Myosin goes for blood

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A major achievement of 20th century cell biology was the identification of the membrane cytoskeleton in mammalian red blood cells (RBCs) (1). This cytoskeletal network is comprised of long, flexible $(\alpha_1\beta_1)_2$ spectrin tetramers that are linked together by short, actin filament-based, junctional complexes to form a 2D, quasi-hexagonal lattice (2, 3). This lattice is then tethered to the RBC's plasma membrane by numerous interactions, most notably the ankyrin B-dependent interaction of spectrin with band 3, an abundant transmembrane protein in RBCs. The RBC's characteristic biconcave shape, as well as its physical properties—strong enough to withstand high shear forces, yet flexible enough to pass through capillaries half its diameter—are made possible by this membrane cytoskeleton. Two remarkable facts about the actin filament-based junctional complexes in this network are that they contain ∼96% of the RBC's total actin, and that the single actin filament present in each complex is always ∼37 nm in length. Precise length control is created by the presence of capping proteins at the barbed and pointed ends of the junctional actin filament (αβ-adducin and tropmodulin, respectively), and two "short" tropomyosin isoforms (TM5b and TM5NM1) that extend along most of the junctional filament's length. Importantly, the uniform length of junctional actin filaments permits just six spectrin attachments per filament, thereby promoting the network's quasi-hexagonal symmetry. While junctional actin filaments are generally considered to be quite static, especially relative to actin filaments in the cortex of typical cells, they do undergo subunit exchange.

When considering actin function in many cellular contexts, nonmuscle myosin 2 (NM2) naturally comes to mind, as these motors are the major actin-based contractile machines in most cell types (4, 5). Consistently, NM2s power a wide range of fundamental cellular processes, including cell migration, cytokinesis, tissue morphogenesis, and epithelial barrier function. Common to all these NM2-based cellular processes is the ability of the myosin to produce contractile force in cooperation with actin filaments. To accomplish this, NM2 assembles into small bipolar filaments composed of ∼30 myosins (i.e., ∼60 motor domains) in which half of the motor domains are present at each end. When these motor domains engage actin filaments of opposing orientation, they drive the sliding of these opposing actin filaments past each other, resulting in contraction (analogous to the muscle sarcomere).

Myosin 2 Regulates the RBC Membrane Cytoskeleton

While previous studies have shown that RBCs contain NM2, its roles, if any, in the structure and function of the RBC's spectrin-actin membrane cytoskeleton have surprisingly not been addressed. Moreover, the function of NM2 in RBCs could differ significantly from its function in "normal" cells, like fibroblasts, given the RBC's unusual actin organization. In normal cells, the F-actin cortex is composed of actin filaments of varying lengths, whose organization ranges from highly branched networks to large, anisotropic assemblies, like stress fibers and the contractile ring of dividing cells. NM2 bipolar filaments commonly function in these more organized regions to create tension within the assemblies and to drive the sliding of longer filaments of opposing orientation past each other. This raises the question whether NM2 filaments in RBCs could pull on the uniformly short, hexagonally spaced, and nonoverlapping actin filaments present in the RBC membrane cytoskeleton.

These and other questions regarding NM2 function in RBCs are now addressed by the recent study of Smith et al. published in PNAS (6). Using qRT-PCR and immunoblots, these authors first show that NM2A is essentially the only NM2 isoform in mature RBCs. This simplifies matters, as the three vertebrate NM2 isoforms (NM2A, NM2B, and NM2C) can coassemble to make mixed filaments of presumably widely different properties (7, 8). Images of RBCs stained with an antibody to NM2A's head/motor domain obtained using a superresolution imaging modality with ∼150-nm XY resolution (i.e., about half the length of the NM2A

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bipolar filament), coupled with nearest-neighbor analyses, suggested that there are ∼70 NM2A bipolar filaments per RBC. To confirm the existence of these bipolar filaments, Smith et al. (6) used RBCs isolated from a mouse in which GFP had been knocked into the NM2A heavy chain (HC) at its N terminus (9). Staining these RBCs with an antibody to the C terminus of NM2A (combined with a red secondary antibody) yielded the signature pattern in superresolution imaging for NM2 bipolar filaments: discreet green-red-green structures that are ∼300 nm end-to-end (7). Moreover, these structures were present in images taken using total internal reflection microscopy (TIRFM), which "sees" only ∼100–150 nm inside the coverslip-bound plasma membrane, arguing that NM2A filaments connect to junctional actin in the spectrin-actin membrane cytoskeleton. Consistent with this idea, the addition of Mg^{2+}ATP to Triton X-100 extracts of RBCs dissociated NM2A from the actin pellet, indicating that NM2A is interacting with RBC F-actin in the "normal" way: that is, in the way consistent with force production.

Smith et al. (6) next sought evidence that NM2A is active in RBCs. Cells regulate the activity of NM2 by regulating where and when NM2 monomers are assembled into bipolar filaments through the action of multiple regulatory light-chain (RLC) kinases (4, 5). RLC phosphorylation activates NM2s by converting them from folded monomers, which are mechanically silent and incapable of assembling into filaments, into extended monomers, which readily assemble into filaments and are capable of walking on F-actin. Consistent with RBC NM2A being in filaments and being mechanically active, the RLC of NM2A in lysates of RBCs grown in the presence of radioactive phosphate was labeled. Interestingly, the NM2A HC was also labeled. Moreover, the HC phosphate content of NM2A in the Triton X-100 pellet was lower than the HC phosphate content of NM2A in the detergent-soluble fraction. Given that NM2 HC phosphorylation usually drives filament disassembly (4, 5), this result argues that those NM2A molecules bound to the spectrin-actin lattice are more stably self-associated into filaments.

In a final series of experiments, Smith et al. (6) tested NM2A's physiological significance in RBCs using the cell-permeant, small-molecule inhibitor blebbistatin (BB) (10). BB selectively inhibits all NM2 isoforms by stabilizing their motor domains in a weak actin-binding state, effectively dissociating the myosin from actin. Not only has BB provided a wealth of information regarding NM2 function in diverse cell types by providing temporal control of NM2 function, it makes possible studies of NM2 function in RBCs, where alternative approaches like the expression of RNAi are not possible.

Consistent with BB's mode of action, initial experiments showed that RBCs treated with BB showed less NM2A near the plasma membrane in TIRFM images. More importantly, BB treatment caused RBCs to elongate and to exhibit a variable loss of biconcavity. These changes argued that NM2A-based contractility exerts force on the RBC membrane cytoskeleton to "pull" the RBC into its normal biconcave shape. Given the changes in RBC membrane curvature seen with BB treatment, Smith et al. (6) next asked if inhibiting NM2A alters local membrane deformability by measuring nanoscale membrane fluctuations in the RBC's plasma membrane using light scattering. Indeed, BB treatment increased the magnitude of these oscillations, as well as their coefficient of variation. These observations argued that contractile forces exerted by NM2A filaments on the RBC's membrane cytoskeleton promote a tension in the RBC plasma membrane that dampens spontaneous membrane oscillations and reduces local membrane deformability.

Finally, as a measure of global RBC deformability, Smith et al. (6) measured the rate of RBC flow through a microfluidic device with a 5-μm diameter (smaller than a RBC's 8-μm diameter). BB treatment caused RBCs to flow through this device more rapidly. Moreover, BB-treated RBCs exhibited greater shear-induced elongation when passing through wider channels. Therefore, NM2A controls global as well as local RBC deformability, which has important implications for the ability of RBCs to deform in capillaries and resist shear stress in large arteries.

While the changes exhibited by RBCs following NM2 inhibition described above are both significant and mechanistically important, one additional observation made by Smith et al. (6) draws a clear distinction between the magnitude of NM2A's role in the RBC membrane cytoskeleton and its role in most other cellular contexts. This observation is that BB treatment does not alter the RBC's membrane cytoskeleton in any major way. Specifically, TIRFM images of phalloidin-stained RBCs showed no measurable change in the intensity of subplasma membrane F-actin staining following BB treatment. This result indicates that NM2A is not required to form or maintain the RBCs spectrin-actin network, at least at a gross level. While subtler (and important) changes in this membrane cytoskeleton must be occurring following NM2A inhibition (see below), this general observation contrasts with the very dramatic changes in actin network structure/function seen in most cells following BB treatment (e.g., abrupt dissolution of stress fibers, cytokinesis failure, rapid loss of focal adhesions) (11, 12). This significant difference argues that NM2 contractility in RBCs serves primarily to modulate (albeit in important ways) a stable membrane cytoskeletal network, rather than to drive its formation and steady-state maintenance. That said, the generality of these results in RBCs should not be underestimated, as similar membrane cytoskeletons (i.e., plasma membrane-attached, spectrin-based lattices linked together by specialized actin strictures) have been identified in other cell types, including neurons (13).

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Unanswered Questions

As with all good studies, the work of Smith et al. (6) raises lots of great questions. One fascinating question revolves around the fact that the density of NM2A filaments in the RBC's membrane cytoskeleton (0.5/μm^2) appears to be much lower than the density of the actin-based junctional complexes (250/μm^2). How then does NM2A contractility exert such a large effect on the RBC's shape and membrane properties? Presumably, the tension/contraction exerted by relatively sparse NM2A bipolar filaments is readily transmitted across a significant fraction of the surrounding 2D membrane cytoskeleton. The extent to which this can occur will depend significantly on the elastic properties of the 2D spectrin-actin network, and the degree to which it is normally extended. Relevant to this question, recent imaging of this network using a superresolution imaging modality with 25-nm XY resolution showed an ∼80-nm junction-to-junction distance (14). This is consistent with spectrin tetramers being relaxed rather than stretched, which leaves room for both extension and compression during the RBC's transit through the body. Determining the

orientation and distribution of NM2A filaments relative to junctional complexes at this resolution, as well how spectrin tetramer extension changes locally and globally following BB treatment, should provide important insight into how NM2A contractility controls RBC shape and deformability. Many myosin-centric questions also remain. For example, tropomyosins can activate, inhibit or have no effect on NM2 depending on the specific tropomyosin and NM2 isoform (15), raising questions about the effect the two tropomyosin isoforms present on junctional actin may have on NM2A mechanochemistry. Additionally, it will be important to define the role played by RLC kinases, like Rho kinase [whose inhibition Smith et al. (6) showed largely phenocopies BB treatment] in the assembly and maintenance of NM2A bipolar filaments in RBCs. Does NM2A's role in controlling local and global RBC membrane deformability vary under different physiological or pathological conditions downstream of differential RLC kinase activation? We eagerly await the answers to these and many other interesting questions.

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