Actin-Crosslinking Protein Regulation of Filament Movement in Motility Assays: A Theoretical Model

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ABSTRACT The interaction of single actin filaments on a myosin-coated coverslip has been modeled by several authors. One model adds a component of "frictional drag" by myosin heads that oppose movement of the actin filaments. We have extended this concept by including the resistive drag from actin crosslinking proteins to understand better the relationship among crosslinking number, actin-myosin force generation, and motility. The validity of this model is supported by agreement with the experimental results from a previous study in which crosslinking proteins were added with myosin molecules under otherwise standard motility assay conditions. The theoretical relationship provides a means to determine many physical parameters that characterize the interaction between a single actin filament and a single actin-crosslinking molecule (various types). In particular, the force constant of a single filamin molecule is calculated as 1.105 pN, approximately 3 times less than a driving myosin head (3.4 pN). Knowledge of this parameter and others derived from this model allows a better understanding of the interaction between myosin and the actin/actin-binding protein cytoskeleton and the role of actin-binding proteins in the regulation and modulation of motility.

INTRODUCTION

Actin binding proteins represent a large family of proteins that affect the structure of the actin network of nonmuscle cells, including proteins that form crosslinks among actin filaments (Stossel et al., 1985; Pollard and Cooper, 1986). The actin network resulting from filament crosslinking provides structure for the cell and is responsible for many important cellular functions. In particular, reversible and irreversible crosslinking may provide an important mechanism for regulation of these cellular functions, which would vary with different relative crosslinking affinities and strengths, crosslinking time, and inhibition of crosslinking by second-messenger molecules or protein-protein competition. These different crosslinking parameters, which vary among each type of actin-crosslinking protein and even between the same actin-crosslinking protein from different species, would provide the cell with an array of modulators of actin network structure to effectively and selectively regulate cellular activity. This effect of actin-crosslinking proteins on the structure of the nonmuscle, actin cytomatrix and the resulting effect on contractile activity has been studied for over two centuries in live cells, cell extracts, three-dimensional reconstituted systems (see Taylor and Condeelis (1979) for a review).

In recent years, two-dimensional motility assays of myosin function have been developed allowing direct visualization of the movement of myosin-coated beads on an actin filament substrate (Sheetz and Spudich, 1983) or actin filaments on a myosin substrate (Kron and Spudich, 1986). These two-dimensional assays provide the ability to study and define molecular relationships at the single molecule level under a variety of well defined conditions (see Huxley (1990) for a review). These motility assays have provided, in particular, extensive information on actin/myosin force generation. Many workers have modeled this actomyosin interaction (Harada et al., 1990; Warshaw et al., 1990; Uyeda et al., 1990; Toyoshima et al., 1990; Tawada and Sekimoto, 1991) to explain and predict experimental results.

We recently adapted one of these motility assays to study how actin-crosslinking proteins may regulate force generation and contraction by actin and myosin (Janson et al., 1992). We now propose a detailed theoretical model, based on the work of Tawada and Sekimoto (1991), to explain the interactions among actin, myosin, and actin-crosslinking proteins. This model lends credence to our previous results, provides a theoretical basis for the determination of biochemical parameters for actin crosslinking proteins, and offers insight into how the variety of actin-crosslinking proteins found in nonmuscle cells may finely regulate cellular processes. Below, we establish several basic assumptions and general characteristics of actin/myosin/actin crosslinking protein interactions in this motility assay, establish a general equation for this relationship, and offer examples of our own and other's work to support this theory.

GLOSSARY OF PARAMETERS (IN ORDER OF APPEARANCE)

ρ_x	Density of specified molecule x
A_x	Area of one molecule x on coverslip surface
B	Molar ratio of myosin molecules per crosslinking molecule
X	General molecule
P_x	Probability of actin filament interacting with a molecule x on the coverslip surface

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R_x	Radius of contact for molecule x
w_x	Two-dimensional bandwidth for molecule x
N_x	Number of molecules x that may interact with a unit length of actin filament
[X]	Concentration of example molecule
K_{d-x}	Dissociation constant of binding for stated molecule x
K_i	Dissociation constant of binding for inhibitory molecule
[I]	Concentration of inhibitory molecule
T_x	Duration time of interaction between an actin filament and molecule x
f_x	Force of molecule x on movement of actin filament
E_x	Elastic stiffness constants of molecule x
f_s	Coefficient of hydrodynamic viscous drag by assay buffer
L	Length of actin filament
η	Assay buffer viscosity
R_a	Radius of actin filament
V	Velocity of actin filament with attached crosslinking molecules
V_{max}	Velocity of actin filament with no attached crosslinking molecules
F_x	Active force (driving or resistive) of molecule x
C	Constant factor from simplified final equation, including effects of myosin heads and actin-crosslinking proteins

Parameter subscripts used throughout this model

myo	Total myosin molecules that may be in one of three states, driving, rigor, or weakly attached
d	Driving myosin head
r	Rigor myosin head
w	Weakly attached myosin head
u	Unattached myosin head
xlink	General crosslinking molecule
filamin	Filamin molecule
α-actinin	α-actinin molecule
x	General molecule

GENERAL THEORETICAL CONSIDERATIONS

Factors affecting actin filament velocity

The velocity of actin filaments could be dependent on seven parameters (Factors a–g), which are discussed in detail below.

Factor (a): The density of myosin and crosslinking molecules on the coverslip surface

The total density of myosin, ρ_{myo}, may be determined in several ways, including determination of comparative protein concentration (Harada et al., 1990) or activity (Uyeda et al., 1990; Toyoshima et al., 1990), analysis of electron micrographs of the assay surface (Harada et al., 1990; Kron et al., 1991), or empirically from previously established binding relationships (Uyeda et al., 1990; Toyoshima et al., 1990). The maximum number of molecules that may bind on the surface would also be limited because of space constraints. Assuming that a single myosin head occupies a 0.011×0.011 μm^2 area ($A_{myo} \approx 0.000121$ μm^2) of coverslip space (Harada et al., 1990), $\rho_{max-myo} = 8264$ myosin heads/μm^2.

ρ_{myo} may be further defined as the combined densities of active driving myosin heads, ρ_d, rigor myosin heads, ρ_r, weakly attached myosin heads, ρ_w, or unattached heads, ρ_u. These species correspond to AM·ADP+Pi, AM, AM·ATP, and M, respectively, seen in Fig. 1. The probability of myosin heads being in one of the three possible attached and one detached state is shown in Table 1. Rigor myosin heads, AM, may be either ATP-deficient or permanently inactive. These inactive myosin heads can inhibit filament movement even at small concentrations (Umemoto and Sellers, 1990). Weakly attached myosin heads, AM·ATP, are present after a productive myosin cycle but before release of the actin filament. Unattached heads, M, are not in contact with an actin filament.

ATP concentration and ATPase activity affect the rate of transition between states of a myosin head (Taylor, 1979; Warshaw et al., 1990; Homsher et al., 1992). Half-maximal actomyosin ATPase activity is seen at approximately 4 μM (Kron and Spudich, 1986). However, if the ATP concentration used in the assay is less than necessary for half-maximal velocity (~60 μM for this assay (Kron and Spudich, 1986)), increasing AM and AM·ATP and, therefore, ρ_r and ρ_w will impede filament movement. Normal assay conditions include an ATP concentration well above this limiting value ($\geq$1 mM), although this factor may be important when ATP concentration is limiting. Unphosphorylated heads (smooth or nonmuscle myosins) may also be characterized as weakly attached heads because they bind to actin but have a very slow ATP turnover rate. This binding and lower ATPase activity would create a finite inhibitory force against filament movement. This inhibition is lessened if unphosphorylated and phosphorylated myosins are copolymerized (Warshaw et al., 1990). The increasingly important effect of resistive forms of myosin on filament velocity and step size have been reported recently (Uyeda et al., 1991; Higuchi and Goldman, 1991; Homsher et al., 1992).

Space constraints must also be considered when two or more different types of molecules are introduced onto the surface (e.g., myosin and crosslinking molecules) according to the general relationship below for number of molecules in

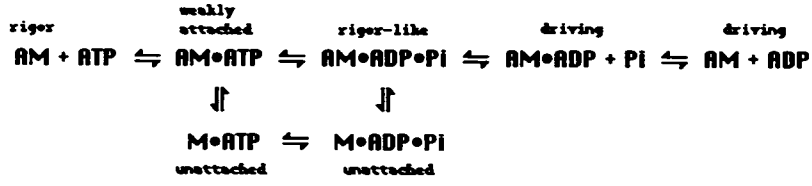


FIGURE 1 Pathway of actin/myosin force generation. Figure illustrates biochemical steps involved in ATP hydrolysis and force generation. Actin-myosin interaction states (rigor, weakly attached, rigor-like, driving, and unattached) are noted.

TABLE 1 States of myosin and crosslinking molecules

Molecule	State	Probability	Function
MYOSIN (attached)	Driving	P_d	Translocating force [f_d]
	Holding/rigor	P_r	Frictional drag [f_r]
	Holding/weak	P_w	Frictional drag [f_w]
MYOSIN (detached)		$1 - P_d - P_r - P_w$	No effect
CROSSLINKER	Attached/holding	P_x	Frictional drag [f_x]
	Detached	$1 - P_x$	No effect

a square micron.

$$nA_{xlink} + nBA_{myo} = 1\ \mu m^2,$$

where n is the total number of crosslinking molecule in the 1 μm^2 area, A_{xlink} is the area occupied by that crosslinking molecule, and B is the molar ratio of myosin to the crosslinking molecule. For example, $A_{filamin} \approx 0.000036\ \mu m^2$ (Gorlin et al., 1990) and $A_{\alpha\text{-actinin}} \approx 0.000012\ \mu m^2$ (Suzuki et al., 1976). Crosslinking molecules on the coverslip may be in one of two states, attached/holding or detached, whose probabilities are also shown in Table 1. The state of the crosslinking molecule is dependent on regulatory factors such as calcium, pH, phosphorylation, ionic strength, and temperature (Stossel et al., 1985; Pollard and Cooper, 1986).

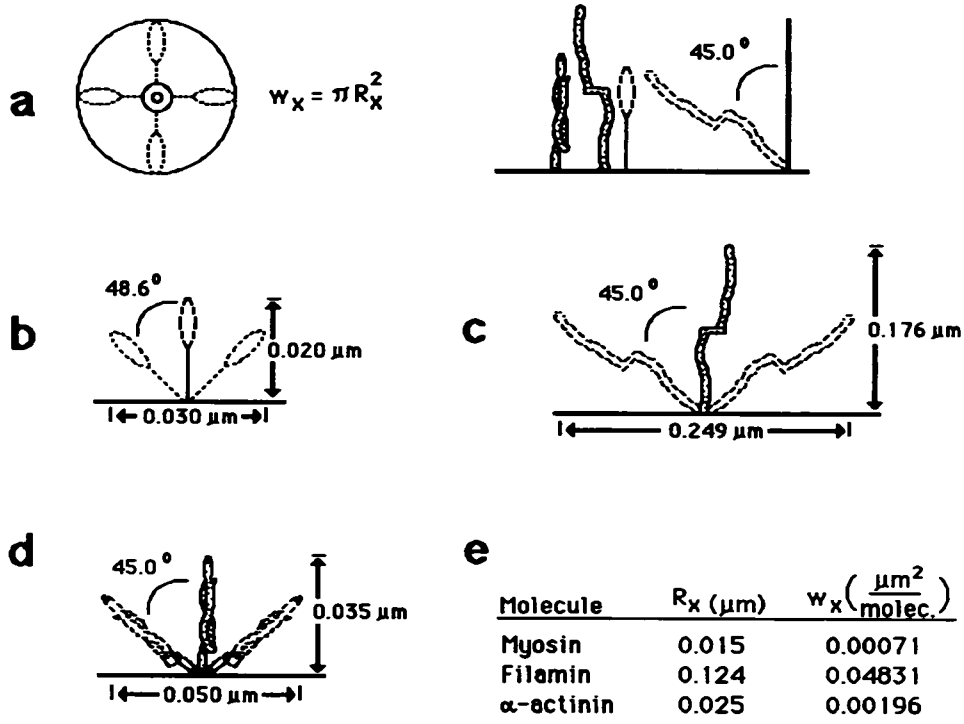
Factor (b): The probability that an actin filament can interact with selected molecules

The chance of interaction between an actin filament and either myosin or a crosslinking molecule with coverslip density ρ_x may be effectively predicted by the bandwidth model (Harada et al., 1990; Uyeda et al., 1990) as

$$P_x = w_x \rho_x,$$

where w_x is the area of the circular band in which the molecule, x, can interact with the actin filament on the two-dimensional coverslip (x- and the y-planes). w_x is determined by calculating the circular area accessible to a tethered crosslinking molecule $w_x = \pi(R_x)^2$, where R_x is the radius of the circle in microns in which molecule X can contact an actin filament (Fig. 2 *a*). w_x carries the dimensions μm^2/molecule. Studies by Toyoshima et al. (1990) show that R_{myo} is approximately 0.015 μm; therefore, $w_{myo} = 0.00071\ \mu m^2$/myosin (Fig. 2, *b* and *e*). Toyoshima and co-workers also note that ρ_{myo} is effectively zero (determined by ATPase measurement) when HMM concentrations of ≤ 5 μg/ml are used, producing no filament movement. This lower concentration value and the resulting myosin density must be considered for specialized applications of this assay and this model.

The bandwidth for actin crosslinking proteins (e.g., filamin and α-actinin) is not known. However, reasonable estimates may be made using values from the myosin molecule.



e

Molecule	R_X (μm)	w_X ($\frac{\mu m^2}{molec.}$)
Myosin	0.015	0.00071
Filamin	0.124	0.04831
α-actinin	0.025	0.00196

FIGURE 2 Circular bandwidths of myosin, filamin, and α-actinin. (*a*) General illustration of concept of circular bandwidth illustrating area in which immobilized molecule could interact with actin filaments. Maximum circular area is approached at approximately 45° tilt, beyond which interactions with actin are inhibited by neighbor molecules (e.g., α-actinin, filamin, myosin). (*b–d*) Illustration of circular bandwidth parameters for myosin (*b*), filamin (*c*), and α-actinin (*d*) used in sample calculations. (*e*) Table of circular bandwidths for example molecules, including R_x and w_x.

One may calculate that a myosin head swings approximately 45° (41.4° calculated) from vertical based on R_{myo} and the known chord length of the myosin head, which is ~0.020 μm (see Fig. 2, *a* and *b*). Actin crosslinking proteins may also, presumably, sway ~45° when attached to the coverslip surface before their ability to contact an actin filament would decrease because of neighbor molecules and/or limited access to the active crosslinking site (see Fig. 2 *a*). The maximum active radius for filamin and α-actinin would be $R_{filamin} = 0.124$ μm/filamin and $R_{\alpha\text{-actinin}} = 0.025$ μm (Fig. 2, *c* and *d*). Therefore, $w_{filamin} = 0.04831$ μm²/filamin and $w_{\alpha\text{-actinin}} = 0.00196$ μm²/α-actinin (Fig. 2 *e*), respectively.

Factor (c): Number of myosin heads and crosslinking molecules that interact with an actin filament per unit length

Although myosin or actin-crosslinking molecule densities may be sufficiently high so that $P_x \sim 100\%$, other factors can limit the actual number of interactions, including the effective association constants of the actin-binding molecule and the limited number of binding sites for each actin-binding molecule. The number of molecules bound may be defined as

$$N_x = \frac{N_{max-x}[X]}{[X] + K_{d-x}}$$

using basic binding relationships. This relationship may not apply directly to the two-dimensional substrate used for this motility assay. However, on a two-dimensional surface, [X] would be at least proportional to the density, ρ, of the molecule. Additionally, a two-dimensional K_{d-x} may be approximated by converting the K_d for a three-dimensional solution to [molecules/μm³]$^{2/3}$, using the conversion factors

$$[(\mu\text{mol/l}) \times (1/10^3\ \text{l/ml}) \times (\text{ml/cm}^3) \times (\text{cm}/10^4\ \mu\text{m})^3$$
$$\times (\text{mol}/10^6\ \mu\text{mol}) \times 6.02 \times 10^{23}\ \text{molecule/mol}]^{2/3},$$

where the final superscript represents the binding affinity for the crosslinking molecule, attached to a two-dimensional substrate with *x*- and *y*-dimensional freedom (i.e., two out of three dimensions of freedom). Therefore, for the activity assay,

$$N^* = \frac{N_{max-x}\rho_x^*}{\rho_x^* + K_{d-x}^*},$$

where * indicates the analagous two-dimensional value.

The number of myosin heads or crosslinking molecules in contact with an actin filament depends on the maximum number of binding sites per unit length of filament, the density of the molecule (if this is a limiting value), and the dissociation constant of the binding to actin. One myosin head can theoretically interact with each actin monomer, making $N_{max-myo} = 361$ per μm of actin filament. Steric hinderance from high densities of myosin molecules and the effective association constants of the three myosin head states would lower this value. Yanagida et al. (1985) have established that in this assay a maximum of ~80 myosin heads interact with a thin filament of 2.7 μm in length in this assay (1 head/0.032 μm). Therefore, $N^*_{max-myo} \sim 30$ heads/μm actin. This measured maximum number of myosin heads, which is far below the theoretical maximum, may explain why surprisingly small amounts of myosin can achieve maximum actin filament velocity because additional myosin heads, although present and functional, could not contact the actin filament.

Actin-crosslinking molecules often interact with actin filaments at discrete locations, which differ among individual molecules, limiting the number of these crosslinkers per unit length of actin filament (Stossel et al., 1985; Pollard and Cooper, 1986). $N^*_{max-xlink}$ and $K^*_{d-xlink}$ must be determined, therefore, for each crosslinking molecule. For example, $N^*_{max-filamin} \sim 27.75$ filamin molecules per μm of actin filament and $K_{d-filamin} \approx 1$ μM (Gorlin et al., 1990) and $N^*_{max-\alpha\text{-actin}} \sim 148$ α-actinin molecules per μm of actin filament (Blanchard et al., 1989) and $K_{d-\alpha\text{-actinin}} \approx 33.4$ μM for *Dictyostelium discoideum* α-actinin (Simon et al., 1988). Direct competition among myosin and these crosslinking molecules probably does not exist because they normally bind to different areas of the actin filament. However, competition among different actin-crosslinking molecules may limit N^*_{xlink} for these proteins. This inhibition may be competitive, noncompetitive, or uncompetitive and may be modeled for each type of competition as illustrated below.

$$\text{Competitive:} \quad N^* = \frac{N_{max-x}\rho_x^*}{\rho_x^* + K^*_{d-x}(1 + [I]/K_i^*)}$$

$$\text{Noncompetitive:} \quad N^* = \frac{N_{max-x}\rho_x^*(1 + [I]/K_i^*)}{\rho_x^* + K^*_{d-x}}$$

$$\text{Uncompetitive:} \quad N^* = \frac{N_{max-x}\rho_x^*(1 + [I]/K_i^*)}{\rho_{x^*} + K^*_{d-x}(1 + [I]/K_i^*)}$$

Factor (d): The duration time of interaction between the actin filament and a selected molecule

Velocity of actin filaments will be directly affected by the amount of time that it is in contact with the myosin molecule (in any of three states) and/or the actin crosslinking molecule. The total actin-myosin interaction time is due to time propelled by a driving myosin head (T_d), or held back by frictional drag of a rigor (T_r) or a weakly attached myosin head (T_w). One may estimate this time by examining on/off rates of each state of the myosin molecule and crosslinking molecules. During force generation (T_d), a myosin head may interact with an actin filament ~1000 times per second or, alternatively, the time of each actin-myosin interaction would be 1 millisecond (Siemankowski et al., 1985). This value is supported by close agreement with the work of Huxley (1974), who determined the time of interaction to be 1–2 ms. A value of 1 ms will be used, therefore, in this model for T_d. T_r may be of almost infinite duration if rigor (ATP-deficient) conditions are maintained. Normally, however, excess ATP is present in motility assays and $T_r \sim 0$. Tawada and Sekimoto (1991) have determined that the time of weak

attachment of actin filaments to myosin heads after a productive power cycle, T_w, is ~20–75 μs. The total time of interaction of an individual myosin head with an actin filament, therefore, would be ~1.020–1.075 ms, assuming nonrigor conditions.

Duration time for actin-crosslinking proteins (T_{xlink}) is often incorrectly modeled as an infinite period, suggesting that these molecules are similar to covalent crosslinks. Infinitely long crosslinks would effectively freeze the cytoskeleton in a constant structure and would be detrimental to the cell (e.g., phalloidin stabilization of actin filaments, taxol stabilization of microtubules). Although some actin-crosslinking molecules do create long-term crosslinks (e.g., filamin > 100 s) (Janmey et al., 1990), many actin-crosslinking molecules have extremely short duration times of crosslinking (e.g., α-actinin < 1 s). A cell may use different crosslinking proteins with different crosslinking duration times to modulate and/or regulate cellular processes (Sato et al., 1987; Janson and Taylor, 1993). The parameter T_{xlink}, therefore, would be of paramount importance for the determination of the effect of actin/myosin/actin-crosslinking molecule interactions and for the resulting regulation of motility.

Factor (e): The positive driving and negative drag forces of selected molecules

The force of a molecule on an actin filament may be defined as $f_x = E_x T_x/2$ (Tawada and Sekimoto, 1991). A driving myosin head will have a positive driving force. Weakly attached and rigor myosin heads and actin crosslinking molecules will have a negative force, although the effect of weakly attached heads has been shown to be minimal (Homsher et al., 1992). Unattached myosin heads produce no force on the actin filament. Elegant studies by Kishino and Yanagida (1988) have established that the force of a single driving myosin head was no less than 0.8 pN (see also Huxley, 1990, for reported correction) for intact myosin and the S-1 fragment. Recent work by Finer et al. (1994) using more exact methods indicate that the actual value is ~3.4 ± 1.2 pN for a single HMM molecule. Therefore, for a single HMM head, $f_d = E_d T_d/2 \sim 3.4$ pN. Additionally, one may calculate that E_d, the elastic force constant for a single HMM head is 6800 pN/s, assuming $T_d = 0.001$ s (see above). Force constants have not been determined for actin-crosslinking proteins, although the modification of the motility assay described in Janson et al. (1992) indicates that such a measurement is possible. If E_r and E_w are the elastic stiffness constants of the myosin head in rigor and weakly attached states, respectively, and E_x is the stiffness constant for a crosslinking molecule, one can express the other force constants as

$$f_r = E_r T_r/2 \qquad f_w = E_w T_w/2 \qquad f_{xlink} = E_{xlink} T_{xlink}/2.$$

Factor (f): Actin filament length

Movement of actin filaments has been noted to be independent of filament length from 40 nm to >15 μm (Takiguchi et al., 1990; Toyoshima et al., 1990; Uyeda et al., 1990; Harada et al., 1990). Very short filaments (<40 nm) tend to dissociate from or remain bound to the substrate (Harada et al., 1990). These facts agree well with the measurements of Yanagida et al. (1985), who noted that a maximum of 1 head may bind to approximately every 32 nm of actin filament. Actin filaments that are somewhat below ~40 nm in length may not be able to interact with even one myosin head, allowing them to float free or become attached to the coverslip. Above 40 nm, maximum velocity of the actin filament is achieved with just a few myosin heads. Additional actin filament length and the additional myosin heads that may then associate with the filament, therefore, would not change the filament velocity.

Factor (g): Viscosity, ionic strength, pH, and temperature of assay solution

As actin filaments translocate through the buffer solution, they may experience viscous drag that impedes their movement. Addition of methyl-cellulose to increase the solution viscosity and stabilize actin-myosin interactions (Kron et al., 1991) may additionally affect filament velocity. This drag force is defined by Tawada and Sekimoto (1991) as the coefficient of hydrodynamic viscous drag, f_s. An actin filament may be modeled as a string of connected hydrodynamic spheres of total length L. Therefore, $f_s = L \times 2\pi\eta/[\ln(L/R_a) - 0.5]$, where η = solvent viscosity and R_a = radius of an actin filament. Elegant work by Homsher et al. (1992) has shown a dramatic dependence of actin filament velocity when normal assays conditions are changed, including pH (≤7.0 and ≥8.0), temperature (<20°C) and/or ionic strength (some effect between 50–150 mM, strong effect <50 mM). Standard assay solution conditions are approximately 1 mM Mg·ATP, 1 mM Mg^{2+}, 10 mM MOPS (pH 7.35), 0.1 mM K_2·EGTA, and 20 mM KCl, resulting in a final ionic strength of 35 mM (Homsher et al., 1992). Any changes from these conditions would have to be incorporated into the affected parameters of this model, including f, K_d, E, T, and w for all molecules involved.

MODEL OF ACTIN-CROSSLINKING PROTEIN EFFECT ON IN VITRO ACTIN-MYOSIN MOVEMENT

General expression

The interaction between an actin filament and myosin and actin-crosslinking proteins on a glass coverslip can be modeled as $V = V_{max}\{F_{myo-d}/F_{xlink}\}$, or the velocity of the actin filament is equal to the original velocity modulated by the ratio of myosin driving force and crosslinker resistive force. From Tawada and Sekimoto (1991), the general expression for interaction among an actin filament, myosin heads in the three different states, and a single population of actin-crosslinking molecules in defined solution can be expressed as

$$V = V_{max}\left(\frac{f_d P_d \rho_d}{f_s + f_r P_r \rho_r + f_w P_w \rho_w + f_{xlink} P_{xlink} \rho_{xlink}}\right), \qquad (1)$$

In expanded form (from above),

$$V = V_{max}\left\{\frac{f_d(w_d\rho_d)\rho_d}{\frac{L2\pi\eta}{\ln(L/R_a)-0.5}+\left(\frac{E_rT_r}{2}\right)(w_r\rho_r)\rho_r+\left(\frac{E_wT_w}{2}\right)(w_w\rho_w)\rho_w+\left(\frac{E_{xlink}T_{xlink}}{2}\right)(w_{xlink}\rho_{xlink})\,\rho_{xlink}}\right\} \tag{2}$$

Multiplying both sides by L/L, the expression becomes

$$V = V_{max}\left\{\frac{f_d(w_d\rho_d)L\rho_d}{\frac{L^22\pi\eta}{\ln(L/R_a)-0.5}+\left(\frac{E_rT_r}{2}\right)(w_r\rho_r)L\rho_r+\left(\frac{E_wT_w}{2}\right)(w_w\rho_w)L\rho_w+\left(\frac{E_{xlink}T_{xlink}}{2}\right)(w_{xlink}\rho_{xlink})L\rho_{xlink}}\right\}. \tag{3}$$

Because $L\rho_d$, $L\rho_r$, $L\rho_w$, and $L\rho_{xlink}$ are the number of molecules per length of actin filament (N_d, N_r, N_w, N_{xlink}, respectively, limited by N_{max-x} for each molecule), this expression may be rewritten as

$$V = V_{max}\left\{\frac{f_d(w_d\rho_d)N_d}{\frac{L^22\pi\eta}{\ln(L/R_a)-0.5}+\left(\frac{E_rT_r}{2}\right)(w_r\rho_r)N_r+\left(\frac{E_wT_w}{2}\right)(w_w\rho_w)N_w+\left(\frac{E_{xlink}T_{xlink}}{2}\right)(w_{xlink}\rho_{xlink})N_{xlink}}\right\} \tag{4a}$$

and using the definition of N_x

$$V = V_{max}\left\{ f_d(w_d\rho_d)\left(\frac{N_{max-d}\rho_d}{\rho_d+K_{d-d}}\right)\Big/\left[\frac{L^22\pi\eta}{\ln(L/R_a)-0.5}+\left(\frac{E_rT_r}{2}\right)(w_r\rho_r)\left(\frac{N_{max-r}\rho_r}{\rho_r+K_{d-r}}\right)+\left(\frac{E_wT_w}{2}\right)(w_w\rho_w)\left(\frac{N_{max-w}\rho_w}{\rho_w+K_{d-w}}\right)\right.\right.$$
$$\left.\left.+\left(\frac{E_{xlink}T_{xlink}}{2}\right)(w_{xlink}\rho_{xlink})\left(\frac{N_{max-xlink}\rho_{xlink}}{\rho_{xlink}+K_{d-xlink}}\right)\right]\right\} \tag{4b}$$

Note: * has been dropped from all N', ρ, and K terms in Eqs. 4a, 4b and all subsequent discussion and expressions for simplicity and clarity.

For samples with more than one type of actin-crosslinking molecule, the expression for $f_{xlink}P_{xlink}\rho_{xlink}$ may be easily extended. If two or more actin-crosslinking molecules are included on the coverslip or in the solution, competition may arise for actin-crosslinking sites. If such a situation exists, N_{xlink} would be expressed as indicated above for the determined type of competition. An example for two actin-crosslinking proteins, xlink-1 and xlink-2, which show competitive binding of actin filaments, is shown below (5a). Additional actin-crosslinking proteins and different types of competition may be easily modeled as described above.

$$V = V_{max}\left\{ f_d(w_d\rho_d)\left(\frac{N_{max-d}\rho_d}{\rho_d+K_{d-d}}\right)\Big/\left[\frac{L^22\pi\eta}{\ln(L/R_a)-0.5}+\left(\frac{E_rT_r}{2}\right)(w_r\rho_r)\left(\frac{N_{max-r}\rho_r}{\rho_r+K_{d-r}}\right)+\left(\frac{E_wT_w}{2}\right)(w_w\rho_w)\left(\frac{N_{max-w}\rho_w}{\rho_w+K_{d-w}}\right)\right.\right.$$
$$+\left(\frac{E_{xlink-1}T_{xlink-1}}{2}\right)(w_{xlink-1}\rho_{xlink-1})\left(\frac{N_{max-xlink-1}\rho_{xlink-1}}{\rho_{xlink-1}+K_{d-xlink-1}(1+[I]/K_{i-xlink-2})}\right) \tag{5a}$$
$$\left.\left.+\left(\frac{E_{xlink-2}T_{xlink-2}}{2}\right)(w_{xlink-2}\rho_{xlink-2})\left(\frac{N_{max-xlink-2}\rho_{xlink-2}}{\rho_{xlink-2}+K_{d-xlink-2}(1+[I]/K_{i-xlink-1})}\right)\right]\right\}$$

Actin-binding proteins also may be added to the motility assay that effectively compete for actin-crosslinking sites but do not form crosslinks themselves (e.g., tropomyosin; see Janson et al., 1992). These added proteins would not hinder actin filament movement but would inhibit actin crosslinking. The effect of these molecules on filament velocity, therefore, would only be noted in the N_{xlink} term as shown below for the three types of competition.

Competitive

$$V = V_{max}\left\{ f_d(w_d\rho_d)\left(\frac{N_{max-d}\rho_d}{\rho_d+K_{d-d}}\right)\Big/\left[\frac{L^22\pi\eta}{\ln(L/R_a)-0.5}+\left(\frac{E_rT_r}{2}\right)(w_r\rho_r)\left(\frac{N_{max-r}\rho_r}{\rho_r+K_{d-r}}\right)\right.\right.$$
$$\left.\left.+\left(\frac{E_wT_w}{2}\right)(w_w\rho_w)\left(\frac{N_{max-w}\rho_w}{\rho_w+K_{d-w}}\right)+\left(\frac{E_{xlink}T_{xlink}}{2}\right)(w_{xlink}\rho_{xlink})\left(\frac{N_{max-xlink}\rho_{xlink}}{\rho_{xlink}+K_{d-xlink}(1+[I]/K_i)}\right)\right]\right\} \tag{5b}$$

Noncompetitive

$$V = V_{max}\left\{f_d(w_d\rho_d)\left(\frac{N_{max-d}\rho_d}{\rho_d + K_{d-d}}\right) \Big/ \left[\frac{L^2 2\pi\eta}{\ln(L/R_a) - 0.5} + \left(\frac{E_r T_r}{2}\right)(w_r\rho_r)\left(\frac{N_{max-r}\rho_r}{\rho_r + K_{d-r}}\right) + \left(\frac{E_w T_w}{2}\right)(w_w\rho_w)\left(\frac{N_{max-w}\rho_w}{\rho_w + K_{d-w}}\right) + \left(\frac{E_{xlink} T_{xlink}}{2}\right)(w_{xlink}\rho_{xlink})\left(\frac{N_{max-xlink}\rho_{xlink}(1 + [I]/K_i)}{\rho_{xlink} + K_{d-xlink}}\right)\right]\right\} \quad (5c)$$

Uncompetitive

$$V = V_{max}\left\{f_d(w_d\rho_d)\left(\frac{N_{max-d}\rho_d}{\rho_d + K_{d-d}}\right) \Big/ \left[\frac{L^2 2\pi\eta}{\ln(L/R_a) - 0.5} + \left(\frac{E_r T_r}{2}\right)(w_r\rho_r)\left(\frac{N_{max-r}\rho_r}{\rho_r + K_{d-r}}\right) + \left(\frac{E_w T_w}{2}\right)(w_w\rho_w)\left(\frac{N_{max-w}\rho_w}{\rho_w + K_{d-w}}\right) + \left(\frac{E_{xlink} T_{xlink}}{2}\right)(w_{xlink}\rho_{xlink})\left(\frac{N_{max-xlink}\rho_{xlink}(1 + [I]/K_i)}{\rho_{xlink} + K_{d-xlink}(1 + [I]/K_i)}\right)\right]\right\} \quad (5d)$$

Simplified expression for standard assay conditions

Equations 4b and 5a–d allow the determination of the velocity of actin filaments in the presence of a single actin-crosslinking protein or a number of actin-crosslinking proteins, assuming that the parameters E_{xlink}, T_{xlink}, w_{xlink}, ρ_{xlink}, $N_{max-xlink}$, $K_{d-xlink}$, $[I]$, and K_i can be calculated or approximated for each crosslinking molecule involved. However, under conditions considered to be standard for the two-dimensional motility assay, a number of the previously defined factors (see above) are negligible or nonexistant, including

Factors (a, b, d): the interaction of rigor and weakly attached myosin II heads with actin filaments

As noted above, rigor heads may take the form of either ATP-deficient or permanently inactive. The concentration of ATP normally used in this assay, ~1 mM, is 16.7 times greater than the half-maximal activity of actomyosin ATPase. ATP-deficient rigor heads would be essentially nonexistant with this ATP concentration. Addition of unlabeled actin filaments to assays (Umemoto and Sellers, 1990), which blocks inactive rigor heads but does not interfere with labeled filament motility, may be used to eliminate such molecules. However, caution should be used because addition of unlabeled actin filaments could affect binding among actin-crosslinking molecules and labeled actin filaments, creating velocities not indicative of the true crosslinking inhibition. By maintaining normal ATP concentrations or blocking inactive rigor heads, one may assume that T_r and $\rho_r \sim 0$.

Unphosphorylated myosin heads would remain as weakly attached heads because light chain kinases are not normally included in the assays. As previously noted, the time of interaction of weakly attached myosin heads with actin, T_w, is ~20–75 μs (Tawada and Sekimoto, 1991). This value is significantly shorter than the duration of a myosin power stroke ($T_d \sim$ 1–2 ms $\gg T_w \sim$ 0.075–0.020 ms). With respect to the total actin-myosin interaction time, T_w may be assumed to be negligible, i.e., $T_w \sim 0$. As a result, P_r and $P_w \sim 0$ and $P_d \sim 1$ (see Table 1).

Factor (c): limiting number of myosin heads and crosslinking molecules that interact with an actin filament per unit length

Under normal assay conditions, the number of driving myosin heads available to an actin filament is a constant (i.e., the effect of K_{d-d} is negligible) and $N_d = N_{max-d}$ is always equal to 30 (see (c)). One can define F_{myo-d}, the active translocating force as $f_d\,(w_d\rho_d)\,(N_d)$, which therefore, would be a constant (e.g., 30 driving myosin heads/μm × 3.4 pN/driving myosin head = 102 pN/μm for skeletal muscle myosin). An analagous expression for the active force of myosin has been used by Pate and Cooke (1989) and Uyeda et al. (1990).

Factor (f): actin filament length

Actin filament lengths are normally far above the limiting value of 40 nm (Harada et al., 1990). Studies cited above indicate no length dependence of filament velocity or percentage of filament moving within the normal filament length distribution.

Factor (g): viscosity, ionic strength, pH, and temperature of assay solution

Several studies have shown that the effect of solution viscosity is minimal or nonexistant (≤3% of total drag) under normal assay conditions (Uyeda et al., 1990; Tawada and Sekimoto, 1991). One may assume, therefore, that $f_s \sim 0$. Standard assay conditions are considered to be approximately 1 mM Mg·ATP, 1 mM Mg^{2+}, 10 mM MOPS (pH 7.35), 0.1 mM K_2·EGTA, and 20 mM KCl, resulting in a final ionic strength of 35 mM and a temperature ~20°C (Homsher et al., 1992). As noted above, changes in standard conditions, in particular pH and ionic strength, can greatly

affect actin/myosin interactions and potentially could affect actin/actin-crosslinking interactions. These changes would have to be taken into account for proper application of this model to particular assays systems.

$F_{myo-d} = f_{myo-d}P_{myo-d}N_{myo-d}$ is constant for most experimental situations because all factors are either unchanged or have only negligible changes (see above). All terms in $F_{xlink} = f_{xlink}P_{xlink}N_{xlink} = (E_{xlink}T_{xlink}/2)(w_{xlink}\rho_{xlink})N_{max-xlink}\cdot\{\rho_{xlink}/(\rho_{xlink} + K_{d-xlink})$ are constants for a given crosslinker type, except ρ_{xlink}. Therefore, F_{xlink} changes with changing ρ_{xlink} (i.e., the inverse of the Myosin:Crosslinker ratio) and, in turn, changes $\{f_{myo-d}/F_{xlink}\}$ and, therefore, V. With these conditions established, one can greatly simplify the expression for the velocity of actin filaments in the presence of varying amounts of an actin-crosslinking protein as

$$V = V_{max}\left\{\frac{F_{myo-d}}{F_{xlink}}\right\}$$

$$= V_{max}\left\{F_{myo-d}\Big/\frac{(E_{xlink}T_{xlink}/2)(w_{xlink}\rho_{xlink})N_{max-xlink}\rho_{xlink}}{\rho_{xlink} + K_{d-xlink}}\right\}.$$

By defining a Constant,

$$C = \frac{F_{myo-d}}{(E_{xlink}T_{xlink}/2)w_{xlink}N_{max-xlink}},$$

this expression may be expressed as

$$V = V_{max}\left\{\frac{C}{\rho_{xlink}^2/[\rho_{xlink} + K_{d-xlink}]}\right\}$$

$$= V_{max}\left\{\frac{C[\rho_{xlink} + K_{d-xlink}]}{\rho_{xlink}^2}\right\}. \quad (6)$$

DISCUSSION

Analysis of predicted relationship (V vs. ρ_{xlink})

The predicted curve from Eq. 6 results in three separate regions outlined below of a changing relationship between V and ρ_{xlink} because of the relationship between ρ_{xlink} and $K_{d-xlink}$ as the relative amount of crosslinker decreases (Fig. 3 *a*).

Region (1)

At very high or infinite crosslinker concentration, the effect of ρ_{xlink} is high and $\rho_{xlink} \gg K_{d-xlink}$. Therefore, $K_{d-xlink}$ is negligible and $V = V_{max}\{C/(\rho_{xlink})^2/\rho_{xlink})\} = V_{max}\{C/(\rho_{xlink})\}$, which produces a straight line. As ρ_{xlink} and F_{xlink} go to infinity, the driving force of the myosin heads becomes negligible and the velocity $V = V_{max}\{\text{Constant}/\infty\} = 0$, i.e., no movement.

Region (2)

At a majority of crosslinker concentrations (Region 2), ρ_{xlink} and $K_{d-xlink}$ are of comparable values and $V_{max} = \{C[\rho_{xlink} + K_{d-xlink}]/(\rho_{xlink})^2\}$, resulting in essentially a parabolic relationship (Note: because Myosin:Crosslinker values, used in Fig. 3, are the inverse of ρ_{xlink}, Region 2 appears as x^2, rather than $1/x^2$).

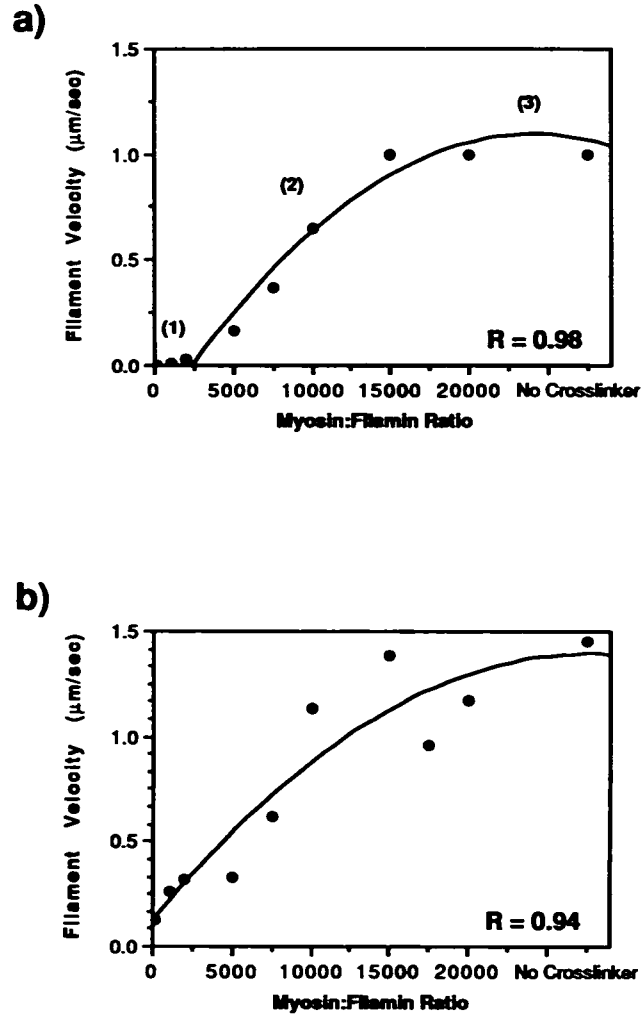


FIGURE 3 Theoretical and experimental calculations. (*a*) Calculation based on the theoretical equation $V = V_{max}\{C[\rho_{xlink} + K_{d-xlink}]/\rho_{xlink}^2\}$, modeling the interaction between single actin filaments and myosin II molecules and a single type of actin-crosslinking protein bound to a two-dimensional substrate. The value ρ_{xlink} is the inverse of Myosin:Crosslinker, graphed above; therefore, as the ratio of Myosin:Crosslinker increases, relative crosslinker concentration and ρ_{xlink} (density of crosslinker molecule) decreases. V_{max} and $C = [F_{myo-d}/(E_{xlink}T_{xlink}/2)w_{xlink}N_{max-xlink}]$ are constants. Each region (1)–(3) discussed in the text is identified. Data were fit using second-degree polynomial with correlation value $R = 0.98$. (*b*) Fit of data from Janson et al. (1992) showing effect of filamin crosslinking on actin filament velocity using the HMM fragment of skeletal muscle myosin II. Data were fit using second-degree polynomial with correlation value $R = 0.94$.

Region (3)

As crosslinker concentration decreases further, $\rho_{xlink} \ll K_{d-xlink}$, F_{xlink} goes to 0, and V approaches the maximum and limiting value V_{max} (Fig. 3 *a*, Region 3). At $\rho_{xlink} = 0$ and $V = V_{max}$ because the effect of crosslinking proteins is no longer relevant and the equation converts to the standard

Tawada and Sekimoto format. Thus, $\lim_{p\text{xlink}\to 0}(V) = V_{max}$; in effect, if no crosslinker is present, the velocity of the actin filament is equivalent to its normal value. Tawada and Sekimoto (1991) provide a similar limit in their model as $P_d \to \infty$ and $P_b \to 0$.

One may also compare the theoretical model to actual data that examines the effect, with constant amounts of myosin, of different amounts of crosslinker on the movement of single actin filaments (Fig. 3 *b*). Using data published in Janson et al. (1992), a parabolic fit results in a correlation value of $R = 0.94$, approximately equal to the theoretical value of 0.98 seen in Fig. 3 *a*. The three regions discussed above from the model equation, including (1) linear, (2) parabolic, and (3) maximum velocity, may be seen in the experimental data, although Region (1) occurs at a very low Myosin:Filamin Ratio (high filamin concentration) of ~100:1 (see Janson et al., 1992) and is not readily evident in this graph.

Determination of $f_{filamin}$ from motility assay data

By knowing V at a number of myosin:filamin molar ratios (Janson et al., 1992) and knowing or calculating $T_{filamin}$, $P_{filamin} = w_{filamin}\rho_{filamin}$, $N_{max-filamin}$, $\rho_{filamin}$, and $K_{d-filamin}$, one may determine an approximate $E_{filamin}$ and, therefore, the resistive force, $f_{filamin}$, of a single crosslinking filamin molecule. Using Eq. 6 and the parabolic fit of data from Janson et al. (1992), and assuming $T_{filamin} = 100$ s (Janmey et al., 1990; see Factor (d) above), $E_{filamin} = 0.0221 \pm 0.0025$ pN/s·filamin and $f_{filamin} = 1.105$ pN. Therefore, the value for the resistive force of a single filamin molecule (1.105 pN) is roughly 3 times less than the force of a single driving myosin head (~3.4 pN) (Finer et al., 1994), or, three filamin molecules would be necessary to stop actin filament movement resulting from one myosin head. This calculation is based on the assumption that all molecules are defined as in contact with the filament (i.e., $P_x = w_x\rho_x = 1$).

However, in the motility assay and in native muscle, several other factors must also be considered and may change the relative force of myosin and a crosslinking molecule such as filamin. The resistive force that a crosslinking molecule creates results from the force it produces, the chance that it interacts with an actin filament, and the number of interactions over a certain increment of time. Myosin cycles approximately every 1 ms; filamin binding lasts at least 100 s. Therefore, in a 1-s period a single myosin would release its driving force 1000 times, whereas the resistive force of filamin would persevere. If this time duration factor is considered, the force for driving or resistive molecules may be defined as $F_x = f_x(w_x\rho_x)(N_x)\partial T_x = f_x N_x T_x(w_x\rho_x$ defined as equal to 1 as above). To determine the relative force values of myosin and filamin, one can determine when the driving forces of myosin would equal the resistive force of filamin or, expressed mathematically,

$$F_{myo} = [f_{myo}\, N_{myo}\, T_{myo}] = F_{xlink} = [f_{xlink}\, N_{xlink}\, T_{xlink}].$$

Values from such an analysis (see Table 2) indicate that a molar ratio of 75:1 Myosin:Filamin molecules are necessary such that $F_{myo} = F_{filamin}$. A ratio lower than 75:1 would result in no actin filament movement, whereas a ratio higher than 75:1 would result in increasing movement. This independently determined value agrees very well with the result of Janson et al. (1992), which indicates that $F_{myo} = F_{filamin}$ occurs around 100:1 Myosin:Filamin for HMM.

TABLE 2 F_{myosin} vs. $F_{filamin}$ at varying myosin:filamin molar ratios

Molar ratio (myosin:filamin)	F_{myosin}	$F_{filamin}$	Ratio
1:1	0.0035	0.26	0.013
75:1	0.26	0.26	1
100:1	0.35	0.26	1.35
1000:1	3.5	0.26	13.46
10000:1	35	0.26	134.62
20000:1	70	0.26	269.23

Values calculated for F_{myo} and $F_{filamin}$ for a normalized 1-s period using general equation $F_x = f_x N_x T_x$ (see Discussion) and the following values for myosin and filamin: $f_{myo} = 3.4$ pN, $T_{myo} = 0.001$ s, $f_{filamin} = 1.105$ pN, $T_{filamin} = 1$ s (note: because filamin maintains a crosslink for ≥100 s, the crosslink duration time for a filamin molecule, $T_{filamin}$, would last for the entire 1 s being considered). N_{myo} and $N_{filamin}$ are determined from the molar ratio indicated. The units of F_{myo} and $F_{filamin}$ are pN over the 1 s time interval.

Similar determinations for other crosslinking proteins (e.g., α-actinin) can be performed using the same methods. The reversal of crosslinking by ions or other actin-binding proteins may also be theoretically modeled as described above. However, one must take special care to determine which parameter(s) would be affected. Addition of calcium to samples containing α-actinin does not directly affect $N_{\alpha\text{-act}}$. Instead, $\rho_{\alpha\text{-act}}$, the density of attached "active" α-actinin crosslinking molecules which may interact with actin filaments, would be affected. Tropomyosin directly competes for filamin actin-binding sites, thereby effectively decreasing $N_{filamin}$ but not affecting $\rho_{filamin}$. Therefore, the effect on actin movement results from direct competition between filamin and tropomyosin binding. As an example, the competition between filamin and tropomyosin (Tm) can be modeled as follows:

$$V = V_{max}\left\{F_{myo-d}\Big/\left[\left(\frac{E_{filamin}T_{filamin}}{2}\right)(w_{filamin}\rho_{filamin}) \times \left(\frac{N_{max-filamin}\,\rho_{filamin}}{[\rho_{filamin} + K_{d-filamin}](1 + [Tm]/K_{i-Tm})}\right)\right]\right\}.$$

SUMMARY

A theoretical model for an actin-myosin-based motility assay has been developed that considers the driving force of myosin molecules *and* the resistive force of any type of actin crosslinking molecule on individual actin filaments. Velocity values are negligible at high relative crosslinker concentrations, rise parabolically at medium relative crosslinker concentrations, and plateau at approximately V_{max} at low relative crosslinker concentrations. The nonlinear relationship between myosin:crosslinker molar ratio and filament velocity

is expected to be caused by the nonlinear nature of probabilities of interaction and the effect of affinities of crosslinker for actin. When pertinent, competition between actin-binding proteins or the effects of inhibitory molecules also lends complexity to the nonlinear relationship.

Calculations using the theoretical relationship and data from Janson et al. (1992) suggest that each filamin molecule produces a resistive force of 1.105 pN with an elastic force constant of 0.0221 pN/s. From this result, one may determine that a molar ratio of 75:1 myosin molecules to filamin molecules would result in a balance between driving forces of the myosin heads and the resistive force of the filamin molecule. This theoretical model and the predicted relationship among actin, myosin, and filamin crosslinking agree very well with the previous data from Janson et al. (1992), offering independent support for its validity. Parameters for other actin crosslinking proteins can be determined in a similar manner.

Actin crosslinking proteins play an important modulatory and/or regulatory role in motility processes (Janson et al., 1991; Janson and Taylor, 1993). Unfortunately, the determination of certain biochemical parameters for various actin crosslinking proteins, particularly those parameters that indicate the relative strength of an actin crosslinking protein in these modulatory or regulatory processes, is often difficult. The theoretical model coupled with the technique of Janson et al. (1992), based on the actin motility assay originally developed by Kron and Spudich (1986), allows these parameters to be calculated in a relatively easy manner.

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