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Identification of the main ubiquitination site in human erythroid α-spectrin

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Abstract Erythroid spectrin is the main component of the red cell membrane skeleton, which is very important in determining the shape, resistance to mechanical stresses and deformability of red cells. Previously we demonstrated that human erythroid α-spectrin is ubiquitinated in vitro and in vivo, and using recombinant peptides we identified on repeat 17 the main ubiquitination site of α-spectrin. In order to identify the lysine(s) involved in the ubiquitination process, in the present study we mutated the lysines by site-directed mutagenesis. We found that ubiquitination was dramatically inhibited in peptides carrying the mutation of lysine 27 on repeat 17 (mutants K25,27R and K27R). We also demonstrated that the correct folding of this protein is fundamental for its recognition by the ubiquitin conjugating system. Furthermore, the region flanking lysine 27 showed a 75% similarity with the leucine zipper pattern present in many regulatory proteins. Thus, a new potential ubiquitin recognition motif was identified in α-spectrin and may be present in several other proteins. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: α-Spectrin; Site-directed mutagenesis; Leucine zipper pattern ubiquitination; Protein folding recognition

1. Introduction

Ubiquitin is a polypeptide of 76 amino acids, which is highly conserved and found both free and covalently bound to target proteins. Conjugation of ubiquitin to the substrate proceeds via a three-step mechanism. Initially, ubiquitin is activated in its C-terminal glycine by ATP and the ubiquitin activating enzyme (E1). Following activation, a ubiquitin conjugating enzyme (E2) transfers ubiquitin from E1 to the substrate via a member of the ubiquitin–protein ligase family (E3). Ubiquitin is usually transferred to an ε-amino group of a Lys residue of the protein substrate to generate an isopeptide bond [1]. Within the ubiquitin system, substrates are recognized by different E3s or E2/E3 complexes [2]. Some proteins are recognized via primary signals in their sequence, while others must undergo post-translational modifications such as phosphorylation, or associate with other polypeptides such as molecular chaperones prior to recognition by the appropriate ligase. Multiubiquitination of some proteins determines their degradation by the 26S-protease complex [3]. In other cases ubiquitination takes part in functional regulation of a substrate protein [4,5]. In mature erythrocytes the activity of the ubiquitin mediated proteolytic system is lost and several enzymes, in particular the ubiquitin conjugating enzymes (E2), show a decrease during red blood cell maturation. However, substantial levels of ubiquitin–protein conjugates persist in erythrocytes indicating that at least some enzymes capable of bringing about conjugate formation remain [6].

Previously, we demonstrated that red blood cell α-spectrin is a specific physiological substrate for ubiquitination [7–9] and, using recombinant peptides, we found that repeats 17 and 20 are actively ubiquitinated, at least in vitro [10].

Spectrin is the main constituent of the erythrocyte membrane skeleton and represents about 25% in weight of the total red cell membrane proteins [11]. Spectrin is composed of two elongated subunits, α and β chains (280 and 246 kDa, respectively), non-covalently associated side-to-side in an antiparallel orientation to form heterodimers [12]. Spectrin heterodimers associate head-to-head to form tetramers, which in turn constitute the long, flexible filaments of the network. Spectrin also forms non-covalent associations with other proteins of the erythrocyte skeleton, such as ankyrin, band 4.1 [13], actin, adducin and tropomyosin [14,15]. The α-spectrin chain consists of 22 repeating segments, whereas the β-spectrin chain consists of only 17 repeats. The repeating segments of α-spectrin, which are about 106 amino acids long, show sequence identity of about 20% but some residues are highly conserved, notably leucine and tryptophan at positions 26 and 45 of the repeats [13,15]. Recombinant DNA techniques have been used to produce fragments of spectrin that can serve as models for the native protein. A number of different spectrin fragments from several species and tissues have already been prepared and investigated by various techniques [16–18]. The first detailed structural model for one spectrin repeat unit was based on the X-ray crystal structure of the 14th segment of *Drosophila* α-spectrin, which revealed three helices (A, B and C) aligned side by side, with helices A and C parallel and helix B antiparallel [19]. More recent structural models of two connected repeats of chicken brain α-spectrin revealed that the helices C and A of adjacent units are connected by a linker region, which is α-helical [20].

In a previous study we used different recombinant peptides to determine which repeats within the α-spectrin chain are ubiquitinated. Peptide α16–17 (containing the entire repeat 17 and part of repeats 16 and 18 of α-spectrin) was the shortest highly ubiquitinated peptide identified in in vitro assays. In

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Abbreviations: GST, glutathione-*S*-transferase; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; PMSF, phenylmethylsulfonyl fluoride

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the present study we used site-directed mutagenesis techniques to search for the lysine(s) directly involved in ubiquitin binding on this molecule in order to localize the structural motif involved in the recognition of α-spectrin by the E2s–E3s enzymes.

Interestingly, the identified sequence is 75% similar to a pattern present in many regulatory proteins and thus could represent a new recognition site for protein ubiquitination and turnover.

2. Materials and methods

2.1. Materials

Pfu DNA polymerase was from Stratagene. Restriction enzymes were from Gibco-BRL. Bacterial strain JM 109 was from Promega. The glutathione–Sepharose 4B affinity column was from Amersham Pharmacia Biotech. All other chemicals were purchased from Sigma.

2.2. Construction of the wild type peptides

Recombinant cDNA encoding α-spectrin fragment α16–17 was obtained by polymerase chain reaction (PCR) amplification of human α-spectrin cDNA clone α14–17, kindly provided by D.W. Speicher (Wistar Institute, Philadelphia, PA, USA). Pfu DNA polymerase was used under standard conditions in the PCR [21]. The oligonucleotide primers used for cDNA amplification were 5′-GGGGGAATTC-TAAAGCTCAATGAGGCCAGTCG-3′ (forward primer) and 5′-GGGGCCATGGTTATAGGGATTCTTCCAACTTAAG-3′ (reverse primer). Three small recombinant cDNA sequences were sub-cloned by PCR amplification from clone α16–17. These sequences coded for three peptides referred to as A, B and C, respectively, from L13 to D41, from L18 to F36 and from H22 to L32, according to the amino acid sequence of α-spectrin repeat 17 (Table 1). The oligonucleotide primers used for cDNA amplification were: peptide A, 5′-AAGC-GAATTCTGAATGTCCAAGA-3′ (forward primer) and 5′-TCCAC-CATGGTTAATCATCTAGATCCTGG-3′ (reverse primer); peptide B, 5′-GTCCGAATTCTGGCAGCTGCACAC-3′ (forward primer) and 5′-CATCCCATGGTTAGAAGAACTGGAACAAGG-3′ (reverse primer); peptide C, 5′-GCAGGAATTCACCACGAAAAATT-GAA-3′ (forward primer) and 5′-GGAACCATGGTTACAAGGCA-TAGGCC3′ (reverse primer). All the primers contained restriction enzyme sites for *Eco*RI (forward primers) and *Nco*I (reverse primers) (underlined sequences). The restriction sites in the reverse primers were preceded by a stop codon. The purified PCR amplified DNAs were cut with the appropriate enzymes and directionally cloned into the restricted pGEX-KG vector [22] using standard techniques. Transformation was performed in *Escherichia coli* strain JM 109. The reliability of the PCR reactions was subsequently confirmed by direct DNA sequencing and the peptides were expressed as glutathione-*S*-transferase (GST) fusion protein.

2.3. Site-directed mutagenesis

Mutations were introduced in the α16–17 construct according to the site-directed mutagenesis method known as long primer unique site elimination (LP-USE) mutagenesis [23] and modified as previously described [24]. Mutations were introduced using two kinds of mutant primers: (i) the selection primer (forward primer) GGTTCC-GCGTGGTACCCCGGGAATTTCC, which eliminates the unique *Bam*HI site by converting it into a *Kpn*I site (underlined sequence); (ii) the other primers (reverse primers) contained the desired mutations (Table 2). A total of 10 recombinant peptides containing 16 mutated lysines were produced for this study. All clones were sequenced to verify the presence of the mismatched bases and the fidelity of the remaining sequence.

Table 1
Amino acid sequence of peptides A, B and C

Peptide	Amino acid sequence
A	LNVQELAAAHHEKLK**K**EAYALFQFFQDLDD
B	LAAAHHEKLK**K**EAYALFQFF
C	HHEKLK**K**EAYAL

Lysine 27 is in bold.

2.4. Expression and purification of wild type or mutant peptides

Overnight cultures were diluted 1:15 in Terrific Broth medium containing 100 μg/ml ampicillin and grown at 37°C to an optical density of 0.5–0.7 at 600 nm. The cultures were diluted again 4:5 and incubated for a further 15 min before induction with 0.5 mM isopropyl β-D-thiogalactopyranoside. Cells were induced for 2 h. After centrifugation the bacterial pellets were frozen and stored at −20°C. GST fusion proteins were purified as described previously [25]. Briefly, cell pellets from 1 l of culture were resuspended in 30 ml of 20 mM phosphate buffer pH 7.3, 150 mM NaCl, 1% Triton X-100, containing 1 mM EDTA, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) or phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 2 μg/ml pepstatin, 100 U/ml aprotinin, 1 mM β-mercaptoethanol and lyzed by sonication. After centrifugation, the cleared lysates were loaded onto a reduced glutathione–Sepharose 4B column. GST fusion proteins were eluted with 50 mM Tris pH 8.0 containing 10 mM glutathione.

2.5. Ubiquitin conjugation assays

Ubiquitin conjugation assays were performed essentially as described [7] with slight modifications. In a final volume of 30 μl, the reaction mixture contained 80 mM Tris pH 7.6, 4 mM $MgCl_2$, 1 mM dithiothreitol, 3.5 mM ATP, 14 mM creatine phosphate, 2.2 μg of creatine phosphokinase, 13 μg of fraction II, 5 μM ^{125}I-Ub, 1 mM PMSF, 1 mM AEBSF, 1 μM leupeptin, 1 μM pepstatin and 50 pmol of purified GST fusion protein. Following 1 h of incubation at 37°C, the mixture was boiled for 3 min in Laemmli sample buffer containing 2% β-mercaptoethanol and then electrophoresed by SDS–PAGE. Gels were stained, dried and autoradiographed.

2.6. Other procedures

SDS–PAGE was performed as described by Laemmli [26]. Gels were stained with Coomassie Brilliant Blue R-250. Protein concentrations were determined by absorbancy at 280 nm using extinction coefficients calculated from sequences. Alternatively, protein concentrations were determined by the method of Bradford [27]. Quantitative

Table 2
Design of mutant recombinant GSTα16–17 peptides

Repeat	Mutations	Mutated reverse primers
17	K3,4,5R	GTTGACATTATCT**C**TT**C**TC**C**TCACAATCTGATC
17	K25R	GGCCTCTTTCAAT**C**TTTCGTGGTGTGCAG
17	K27R	GGCATAGGCCTCT**C**TCAATTTTTCGTGG
17	K25,27R	GGCATAGGCCTCT**C**TCAAT**C**TTTCGTGGTGTGCAG
17	K49R	CACTCGTATCAAC**C**TCTCCTCTATCCAG
17	K70,71,73R	CCCCTCTAGGCGT**C**TGTGC**C**TC**C**TCAGCAAGTTCTGAACC
17	K95,97,99R	CCCCACAGCAGCC**C**TGTCT**C**TCAGC**C**TCTCTGCCATATCC
18	K14,16R	CCTTGGCCAACTCT**C**TGAGC**C**TCTCCCAGTGTTC
18	K50R	AGTCCTCGGGCC**C**TGGCCAACTCTTTG
18	K25R	GGGATTCTTCCAAC**C**TAAGTCCTCGGGC

Lysines 3, 4, 5, 25, 27, 49, 70, 71, 73, 95, 97 and 99 on repeat 17 and lysines 14, 16, 20 and 25 on repeat 18 correspond, respectively, to lysines 1685, 1686, 1687, 1707, 1709, 1731, 1752, 1753, 1755, 1777, 1779, 1781, 1802, 1804, 1808 and 1813 on the entire α-spectrin sequence.
Mutated nucleotides are in bold.

determinations of protein bands in Coomassie Blue stained gels were performed using an LKB Ultroscan XL laser densitometer. The gels (stained and dried) were also analyzed with a GS-250 Molecular Imager (BioRad) to evaluate the radioactivity incorporated into the GSTα16–17 mutant peptides.

3. Results

3.1. *Expression and purification of wild type and mutant α-spectrin peptides*

In a previous study we constructed the recombinant α16–17 peptide as a reliable model of the entire α-spectrin [10]. The recombinant peptide α16–17 includes a region of α-spectrin ranging from lysine 25 of repeat 16 (K1601) to leucine 30 of repeat 18 (L1818), according to the published human α-spectrin sequence [28]. Moreover, this peptide is formed by two homologous conformational units which allow it to fold correctly and as a stable structure [16]. In this study we constructed ten mutants in an attempt to identify the lysine(s) involved in ubiquitin conjugation. The GSTα16–17 wild type or mutant peptides were highly expressed and soluble in bacteria, but differed slightly in their yield. Yields of the purified fusion proteins ranged from 20 mg/l of bacterial culture for wild type GSTα16–17, to 5 mg/l for mutant 17K70,71,73R. Peptides A, B and C were fragments of the α-spectrin repeat 17 (see below). Although the yields of these peptides were comparable to those of wild type GSTα16–17 peptide, it is likely that they did not fold correctly in the α helix structure because they represent only a part of the repetitive unit. After elution from the glutathione–Sepharose 4B affinity column, all of the peptides had an apparent molecular mass in SDS–PAGE in agreement with the calculated value. Gel scan analysis of band intensities indicated that the samples were between 55 and 85% pure. The peptides were utilized in ubiquitin conjugation assays without other purification step.

3.2. *Identification of lysines susceptible to ubiquitination on GSTα16–17*

We had previously shown that the GSTα16–17 peptide could be ubiquitinated, whereas GSTα15–16 and GSTα17 could not. Since ubiquitin binds to proteins via lysine residues, we used site-directed mutagenesis to substitute lysines potentially involved in the ubiquitination of the GSTα16–17 peptide. Spectrin fragment α16–17 contains 22 lysines, the first five of which were not, however, considered potential targets for ubiquitin conjugation because they are located in repeat 16, which is entirely included in the non-ubiquitinated GSTα15–16 peptide [10]. Therefore, we considered that GSTα16–17 peptide contained lysines potentially susceptible to ubiquitination in repeat 17 at positions 3, 4, 5, 10, 25, 27, 49, 70, 71, 73, 95, 97, 99 and in repeat 18 at positions 14, 16, 20, 25 according to the published human α-spectrin sequence [28]. Comparison of the sequences of mouse and human erythrocyte α-spectrin revealed conserved lysines in repeat 17 at positions 4, 25, 27, 49, 70, 71, 73 and 99 and in repeat 18 at positions 14, 16 and 20. Despite the fact that non-ubiquitinated GSTα17 peptide contained lysines 25–99 in repeat 17 and lysines 14–25 in repeat 18 [10], we mutated all of the lysines to arginines, with the exception of lysine 10, either individually or in groups when clustered close together. Arginine was chosen so as not to change the charge of the protein. The mutants generated are as indicated in Fig. 1 and Table 2. Next, we investigated the effects of the Lys to Arg mutations on the ubiquitination of the GSTα16–17 peptide. Ubiquitination assays were performed as described in Section 2. ^{125}I-Ub incorporation in GSTα16–17 wild type and mutant peptides was quantified using a Molecular Imager. The counts were normalized with the real contents of proteins in the Coomassie stained gel before comparison. In all experiments the ubiquitination level of wild type GSTα16–17 was taken as 100%. In a first series of experiments this level of ubiquitination was not significantly affected by mutations on lysines 3, 4, 5, 49,

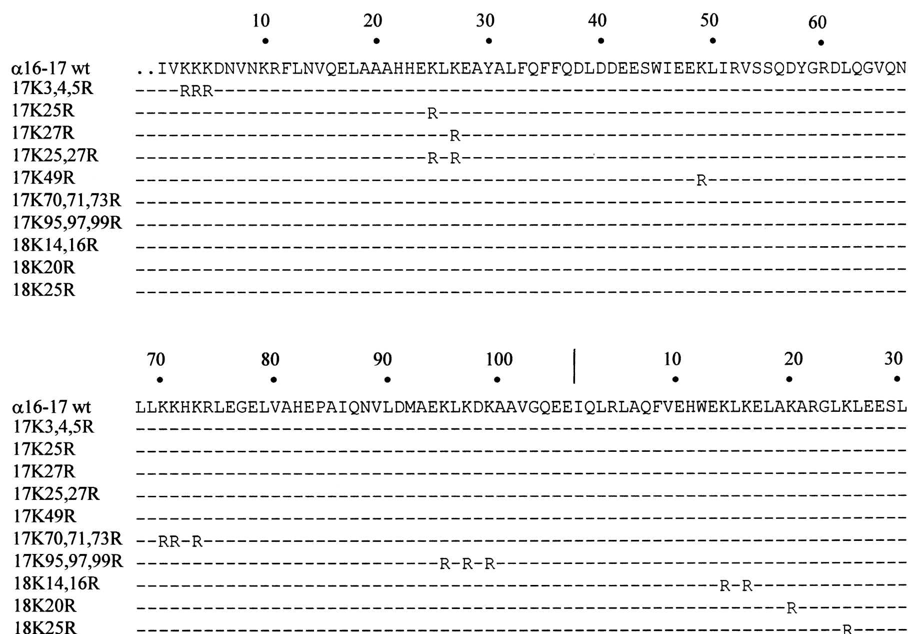

Fig. 1. Partial amino acid sequence of wild type GSTα16–17 peptide and the mutant peptides produced for this study. The sequence starts at the beginning of repeat 17 and stops at the end of the peptide (Leu 30 of repeat 18 according to the published human α-spectrin sequence). Hyphens indicate unchanged residues.

Fig. 2. Ubiquitination of wild type GSTα16–17 or mutant GSTα16–17 peptides. The mutant peptides were tested in ubiquitin conjugation assays as described in Section 2. The ubiquitination of the mutant peptides is shown as a percent of wild type GSTα16–17. The values are the means of several independent experiments ± S.D.

70, 71, 73, 95, 97 and 99 in repeat 17 (mutants K3,4,5R, K49R, K70,71,73R and K95,97,99R) and on lysines 14, 16, 20 and 25 in repeat 18 (mutants K14,16R, K20R and K25R). In contrast, replacement of lysines 25 and 27 together in repeat 17 with arginines (mutants K25,27R) markedly attenuated the conjugation of ^{125}I-ubiquitin on the GSTα16–17 peptide (Fig. 2). Subsequently we mutated lysines 25 and 27 separately in order to determine whether ubiquitination required one specific lysine or both. Mutant K25R was ubiquitinated in the same way as the wild type, whereas mutant K27R was much less ubiquitinated. These findings indicate that lysine 27 serves as the primary Ub acceptor of α-spectrin repeat 17. In order to define the repeat 17 primary sequence involved in the E2/E3 recognition, we constructed three small peptides, referred to as A, B and C, respectively, consisting of a sequence of 29, 19 and 11 amino acids on repeat 17. Each sequence contained lysine 27 at its center. The ubiquitination assay using these peptides as substrate showed no evidence of ubiquitin binding, indicating that the secondary structure of α-spectrin is very important for the interaction with the enzymatic system of ubiquitin conjugation.

3.3. *Sequence homology of the α-spectrin ubiquitination site*

In an attempt to find some protein patterns homologous to the region flanking lysine 27, we searched the PROSITE database using PROSCAN. A search with a 30 amino acid sequence containing lysine 27 gave some similarities to various protein patterns. The most interesting was a 75% similarity of the α-spectrin fragment LAAAHHEKLKEAYALFQFFQDL (lysine 27 is underlined) with the leucine zipper pattern.

4. Discussion

Although the enzymatic pathway for ubiquitin conjugation is well characterized, the way in which proteins are selected for ubiquitination is only beginning to be clarified. In addition to providing a lysine residue(s) which can be ubiquitinated, the substrate proteins contain elements that are targeted directly by the ubiquitination apparatus, which in most cases is expected to be a specific E2/E3 complex [2]. In the present study we have demonstrated a critical requirement for the presence of lysine 27 of repeat 17 for ubiquitination of human erythroid α-spectrin recombinant peptide. Although ubiquitination was dramatically inhibited in mutant K25,27R and K27R, it appears not to be totally abrogated (see Fig. 2). This fact may be explained with two hypotheses: (i) ubiquitination can also occur at other sites, albeit much less efficiently and (ii) endogenous AGA codons or other AGA codons introduced by mutagenesis are subject to low frequency misincorporation of lysine for arginine in *E. coli*, as previously described by You et al. [29]. The presence of lysine 27 of repeat 17 in recombinant peptides is not sufficient to promote ubiquitin conjugation. In fact, peptides GSTα15–16 and GSTα17 contained this lysine but were not ubiquitinated

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                    10         20         30         40         50
SPCA_HUMAN IVKKKDNVNK RFLNVQELAA AHHEKLKEAY ALFQFFQDLD DEESWIEEKL IRV
SPCA_MOUSE IEEKMNGVNE RFENVQSLAA AHHEKLKETY ALFQFFQDLD DEEAWIEEKL LRV
SPCA_DROME IQEKRQSINE RYERICNLAA HRQARLNEAL TLHQFFRDIA DEESWIKEKK LLV
SPCN_HUMAN VKDKRDTING RFQKIKSMAA SRRAKLNESH RLHQFFRDMD DEESWIKEKK LLV
SPCN_RAT   VKEKRDTING RFQKIKSMAT SRRAKLSESH RLHQFFRDMD DEESWIKEKK LLV
SPCN_CHICK VKDKRETING RFQRIKSMAA ARRAKLNESH RLHQFFRDMD DEESWIKEKK LLV
           :  * : :*  *:  :  :*:  :: :* *:    * ***:*:  ***:**:**  : *

                 60         70         80         90        100
SPCA_HUMAN SSQDYGR DLQGVQNLLK KHKRLEGELV AHEPAIQNVL DMAEKLKDKA AVGQEE
SPCA_MOUSE SSQDYGR DLQSVQNLLK KHKRLEGELV AHEPAVQNVL DTAESLRDKA AVGKEE
SPCA_DROME GSDDYGR DLTGVQNLKK KHKRLEAELG SHEPAIQAVQ EAGEKLMDVS NLGVPE
SPCN_HUMAN GSEDYGR DLTGVQNLRK KHKRLEAELA AHEPAIQGVL DTGKKLSDDN TIGKEE
SPCN_RAT   SSEDYGR DLTGVQNLRK KHKRLEAELA AHEPAIQGVL DTGKKLSDDN TIGQEE
SPCN_CHICK SSEDYGR DLTGVQNLRK KHKRLEAELA AHEPAIQGVL DTGKKLSDDN TIGKEE
            *:**** **  **** * ****** **  :****:*  *  :  : * *    :*  *
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Fig. 3. Comparison of sequences of α-spectrin repeat 17. Erythroid and non-erythroid α-spectrin of different species with known sequences are compared. '*' indicates perfect matches and ':' indicates a high similarity between the sequences. Lysine 27 in the human sequence is underlined.

[10]. These data indicate that some amino acid residues flanking this lysine are necessary for recognition of the substrate and subsequent ubiquitination. In an attempt to define the sequence flanking lysine 27 involved in the conjugation process, we constructed three smaller peptides (A, B and C) containing this lysine in the middle of their sequence. These peptides were only part of repeat 17 and we expected that their secondary structure would be lost or severely compromised, as described by Winograd et al. [17]. None of these peptides were ubiquitinated in vitro, suggesting that not only the primary sequence is important in the recognition of α-spectrin by the enzymatic system of ubiquitin conjugation but also the correct folding of the molecule. Lysine 27 is located in a region between helices C and A of the triple helical structure of the repetitive unit. In the crystal structure, helices C and A appeared to be uninterrupted [20]. In solution, however, the flexibility between segments appears to involve some disruption of this continuous helix [30]. Recent hydrodynamic studies of human erythroid α-spectrin fragments support the hypothesis that there is a flexible and non-helical linking region between rigid segments [31]. Lysine 27 is located in this region and it could be interesting to verify the involvement of ubiquitination in regulating the flexibility of the molecule. Interestingly, comparison (using the LALIGN program) between a 30 amino acid sequence flanking this lysine and the entire α-spectrin sequence showed an identity of 36.7% with the homologous part of repeat 20, probably the second locus of ubiquitination, as we shown previously [10]. In all repetitive units of α-spectrin, lysine 27 is present only in repeats 17 and 6 but in this case the identity of sequences flanking this lysine was only 30%. Sequence comparison of non-erythroid and erythroid repeat 17 α-spectrins of different species showed low similarity in the region containing lysine 27 (Fig. 3). In fact, in this region only human and mouse erythroid α-spectrin can be considered very homologous (about 93% identity). Interestingly, mouse α-spectrin is also ubiquitinated in vitro (not shown). Moreover, in *Drosophila* α-spectrin and in non-erythroid spectrins, lysine 27 is not present. Previously we had shown that non-erythroid chicken α-spectrin was not ubiquitinated in vitro [8] and our current data may explain this finding. In conclusion, the data presented seem to indicate that there is no strict sequence consensus motif for ubiquitination in α-spectrin but rather a conformational recognition mechanism where the position of a critical lysine seems to be important.

A further sequence homology search of the α-spectrin ubiquitination site identified a similarity with the leucine zipper pattern. The leucine zipper consists of a periodic repetition of leucine residues at every seventh position. The segments containing these periodic arrays of leucine residues seem to exist in an α-helical conformation. The leucine zipper pattern is present in many gene regulatory proteins, many of which, curiously, are ubiquitinated [32]. In particular c-*jun* could be ubiquitinated in this region [33]. Such similarities may indicate certain homologies in parts of sequences of completely different ubiquitinated proteins.

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