Title

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Journal

Annals of Biomedical Engineering, 31(11)

ISSN

0090-6964

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Publication Date

2003-12-01

DOI

10.1114/1.1635820

Peer reviewed

Protofilament and Hexagon: A 3-D Mechanical Model for the Junctional Complex in the Erythrocyte Membrane Skeleton

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Running title: Protofilament and Hexagon

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ABSTRACT

It is a long-standing mystery why erythrocyte actin filaments in the junctional complex (JC) are uniformly ~37 nm and the membrane skeleton consists of hexagons. We have previously proposed that a "molecular ruler" formed by E-tropomodulin (E-Tmod) and tropomyosin (TM) 5 or 5b functions to generate protofilaments of 12 G actin under mechanical stress. Here we illustrate that *intrinsic* properties of actin filaments, e.g., turns, chemical bonds, and dimensions of the helix, also favor fragmentation into protofilaments under mechanical stress. We further construct a mechanical model in that a pair of G actin is wrapped around by a split α and β spectrin, which may spin to two potential positions, and stabilize to one when the tail end is restricted. A reinforced protofilament may function as a mechanical axis to anchor 3 (top, middle and bottom) pairs of Sp. Each Sp pair may wrap around the protofilament with a wide dihedral angle (~166.2^o) and a minimal axial distance (2.75 nm). Such 3 Sp pairs may spiral down (right-handed) the protofilament from the pointed end with a dihedral angle of ~55.4^o in between Sp pairs. This first 3-D model of JC may explain the hexagonal geometry of the erythrocyte membrane skeleton.

Key words: Actin, Protein 4.1, Spectrin, Tropomodulin, Tropomyosin.

INTRODUCTION

The actin filaments comprise one of the most important cytoskeletons in animal cells. They are organized into arrays of contractile bundles, gel-like networks, or tight parallel bundles, often with enormous variations in length. The actin filaments in the membrane skeletal network of human erythrocytes, however, are uniformly ~37 nm (Fig. 1a).[37] These short actin filaments are referred to as "protofilaments." Interestingly, each protofilament located at the center of a junctional complex (JC) is (mostly) associated with six $\alpha\beta$ spectrin heterodimers (Sp). Furthermore, each lattice of the hexagonal network is made out of a spectrin tetramer, formed by head-to-head association of two Sp from adjacent JC. Therefore, this thin skeletal network underneath the lipid bilayer has a distinct hexagonal feature. Since this network governs the elastic deformation of erythrocytes and ensures the integrity of the lipid bilayer, understanding how protofilaments are generated and how hexagons are constructed in the membrane skeletal network is fundamentally important.

The remarkably regular organization of the erythrocyte membrane skeleton is a curious and important mystery. To establish a hexagonal structure with ~6 Sp radiating from a central short protofilament, nature must have robust mechanisms for controlling the length of protofilaments and organizing Sp upon them.

The main purposes of this article are twofold: (1) to illustrate a new mechanism by which a protofilament is generated, based on the *intrinsic* properties of the actin filaments; and (2) to propose a 3-D model for the JC, in which a protofilament functions as a mechanical axis for six cable-like Sp, to build a basic unit for the hexagonal organization. The mechanical model for JC proposed here (#1 manuscript) was further developed into a numerical model (#2 manuscript), which was then used to predict the motion and configuration of a protofilament and the tension of each of the

associated 6 Sp in response to various mechanical stresses (#3 manuscript). As a result, the biomechanics of JC in membrane skeletal network will be understood in greater detail at the molecular level.

The main molecules in the current model are actin and spectrin, with tropomyosin (TM), erythrocyte tropomodulin (E-Tmod), and protein 4.1 that are incorporated to stabilize a particular spectrin/actin assembly. Additional proteins, such as glycophorin C and adducin, etc., may be incorporated in a more complete model in the near future.

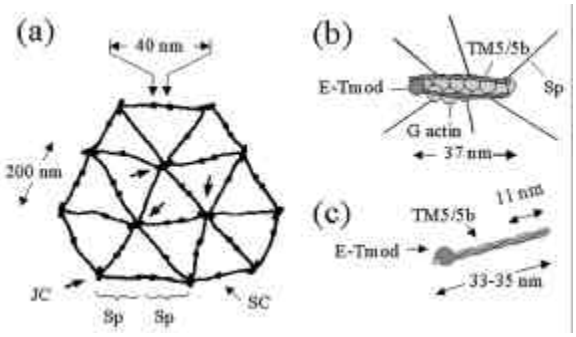

FIGURE 1. Schematic drawings of the hexagonal network the erythrocyte membrane skeleton (a), a junctional complex (b), and an E-Tmod/TM complex (c). (a) is an expanded hexagonal network, based on electron micrographs of erythrocytes in hypotonic solution;[22] (b) shows a protofilament of ~37 nm associated 2 TM, an E-Tmod and 6 Sp; and (c) shows an E-Tmod/TM complex as a "molecular ruler" for the protofilament. JC, junctional complex, 200 nm apart; SC, suspension complex, 40 nm apart; TM5/5b, tropomyosin isoform 5 and 5b homo- or heterodimer.

G actin is a globular protein of ~42 kDa consisting of 375 residues. It is composed of two major domains, the smaller divided into subdomains 1 and 2, and the larger divided into subdomains 3 and 4.[21] It has a tendency to assemble into a filamentous structure (F-actin), with an apparent double helix. The structure of F-actin, or actin filament of α actin, has been determined by electron microscopy,[4] and that of αG actin with Dnase I by X-ray crystallography.[21] β G actin is generally similar to α G actin in structure, as revealed by crystalline profilin-β actin, but with several significant local changes and an overall 5^{o} rotation between its two major domains.[33] The domain rotation may represent the native structure or a conformation induced during formation of the ribbon contact. An atomic model of F-actin has been described using αG actin Dnase I crystallographic data.[20] Using high-resolution electron cryomicroscopy of a cofilin-actin filament, helical reconstruction of F-actin and cofilin-actin have also been reported.[8] The biophysical properties of the actin filament network[51] and single actin filaments, such as stretching, bending and twisting have been extensively studied. Polymerization and depolymerization of the actin filament occur at both ends: faster at the barbed end and slower at the pointed end (for review of actin see [1,40]). There are 6 distinct, yet highly conserved, actin isoforms in humans. In erythrocytes, the gene product is that of the non-muscle or cytoplasmic β actin gene.[31]

There are several actin-binding proteins that regulate the assembly of actin and its interactions with other proteins. One of them is TM, which is a rod-like molecule consisting of long coiled coils. TM may be classified into high (HMW) and low molecular weight (LMW) TM. The former generally has 284 residues and is ~42 nm in length, and the latter 248 residues and ~34 nm (for review of TM see [22]). In human erythrocytes, major TM isoforms are TM5[44] and TM5b,[43] gene products of the γ- and α-TM genes, respectively. Interestingly, both TM5 (29.0 kDa) and TM5b (28.7 kDa) are of LMW TM and capable of forming homodimers or heterodimers with each other. One additional important feature shared by TM5 and TM5b is their high affinity toward both actin and E-Tmod, allowing these three molecules to form

stable complexes.[43] TM in the text refers to the homodimer or heterodimer of TM5/5b, unless stated otherwise.

Human E-Tmod is a 40.6-kDa protein consisting of 359 residues.[42] It is a TM-binding[14] and an actin-capping protein that blocks the elongation and depolymerization of the actin filaments from the pointed or slow-growing end.[48] Although originally identified in human erythrocytes, E-Tmod is also expressed in other cells and tissues, such as cardiac and skeletal muscles (for review see [15]). Both human and mouse *E-Tmod* genes have been cloned and characterized. Three E-Tmod homologs of similar size derived from distinct genes (i.e., Sk-Tmod, N-Tmod, and U-Tmod) exist in humans and mice.[12] The finding that target disruption of the *E-Tmod* gene resulted in embryonic death in mice indicates that the capping function of E-Tmod cannot be compensated by other Tmod homologs.[10]

In vitro and *in vivo* studies have revealed expression, localization, interaction, function, and disease relevance of several actin, TM, and Tmod isoforms. While some principles found in striated muscles may be applicable to non-muscle cells, different isoforms in many cases are utilized to build different structures for distinct functions. For example, in skeletal muscles, the sarcomeric thin filament is long and uniform in length (~1 μm) and composed of α actin; TMs are α-TM and β-TM of HMW; and Sk-Tmod is expressed in addition to E-Tmod (in certain types of muscles and in certain species). The N-terminus of nebulin, a long molecule that spans the entire length of the thin filament, binds to E-Tmod, [26] providing a possible mechanism by which skeletal muscles regulate the length of their thin filaments. Nebulin, however, is not expressed in erythrocytes or cardiomyocytes.

Several models/sketches for JC in the erythrocyte membrane skeleton had been reviewed[15] and briefly compared.[41] In fact, the view of protofilaments with a typical length of ~37 nm is slightly controversial.[15] Different numbers of G actin (e.g., 18 or 12) and Sp (e.g., 6, more, or less) or different lengths of the actin filaments (e.g., ~60 or ~37 nm) have been proposed.

We have previously proposed that the complex formed by E-Tmod and TM5/5b (stands for their homo- or hetero-dimer) (Fig. 1c) may function as a "molecular ruler" for protofilaments in erythrocytes.[41,43] We reasoned that the high affinities of TM5/5b toward both F-actin and E-Tmod, and the given length of TM5/5b make the ruler complex suitable to protect the actin filament, under stress, of one TM length, which is equivalent to 12 G actin. We then assumed the ratio of 2:1 G actin:Sp produces six associated Sp for the hexagonal geometry.[43] Our previous "molecular ruler" mechanism, however, did not consider contributions made by some of the intrinsic properties of the actin filament. Furthermore, we assumed that a pair of G actin is associated with one Sp without going into detail as to how that is achieved.

This article takes a fresh start and a closer look at a single naked actin filament at the molecular level to illustrate a new mechanism by which a protofilament is generated from a mechanical point of view. We then review the role of E-Tmod/TM complex, a previously proposed molecular ruler, in stabilizing, reinforcing, and generating a protofilament. We further consider several possible modes by which one protofilament may be associated with 6 Sp. One mode may allow a protofilament to function as a mechanical axis for 3 pairs of Sp (top, middle, and bottom) to span a full

circle around the axis. We also consider how suspension complexes (SC, in Fig. 1a) facilitate the head-to-head association of Sp in completing the hexagonal geometry of the membrane skeleton. Overall this investigation reveals the beauty of a JC as a mechanical design, explains the hexagonal geometry of the membrane skeletal network, and makes the biomechanics of erythrocyte membranes more understandable at the molecular level.

METHODS

G Actin

Based on the model of F-actin consisting of α G actin developed by Bremer and Aebi,[3] each G actin may fit into a cube with dimensions of 5.5 nm x 5.5 nm x 3.5 nm. These dimensions are used to analyze the mechanical stress and dihedral angles among G actin with respect to the long axis of the protofilament. The orientation of each G actin is further identified by 4 subdomains, both the front and back of them.[3] In the text, G actin refers to α G actin unless specified otherwise.

Actin Protofilament

The 3-D view of a long actin filament (Fig. 2) was generated using the AC3D software (Version 3.6, Inivis Ltd, UK), in which the actin filament was represented as a helical structure with 12 G actin per left-handed 180^o turn, and a repeat of 35.75 nm.[20] In the mechanical analysis we treat the actin filament as a single helix (Fig. 2), even though because the dihedral angle between one G actin and the next is 166.154^o (166.2^o used in the rest of the text)[20] from the pointed end to the barbed end (see Fig. 3a), the actin helix morphologically appears as two right-handed steep helices, which twine gradually round each other (see Fig. 3b). For clarity in the model we name each G actin based on the apparent strand (i.e., a or b strand) it is in, and the position it has from the pointed end to the barbed end.

Maximum Stress in Bending

The in-plane bending stress on a helicoidal beam of rectangular cross-section, when it is bent in the *x*-*y* plane (Fig. 2), can be calculated approximately as:[16]

$$\sigma = (M/I)\, y \qquad (1)$$

where σ is the bending stress (in pN/nm^2) along the *x* axis, M is the moment (in pN-nm), I is the moment of inertia ($bh^3/12$ in position 2 or $hb^3/12$ in position 1 or 3 in Fig. 2, in nm^4), and *y* is the distance from the neutral axis (*h*/2 in position 2 or *b*/2 in position 1 or 3 in Fig. 2, in nm).

When *y* is measured to the extreme fiber (i.e., top or bottom surface, C) of the cross section, the maximum stress, σ_{max}, can be calculated as:

$$\sigma_{max} = (M/I)\, C = M\,(C/I) = M/S \qquad (2)$$

where S is the elastic section modulus (in nm^3).

$$S_2 = bh^2/6 \text{ for position 2}$$
$$S_1 = S_3 = hb^2/6 \text{ for position 1 and 3} \qquad (3)$$

When *h* is larger than *b*, Eq. (3) shows that the elastic section modulus (S_2) at position 2 is larger than that (S_1 or S_3) at position 1 or 3. Therefore, it can be shown from Eq. (2) that the maximum stress (σ_{max}) at position 1 or 3 is greater than that at position 2.

Spectrin Heterodimer (Sp), Tetramer, and Tail

The α and β spectrin subunits are arranged anti-parallel to each other, with the tail end associated with the protofilament, consisting of the N-terminus of β spectrin and the C terminus of α spectrin.[39] A spectrin tetramer is formed by the head-to-head association between two Sp, with the C terminus of β spectrin binding to the N terminus of α spectrin[46] in each strand. The tail end of Sp associates with the protofilament by having a G actin binding site on β spectrin at its N-terminus. This site is adjacent to a protein 4.1 binding site and followed by 17 triple helical repeats. The C-terminal 13 residues of α spectrin also contribute to the binding of Sp to the JC, but no G actin binding site has been identified on α spectrin.[47] Therefore, an Sp tail is treated in the model as a tie made by α (N-terminus) and β (C-terminus) spectrin. The tying may strengthen the αβ spectrin interaction, resist separation from actin, and become bulky when it is associated with protein 4.1.

b Spectrin Binding Site on G Actin

Since the β spectrin binding site on G actin has not been identified, its position is not specified in the model, but is treated topologically consistently among all G actin. Therefore, the β spectrin binding sites between two adjacent G actins have a dihedral angle of ~166.2^o with respect to the long axis of the filament (see Fig. 4). Each bound β spectrin (e.g., β_3) is placed in a cleft formed by 3 consecutive G actin (e.g., 3a, 3b, and 4a), and numbered according to the middle G actin (e.g., 3b). The binding site of α-actinin, whose triple helical repeats resemble the first repeats of β spectrin (and the last repeats of α spectrin),[6] has been mapped to the outer face of subdomain 2 and contacts subdomain 1 of two neighboring G actin along the long-pitch helical strand of F-actin.[28] A recent study also positioned spectrin-like molecules (utrophin and dystrophin) to the similar location.[32]

RESULTS AND DISCUSSION

Creation of Protofilament

When long actin filaments are subjected to mechanical stress, such as in-plane bending (this study) or other stress modes,[36] they would more likely fragment into ~36 nm length than any other. This may be due to (1) the actin helix has an intrinsic property of ~36 nm per 180^o turn,[20] (2) only weak non-covalent bonds (without strong covalent bonds) exist between individual G actin subunits, and (3) the cross-section dimensions of the helix (*y*- and *z*-axes, Fig. 2) are not equal.

Maximum Stress

Electron micrographs of long actin filament revealed a successive narrow (7-nm diameter) and wide (9-nm diameter) view of the helix that repeats every ~36 nm with an 180^o rotation.[4] The reason why a long helix breaks at the ends of a 36-nm turn, the narrowest viewpoints (positions 1 and 3, Fig. 2), rather than in the middle (position 2) or anywhere in between, may attribute to the finding that the maximum stress (σ_{max}) is the highest at these two positions. Assume the cross section of the actin filament at the maximum height, position 2, is rectangular with an area of h x b, where h is the height (9 nm, the wide view point) and b is the width (7 nm, the narrow view point). Here an analogy of a helicoidal beam of rectangular cross sections is being used to illustrate how chemical bonds will be loaded in different cross sections to carry a given

moment. Fig. 2 illustrates that the maximum stress for the bending in the *x*-*y* plane (i.e., bending about the *z* axis) is the greatest where the height in the *y* direction is the smallest (the narrowest view points) at positions 1 and 3. The maximum stress at the bottom of the cross section, σ_{max}, facing the force at position 2 is $6M/bh^2$ (see Methods). At positions 1 and 3, the two ends of a protofilament, their cross sections are the same as that at position 2, except that they are 90^o from that at position 2. The values of σ_{max} at the bottom of their cross sections are, therefore, $6M/hb^2$. As the result, the ratio of maximum stress at positions 1, 2, and 3 would be h/b:1:h/b, which is approximately 1.29:1:1.29. The filament, therefore, would preferentially break at positions 1 and 3, creating a protofilament of ~36 nm. If *h* were equal to *b*, σ_{max} would be the same along the entire filament. In that case, there would be no preferred break points, and no protofilament would be generated. Other possible mechanical stresses that may also contribute to the breaks at the positions 1 and 3, and how the detailed contour surface between G actin[34] may affect the 3-D fracture in the in-plane bending are being investigated.

Our hypothesis that the intrinsic properties of the actin filaments may favor the formation of protofilaments by mechanical stress is supported by the analysis of maximal stress along the actin filament in one mode (i.e., in-plane bending) reported here. It is also supported by a report[36] in which several ~37 nm long fragments along the filamentous actin were imaged by cryoatomic force microscopy. Shao et al.[36] presumed that the filaments were fragmented before being imaged by AFM, and that the ~37 nm periodicity of fragmentation was due to periodic surface interactions. Although the assumed forces applied to the actin filament (i.e., the surface tension of the solution that flattened the filament to the mica surface, and the electrostatic interaction that maximized the contact area) and the temperature (e.g., 80 to 85 K), etc., in this case may not apply directly to actin filaments in living cells, the profile of the maximal stress that led to ~37 nm fragments is important and deserves to be analyzed.

Non-Covalent Bonds Between G Actin

The breaks at the maximum stress may occur between G actin subunits, not within G actin. When covalent and non-covalent bonds are equally loaded, non-covalent ones may break first. Multiple weak non-covalent bonds between G-actin, without stronger peptide bonds that exist within G actin, may allow the breakage between G actin to occur. If covalent bonds were to exist in between subunits in the helix, like in the case of DNA, the energy would be largely used to deform the filament (within and between subunits) before the filament breaks.

Phases of TM

A long actin filament partially coated by LMW TM, e.g., TM5 or 5b,[43,44] may also be a good source for protofilament production. It is known that TM5 and 5b are ~33-35-nm long (Fig. 1c), which is equal to or slightly shorter than that of the ~36 nm per 180^o turn of the actin filament. As illustrated in Fig. 3, a TM may be able to position in one of the 2 grooves of the actin filament, which spans 180^o from one end to the other (Fig. 3). TM5 and TM5b in the grooves, therefore, may not only be in phase with the protofilament in length, but also in phase with the angle of the turn. Such TM, therefore, may reinforce along one protofilament where the values of σ_{max} may already be lower than that at positions 1 and

3, but offers no protection at positions 1 and 3 where the σ_{max} is the greatest, thus facilitating the fragmentation of the actin filament into protofilaments.

Retention of a Protofilament

When naked protofilaments or TM-coated protofilaments of ~36 nm are generated by mechanical stress, they would quickly lose their hallmark length, unless there are mechanisms to retain them. That is due to their ability to reassemble themselves as units, by annealing, or to undergo polymerization/depolymerization by adding or subtracting individual G actin at either end.

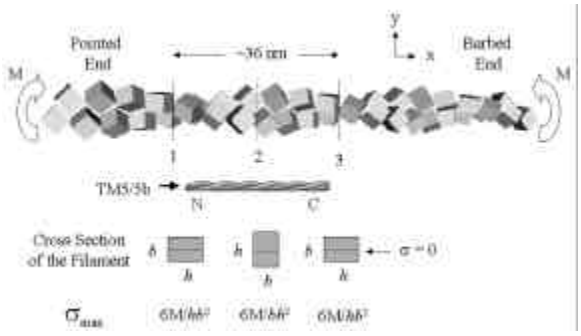

FIGURE 2. Bending of a long helical actin filament may create a short protofilament of ~36 nm. Each box in the long actin filament represents a G actin subunit. Two arrows at the pointed and barbed ends indicate the bending of the filament in the plane of *xy*. Three cross sections (in the plane of *yz*) of the actin filament at the two ends of (positions 1 and 3) and the mid point (position 2) of the ~36-nm and 180-degree turn are shown. At position 2, *h* is the height and *b* is the width. The dash lines in the middle of cross sections are the neutral axis where the stress is zero. The maximum stress, σ_{max}, at the bottom of the helical actin filament is the greatest at positions 1 and 3, and the lowest at position 2. The *xyz* coordinates are shown.

There are several proteins capable of capping either the barbed or the pointed end of the actin filament. E-Tmod, a pointed end capping protein, binds to the N-terminal residues 7-14 of TM5.[45] When a rod-like TM5 of 33-35 nm is bound with an E-Tmod (approximately the same molecular weight as a G actin) (Fig. 1b), the complex may position itself at the pointed end of the protofilament and prevent the polymerization from that end. Under this condition, the barbed end may be free to grow if not capped (e.g., by adducin or gelsolin) and a pool of G actin above the critical concentration is available. However, newly grown segments would be fragmented again given the stress and strain of erythrocytes during circulation. Therefore, under this condition, if all TM are saturated by E-Tmod, no growth at either end would occur.[43] In solutions of pure actin, filaments assume an exponential distribution of lengths that is well described by assuming a uniform probability of fragmentation along a filament balanced by annealing.[35] The importance of annealing in the length control of actin filaments further supports the importance of E-Tmod/TM complex in the retention of protofilaments.

In definitive erythrocytes, the formation of protofilaments may take place during the maturation of reticulocytes. Reticulocytes in the initial stage are rigid, despite the fact that they have enucleated, and take about two days to mature into highly deformable erythrocytes.[7] The deformation is especially drastic as they go through small capillaries or enter narrow slits of the spleen sinusoids whose dimensions may be smaller than that of erythrocytes. It is likely that during the first stage of reticulocytes, long actin filaments of the cytoskeleton may be fragmented into many protofilaments, which can be used as beams of uniform length to build the thin membrane skeletal network.

Intrinsic Helix Mechanism vs. Molecular Ruler Mechanism

In erythrogenesis, it is interesting to consider whether a filament is more likely to experience pure bending over a small stretch to liberate a ~36 nm section or some

stochastic spike somewhere that breaks the filament in two. The final consequence, however, may be similar as eventually the filament may break into protofilaments either by the intrinsic "helix" mechanism or the external "ruler" mechanism. The calculation for σ_{max} (see Methods and Fig. 2) may also be applicable to the ruler mechanism, which goes like this: If a long filament breaks into two, each pointed end of the filament may be associated with a complex of E-Tmod/TM5 or E-Tmod/TM5b. When TM protects only the terminal segment of ~33-35 nm but not at position 3 where σ_{max} would be the greatest (Fig. 2, also under *Phases of TM*), and E-Tmod prevents the filament from annealing or polymerization at the pointed end, at the end, only protofilaments of ~36 nm reinforced by "molecular rulers" may survive under mechanical stress.

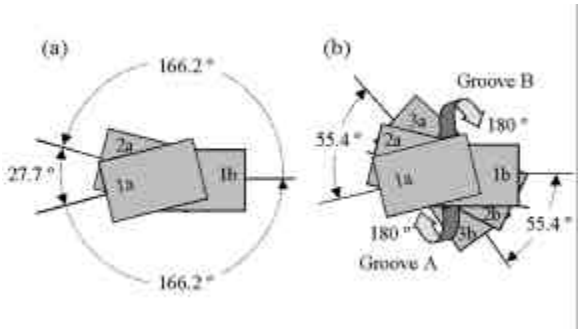

FIGURE 3. Dihedral angles between G actin subunits with respect to the long axis of the protofilament. (a) shows the actin filament as a left-handed strand with a dihedral angle of ~27.7^o between G actin 1a and 2a. (b) shows the actin filament as two right-handed strands with a dihedral angle of ~55.4^o between G actin 1a and 3a or between G actin 1b and 3b. The ~180^o turns of the grooves A and B are also indicated.

It has long been suggested that the uniform length of actin filaments in erythrocytes was the result of the actin complexing with tropomyosin.[37] However, it was not at all clear why the complex should stop with only one tropomyosin molecule. The classic (but untested) hypothesis for controlling protofilament length is that tropomyosin provides a template and recruits capping proteins at either end to control filament growth. The specific "molecular ruler" hypothesis we have previously proposed was that TM5 or TM5b isoforms of ~33-35 nm provide a template and recruit capping protein E-Tmod at the pointed end, and the complex protects actin filaments of ~37 nm under mechanical stress.[41,43] We have now generated recombinant proteins of TM5, which have been mutated to different lengths, in order to test the "molecular ruler" hypothesis. This would put two specific mechanisms that we have proposed (i.e., the helix mechanism and the ruler mechanism) into direct test and perhaps be able to quantify their relative importance in controlling the length of protofilaments.

Six Pairs of G Actin

A mechanically generated protofilament would be a helix consisting of 12 or 13 G actin. Based on previous findings that there is a left handed rotation of ~166.2^o from one G actin to the next,[20] Fig. 3a and 3b show the top view of first 3 and 5 G actin in a protofilament with the pointed end at the top, respectively. Each G actin is drawn as a rectangle.[3] The straight line radiating out from the midpoint of each G actin represents the dihedral angle of G actin with respect to the long axis of the protofilament. Fig. 3a shows the actin filament as a single left-handed strand; Fig. 3b shows the same structure appears as two right-handed strands. All G actin in one apparent right-handed strand are labeled "a"; the others "b", and numbered 1 to 6 or 7 from top to bottom.

Here we define a "pair" of G actin as the two G actin, one from each apparent strand, e.g., 1a and 1b, which are staggered by 2.75 nm. Thus a protofilament of 13 G actin may

consist of 6 pairs of G actin: 1a/1b, 2a/2b, 3a/3b, 4a/4b, 5a/5b, and 6a/6b, plus 7a. Fig. 3a illustrates the inclusion angle of ~166.2^o within a pair of G actin with respect to the long axis of the protofilament, and the inclusion angle of ~27.7^o between two closest G actin in the same strand (e.g., 1a and 2a, with 1b in between). Repeating this arrangement, the inclusion angle between 1a and 3a or 1b and 3b may become ~55.4^o (Fig. 3b). The two grooves of the protofilament, each having a right-handed rotation of 180^o, are also indicated. The side view of groove A looks at the front of G actin 1a and the back of 1b; and that of groove B looks at the back of G actin 1a and the front of 1b. Even though the inclusion angle of ~166.2^o is used here to construct the model, it is understood that variable twist (± 5-6^o) between adjacent actin subunits in a filament does indeed exist, not only in the naked actin filament, but also in actin filament decorated with different actin binding proteins and actin filament within actin bundle.[18,13,27]

Modes of Sp Association with Protofilament

In order for the membrane skeleton to have a hexagonal arrangement, i.e., 6 Sp per protofilament, six Sp must either associate with 6 well-defined pairs of G actin, or six Sp must associate randomly with 6 G actin and leave the other 6 or 7 G actin unbound. Three possible modes are presented in Fig. 4. The top view shows the dihedral angles among G actin occupied by Sp, and the side view shows the vertical relationship among them. For clarity, the 180^o turn of the protofilament (from 1a to 7a) is not drawn in the side view. All putative positions of Sp binding sites among all G actin (~166.2^o from one G actin to the next, e.g., 1a to 1b) are indicated by arrows, occupied (closed) or unoccupied (open). Mode 1 is random and produces various dihedral angles among 6 Sp. More accurately, these angles are of those (shaded) G actin occupied by Sp. On the other hand, both Modes 2 and 3 assume the regularity that there is only one Sp associated with one pair of G actin. Modes 2 and 3, however, differ in the choice of G actin within the pair to which Sp binds. In Mode 2, all 6 Sp are bound to either 6 "a" or 6 "b" G actin. In Mode 3, 6 Sp are bound to 3 "a" and 3 "b" G actin, alternating between the 2 apparent strands from one end to the other (i.e., 1b, 2a, 3b, 4a, 5b and 6a. Mode 2 may result in dihedral angles of 6 or 7 Sp spanning ~138.5^o or ~166.2^o (27.7^o x 5 or 6), approximately half circle around the long axis of the protofilament. Each of the 6 Sp may be evenly spaced, both axially and angle wise, along one side of the protofilament. Mode 3, on the other hand, may allow dihedral angles of 6 Sp to span almost evenly in a full circle of 360^o: 5 dihedral angles of ~55.4^o and one ~83.0^o. The 6 Sp may be arranged into 3 back-to-back clusters at the top, middle, and bottom regions of the protofilament. An additional Sp may be associated with G actin 7b, which would have the same dihedral angle as 1a, and would result in a heptagon with 6 angles of ~55.4^o and one ~27.7^o. Heptagons exist in ~8% of the configurations in the membrane skeleton.[23]

If variable random twist between adjacent G actin is taken into consideration, the 6 dihedral angles in Mode 3 may deviate slightly from 5 angles of ~55.4 and one angle of ~83.0, with a possibility of making them more similar to each other.

Three Pairs of Sp

Mode 3 may also allow the classification of 6 Sp into 3 Sp pairs: Sp1/2, Sp3/4, and Sp5/6, in which the dihedral angle within a Sp pair (e.g., Sp1 radiating out from 1b, and Sp2 from 2a) may be the widest (~166.2^o)

and the axial distance within a Sp pair (e.g., Sp1 and Sp2) may be the minimum (i.e., 2.75 nm).

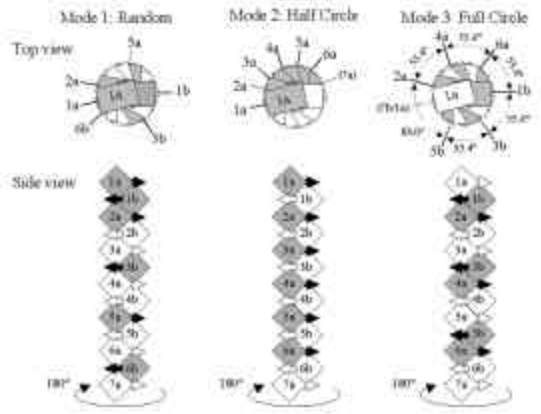

FIGURE 4. Three possible Modes of 6 G actin bound by 6 Sp in a protofilament. Both the top view (the pointed end at the top) and side view (without 180° turn for clarity) are presented. Note the β spectrin binding site (arrow) on G actin is assumed to have polarity. The open arrows are those not occupied by β spectrin; the closed ones are. In Mode 1, the distribution of occupied G actin is random, and so are their dihedral angles; in Models 2 and 3, the occupied G actin are in order. In Mode 2, the 6 occupied G actin are evenly distributed, resulting 6 β spectrin binding sites spanning half of the circle around a protofilament. In Mode 3, 6 occupied G actin are clustered in a back-to-back fashion, resulting 6 β spectrin binding sites spanning the complete circle of a protofilament.

One Sp Wraps Around One Pair of G Actin

It is known that each Sp has only one actin-binding site located at the very N-terminal end of the β spectrin. The important question, therefore, is how one Sp binds to a pair of G actin in a protofilament. A wrap around model is proposed here.

It has been established that the tail end of an Sp is formed by the N-terminus of β spectrin and the C terminus of α spectrin.[39] It is possible that each Sp may split near the tail end, allowing individual α spectrin and β spectrin to wrap around a pair of G actin, as shown in Fig. 5a. Since only β spectrin has an actin-binding site, we proposed that G actin 1b, 2a, 3b, 4a, 5b, and 6a are for β spectrin to bind to (as in Mode 3); and the other G actin in the pair namely 1a, 2b, 3a, 4b, 5a, and 6b are for α spectrin to "hook on". Without specific binding between α spectrin and G actin, slipping of α spectrin on the surface of G actin may be allowed. This feature may allow the rolling of a protofilament, and the tension of an Sp to be equilibrated in its full length in response to mechanical stress.

Pairing of Sp

Fig. 5b shows a closer look of how a pair of G actin (e.g., 3a and 3b) may be wrapped around by a α spectrin and β spectrin, and how a pair of Sp (e.g., Sp3 and Sp4) may be oriented relative to each other. To emphasize the relative position of G actin in a pair, which is almost back-to-back (~166.2°), the 4 subdomains of G actin are shown. Both front and the rear (R) sides are indicated. Spectrin β3, for example, may be positioned in the cleft formed by G actin 3a, 3b, and 4a, and is named based on its relative position to 3b (the 3rd G actin pair). It may be oriented from the tail end to head by going, for example, away from us into the wall. Spectrin β4, positioned in the cleft formed by 3b, 4a, and 4b, would then come out of the plane toward us. By the virtue of forming a tie at the tail end, spectrin α3 and α4 would have to orient themselves from the tail end to the head end by going into and out of the wall, respectively. Therefore, Sp3 would orient itself away from us, and Sp4 toward us, with a dihedral angle of ~166.2° between them.

This wrap around model would allow every one of the 12 G actin to participate in the

spectrin-actin interaction, even though only half of them may have their β spectrin binding sites correctly oriented and occupied by β spectrin. It would also allows every one of the 12 spectrin monomers to participate in the interaction, even though only half of them (i.e., 6 β spectrin) may establish specific binding with G actin. The proposed partial separation between α and β spectrin, in order to wrap around a pair of G actin, agrees with the weak interaction revealed by electron microscopy a few domains away from the tail end.[38]

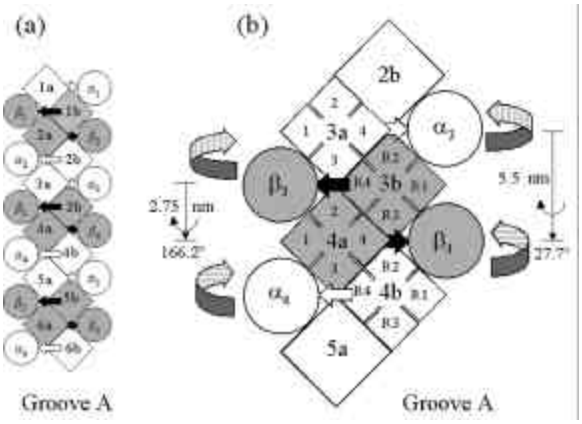

FIGURE 5. The wrap around and swing model. (a) shows that each of the 6 pair of G actin may be wrapped by one α and β spectrin. (b) shows the proposed relationship between α and β spectrin and G actin pairs with a greater detail with the front and rear (R) sides of 4 subdomains indicated. The α and β spectrin may split near the tail end and swing around a pair of G actin. The closed and open arrows indicate proposed occupied and unoccupied β spectrin binding sites, respectively. The space created by a difference of ~27.7° and 5.5 nm (e.g., between G actin 3a and 4a) may be too small for two bulky ends of Sp, but that of ~166.2° and 2.75 nm (e.g., between G actin 3a and 3b) or ~55.4° and 11 nm (e.g., between G actin 3a and 5a) may be sufficient. It is likely that each of the bulky ends (including protein 4.1) may have a dimension of ~5.5-11 nm in height, and may be positioned in one of the grooves.

The wrap around model may increase the contact area between Sp and G actin, decreasing the chance to detach an Sp from the protofilament or pull a G actin out of the protofilament. In addition, a pair of Sp opposing each other with ~166.2° may actually tighten two apparent strands of the protofilament with increased tension. This model may also allow 2 TM, one in each groove of the protofilament, to be presumably inside the loop of an Sp. "The compatibility studies on TM and Sp have shown that TM from the erythrocyte membrane binds F-actin effectively in the presence and absence of Sp. The bound Sp/actin, however, was reduced from approximately 0.06, when TM was absent, to approximately 0.03 when the F-actin was saturated with TM.[25]

Reinforcement by 2 TM

A protofilament of ~36 nm may be bracketed by a pair of TM5/5b. Each TM5/5b in the groove of the protofilament (Fig. 3b and Fig. 5) may wind down 180° vertically from the pointed end (N-terminus of TM) to the barbed end (C-terminus of TM), and may be 180° horizontally away from each other (facing each other), uniting 6 pairs of G actin into one unit. Such TM reinforcement replaces periodic weakness (non-covalent bonds) between G actin with a continuous strength (covalent bonds) within TM along the entire length of a protofilament. Such opposing, longitudinal, winding brackets may further reduce the chance for any individual G actin to be pulled away from the protofilament, when one or more specific Sp is under tension, regardless the direction. In addition, TM is a coiled coil with continuous heptad repeats. Therefore, TM and protofilament may bend rather than break in response to mechanical stress. As a result a protofilament may survive mechanical stresses during erythrocyte deformation.

The Swing of Sp

It is noted that in Fig. 5, α and β spectrin of a given Sp may exchange their positions. The exchange may be done by swinging and

not by flipping. Only by swinging, the parallelism of the direction of β spectrin binding site and β spectrin are preserved. For example, β3 may swing ~166.2° to occupy the β spectrin binding site on 3a. It may swing again, forward or backward, to its original position on 3b. Thus, there may be only one way for an Sp to wrap a given pair of G actin, but an Sp may swing to 2 possible positions. The N-terminus of β spectrin has 2 calponin-homologous (CH1 and CH2) domains,[2] and calponin is known to have affinity toward TM and actin.[29] Therefore, there exists a possibility that the tail end of an Sp may be further stabilized by a TM. So when an Sp swings, from one groove of the protofilament to the other, the tail may snap to a new position.

The Tail of Sp

It is known that protein 4.1 stabilizes the binding of Sp to actin.[17] The protein 4.1 binding site has been mapped to the tail end near the N-terminus of β spectrin. Therefore, the tail end may form a large complex when protein 4.1 (~74 kDa, if sphere, the diameter would be ~4.8 nm) is associated.[11] The small dihedral angle of ~27.7° (Fig. 3a) may make the 2 β spectrin bound to 2 consecutive G actin in the same apparent strand (e.g., 3a and 4a in Mode 2 of Fig. 4) to be simultaneously stabilized by protein 4.1 difficult, due to the close proximity of the two bulky ends. On the contrary, the simultaneous stabilization of β spectrin on 2 consecutive G actin (e.g., 3b and 4a, in Mode 3), which are in different apparent strands, may be allowed as the wide (~166.2°) angle may permit two bulky ends, even though they are only staggered by 2.75 nm (Fig. 5b). Swinging Sp3 from 3a position to 3b, or from groove B to A, would make the simultaneous stabilization of Sp3 and Sp4 in a back-to-back fashion possible. Between 3b and 5b, the dihedral angle is ~55.4°, and the axial distance is 11 nm, the space clearance may allow for a bulky tail. The periodicity of 11nm[49] in TM (Fig. 1c) further supports the involvement of TM in the regularity of Sp association with protofilament.

One Rule Converts All Modes to Mode 3

When one Sp forms a bulky end, it probably sets sequentially the order of the Sp occupancy for the rest of the protofilament. Mode 2 may thus be converted to Mode 3, accomplished by swinging Sp1, 3, and 5 to the other positions (Fig. 4) and then being stabilized. The proposed rule governing the configuration of Sp is that "no two β spectrin binding sites in two consecutive G actin of the same apparent strand may be bound by β spectrin *and* stabilized with a bulky tail". By this rule, 3 consecutive G actin bound by β spectrin, like in Mode 1, would not be allowed. Such condition would also not allow the participation by α spectrin in the wrap around model. If any one Sp is not paired, a pentagon would be generated, which exists in about 3% of the configuration in the membrane skeleton.[23]

E-Tmod Capping and Periodicity of TM

Fig. 6a shows the view from groove A where G actin 1a is associated the N-terminus of a TM (including residues 1-9),[9] which extends ~33-35 nm to protect 6 or 7 G actin, from 1a to 7a. Fig. 6b shows the view from groove B where an E-Tmod, which may cap G actin 1a, is associated with another TM (at residues 7-14)[45] and its length extends to protect 6 G actin, from 1b to 6b. Therefore E-Tmod/TM, the proposed molecular ruler, together with another TM in the other groove, may protect 12 or 13 G actin in total. It is possible that the variable twist between G actin may also result in sligth variations in the length per 180° turn

of the actin filament and that of its associated TM.

The presumed 11-nm periodicity of 3 in each TM may coincide with the positions of 3 Sp associated with each groove (e.g., Sp1, 3, and 5 in groove B, and Sp2, 4, and 6 in groove A), further suggests a potential chemical and/or mechanical role for TM in the association of Sp with the protofilament. E-Tmod brings with it a TM binding site, but may not a β spectrin binding site. Therefore, prior to the capping, Sp1 may be able to swing to two potential positions, but only one afterwards. Consequently, the E-Tmod capping followed by the bulky tail restriction in the groove, most likely by protein 4.1, would limit the configuration of 6 Sp to Mode 3.

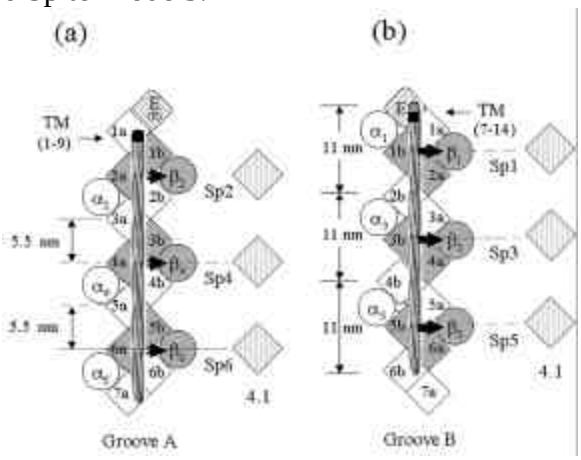

FIGURE 6. The capping of E-Tmod and the periodicity of TM. The groove A view shows the proposed binding of a TM (residues 1-9, based on α-TM)[19] to G actin 1a (a); The groove B view shows the proposed binding of another TM (residues 7-14) to E-Tmod (b). TM is a coiled coil of ~33-35 nm long. The periodicity of 5.5 nm per 180° turn of TM (assumed to be similar to α-TM[49]) coincides with the dimension of a G actin. The assumed periodicity of 11 nm per 360° turn of TM coincides with the proposed positions of the top, middle, and bottom Sp in each groove in Mode 3. Binding of protein 4.1 to the tail of Sp1, 3, and 5 may make them bulky and therefore become restricted in groove B; that to the tail of Sp2, 4, and 6 in groove A. The subdomain of actin that interacts with TM is not specified in the drawing.

Protofilament as Mechanical Axis

In Mode 3, 6 Sp may be classified into 3 pairs, i.e., top, middle and bottom pairs (Fig. 4). To clearly identify 3 pairs of Sp, they are color coded in Fig.7, in green, yellow, and red, respectively. Since every pair of G actin may define one Sp, and two consecutive pairs of G actin may define a pair of Sp, 2 corresponding G actin pairs are similarly color coded, with a line drawn in between each G actin pair. At this point it would be useful to think of the protofilament as if it were a freestanding pole and 3 Sp pairs were 6 cables supporting the pole. The 6 cables may support the pole by tying it down to the ground, the lipid bilayer, forming a hexagonal configuration. The pitch angle dynamics of a protofilament, relative to the plane of lipid bilayer, when 3 associated pairs of Sp are under various stress conditions is investigated and reported in manuscripts # 2 and #3.

A Hexagon

The 3-D illustration of Mode 3 in Fig. 7a shows Sp1, 3, and 5 spiraling down on one side, and Sp2, 4, and 6 spiraling down on the other side of the protofilament. The 6 places where 3 pairs of Sp tie down to would be SC in the membrane skeleton. The SC may be numbered (clockwise, SC1, 3, 5, 2, 4, and 6) based on the order of Sp in 3 pairs, which, in term, may be determined by the order of G actin in 6 pairs within the protofilament. Thus the hexagon may be divided into the "odd" and the "even' side, based on the assignment of Sp. In an equal-biaxial extension, these 6 SC would form a regular hexagon (without physical edges). The dihedral angle between 2 Sp on the same side of the hexagon would be ~60°, and that within each of Sp pairs would be ~180° with a minimal axial distance. Such arrangement minimizes torque on the protofilament.

Furthermore, the variable twist between G actin subunits (± 5-6^{o}) and the flexibility of the 4 subdomains of G actin may allow some energy to be absorbed when 3 pairs of Sp associated with a protofilament are subject to mechanical stress. It has been reported that the actin subunits may randomly exist in different discrete states of "twist", with a significant energy barrier separating these states.[30] The molecular basis for the hexagon proposed here for the erythrocyte membrane skeleton may be a prototype, which may be modified to understand other cytoskeletal structures involving different actin binding proteins.

Suspension Complex (*SC*)

The basic unit of E-Tmod/TM/actin is expected to be suspended underneath the lipid bilayer, not only because the protofilament itself is associated with glycophorin C,[50] a single-transmembrane-domain protein, but also because each of the 6 associated Sp binds distally to a SC (Fig. 1a) in the membrane. The SC consists of ankyrin, band 3 and band 4.2, where band 3 is a multiple-transmembrane-domain protein, band 4.2 is acetylated (so is tightly membrane bound), and ankyrin has a β spectrin binding site. Single unit of E-Tmod/TM/actin with 6 Sp, therefore, may be suspended underneath the lipid bilayer and free to rotate, diffuse, or collide with other units (Fig. 7b).

The Head of Sp

Even though the Sp heads may be as far as ~100 nm away from the JC, they are near the plane of lipid bilayer or the SC (< 20 nm) because of the ankyrin-binding sites are near their heads (Fig. 1a). The presence of membrane association domains (MAD) in Sp,[24] further ensures that their heads are very close to the lipid bilayer. Therefore, when units of E-Tmod/TM/actin/Sp (Fig. 7b) collide with each, all the heads are more or less in the same plane, regardless which G actin pair with which they are associated, thus increasing the chance of forming the head-to-head association.

Hexagonal network

The head-to-head association of Sp underneath the lipid bilayer is non-covalent, so the association can be broken and reformed. Between one E-Tmod/TM/actin/Sp and its neighbors any tangled tetramers may be rearranged until the hexagonal arrangement is in order. Perhaps, the tetramers that are not associated in order like those in Fig. 7, would bear larger tension and therefore break, given a second chance to reform the head-to-head association. Eventually these assemblies coalesce to form the erythrocyte membrane cytoskeleton with a hexagonal geometry, a geometry that has been revealed by EM since 1985[5] (Fig. 1a).

Summary

Both intrinsic properties of the actin filament (i.e., 180^{o} turn per ~36 nm, non-covalent bonds in between G actin subunits, and unequal dimensions of the cross section) and the externally associated "molecular ruler" favor the formation of protofilament. Once long actin filaments are converted to protofilaments and retained by E-Tmod/TM, the formation of the hexagonal actin-Sp based network is on its way. The sequence of events may be as follows: During the initial stage of reticulocyte maturation, long helical actin filaments, naked or partially coated with TM5 or 5b, or those whose pointed end are associated with a molecular ruler (i.e., E-Tmod/TM5 or E-Tmod/TM5b) may be fragmented into ~36 nm in response to mechanical stress. A protofilament

reinforced by 2 TM, one in each groove, and capped by an E-Tmod may be a stable unit containing 6 pairs of G actin.

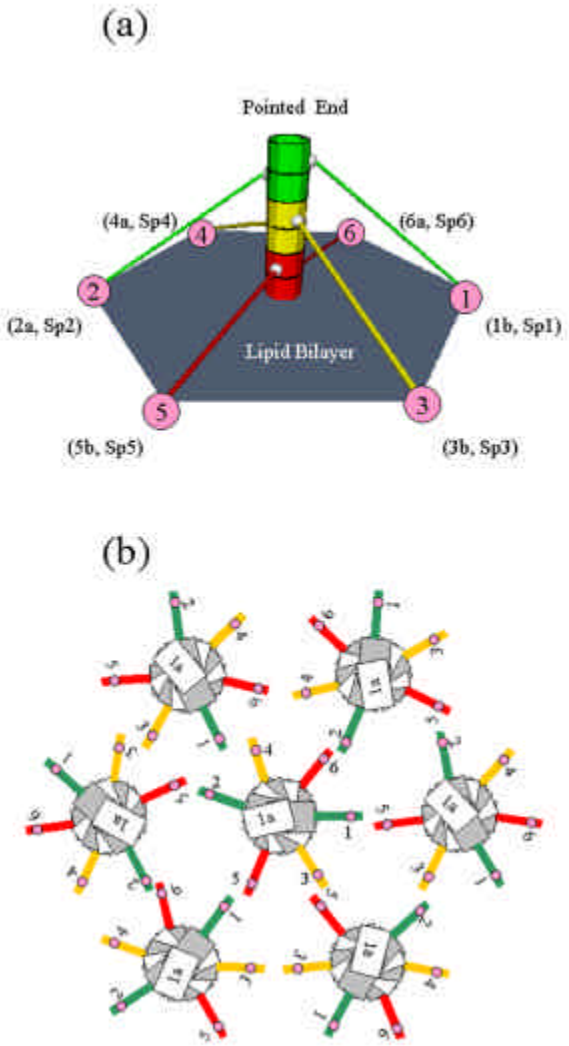

FIGURE 7. A 3-D presentation of a JC and a top view of six plus one JC in the membrane. (a) shows a protofilament that may function as the mechanical axis for 3 pairs of Sp. Each of the 6 Sp may connect to a SC in the lipid bilayer, forming a small hexagon. Both Sp pairs (top, middle, and bottom) and G actin pairs to which Sp pairs are attached are color-coded. Lines separate same colored G actin pairs into two, one for each Sp to attach. The hexagon may be defined by the position of SC, which has no physical edges, meaning no proteins serve to connect SC_{1-6}. Note the relative positions of Sp1 to Sp6 on the protofilament, their dihedral angles and the order of SC in the hexagon. In (b) 6 peripheral JC and one central JC may rotate in various orientations to connect and form a large hexagon. All six edges and the lattices connecting six corners to the center of the hexagon are made out of spectrin tetramers. Note all Sp heads are presumably near SC and the plane of lipid bilayer, facilitating the collision among the heads.

Each pair of G actin may be wrapped around by a α and a β spectrin, whose tail end may spin to two positions, but restrict to one after protein 4.1 association. Such molecular organization, may dictate 3 well-defined pairs of Sp to the top, middle, and bottom regions of the protofilament. Each pair of Sp wraps around the helical filament with a widest dihedral angle and a minimal axial distance in between. Three pairs of Sp spiraling down on two sides of the protofilament, which serves as a mechanical axis, suspend each unit onto the lipid bilayer through 6 SC. Such hexagonal units (i.e., protofilaments each capped at the pointed end by E-Tmod, reinforced longitudinally by a pair of TM, wrapped around horizontally by 3 pairs of Sp, and connected distally with 6 SC through 6 Sp) may be the basic building blocks for the erythrocyte membrane skeleton. These building blocks may rotate, diffuse, and collide in the plane of lipid bilayer, forming spectrin tetramers by head-to-head association between Sp from neighboring units, and completing the erythrocyte membrane skeleton network with a distinct hexagonal feature.

ACKNOWLEDGMENTS

This work was supported by NIH Research Grant PO1HL-43026-6 from the National Heart, Lung, and Blood Institute (Project 2, LAS). We would like to thank Drs. Petr Krysl and Chia-Ming Uang in the Department of Structural Engineering at UCSD for advice concerning models of macroscopic helical rods and critical reading of the manuscript. Carlos Vera was supported by a pre-doctoral fellowship from UCMEXUS-CONACYT, and a post-doctoral fellowship from Sung's Molecular Bioengineering Lab. Lanping Amy (Tseng) Sung wishes to dedicate this paper to her late father Mr. Cheng-Fa Tseng who once served as Deputy Chief Engineer in Taiwan Sugar Corporation.

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