub. This particular ubiquitin derivative, although a substrate for the ubiquitin-conjugating system, is unable to form multiubiquitin chains (38). After SDS-PAGE, the digested ^{125}I-meUb-α-spectrin was processed for Western blotting analysis as in the experiment described above (Fig. 2*A*). In the autoradiogram (Fig. 3, *lane B*), the two bands of M_r 40,000 and 29,000 were present as in the experiment with ^{125}I-Ub (Fig. 1*B, lanes 1* and *2*). An additional band of M_r 38,000 was also found. The IgG against αV domain recognized the ubiquitinated peptide of M_r 40,000 (Fig. 3, *a*), whereas the IgG anti-αIII recognized the ubiquitinated peptides of M_r 29,000 (Fig. 3, *c*) and M_r 38,000 (Fig. 3, *b*), confirming the data obtained with ^{125}I-ubiquitin. Moreover, the M_r 38,000 peptide plus the peptide of M_r 29,000 contained the same radioactivity found in the M_r 29,000 band of the previous experiment in which ^{125}I ubiquitin was used.

Coomassie Blue staining of digested spectrin did not reveal any difference using either the ^{125}I-Ub or ^{125}I-meUb derivatives in the conjugation assay. However, some differences in the M_r 40,000 range were evident in the films obtained by probing the membranes with IgG anti-αIII and anti-αIV (Fig. 2*B, lanes 6–8*; Fig. 3, *lanes 6–8*). These minor differences were not further investigated and could be due to different conformations of α-spectrin when Ub chains are bound, as well as to the relative proteolytic susceptibility of multiubiquitinated *versus* monoubiquitinated α-spectrin. Interestingly, when ^{125}I-meUb was used, the two radioiodinated bands of M_r 36,000 and 25,500 were no longer present. Thus, it must be concluded that when using fraction II from rabbit reticulocytes, the cytoskeletal protein α-spectrin is multiubiquitinated, at least *in vitro*, and that the peptides of M_r 36,000 and 25,500 correspond to tetra and tri multiubiquitin chains.

Immunoprecipitation of Ubiquitinated α-Spectrin Peptides—To directly demonstrate that ubiquitin is bound to the αIII and αV domains of α-spectrin, we used a second approach, as described in the scheme of Fig. 4*A*. Endoprotease Lys C-digested spectrin was divided into five identical aliquots, each of which was incubated with IgG against each domain of α-spectrin. Five columns of immobilized protein A were used to retain IgG. Free ubiquitin and digested peptides not recognized by IgG were removed during the washing steps of the columns. The proteins retained and eluted from the protein A columns were collected and counted in a gamma counter. As shown in Fig. 4*B,* eluates from columns receiving IgG anti-αI and anti-αII domains showed a very low radioactivity, probably due to some nonspecific interaction between free ^{125}I-Ub and the col-



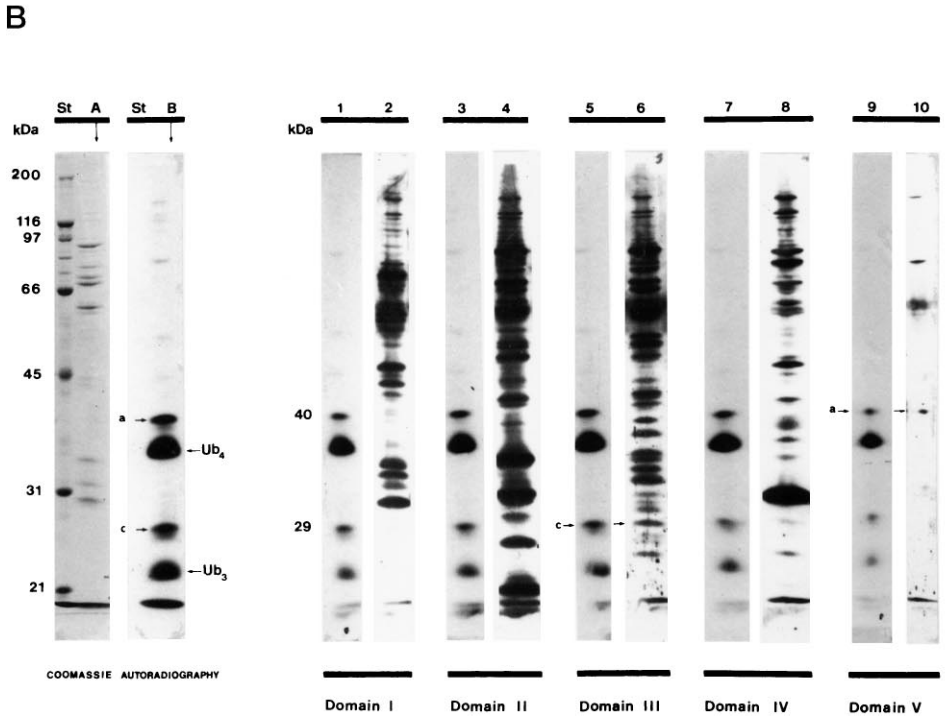


FIG. 2. **Identification of α-spectrin ubiquitin binding sites.** *A,* scheme of the procedure used. Erythrocyte membranes (900 μg) were incubated with ^{125}I-Ub (5 μM) in the presence of ATP (3.5 mM) and fraction II (800 μg) in a final volume of 3.1 ml of incubation mixture. The membranes were then centrifuged at 16,000 × *g* and washed twice with phosphate-buffered saline, pH 7.4, containing 1 mM PMSF and 0.2 mM AEBSF to eliminate fraction II proteins and unbound ^{125}I-Ub. Crude spectrin was extracted with a low ionic strength buffer. Extracted crude spectrin was then dialyzed to eliminate antiproteolytic agents, and endoprotease Lys C was added. After 3 h of digestion, polypeptides of different molecular weights were obtained. A portion of the digestion products of crude spectrin was separated by SDS-PAGE and analyzed by autoradiography. Another part of the digestion products was divided into five aliquots and used for Western blotting analysis. An autoradiogram of the

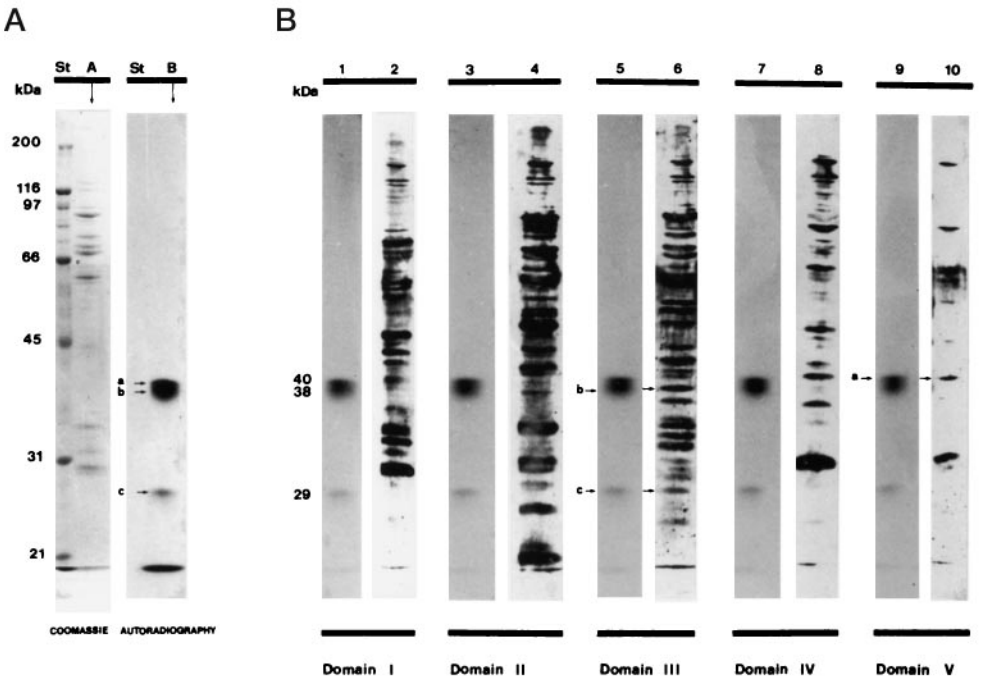


FIG. 3. **Identification of α-spectrin ubiquitin binding sites using ^{125}I-meUb in the conjugation assay.** The procedure used was essentially that described in the legend to Fig. 2 using ^{125}I-meUb instead of ^{125}I-Ub. High molecular weight standards (*lane St*) and the ^{125}I-ubiquitinated α-spectrin digest (*lane A*) were separated by SDS-PAGE and stained with Coomassie Blue. The relative autoradiogram was obtained (*lane B*). *Odd numbers,* autoradiograms of nitrocellulose membranes; *even numbers,* films obtained by ECL when the nitrocellulose membranes were probed with a specific antibody against each of the five α-spectrin domains. *a,* IgG against αV domain recognized the ubiquitinated peptide of M_r 40,000; *b* and *c,* IgG anti-αIII recognized the ubiquitinated peptides of M_r 38,000 (*b*) and M_r 29,000 (*c*).

umn. Eluate from the column with bound IgG anti-αIV domain showed a higher radioactivity than the latter eluates, whereas only the eluates from columns with bound IgG anti-αIII and anti-αV domains showed a strong radioactivity. This experiment provides direct proof that the ubiquitinated peptides of α-spectrin are specifically recognized by IgG anti-αIII domain and anti-αV domain of α-spectrin. Thus, it must be concluded that ubiquitin binding sites of α-spectrin are present in these two domains.

Ubiquitination of Non-Erythroid Spectrin-like Protein—Brain spectrin (fodrin) was used as substrate for an *in vitro* conjugation assay using ^{125}I-Ub in the presence of rabbit reticulocyte fraction II. Conjugation was stopped at 120 min with sample buffer, and the sample was boiled for 5 min and electrophoresed in SDS-polyacrylamide gels, stained, dried, and autoradiographed. No radioactive bands were found at the expected molecular weight of fodrin (M_r 260,000 and 225,000), indicating that this protein is not ubiquitinated, at least *in vitro.*

DISCUSSION

Spectrin is the principal component of the erythrocyte membrane skeleton and plays a dominant role in determining such mechanical properties of the erythrocyte as elasticity and deformability (47). Membrane equilibrium depends on the structural integrity of the skeletal proteins and on normal molecular interactions between the cytoskeletal proteins and membrane. Moreover, the binding of cytosolic components such as enzymes and hemoglobin to cytoskeletal proteins can play a role in membrane stability. Among the factors that may serve as regulators of cytoskeletal organization is protein phosphorylation-dephosphorylation (36). β-Spectrin has been reported to be a substrate for cytosol and membranous casein kinases. In particular, this chain contains a cluster of six phosphorylation sites. Phosphorylation of spectrin has been shown not to affect either dimer-dimer associations (48) or spectrin binding to ankyrin *in vitro* (49). However, phosphorylation affects spectrin inextractability from "inside-out" vesicles (50) and modulates the mechanical function and stability of the intact membrane structure (51). Other mediators, such as Ca^{2+} and calmodulin, can also regulate membrane stability (52). Interestingly, free calmodulin can be ubiquitinated in a Ca^{2+}-dependent manner and subsequently degraded, a process which could act as a control mechanism for all free calmodulin in excess (53). Recently, we described and characterized a new posttranslational modification of erythrocyte α-spectrin in which ubiquitin binds covalently to the α-spectrin chain (28).

In this report, using different approaches, we demonstrate the existence of two binding sites for ubiquitin on α-spectrin.

nitrocellulose membranes was obtained, after which each of the five nitrocellulose membrane lanes was probed with an antibody against each domain of α-spectrin. Goat anti-rabbit IgG horseradish peroxidase conjugate (*Goat anti-rabbit HRP conjugate*) was used as second antibody and ECL as the detection system. Overlapping of the autoradiograms and films obtained by ECL was used to determine which antibody was able to recognize the radiolabeled polypeptides. *B,* immunochemical identification of α-spectrin ubiquitin-binding sites. One fraction (35 μg) of ^{125}I-ubiquitinated α-spectrin digest was processed for SDS-PAGE, stained with Coomassie Blue (*lane A*) and autoradiographed (*lane B*). The other fraction was divided into five aliquots (each of 35 μg), processed for SDS-PAGE, and electroblotted onto five different nitrocellulose membranes. Nitrocellulose membranes were dried and autoradiographed (*odd numbers*). Then each of them was probed with a specific antibody against each of the five α-spectrin domains (*even numbers*). Dilution of the first antibody was: IgG anti-αI domain, 1:7,500; IgG anti-αII domain, 1:10,000; IgG anti-αIII domain, 1:5,000; IgG anti-αIV domain, 1:40,000; and IgG anti-αV domain 1:7,500. The autoradiograms and the films obtained by ECL were overlapped to identify the ubiquitinated peptides. *a,* radioiodinated peptide of M_r 40,000 specifically recognized by IgG anti-αV (*lanes 9* and *10*). *c,* radioiodinated peptide of M_r 29,000 recognized by IgG anti-αIII (*lanes 5* and *6*).

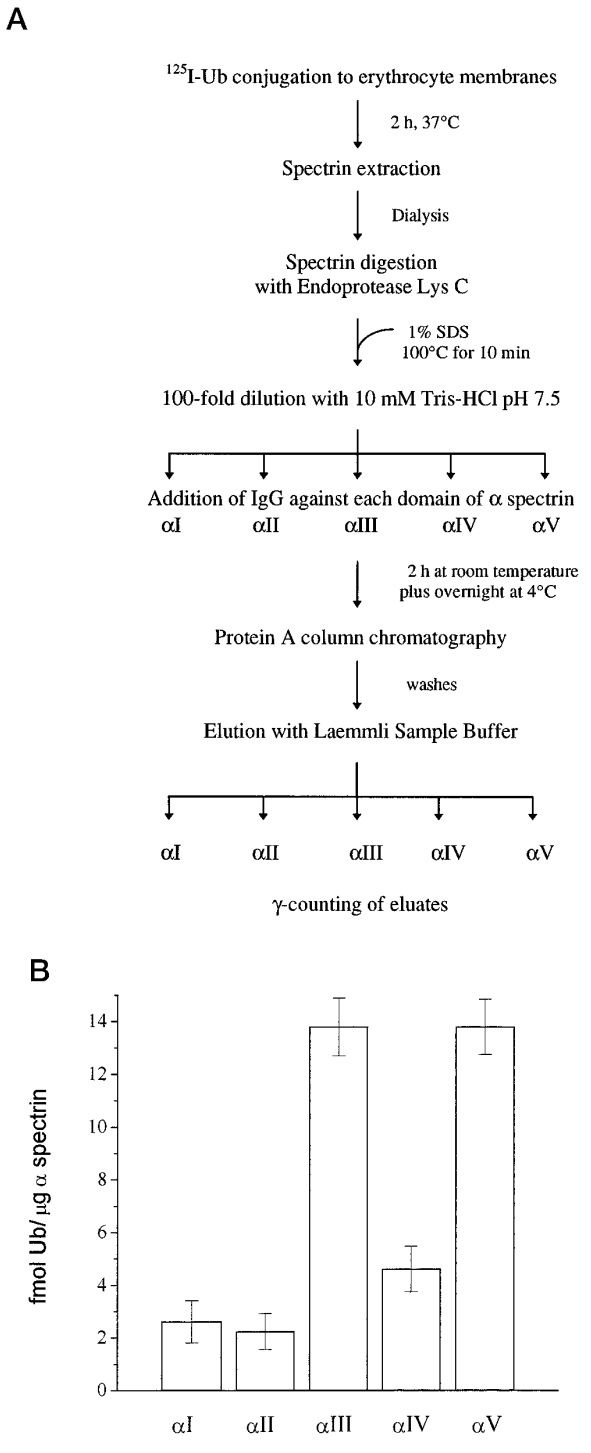


FIG. 4. **Immunoprecipitation of ^{125}I-Ub-α-spectrin peptides.** *A,* scheme of the procedure used to immunoprecipitate the ^{125}I-ubiquitinated α-spectrin digest. Conjugation of ^{125}I-Ub and human erythrocyte membrane spectrin extraction and digestion were performed as described under "Experimental Procedures." The digestion was stopped with 0.2 mM AEBSF, 1% (w/v) SDS and boiled for 10 min. The sample was then diluted 100-fold to lower the SDS concentration to 0.01%, divided into five aliquots of 35 μg each, and incubated with IgG against each domain of α-spectrin. A 5-fold excess (mol/mol) of specific IgG with respect to each trypsin-resistant domain was used. Each sample was incubated overnight with protein A, before loading antigen-IgG-protein A complexes into five different columns and washed with 10 mM Tris-HCl, pH 7.5. Five different eluates were obtained using 0.2 M glycine, pH 2.0, and counted in a gamma counter. *B,* the histogram shows the result obtained by gamma counting of eluates from the five columns of immobilized protein A. The *x* axis indicates which domain was immunoprecipitated using specific polyclonal IgG. The values are the mean of three experiments; *bars,* S.D.

Digestion with endoprotease Lys C of ubiquitinated spectrin revealed the presence of two ^{125}I-Ub peptides of M_r 40,000 and 29,000. As shown by Western blotting, these radiolabeled peptides were recognized by polyclonal IgG anti-αV domain and anti-αIII domain, respectively, indicating that these two peptides are the sites of ubiquitination on red blood cell α-spectrin. It could be speculated that α-spectrin ubiquitination on domains III and V may play a role in membrane stability as found for other mediators of red blood cell membrane. The αIII domain is not associated to any known function, whereas the αV domain contains the nucleation site for association with the β chain (32) and is involved in Ca^{2+} binding (54) and, with the βIV domain, participates in the interaction with actin and protein 4.1 (55). Interestingly, the αV domain is involved in the ubiquitination of α-spectrin and contains repeats 20 through 22, which exhibit atypical features. In fact, there is insertion of several amino acids into the repeats 20 and 21. Moreover, repeat 22 has a reduced homology to a typical spectrin repeat. Moreover, the nucleation site present in the αV domain is not only responsible for the initial α-β spectrin binding but also controls the side-to-side register of the many homologous repeats in both subunits. An unusual feature of the nucleation regions is that three of the repeats (two in the α and one in the β subunits) have an eight-residue insertion in the normal 106-residue repeat unit (32). These eight-residue insertions, which contain a lysine residue, might confer unique conformational properties upon the nucleation site, and the ubiquitination of domain αV might play a role in this context. The erythroblast-to-erythrocyte maturation process is accompanied by changes in the composition and properties of the plasma membrane. Furthermore, mature erythrocytes are incapable of ubiquitin/26 S proteosome-dependent degradation (56). Thus, ubiquitination of α-spectrin could play two different roles during erythrocyte maturation. In erythroblasts, the amount of α-spectrin synthesized exceeds by more than 3-fold the amount assembled on the membrane, and the excess unassembled peptides are rapidly degraded (57). It could be speculated that the binding of ubiquitin to α-spectrin in erythroblasts involves subsequent degradation. It is important to note that the sequence of α-spectrin contains a glutamic residue in the first position that may act as a secondary destabilizing residue according to the N-end rule (58) when spectrin is in the unassembled form. The second determinant, a specific internal lysine residue, could be the first lysine located at position 5 or 15 (domain I) of the α-spectrin sequence. Excess hemoglobin subunits are subject to an analogous targeted degradation in thalassemia (59). Because, as shown in this report, ubiquitination occurs in mature red blood cells on the αIII and αV domains, and thus quite far from the αI domain (moreover, these cells are incapable of ubiquitin/26 S proteosome-dependent degradation), the ubiquitination process of assembled α-spectrin probably has a different role in these cells than in erythroblasts. Ubiquitin itself and/or multiubiquitin chains could have a potential function as conformation-perturbing devices when conjugated to cytoskeletal proteins, given their orientational flexibility and reversibility (60). Thus, we suggest that the ubiquitination of α-spectrin in mature erythrocytes should be considered a new posttranslational event with a regulatory role in spectrin function rather than a signal for α-spectrin degradation. In fact, ubiquitination is a dynamic process (28), the linkage is covalent, and a significant amount (3% of the total α-spectrin chain) is continuously ubiquitinated in erythrocytes. As reported previously for globin-spectrin complex formation during erythrocyte senescence (61), such an amount could account for a significant change in membrane deformability. We also investigated whether other proteins belonging

to the spectrin superfamily are ubiquitinated. Brain α-fodrin was not found to be a substrate for ubiquitin in an *in vitro* assay. This non-erythroid spectrin and erythroid α-spectrin have very similar sequences (54% identity) throughout their entire length (62), but interestingly, fodrin at its C-terminal has an atypical sequence of 150 residues in repeat 22 (αV domain), and the identity of the 37 residues at the very C-terminal is less than 10%. Moreover, fodrin differs considerably from erythrocyte α-spectrin in repeats 11 and 12 (αIII domain) and possesses a calmodulin-binding site in the latter repeat that is absent in α-spectrin (63). Thus, erythrocyte α-spectrin and brain fodrin differ mainly in the domains found to be susceptible to ubiquitination. It would be interesting to examine whether α-actinin, another protein of the spectrin superfamily, is ubiquitinated. In particular, repeats 20 through 22, together with the nonrepeat C terminus of α-spectrin, are highly homologous with the C terminus of α-actinin (64). Preliminary studies now in progress in our laboratory indicate that α-actinin is ubiquitinated, at least *in vivo*. To date, no information is available on the function of the αIII domain of α-spectrin, but because the carboxyl terminus of the α-spectrin subunits (αV domain) is involved in the binding of the β-spectrin chain to form the α-β dimer and is an important site for many mediators in red blood cells, the ubiquitination of this cytoskeletal protein may be of physiological significance.

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Identification of α-Spectrin Domains Susceptible to Ubiquitination

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