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KINETIC DETERMINATION OF TALIN-ACTIN BINDING

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Smooth muscle talin prepared from chicken gizzard binds to skeletal muscle actin in vitro. The stoichiometry of 1:3 for talin : fluorescent labelled G-actin was confirmed by steady state titration and viscosity measurements under non-polymerizing conditions. The binding constant (K_d) of talin and G-actin was determined by continuous fluorescence titration and gave a value of approx 0.3µM. The association rate constant of talin and fluorescent labelled G-actin of approx $7x10^6$ M^{-1} x s^{-1} was ascertained by the stopped flow method; the dissociation rate constant was calculated at approx 2-3 s^{-1}.

Talin may be involved in linking the cytoskeleton to the plasma membrane (1). For platelet talin, we have recently shown that this protein reconstituted into membrane bilayers selectively interacts with lipids (2). On the other hand, platelet talin interferes with actin polymerization by acting as a nucleation promoting protein (3).

We have isolated talin from smooth muscle following the method of Molony et al. (4) and report here its direct binding to skeletal muscle actin in vitro and the kinetic parameters which we have determined for this interaction.

MATERIALS AND METHODS

G-actin preparation and labelling:

Muscle G-actin was prepared from acetone powder of rabbit back muscle using the method of Spudich and Watt (5), as modified by Eisenberg and Kielley (6), followed by gel filtration on Saphadex G-200. Monomeric actin was stored on ice in a buffer containing 5mM Tris-HCl, 0.2mM DTT, 0.1mM $CaCl_2$ and 0.01% NaN_3, pH 8.0 (G-buffer). The concentration of monomeric actin was determined from the absorbance at 290nm using an extinction coeffcient of 0.617 mg^{-1} x ml x cm^{-1}. G-actin was labelled with 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole (NBD) for fluorescence measurements following the protocol of Detmers et al. (7).

Abbreviation: NBD = (7-chloro-4-nitro-benzeno-2-oxa-1,3-diazole).

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chloramphenicol and 1mM of acetyl-CoA at 37 °C for 5 hrs. The reaction products were separated by thin layer chromatography (TLC), and the CAT activity was calculated from the radioactivity of ^{14}C spots of origin and acetylated chloramphenicol on the TLC plates using AMBIS imaging analysis system.

Preparation for RNA and Northern Hybridization
Cellular RNA was prepared from cells using guanidine hydrochloride procedure and was fractionated in 1% agarose gels containing formaldehyde and transfered onto nitrocellulose filters as described (4). Hybridization was carried out in 50% formamide at 42 °C, and the filters were extensively washed in 0.1x SSC containing 0.5% SDS at 50 °C. Purified 1.8-kb insert of RSV-c-*jun* plasmid (16) was labelled with ^{32}P by using Multiprime labelling kit (Amersham), and used as the hybridization probe.

Cell labeling and Immunoprecipitation
Cells were labeled for 3 hrs in phosphate free DMEM supplemented with 5% calf serum and 700 μCi of [^{32}P]-orthophosphate per ml. The labeled cells were solubilized in modified RIPA buffer (50mM Trishydrochloride [pH 7.4], 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.15M NaCl, 1mM phenylmethylsulfonylfluoride, 10mM sodium pyrophosphate, 10mM sodium fluoride, 4mM EDTA, 2mM sodium vanadate), and the lysates were incubated with antibodies for 1 hr. The immunocomplexes were absorbed to protein A-Sepharose 4B and washed extensively with modified RIPA buffer as described previously (17). Antibodies recognizing the c-Jun protein were purchased from Oncogene Science. The immunoprecipitates were analyzed on a sodium dodecyl sulfate-polyacrylamid (8%) gel followed by autoradiography.

Results

Transactivation of TRE, SRE and CRE by active c-erbB-2
Complementary DNAs of wild type c-*erbB*-2 and its mutant encoding Glu instead of Val-659 within the transmembrane domain were cloned into a mammalian expression vector pCOMlu that utilizes the Harvey sarcoma virus LTR for gene expression (16). Upon transfection into NIH3T3 cells, the mutant c-*erbB*-2 construct, pCOB2A, induced transformed foci (250 focus-forming unit per μg of DNA), while the wild type construct pCOB2N did not induce any transformed foci. To examine the effect of c-*erbB*-2 expression on transcriptional regulation of the genes with TRE, SRE, or CRE, we cotransfected the reporter plasmid TRECAT, SRECAT, or CRECAT into NIH3T3 cells with the c-*erbB*-2 expression plasmid pCOB2A or pCOB2N, or pUC119 as a control. To normalize the transfection efficiency of separate experiments, a construct bearing the human growth hormone gene under the control of the thymidine kinase gene (pTKGH) promoter was also cotransfected with the CAT constructs. When the mutant c-*erbB*-2(active) construct pCOB2A was cotransfected, the CAT activity of every reporter construct was elevated as compared with that from cells cotransfected with the control plasmid (Fig. 1). In contrast, the CAT activity of none of the reporter constructs was enhanced by the wild type c-*erbB*-2(normal) construct pCOB2N. The CAT activities of TRECAT, SRECAT, and CRECAT by active c-*erbB*-2 were 32-, 18-, and 7-fold, respectively, above those by normal c-*erbB*-2 or the basal level. Since the effect of active c-*erbB*-2 on TRECAT was remarkable, we focused our study on dissecting the mechanisms of TRE stimulation.
The c-*erbB*-2 expression vector pCOB2A or pCOB2N was cotransfected into NIH3T3 cells together with pRSVneo. Following selection with G418, two cell clones, B2A-7 and B2-24, which express a high level of mutant and wild-type c-*erbB*-2, respectively, were

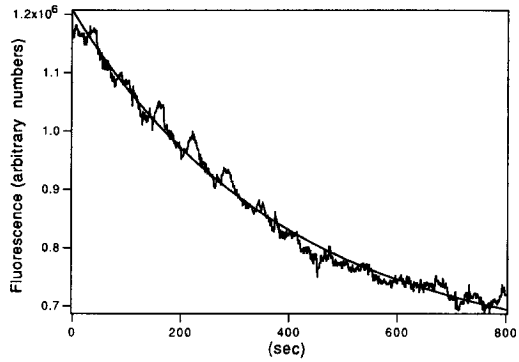

Figure 1. Continuous titration of 0.31mg/ml talin at a rate of 70µl per minute into a 3ml cuvette containing 0.0126 mg/ml NBD labelled G-actin. Buffer conditions: 5mM Tris/HCl, 0.2mM DTT, 0.1mM $CaCl_2$ and 0.01% NaN_3, pH 8.0, 20° C.; Overall K_d approx 0.3µM; Fluorescence changed by a factor of approx 1.7.

fractional saturation of G-actin by talin. α is defined in terms of the fluorescence signal, F as;

$$\alpha = (F_0 - F) / (F_0 - F_\infty),$$

where F_0 and F_∞ are the fluorescence signals for zero and infinite talin concentrations, respectively.

Transient kinetic measurements:

The association rate constant was obtained by the stopped flow method. NBD labelled G-actin was mixed with an excess of talin under pseudo-first order conditions. The fluorescence change could be described by a single exponential with the concentration of talin used. Fig.2 shows a typical result for a

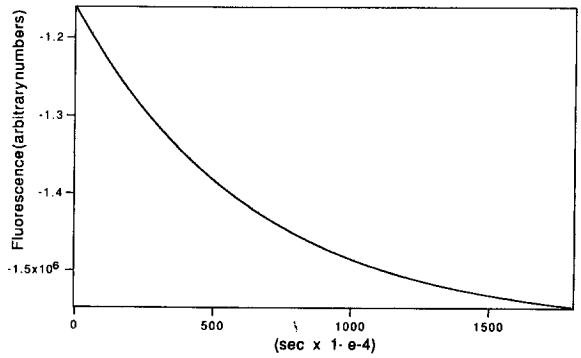

Figure 2. The trace represents the average of ten consecutive measurements in the stopped flow machine. The best fit to a single exponential is shown superimposed. Buffer conditions as in Fig. 1. Protein concentration: 1µM NBD labelled G-actin and 1.5µM talin. The observed rate (k_{obs}) = 17 s^{-1}.

fluorescence transient with a least square best fit exponential superimposed. Assuming scheme I describes the result of this concentration, the observed rate of fluorescence change is calculated by the following equation ($1/\tau = k_{obs}$);

$$\text{(Scheme I)} \qquad 1/\tau = k_{+1}\,([\text{G-Actin}] + [\text{Talin}]) + k_{-1}$$

The calculated rate of association is approx 7×10^6 $M^{-1} \times s^{-1}$. This data is in agreement with the estimated "on-rate" of 7.5×10^6 $M^{-1} \times s^{-1}$ taken from the steady state plot (Fig. 1). The "off-rate" deduced from $Kd = k_{-1}/k_{+1}$ was approx 2-3 s^{-1}.

Breakpoint titration:

The binding stoichiometry of talin to G-actin was estimated by breakpoint titration described by Kouyama and Mihashi (8). In this experiment, 2μM NBD labelled G-actin (large excess of the K_d) was used and small amounts of talin were added until saturation (no further change in fluorescence) was achieved. Fig. 3 shows the breakpoint at approx 0.7μM which gave a talin to G-actin ratio of approx 1:3.

Viscosity measurements:

The low-shear falling ball viscosity technique described by Griffith and Pollard (9) and Fechheimer et al. (10) was used as a semi-quantitative assay to characterize the interaction between talin and G-actin. Using increasing talin

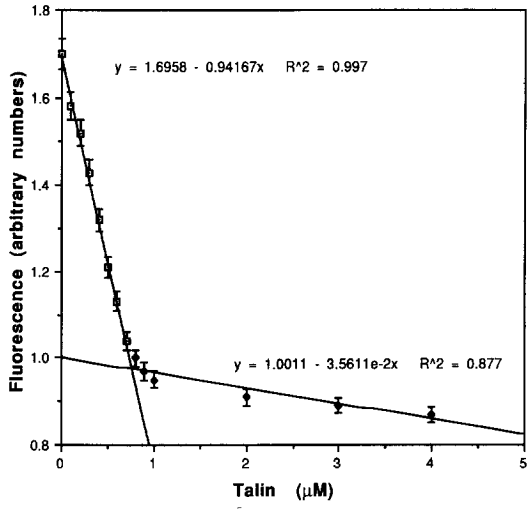

Figure 3. Breakpoint determined by steady state titration of 2μM NBD labelled G-actin and thirteen additions of talin with a total concentration of 4μM. Linear dependence of talin and G-actin at a molar ratio up to approx 0.7μM. Little change in fluorescence on further addition of talin; this indicates binding stoichiometry of approx 1:3. Buffer conditions as in Fig.1.

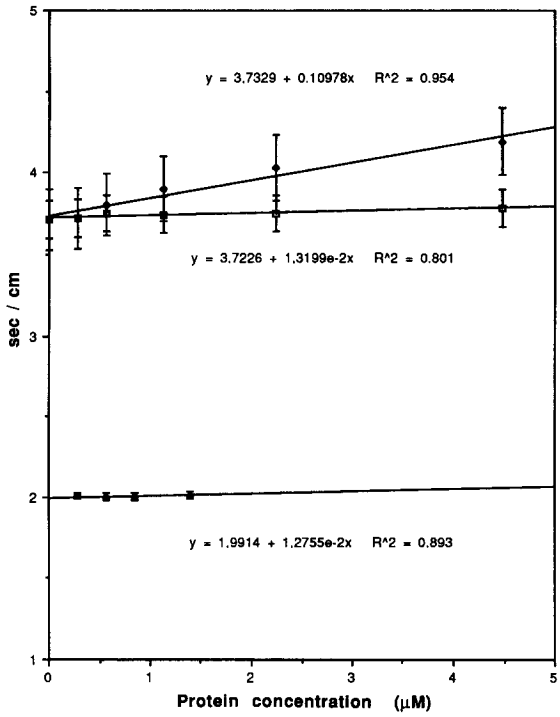

Figure 4. Interaction between talin and NBD labelled G-actin and stoichiometry measured by falling ball at a 15^0 slope. Buffer conditions as in Fig. 1. The molar ratio of talin : G-actin was approx 1 : 3. However, the total protein concentration was the same for G-actin and talin (top slope = 0.10978x), G-actin only (middle slope = 1.3199e-2x) and talin only (bottom slope = 1.2755e-2x).

concentrations at a constant G-actin concentration showed an approx 8-fold increase in viscosity as compared to G-actin on its own at the same total concentration (Fig. 4). This result is intriguing since not only does it indicate the binding between talin and G-actin, it is also consistent with the determined stoichiometry. A binding of 1 : 3 is reflected by comparing the slope of the viscosity graph taking into account the molecular weight ratio of the two proteins. The viscosity at increasing talin concentrations in the absence of G-actin did not change under these buffer conditions.

DISCUSSION

By using stopped flow kinetic and fluorescence titration, we have examined the binding of talin to G-actin in vitro. The stoichiometry of 1 : 3 indicated a more complex binding than a one step mechanism. In a first approach we have, however, determined the overall binding characteristics. Further work will include transient kinetic analysis over a wider concentration range, provided talin can be purified at higher yields.

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