lack of connectivity in the difference maps between the head and tail regions of troponin. This two-site model for the attachment of troponin to tropomyosin is in agreement with earlier biochemical studies of binding[4,15-17] and electron-microscope observations[3]. The head region of troponin appears to bind weakly to tropomyosin under the ionic conditions used here, and we are studying this interaction further.

Our results reported here together with other findings provide new information about the tropomyosin/troponin switch in muscle. A recent model for regulation suggests that it is primarily the troponin complex that holds tropomyosin extended along the actin helix in the 'off' or resting state[6]. We now suggest that troponin would be bound in a strong invariant link to tropomyosin only near the head-to-tail joint of the filaments. A highly α-helical portion of CB2, although not forming a regular three-chain structure[18], is likely to bind through electrostatic interactions to the two-chain coiled coil of tropomyosin. These interactions are not, however, in regions predicted previously by electrostatic considerations[19,20]. The middle portion of TnT might form a flexible link between the tightly bound tail region and the head of the troponin complex. The binding of the head region of troponin would depend on the Ca^{2+} saturation of TnC[16,17,21,22]: at low Ca^{2+} concentration, corresponding to the 'off' state of the switch, the linkages between the subunits are weak, whereas those between TnI and actin, and between TnT and tropomyosin, are relatively strong. In the presence of Ca^{2+}, the head of the troponin complex detaches allowing the movement of tropomyosin to a new position on the thin filament and the binding of force-producing myosin heads. This is the 'on' or 'potentiated' state of the switch (see also ref. 6). Whatever the Ca^{2+} level, the tail region of troponin remains attached to the tropomyosin filament. This invariant linkage strengthens the head-to-tail joint between molecules, enhancing the cooperativity of the thin filament and maintaining the conserved connection of troponin to tropomyosin required for the switching mechanism[8].

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Dependence of the mechanical properties of actin/α-actinin gels on deformation rate

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The cortical cytoplasm, including the cleavage furrow, is largely composed of a network of actin filaments that is rigid even as it is extensively deformed during cytokinesis[1,2]. Here we address the question of how actin-filament networks such as those in the cortex can be simultaneously rigid (solid-like) and fluid-like. Conventional explanations are that actin filaments rearrange by some combination of depolymerization and repolymerization; fragmentation and annealing of filaments; and inactivation and re-establishment of crosslinks between filaments[3-5]. We describe the mechanical properties of a model system consisting of actin filaments and *Acanthamoeba* α-actinin[6-8], one of several actin crosslinking proteins found in amoeba and other cells[4,9]. The results suggest another molecular mechanism that may account for the paradoxical mechanical properties of the cortex. When deformed rapidly, these mixtures are 40 times more rigid than actin filaments without α-actinin, but when deformed slowly these mixtures were indistinguishable from filaments alone. These time-dependent mechanical properties can be explained by multiple, rapidly rearranging α-actinin crosslinks between the actin filaments, a mechanism proposed by Frey-Wyssling[10] to account for the behaviour of cytoplasm long before the discovery of cytoplasmic actin or α-actinin. If other actin-filament crosslinking proteins behave like *Acanthamoeba* α-actinin, this mechanism may explain how the cortex recoils elastically from small rapid insults but deforms extensively when minute forces are applied over long periods of time[1,11,12]

We evaluated the mechanical properties of mixtures of actin and *Acanthamoeba* α-actinin in a cone-and-plate rheometer using a sinusoidal pattern of low amplitude deformations[8,13-15]. As described previously[15], the small strains (deformations) used do not alter the mechanical properties of equilibrated samples of actin.

It is known that α-actinin increases both the rigidity (elasticity, G') and viscosity (η') of actin filament[8], but we show here that the extent of these changes depends strongly on the rate of deformation (Fig. 1). At equilibrium, actin filaments alone at 24 μM form a weak gel (viscoelastic solid) characterized by a frequency-independent rigidity (equilibrium elastic modulus), $G_e = 14 \pm 4$ dyn cm^{-2} (Fig. 1*a*), as shown previously[15]. With the addition of α-actinin, both the dynamic elasticity G' and dynamic viscosity η' of rabbit actin filaments are elevated 40-fold when sinusoidally deformed (strained) at 1.0 Hz. But at low frequencies ($<10^{-3}$ Hz), both parameters approach values for actin filaments alone (Fig. 1*a*).

The material properties of mixtures of polymerized actin and α-actinin at high rates of sinusoidal deformation are attributable to multiple crosslinks between the filaments, as control experiments exclude substantial contributions by the other components in the mixture. First, free α-actinin, free actin filaments and free nonfilamentous actin do not contribute significantly to the mechanical properties of the gel because G' and η' for each alone are at least 10-fold lower at high frequencies ($>10^{-2}$ Hz) (Fig. 1). Second, complexes of α-actinin with actin monomers (if any) do not contribute, because α-actinin does not alter the rheological properties of nonfilamentous actin alone (Fig. 1*b*). Third, the temperature dependence of the elasticity of the mixture of α-actinin with actin filaments is opposite to that of the

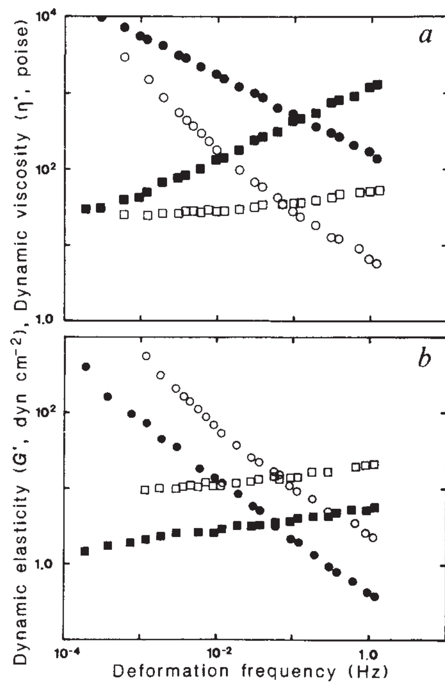

Fig. 1 Effect of *Acanthamoeba* α-actinin on the mechanical properties of actin over a wide range of deformation rates (frequencies). *a*, 24 μM filamentous actin alone (F-actin; white squares, G'; white circles, η') or filamentous actin plus 1.6 μM α-actinin (F-actin + α-actinin, black squares, G'; black circles, η'). *b*, 1.6 μM α-actinin in polymerization buffer (black squares, G'; black circles, η') and 24 μM nonfilamentous actin plus 1.6 μM α-actinin (white squares, G'; white circles, η') in low-salt buffer (0.1 mM $MgCl_2$, 1 mM EGTA, 2 mM PIPES *p*H 7.0, 0.2 mM ATP, 1 mM dithiothreitol (DTT), 0.5 mM NaN_3).

Methods. ***a***, G' and η' were evaluated with a cone-and-plate rheometer (Weissenberg rheogoniometer) for the frequency range 10^{-4}–3 Hz. The sample was deformed by sinusoidally oscillating the bottom plate relative to a freely suspended cone using small displacements that do not disrupt the equilibrium mechanical properties of actin filaments[15]. From the phase and amplitude response of the cone relative to the plate, we calculated G' and η' using linear viscoelastic theory[15,19]. In this type of experiment, the freely suspended cone oscillates in phase with the plate if a solid is present between cone and plate. If a newtonian liquid like water is placed between cone and plate, the cone oscillates −90° out-of-phase with the plate. For a complex material like actin that exhibits both solid-like and liquid-like properties, dynamic elasticity and dynamic viscosity are the in-phase and −90° out-of-phase components of the cone response. These dynamic parameters measure elasticity and viscosity under nondisruptive conditions and have no relation to shear rate dependent parameters such as shear viscosity[15,19]. Gel-filtered actin from rabbit skeletal muscle[30] and *Acanthamoeba* α-actinin[6,7] were equilibrated in polymerization buffer (100 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM PIPES *p*H 7.0, 0.2 mM ATP, 1 mM DTT, 0.5 mM NaN_3) for 1,000 min at 25 °C in the rheometer[15]. The rheometer cone and plate were 10 cm in diameter with a cone angle of 0.01729 rad. The deformation frequency was varied with a constant amplitude of 0.0046 rad for a maximum strain of 0.083[15]. Below a frequency of 6×10^{-2} Hz, actin filaments have a frequency-independent elasticity G_e (see text), characteristic of a viscoelastic solid[15,19]. At 1.0 Hz, α-actinin increases both the G' and η' by 40-fold, but at 6×10^{-4} Hz the mixture was mechanically indistinguishable from actin filaments alone. These data were reproduced for two sets of experiments with two preparations of α-actinin. Both types of samples contained numerous actin filaments shown by electron microscopy[31]. ***b***, G' tends toward zero at low frequency indicating that α-actinin alone is a viscoelastic liquid. G' and η' are identical for α-actinin in polymerization buffer and low-salt buffer. For the mixture of nonfilamentous actin and α-actinin, G' and η' are the same as values for nonfilamentous actin alone indicating that α-actinin does not significantly affect the mechanical properties of nonfilamentous actin for this frequency range.

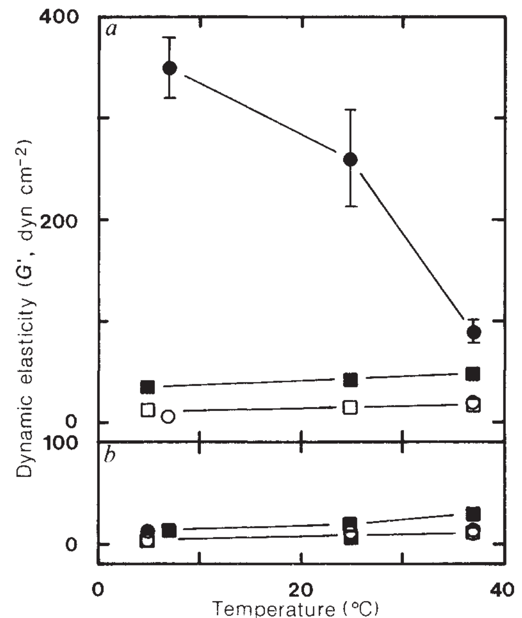

Fig. 2 Temperature effects on dynamic elasticity. 24 μM filamentous actin (G', black squares), 24 μM filamentous actin plus 1.6 μM α-actinin (black circles), 24 μM nonfilamentous actin white squares), 24 μM nonfilamentous actin plus 1.6 μM α-actinin (white circles). These symbols correspond to the calculated means for 2–5 sets of experiments with proteins from different samples of *Acanthamoeba*. Standard deviations were smaller than the space occupied by the symbols, except where designated by error bars. *a*, Deformation frequency = 6×10^{-1} Hz. The mixture of filamentous actin plus α-actinin had higher values of G' and an opposite trend of G' as a function of temperature compared with the other samples, showing that functional crosslinks between actin filaments dominate the mechanical properties for the mixture at high frequencies. *b*, Deformation frequency = 6×10^{-4} Hz. For all samples, G' increased with temperature in a similar fashion.

other components (actin monomers ± α-actinin and actin filaments alone) in the sample (Fig. 2).

The key experiment to elucidate the frequency dependence of the mechanical properties of these gels was to measure the equilibrium binding constant for α-actinin to actin filaments (Fig. 3), so that we could estimate the steady-state association and dissociation rates of the crosslinks. In sedimentation binding experiments, the dissociation constant K_D of the actin filament and α-actinin complex is 26 μM (Fig. 3). From this value we estimated the number of α-actinin molecules bound and the possible exchange rates. First, under the conditions used in Figs 1 and 2, only 35% of the total α-actinin (1.6 μM) is bound to actin filaments; but as the average filament length is probably >5 μm (ref. 16) >50 α-actinins are bound to each filament at any instant. Second, if the association rate constant is assumed conservatively to be $10^5\ M^{-1}\ s^{-1}$, the dissociation rate constant is $>2\ s^{-1}$. The actual value may be one or two orders of magnitude higher, because other proteins that bind to actin filaments such as myosin have association rate constants of 10^6 to $10^7\ m^{-1}\ s^{-1}$. Consequently, at equilibrium the α-actinin in these gels binds to and dissociates from the actin filaments rapidly (2–200 s^{-1}), like the weakly bound intermediates in the actomyosin crossbridge cycle in muscle contraction[17,18]. The difference with myosin, however, is that α-actinin 'cycling' is independent of ATP.

The rapid binding equilibrium of α-actinin with actin filaments means that crosslinks between filaments can rearrange quickly and together with the existence of multiple crosslinks between filaments explains why the mechanical properties of the gel depend on the rate of sinusoidal deformation (Fig. 1).

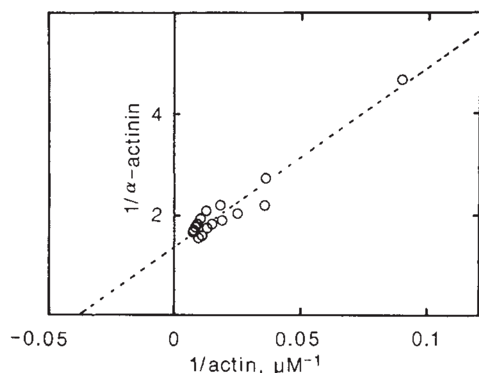

Fig. 3 Dissociation constant for the complex of *Acanthamoeba* α-actinin with actin filaments.

Methods. We incubated 8.5 nmol of α-actinin with 408 nmol [^{14}C]iodoacetamide (NEN, Boston) for 90 min in 10 mM Tris-HCl *p*H 7.5, 1 mM EDTA, 0.1 mM DTT in 1 ml total volume at room temperature. The reaction was stopped with 3 μmol DTT and the products were concentrated to 0.5 ml vol. by dialysing against Aquacide II (Calbiochem, La Jolla) for 60 min, 5 °C. The products were gel filtered on a S-300 column (1.2×47 cm) pre-equilibrated with Tris-EDTA-DTT buffer and radioactivity counted on a Beckman LS 7000 liquid scintillation counter. The labelling varied from 20 to 100% for three batches of α-actinin. The gelation activity was assayed with the 'falling ball' technique[32] and varied from 80 to 150% compared with unlabelled α-actinin. Iodoacetamide-labelled α-actinin (1.6 μM) was incubated for 1,000 min at 25 °C with various concentrations of rabbit muscle actin (5-180 μM) in polymerization buffer (100 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM PIPES *p*H 7.0, 0.2 mM ATP, 1 mM DTT, 0.5 mM NaN_3). The mixtures were centrifuged for 25 min at $1.6\times10^5 g$ in an Airfuge (Beckman) to pellet the actin filaments and the supernatants (70 μl) were assayed for radioactivity to measure the free α-actinin. The amount of α-actinin in the pellet was calculated from the original total and the data are presented as a double reciprocal plot by the method of Brenner *et al.*[17]. These data from two different batches of α-actinin were fit to a linear regression line. The intercept on the *x* axis gives $K_D = 26$ μM.

When force is applied slowly, the α-actinin crosslinks rearrange faster than the displacement of actin filaments relative to each other, so they offer little or no resistance. When force is applied more rapidly than the whole population of crosslinks can rearrange, there will be physical connections between the filaments that make the network rigid. Amazingly, Frey-Wyssling[10] in 1948 proposed a similar model without knowledge of cytoplasmic actin or crosslinking proteins.

Because association and dissociation rates are functions of temperature, the rate of crosslink re-arrangement should also increase with the temperature. This may explain why the rigidity (G') of the gel mixture decreases with temperature, first shown by Abe and Maruyama[8], whereas G' for the individual components increases with temperature (Fig. 2*a*). During low-frequency deformations this effect of temperature on the mixture is not observed because the rate of crosslink re-arrangement is faster than the deformation frequency (Fig. 2*b*). We have recently confirmed every aspect of this model for α-actinin in fluorescence photobleaching recovery experiments (M.S., D. Loftus, J. Cooper, E. Elson, C. Freider and T.P., in preparation).

The effects of α-actinin on the mechanical properties of actin filaments were unexpected because, to our knowledge, similar effects have not been observed for either synthetic polymers or isolated proteins. Addition of covalent crosslinkers to a visco-elastic solid of synthetic polymers results in the elevation of the equilibrium elastic modulus (G_e) rather than only an increase in G' at high frequency[19]. Although actin-filament crosslinking proteins such as α-actinin, actin-binding protein, spectrin and others[4,9] do not covalently link filaments, previous experimental and theoretical studies of filament networks have assumed for simplicity that the crosslinks are permanent[20-23]. In dynamic models of cytoskeletal gels, for example, crosslinking proteins have been modelled as permanent links that are disrupted by regulatory factors such as calcium[22,23]. Our data show that the network of *Acanthamoeba* α-actinin and actin filaments is both rigid and deformable depending on the rate of sinusoidal deformation without requiring regulation by inactivating crosslinks, fragmenting filaments or depolymerizing filaments. This may be particularly important for *Acanthamoeba* α-actinin, as it is not regulated by calcium[7].

Macrophage α-actinin[24] and actin-binding protein[25] are also likely to be in rapid equilibrium with actin filaments because they bind relatively weakly ($K_D = 0.2$–0.5 μM), especially α-actinin in the presence of calcium ($K_D = 1$ μM). As discussed above, the dissociation rate constants may be in the range of 0.02–$10\ s^{-1}$. Consequently, the mechanical properties of actin filaments with these crosslinkers should also depend on the rate of deformation. This notion of dynamic crosslinks is consistent with the recent data of Zaner[14,26] and should become more obvious at lower and lower frequencies.

The dynamic model[10] for α-actinin crosslinking of actin filaments may be applicable to the amoeba cortex and may explain some features of cytokinesis. Like amoebas[7,27] both sea urchin eggs[28] and cultured vertebrate cells[29] have a cortical cytoplasm that is rich in actin filaments and α-actinin. Hiramoto[1] and Peterson *et al.*[12] showed that the cortex of sea urchin eggs and fibroblasts is rigid (elastic) and more resistant to rapid deformations than to slow deformations. For example, although the cleavage furrow generates only minute forces of 3×10^{-3} dyn or stresses between 10 and 50 dyn cm^{-2} over 10–30 min (between 10^{-3} and 10^{-4} Hz), it is capable of grossly deforming the highly rigid cortex[1]. Our data suggest that α-actinin localized to the cortex[27-29] forms an actin gel that resists rapid insults yet deforms with minimal resistance provided that stress is applied for a long time.

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