Four distinct spectrin αII domain polymorphisms are known to occur in several nonwhite populations. Type 1 is essentially the only form found in whites and is also the most common form in nonwhites. In contrast to most other spectrin mutations that are single-base substitutions, two of the αII domain polymorphisms, types 2 and 3, are particularly unusual because they appear to involve 4-Kd insertions relative to type 1. We have identified the mutations responsible for these polymorphisms using biochemical approaches and a computer database of spectrin-domain peptides separated by two-dimensional gels. The type 3 mutation is characterized by an apparent 4-Kd increase in αII domain peptides with no change in β subunits. This apparent molecular weight increase is a sodium dodecyl sulfate (SDS) gel artifact resulting from an Arg → His mutation at residue 22 of the domain. The type 4 polymorphism shows a basic charge shift with no apparent change in molecular weight on gels. This charge shift results from a mutation of Thr → Arg at position 174 of the domain. This mutation appears to be linked to a “silent” mutation at position 130 from an Ile → Val. Support for possible linkage was obtained from analysis of three unrelated donors with the type 2 polymorphism. The type 2 polymorphism shows both the charge shift characteristic of the type 4 mutation and the apparent size shift that defines the type 3 polymorphism. Analysis of type 2 peptides confirmed that the two mutations described above for type 4 as well as the mutation at residue 22 observed in type 3 occur simultaneously in type 2. The observation that the type 2 polymorphism is a composite of the type 3 and 4 mutations is especially surprising because the type 2 polymorphism occurs far more frequently than either the type 3 or 4 forms. The basis for apparent linkage between the mutations at residues 130 and 174, which are encoded by different exons, is also not clear. Identification of the mutations described here permits design of genetic screening analyses that can be applied to larger populations to evaluate this potential linkage.

Identification of the Amino Acid Mutations Associated With Human Erythrocyte Spectrin αII Domain Polymorphisms

By Byron R. DiPaolo, Kaye D. Speicher, and David W. Speicher

SPECTRIN IS A critical, major component of the erythrocyte membrane skeleton. This two-dimensional protein network is responsible for the unique structural integrity as well as elasticity of the red cell. The spectrin α (M₉ = 280,000) and β (M₉ = 246,000) subunits associate side-to-side to form a long flexible rod-like heterodimer, and two dimers associate head-to-head to form tetramers that cross-link short actin filaments. Other major components of the membrane skeleton include Protein 4.1 and ankrysin. This complex lattice is attached to the lipid bilayer through at least two integral membrane proteins, Band 3, and glycoporphin.1-4

Most of the hereditary hemolytic anemias, which include hereditary elliptocytosis (HE), spherocytosis (HS), and pyropoikilocytosis (HPP), involve mutations of either a membrane skeletal protein or one of the associated integral membrane proteins. The majority of molecular abnormalities identified to date involve spectrin mutations.5-8 Most known spectrin defects are single-base substitutions that alter a single amino acid. These mutated sites are most frequently located in either the αI or βI domain and affect tetramer assembly.9-14 Other characterized mutations involve truncations of the β subunit C-terminal region that also destabilizes tetramer formation.15-19 Because of the very large size of the spectrin molecule and the large number of introns,20 most single-base mutations of spectrin have initially been localized to a specific region by analyzing tryptic domains21 before direct identification of the mutation using polymerase chain reaction (PCR) approaches.

In addition to mutations associated with hereditary anemias, a number of nonpathogenic spectrin polymorphisms have been identified using tryptic domain maps,22-25 and others have been identified at the nucleic acid level.26-28 Some of the nucleic acid mutations are silent whereas others code for single amino acid changes. One of the amino acid polymorphisms, designated Spa3241, has been correlated with variable expression of mutant alleles in the heterozygotic state depending on whether the pathogenic mutation is cis or trans to the polymorphism.24 The identification of a critical nucleation site for heterodimer assembly involving the αIV and βIV domains29 suggests that other polymorphisms from these regions could also play a role in variable clinical expression of pathogenic mutations in the heterozygotic state. The potential role(s) of polymorphisms in other regions of the spectrin molecule remain undefined.

Several features of four αII domain polymorphisms identified by Knowles et al22 are particularly interesting. These mutations appear to have no direct relationship with any red blood cell (RBC) disorder. They were initially identified based on altered two-dimensional (2D) gel migration of αII domain peptides from mild trypsin digestions of spectrin. Type 1 was the only form found in whites, and it is the form that was sequenced at both the protein30 and cDNA levels.26 In contrast, all four polymorphisms were detected in an analysis of 14 kindreds of African origin. Two polymorphisms, types 2 (basic charge shift and apparent 4-Kd increase) and 3 (apparent 4-Kd increase only), were particularly unusual because all identified αII domain and subdomain peptides showed this size increase, whereas the
SPECTRIN \( \alpha II \) DOMAIN POLYMORPHISMS

Fig 1. 2D gel and computer map of spectrin domain peptides produced by mild trypsin digestion at 0°C. (A) A Coomassie blue stained 2D gel containing 200 μg of spectrin peptides from a type 1/3 donor. The previously identified major \( \alpha II \) domain peptides \(^{22} \) are indicated for type 1 (T46, T35, T30, and T25; arrow heads) and type 3 (T46 \(^{+} \), T35 \(^{+} \), T30 \(^{+} \), and T25 \(^{+} \); arrows). The brackets include the region of the 2D gel that contains additional \( \alpha II \) peptides. The apparent molecular masses in Kd of the major type 1 peptides are indicated on the right and the pH’s are indicated across the bottom of the gel. The previously reported \(^{22} \) molecular weights will be retained for consistency although several are inaccurate. (B) A computer-generated map of the bracketed region from A that illustrates a composite from the four \( \alpha II \) polymorphisms. \( \alpha II \) peptides are indicated by solid spots labeled with computer-assigned spot numbers that will be used as identifiers.

sizes of neighboring domain peptides were unaffected. A possible 30- to 40-residue insertion would be unusual because most of spectrin is comprised of homologous segments or motifs that have a strongly conserved length of 106 residues. \(^{25,26,30} \) The importance of segment-length conservation is further supported by the observation that a single amino acid insertion in the \( \alpha II \) domain is sufficient to cause elliptocytosis. \(^{14} \) Another striking feature of the \( \alpha II \) polymorphisms is that type 2 appears to be comprised of both mutations observed separately in types 3 (size increase only) and 4 (basic charge shift only). \(^{23} \) but occurs far more frequently than either type 3 or 4. \(^{22} \)

In this study we identified the amino acid mutations associated with the \( \alpha II \) polymorphisms using protein chemical approaches. Location and identification of the apparent molecular weight-related mutation was especially facilitated through use of a computer database for spectrin domain peptides. Computer analysis was used to match and quantify all spots associated with the \( \alpha II \) domain on 2D gel maps. This analysis together with definition of the N-terminal and C-terminal boundaries of all peptides from a domain provides a powerful tool for more precisely locating sites of mutations as illustrated here.

MATERIALS AND METHODS

Spectrin isolation and tryptic domain peptides. Spectrin was isolated from normal and variant donor blood within 24 hours of collection as previously described. \(^{29} \) Spectrin at 1 mg/mL or less was digested with tosyl-phenylalanine chloromethyl ketone (TPCK)-trypsin (Worthington Biochemical Corp., Freehold, NJ) in 20 mmol/L Tris-HCl, 1 mmol/L \( \beta \)-mercaptoethanol, 0.02% NaI, pH 8.0, at 0°C for 90 minutes using an enzyme:substrate ratio of 1:20. Digestion was terminated by the addition of diisopropyl fluoroephosphate (DFP) (1 to 5 mmol/L final concentration) and incubation for 48 hours on ice to ensure complete trypsin inactivation before further use.

Polyacrylamide gel electrophoresis (PAGE) and electroblotting. One-dimensional (1D) polyacrylamide gels were prepared according to Laemmli. \(^{21} \) 2D gel electrophoresis (isoelectric focusing [IEF]/sodium dodecyl sulfate [SDS]-PAGE) was performed essentially by the method of O'Farrell \(^{22} \) with several modifications. Isoelectrofocusing was performed in glass tubes (3 mm × 22 cm) with a gel height of 16 cm using Millipore pH 3-10/2D ampholytes. Electrode buffers were 1 mol/L H\(_{2}\)PO\(_4\) and 1 mol/L NaOH. Gels were prefocused for 30 minutes at 500 V and samples were isofocused for 15,000 volt hours (Vh). The second dimension used a 16-cm long separation gel with a 10% to 17% linear acrylamide gradient. Samples were blotted from 1D or 2D gels onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes as previously described. \(^{33} \)

Computer analysis of 2D domain maps and development of a computer database. Coomassie blue-stained 2D gels of the spectrin tryptic domain peptides were scanned and analyzed using a Molecular Dynamics Model 100A laser scanner (Sunnyvale, CA). Images were processed, matched, and quantified using PDQUEST version 4.0 software (pdi, Inc., Huntington Station, NY). Spot numbers are automatically assigned by the software based on a two-dimensional grid and are used as reference numbers for tracking the 120 to 150 reproducible spots on the domain maps. A database, indexed to the spot number, is used to compile characteristics of each spot.

Affinity purification of \( \alpha II \) domain peptides. The \( \alpha II \) domain peptides were purified from mild trypsic digests of spectrin using a monoclonal antibody (MoAb) \(^{34} \) coupled to Sepharose CI-4B (Pharmacia, Piscataway, NJ). Briefly, 40 mg of a mild trypsin digest of crude spectrin was brought to (final concentration) 100 mmol/L Tris, 300 mmol/L NaCl, 1 mmol/L \( \beta \)-mercaptoethanol, 0.02% Triton X-100 (BioRad Laboratories, Mcllvine, NY), 2 mol/L urea, pH 7.4, and incubated 15 minutes at 37°C before loading onto the column pre-equilibrated with loading buffer (100 mmol/L Tris-HCl, 300 mmol/L NaCl, 1 mmol/L \( \beta \)-mercaptoethanol, 0.02% Triton X-100, 0.02% sodium azide, pH 7.4). Unbound peptides were eluted at 2 mL/min with loading buffer minus Triton. Bound \( \alpha II \) domain peptides were eluted with 100 mmol/L Tris-HCl, 300 mmol/L NaCl, 1 mmol/L \( \beta \)-mercaptoethanol, 0.02% Triton X-100, 0.02% sodium azide, pH 7.4.
mmol/L NaCl, 6 mol/L urea, pH 7.0. Column effluent was monitored at 280 nm and bound fractions were pooled, concentrated, and dialyzed into 20 mmol/L Tris-HCl, 1 mmol/L β-mercaptoethanol, 0.02% NaN₃, pH 7.6.

_Cleavage at tryptophans._ In some experiments peptides were fluorescently labeled on cysteine residues with N-iodoacetyl-N-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS; Sigma Chemical Co, St Louis, MO) as previously described to facilitate detection on gels or blots as well as to identify cysteine-containing fragments. Affinity-purified fluorescent αII peptides from heterozygous donors were separated on 1D 10% gels and blotted to PVDF membranes followed by extraction as described by Stone et al. Cleavage of extracted αII domain peptides at tryptophan residues was accomplished in 50% acetic acid using a 100-fold mol/L excess of 2-(2-nitrophenylsulfonyl)-3-methyl-3-bromoindolenine (BNPS-skatole; Pierce, Rockford, IL) relative to tryptophan for 48 hours.

_Complete trypsin digestion and high performance liquid chromatography (HPLC) purification._ The αII domain peptides from different polymorphisms were compared by HPLC peptide mapping after complete digestion with trypsin either using samples eluted from PVDF membranes or by in situ digestion on nitrocellulose membranes as previously described by Abersold et al. Enzymatic hydrolysis was terminated by acidification with 5% trifluoroacetic acid (TFA) to pH 2.0. Digestion mixtures were separated by reverse-phase HPLC on a Supelco LC-18-DB column (2.1 × 250 mm) (Supelco, Inc, Bellefonte, PA) using a linear gradient from 0.1% TFA to 0.09% TFA in 70% acetonitrile at 0.2 mL/min. Selected peaks were further characterized by amino acid analysis and/or N-terminal sequencing.

_Protein chemical methods._ Automated N-terminal sequence analyses of peptides blotted to PVDF membranes as well as HPLC-purified peptides were performed as previously described. Amino acid compositions were performed using manual derivatization with phenylisothiocyanate (PITC) and HPLC separation essentially as described by Ebert after vapor-phase hydrolysis using 6 N HCl, 1% phenol under argon at 160°C for 1 hour. Samples for mass spectrometry analysis were electroblotted to PVDF membranes from 2D gels, detected by transillumination, extracted into small volumes of 1% TFA/40% acetonitrile, lyophilized, redissolved in 2 to 10 μL, and loaded on a Finnigan MAT Lasermat Mass Analyzer (San Jose, CA).

RESULT

_Characterization of donors._ Most of the structural determinations described below were performed on three unrelated normal volunteer donors of African descent with the following heterozygotic αII polymorphisms: type 1/2, type 1/3, and type 3/4. Two additional unrelated type 1/2 donors were used to confirm mutations found in the initially characterized donor. Several type 1/1 donors were used for 2D gel comparisons and as controls for Western blotting experiments. However, for most comparative chemical cleavages and HPLC mapping experiments, the type 1 peptides from the same donor were used as controls for variant peptide analysis.

_Computer-assisted analysis and use of the 2D gel database._ A typical 2D gel pattern of the tryptic spectrin domain peptides from a type 1/3 donor is shown in Fig 1A. The major type 1 peptides and the corresponding peptides from type 3, which show the apparent 4-Kd molecular weight increase are highlighted. A computer-generated map (Fig 1B) from the bracketed region of the gel shown in Fig...
Fig 3. Detail of the region from the 2D gel computer images containing all domain peptides. The illustrated region is equivalent to the bracketed region in Fig 1A. Spot numbers are illustrated for each map (compare with Fig 1B) and matching spots from each gel have identical numbers within the matchset. The major all domain peptides for type 1, T46, T35, T30, and T25, are indicated with arrowheads (A). The major polymorphic T46, T35, T30, and T25 related spots associated with the type 2 (B), type 3 (C), and type 4 (D) variants are highlighted with arrows. Minor, more basic all domain peptides are enclosed in brackets. Peptides in this group that show charge shifts for types 2 and 4 are indicated using small arrows.

1A summarizes the locations of all αII polymorphism spots because it represents a “composite” from comparison of gels containing all four αII domain polymorphisms. All αII domain-related peptides used in this analysis are shown as filled spots and the computer-assigned “spot number” will be used as identifiers in the following descriptions. These identifiers are useful for correlating spots from different gels and as unique labels for compiling a database containing known information about each peptide (see below). For example, the composite map (Fig 1B) can be correlated with the type 1/3 gel (Fig 1A) as follows: type 1; T46 (spots 3603 and 4602), T35 (5402), T30 (5403), and T25 (4301) and type 3; T46' (3608 and 4606), T35' (6401), T30' (6402), T25' (4304).

The two-dimensional gel pattern shown in Fig 1 is qualitatively similar to that originally observed by Knowles et al., although the increased gel size and modifications to gel conditions described in Materials and Methods permits expanded detection and quantitation of peptides. Using this approach, about 150 reproducible spectrin domain and subdomain peptides can be evaluated when assisted by computer image analysis. As shown here, identification and characterization of some of these minor, but reproducible, spots greatly enhances localization of mutations. With the aid of this 2D gel image analysis, several less prominent αII domain peptides were identified that did not show the 4-Kd molecular weight shifts observed for the major peptides from type 2 and 3 samples. These previously undetected spots on the left side of the gel are also labeled in Fig 1B.

Location of polymorphism changes within the αII domain. The locations of all αII domain peptides, including the less prominent spots and the polymorphism-related shifts, can be visualized and evaluated by Western blotting with a domain-specific antibody as shown in Fig 2. The apparent increased size (types 2 and 3) as well as the basic charge shift (types 2 and 4) are readily apparent for the major αII domain peptides as expected. However, the less prominent spots on the basic side of the gel clearly do not show the apparent molecular weight shift for either the type 2 or 3 polymorphism, but do show a basic charge shift for types 2 and 4. The αII spots shown by Western blotting can be correlated with reproducible, lightly staining Coomassie blue spots showing the same polymorphism shifts (Fig 3). As observed with the Western blots, these bracketed Coomassie blue stained spots do not show a shift in size for types 2 and 3, but do show the charge shift for types 2 and 4 (small arrows). Because some peptides normally show charge heterogeneity caused by staggered tryptic cleavage, some poly-
Table 1. Determination of N-Terminal and C-Terminal Boundaries of αII Domain Peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Spot Numbers†</th>
<th>N-Terminal</th>
<th>C-Terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>T46</td>
<td>3603, 4602</td>
<td>GTOLHEAN...</td>
<td>Complete α II seq30 and charge cluster‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gel migration and charge cluster‡</td>
</tr>
<tr>
<td>T42</td>
<td>2603, 3601</td>
<td>GLAEVQRN...</td>
<td>Mass spectrometry§</td>
</tr>
<tr>
<td>T35</td>
<td>5402</td>
<td>GTOLHEAN...</td>
<td>ND</td>
</tr>
<tr>
<td>T33</td>
<td>3404</td>
<td>GLAEVQRN...</td>
<td>ND</td>
</tr>
<tr>
<td>T30</td>
<td>5403</td>
<td>GTOLHEAN...</td>
<td>Mass spectrometry§</td>
</tr>
<tr>
<td>T25</td>
<td>4301</td>
<td>GTOLHEAN...</td>
<td>Sequence of C-terminal CNBr peptide</td>
</tr>
</tbody>
</table>

Abbreviations: ND, not determined; CNBr, cyanogen bromide.
† Both indicated spots for this peptide produced this sequence.
‡ The two major and an occasionally observed third spot differing by one charge unit arise from heterogeneous tryptic cleavage at an Arg-Arg-Arg sequence at positions 1045, 1046, 1047 of the complete α subunit sequence.26
§ The tryptic cleavage site producing a molecular weight consistent with the mass determination was selected.

Identification of the charge-shift related mutation(s). As shown in Fig 4, the charge shift observed in the type 2 and 4 polymorphisms has to be located within the region between residues 718 to 916 in the complete α sequence. Affinity-purified α II domain peptides from the donors indicated above were used to detect possible mutations in this region. The only non-α II domain peptide contaminating these preparations was at the α III domain 52-Kd peptide, which eluted early when the bound fraction was eluted and could be minimized by selective pooling. The composition of typical affinity-purified samples from heterozygous donors is shown in Fig 5. Because each heterozygous donor used here had an apparent molecular weight increase, the major peptides for each polymorphism could be adequately resolved and hence purified on 1D gels as shown in Fig 5. Peptides from each variant were purified in this manner and electroblotted to either PVDF membranes or to nitrocellulose for subsequent chemical or protease cleavage.

HPLC peptide maps using complete trypsin digestions of domain peptides either in solution after elution from PVDF membranes or in situ on nitrocellulose membranes proved to be the most useful approach for detection of mutations. By comparing maps of analogous peptides from different polymorphisms with type 1 peptides, a one or more peptide shift was observed in each sample. These peptides were sorption mass spectrometry to directly compare the mass of domain peptides showing the size shift with corresponding type 1 or 4 peptides. These determinations were within 100 daltons of each other, which confirmed that the apparent size difference for the type 2 and 3 variants results from artifactual migration on SDS gels resulting from the Arg to His mutation at residue 22. N-terminal sequence analysis of type 4 peptides established that the Arg at this position was unchanged in this variant. These mutations were also confirmed by HPLC mapping comparisons after complete tryptic digestion of peptides from each polymorphism (see below).

Identification of the 4-Kd size shift mutation. Extended N-terminal sequences of type 2 and 3 peptides containing the apparent size increase were performed and at least 45 to 55 residues were unambiguously determined for both variants. The only change relative to the type 1 sequence26,30 was an Arg to His mutation of residue 22 in the domain (residue 701 in the complete α subunit sequence). The possibility that an insertion occurred further from the N-terminal could be eliminated because the sequence runs extended into the T42 peptide that did not show the size shift. A possible internal duplication was eliminated using laser de-
SPECTRIN αII DOMAIN POLYMORPHISMS

Fig 5. αII domain peptides affinity purified on an MoAb column. (A) Coomassie blue stained 10% to 15% gradient gel; lane 1, type 1/1; lane 2, type 1/2; lane 3, type 1/3; 20 μg/lane. (B) Corresponding Coomassie blue-stained 2D gel of a type 1/3 sample, 200 μg. Arrows in both (A) and (B) highlight peptides showing the 4-Kd size increase for the major αII peptides.

Characterized using amino acid analysis and N-terminal sequence analysis. In addition to the Arg to His mutation at residue 22 of the domain described above for types 2 and 3 (size shift), a mutation from Ile to Val at residue 130 of the domain was identified in the type 4 sample. Another peptide shift in the type 4 sample led to identification of a Thr to Arg mutation at position 174 of the domain. The latter mutation is consistent with the basic charge-shift characteristic of this polymorphism whereas the additional conservative substitution of a Val for an Ile was unexpected based on the peptide shifts observed on 2D gels. Possible linkage of these mutations was suggested because the same Thr to Arg and Ile to Val mutations were identified from HPLC tryptic maps of a type 2 sample. This Val was also identified from maps of two additional unrelated type 2 donors but not in any maps from type 1 or type 3 samples. No evidence of other mutations was obtained from these HPLC maps for any of the type 2 or type 4 samples. The only peptide alteration observed on HPLC maps for the type 3 mutation was the loss of a cleavage site resulting from the His to Arg mutation at position 22.

Peptides eluted from PVDF membranes were also cleaved at tryptophans using BNPS-skatole and peptides were separated by 1D SDS gels and reblotted to PVDF membranes. A 27-Kd peptide resulting from cleavage at residue 129 was identified and sequenced. Because residue 130 was the N-terminal residue in this peptide, short sequence runs could be used to determine whether Ile or Val occurred at this position. Sequence analysis of this peptide from the type 3 sample and a type 1 sample unambiguously established that Ile was present in this position, whereas sequencing of this peptide from two of the type 2 samples confirmed the Val substitution.

DISCUSSION

In this study we identified the mutations associated with the four αII domain polymorphisms that are summarized in Fig 6. The known codons from the cDNA sequence and the predicted single-base mutations that would result in the observed amino acid mutations are shown.

The mutation of an Arg to a His at position 22 of the domain is responsible for the observed 4-Kd size increase observed on SDS gels for all peptides that contain this region. Apparently this single amino acid mutation perturbs SDS binding or affects unfolding in SDS that results in this artifactual migration. The apparent linkage of the mutations at residues 130 and 174 is even more surprising. There is no obvious explanation for such a linkage because these two residues are coded by two different exons. Of course the sample size in this analysis is too small to determine whether these mutations always cosegregate and analyses on larger populations are needed. These studies are being pur-
sued using restriction fragment length polymorphism (RFLP) assays (Gallagher et al, manuscript in preparation).

Another surprising aspect of these polymorphisms is the observed high frequency of the type 2 variant. As shown here, three separate mutations define this polymorphism and type 2 is actually a compound type 3-4. The high frequency of this form, usually in the heterozygous state with type 1, suggests a relatively strong selective advantage of this polymorphism because the separate mutations represented by type 3 and type 4 occur at a low frequency. Based on the initial study of 37 individuals of African descent, the allelic frequency was 0.50 for type 1, 0.30 for type 2, 0.07 for type 3, and 0.07 for type 4. While these authors noted the small size of their study as a limitation in evaluating the significance of these frequencies, our random evaluation of an entirely separate pool of donors shows similar polymorphism frequencies. Based on the high frequency of the non-type 1 variants in populations of subtropical origins but not in whites, an obvious possible selective advantage for these polymorphisms could be an increased resistance to malaria or another environmental factor. Initial attempts to detect malarial resistance on several donor samples were variable (data not given). However, it should be noted that even marginal resistance to malaria or another tropical disease or factor may confer sufficient selective advantage to establish prominence of type 2 because these polymorphisms do not show any detrimental aspect. If these initially observed allelic frequencies apply to larger populations, the prominence of type 2 over types 3 and 4 from which it might arise, could indicate that type 2 offers a greater selective advantage than either type 3 or 4.

The interpretations presented above are based on the assumption that the types 2, 3, and 4 mutations evolved from type 1 because it appears to be the most common type in all populations. An alternate explanation for the high frequency of the type 2 variant is that this polymorphism might represent a founder sequence, which developed independently and in parallel with type 1 in early gene pools. Taking this approach, type 4 could have evolved from type 2 by a single mutation and type 3 could have evolved from type 1 as a single mutation.

This study also shows that computer-assisted image analysis together with a developing database of detailed characteristics for spectrin peptides is a powerful tool for more precise location of mutations within spectrin. As noted previously, 2D analysis of spectrin domain maps has been the primary screening method for identification of most spectrin mutations that have been characterized to date. The large number of introns in the spectrin genes together with lack of a good source of mRNA in readily available clinical samples has limited the application of molecular approaches as initial screens for spectrin mutations. The relatively common occurrence of polymorphisms throughout spectrin as well as the fact that most mutations are found in the heterozygous state will probably continue to complicate direct genetic screening of new mutations without initial localization using analysis of domain peptides.

The value of the computer analysis/database approach is most clearly shown by the relative ease of locating the apparent size-shift mutation in the aII domain polymorphisms. This image analysis and associated database offer several advantages over manual comparison of gels and the limited characterization of peptides previously available. The initial report of the aII domain polymorphisms tracked about 50 spots, compared with analysis of about 150 spots using computer image analysis in the present study. The computer-assisted analysis also can detect changes that can be easily overlooked using manual inspection, especially minor spots such as the basic aII domain peptides that played a critical role in determining the location of the size shift related mutation.

The resolution of this technique could be further improved by characterizing additional spots to improve resolution of the peptide map. The recent availability of laser desorption mass spectrometry makes fairly precise determination of the C-terminal boundaries of gel-purified peptides feasible, which dramatically improves the precision of peptide boundary assignments. Laser desorption mass spectrometry analysis is sufficiently accurate to distinguish between most alternative tryptic cleavage sites and to detect the loss or insertion of at least four or more amino acids, but it is currently not capable of detecting point amino acid substitutions in the size range encountered with spectrin domain peptides.

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Identification of the amino acid mutations associated with human erythrocyte spectrin alpha II domain polymorphisms

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