Biochemical and Biophysical Research Communications **271,** 553–557 (2000)
doi:10.1006/bbrc.2000.2653, available online at http://www.idealibrary.com on IDEAL®

Kinetic Determination of Focal Adhesion Protein Formation

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Received April 5, 2000

I examined the binding kinetics between integrin ($\alpha_{IIb}\beta_3$) and purified focal adhesion proteins, including α-actinin, filamin, vinculin, talin, and F-actin. Using static light-scatter technique, I observed affinities of the order talin > filamin > F-actin > α-actinin > (talin when bound to vinculin) which were lower when integrin was complexed with fibronectin. No binding between integrin and vinculin was detected. The calculated dissociation constants (K_d) ranged between 0.4 μM and 5 μM. These results in part confirm previously published data using different methods. The modest affinity with which the focal adhesion proteins interact *in vitro* might be indicative of how cells, e.g., thrombocytes, gain a high degree of versatility and velocity.

Key Words: **focal adhesion protein interactions; static light scattering.**

An important link between the actin cytoskeleton and the plasma membrane, the focal adhesion complex (FAC) consists of a complex of proteins that assemble at sites of attachment of the cell to the extracellular matrix. The transmembrane proteins mediating these contacts are members of the integrin family of extracellular matrix (ECM) receptors. Integrins are heterodimeric proteins that span the plasma membrane bilayer, and the cytoplasmic domain is responsible for linkage of the actin cytoskeleton (CSK) (1). A number of proteins are found in the FAC at the intracellular face of the plasma membrane, including vinculin, talin, α-actinin, and filamin. Which proteins are required for the formation of FAC is still under investigation, and the order and affinity with which these proteins bind integrins is only partly understood.

Abbreviations used: FAC, focal adhesion complex; ECM, extracellular matrix; CSK, cytoskeleton; BSA, bovine serum albumin; LS, light scattering.

[1] This work is dedicated to Dipl. Biol. Irene Sprenger who died tragically in 1999.

A major protein component of focal adhesions is vinculin. Vinculin binds talin, α-actinin, actin, and itself, but not integrin (2–5). As with other components of the focal adhesion complex, vinculin illustrates the apparent redundancy of contacts mediating the connection of actin to the plasma membrane. Talin has been demonstrated to bind the intracellular domains of the integrin β1, β1A, and β1D-chain (6, 7). The binding of talin to integrins may be under the control of phosphorylation, caused for example by interleukin-1-β; talin's function as a linker protein can be reversed by the calcium-dependent protease calpain (8, 9). α-Actinin is an actin cross-linking protein that, in nonmuscle cells, is regulated by Ca^{2+} and forms filament aggregates ranging from bundles to open networks (10). A direct binding of α-actinin to integrin (β1 and β2) has been documented (11, 12), and the importance of this protein *in vivo* is suggested by the finding that injection of α-actinin fragments into living cells disrupts their cytoskeleton (13). In addition to these components of focal adhesions, other proteins are found to be preferentially associated with these structures, including filamin and F-actin (14–16). Sharma *et al.* (17) showed that filamin (ABP-280) binds specifically to the cytoplasmic tail of β2-integrin CD18 subunit; and more recently, Kieffer *et al.* (18) reported direct binding of F-actin to the cytoplasmic domain of the α2-integrin chain. These less abundant proteins may be responsible, in part, for the regulation of the larger focal contact complex.

In this study, the author used kinetic methods on purified focal adhesion proteins to determine their affinity with integrin ($\alpha_{IIb}\beta_3$). These interactions are believed to be essential, e.g. for 'inside ↔ out' signaling during platelet activation and cytoskeletal restructuring (19).

MATERIALS AND METHODS

Protein preparations. Actin was prepared according to procedures of Spudich and Watt (20) from acetone powder obtained from rabbit back muscle; this was followed by a gel filtration step as

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described by MacLean-Fletcher and Pollard (21). The biological activity of the purified actin was tested, using falling ball viscometry, and its concentration was determined at ϵ_{290nm} = 26,460 M^{-1} cm^{-1}. Fractionated G-actin was stored in G-buffer: 0.2 mM Tris/HCl, pH 7.5; 0.2 mM $CaCl_2$, 0.5 mM ATP, 0.2 mM DTT, and 0.005% NaN_3, for no longer than 10 days. For the light-scatter studies, G-actin was polymerized overnight at 4°C in 0.2 mM Tris/HCl, pH 7.5; 100 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.2 mM $CaCl_2$, 0.2 mM DTT, 0.5 mM ATP.

α-Actinin was isolated from turkey gizzard as described by Craig *et al.* (22) and further purified by a hydroxylapaptit column. The purity was determined according to Laemmli (23). The protein was sterile-filtered and kept at 4°C in the dark. The protein concentration was measured by UV spectroscopy, using ϵ_{278nm} = 97,000 M^{-1} cm^{-1}.

Filamin was isolated using the method of Shizuta *et al.* (24). Low ionic strength extraction of chicken gizzard was followed by Mg^{2+} and $(NH_4)_2SO_4$ precipitation, ion exchange, and gel filtration column chromatography. For all experiments, an additional hydroxylapatite column was used to further purify the protein (25). The purity of the filamin was determined using SDS/PAGE. The concentration of purified filamin was measured by UV spectroscopy, using $\epsilon_{278.5nm}$ = 205,000 M^{-1} cm^{-1}, following the method of Bradford (26) with BSA as standard. A molecular mass of 270 kDa for monomeric filamin was used.

Talin and vinculin were isolated from outdated human thrombocytes by the Collier and Wang method (27). After the first ionic exchange column, talin was passed through a gel filtration column, and vinculin was passed through an additional hydroxylapatite column (eluted by a linear gradient from 0.02 M to 0.4 M KH_2PO_4), further purifying each (28). The purity of the proteins was analyzed on SDS mini-slab gels. Protein concentrations were determined according to Bradford (26).

Integrin $\alpha_{IIb}\beta_3$ was isolated from human thrombocytes using the method of Fitzgerald *et al.* (29). Given results of the subsequent binding studies, the procedure was slightly altered in the following way: Triton X-100 was replaced by the non-ionic detergent, *n*-octylpolyoxyethylene (POE). After the final isolation step (Sephacryl S300 column), the purity was determined by SDS/PAGE. Protein concentrations were determined according to (30), a modified method by Lowry.

Fibronectin was purchased from CALBIOCHEM (Fulterton, CA) and used without further purification. The experimental buffer contained 2 mM Tris/HCl, 50 mM KCl, pH 7.5, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.2 mM DTT, and 0.5 mM Na_2ATP; measurements were performed at 20°C. Figure 1 shows all purified proteins on 10 and 7.5% SDS/Page, respectively.

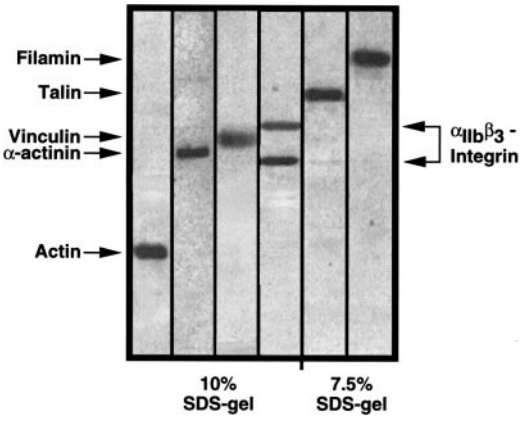

FIG. 1. SDS/PAGE. Actin (~42 kDa), α-actinin (~100 kDa), vinculin (~116 kDa), and integrin (α_{IIb}-chain ~93 kDa; β_3-chain ~136 kDa at 1:1 stochiometry) as well as talin (~205 kDa) and filamin (~250 kDa) were analyzed by 10 and 7.5% SDS–PAGE, respectively. Each lane was loaded with 5 μg protein.

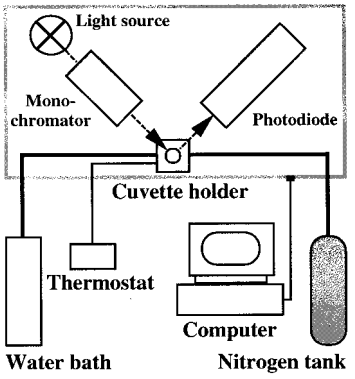

FIG. 2. Spectrophotometer. Schematic diagram of the fluid handling and detection system for the Fluorolog 50B spectrophotometer from Perkin Elmer.

Steady state measurements. Binding studies were carried out in a Perkin Elmer Fluorolog 50B Spectrophotometer (Fig. 2). Briefly, monochromated light was passed through a quartz cuvette—containing the proteins in solution—and detected by a photodiode. The metal cell holder of the 0.3 ml measuring cuvette was controlled thermostatically by an external water bath (±0.1°C). During titration protein solutions were added and gently mixed by hand to prevent the formation of air bubbles. The light-scatter (LS) intensity, I, was recorded at 380 nm and a 90° angle to the incident light. This wavelength was chosen to avoid interference from protein adsorption at 280 nm. Since light at 90° angle can measure the amount of protein bound and the protein of reflected light in the light path is measured at 380 nm, unbound protein has little influence on the detected signal. Data were collected on an Apple Macintosh IIfx computer and later transferred to a Power Macintosh 7300/200 for data analysis.

Assuming the binding of A and B is in rapid equilibrium, the overall equilibrium constant of C, K is defined by the following relation:

$$A + B \overset{K}{\leftrightarrow} C; \quad K = [C]/[A]^*[B], \qquad [1]$$

where A is integrin ($\alpha_{IIb}\beta_3$), or integrin complexed with fibronectin at a molar ratio of 1:1; B is α-actinin, filamin, talin, vinculin, F-actin, or fibronectin; C is protein complex; K is overall equilibrium constant, and the dissociation constant, K_d is determined from the relation K_d = 1/K. The protein concentration is given in parenthesis. With increasing protein concentration of B, an augmentation of the light scatter intensity, I should be observed. This behavior is expected as the equilibrium constant, K represents the relation of [C]/[A]*[B] and since higher protein concentration of B should lead at constant protein concentration of A to increased protein complex formation C which is proportional to the light scatter intensity, I.

Data obtained from steady-state titrations were corrected for any dilution effects from the added protein and then fitted by a nonlinear least-squares fitting routine to the following equation (31): $\beta^2[A]_0 - \beta([A]_0 + [B]_0 + K_d) + [B]_0 = 0$, where $[A]_0$ is the starting concentration of integrin ($\alpha_{IIb}\beta_3$), or integrin complexed with fibronectin at a molar ratio of 1:1, $[B]_0$ is the total concentration of α-actinin, filamin, talin, vinculin, F-actin, or fibronectin added, K_d is the dissociation constant, and β is the fractional saturation of A by B. β is defined in

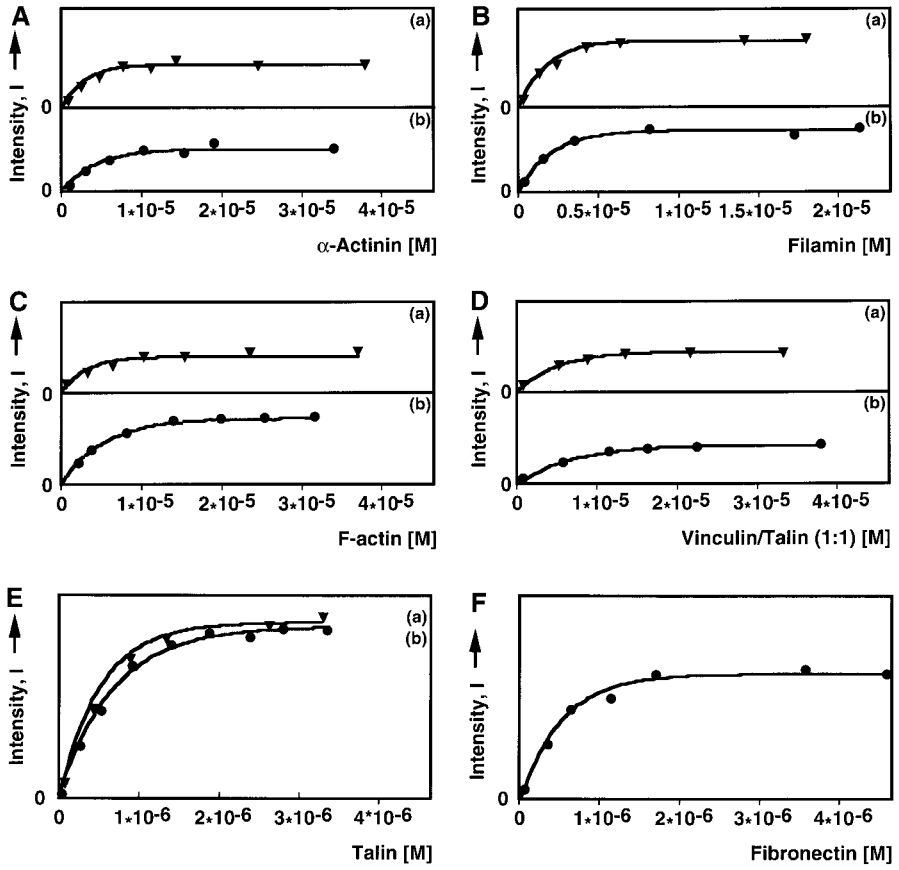

FIG. 3. Titration curves. Titrations were performed in the spectrophotometer at 380 nm by adding (A) α-actinin, (B) filamin, (C) F-actin, (D) vinculin complexed with talin at a molar ratio of 1:1, (E) talin, and (F) fibronectin into a quartz cuvette containing 0.3 ml of 3 μM integrin (a), or 3 μM integrin and fibronectin at a molar ratio of 1:1 (b), and 2 mM Tris/HCl, 50 mM KCl, pH 7.5, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.2 mM DTT, 0.5 mM Na_2ATP at 20°C. The data were analyzed as described in the text and the solid line is the best fit. Data shown are means of triplicate experiments; SD $<$ 5%.

terms of I, as $\beta = (I_0 - I)/(I_0 - I_\infty)$, where I_0 and I_∞ are the intensity for zero and infinite concentrations of B, respectively.

RESULTS

Equilibrium binding measurements. Steady state kinetics was used to demonstrate the protein–protein binding sequence in this multiprotein system. The experimental approach to this investigation of the equilibrium between purified integrin ($\alpha_{IIb}\beta_3$) and other focal adhesion proteins was straightforward. The high signal-to-noise ratio of the light-scatter intensity, I, allowed the direct measurement of the dissociation constant, K_d using titration (see Eq. 1). Figures 3A–3F show the hyperbolic dependence of the LS signal with respect to total focal adhesion protein concentration, and the line gives the best fit to the data. Table 1 shows the K_d for all protein species interacting with integrin or integrin complexed with fibronectin at a molar ratio of 1:1. The results indicate an increase in molar affinity in the order of talin $>$ filamin $>$ F-actin $>$ α-actinin $>$ (vinculin complexed with talin). However, the affinity for the integrin-fibronectin complex was lower compared to integrin; this may be an indication of the signaling path, e.g. in platelets. The K_d calculated for α-actinin compares well with data published by Otey *et al.* (11), using solid phase binding assay. Recently, Pavalko and LaRoche (12) have shown that activation of neutrophils induces the interaction between integrin β2 subunit CD18 and α-actinin. Using gel filtration, Horwitz *et al.* (6) described a linkage between fibronectin, CSAT antigen—an integral membrane glycoprotein complex, i.e., integrin β1—and talin, and reported a K_d for CSAT antigen and talin of ~0.7 μM. More recently using solid phase binding assay, Knezevic *et al.* (7) reported a direct interaction between integrin $\alpha_{IIb}\beta_3$ and talin with an even higher affinity (K_d = 0.015 μM). In the present study, the steady-state kinetic method showed, the K_d to be approximately 0.4 μM. In spite of the amount of research elucidating the binding of integrins with focal adhesion proteins, only a few dissociation constants have been published. Mueller (32) found that for fibrinogen binding to integrin $\alpha_{IIb}\beta_3$

TABLE 1

K_d for the Binding of Proteins to Integrin and Integrin-Fibronectin Complex

Proteins	$K_d = 1/K$ (μM)	
	Integrin	Integrin/ Fibronectin (1:1)
Fibronectin	0.4	—
Talin	0.4	0.6
Filamin	1.2	1.5
F-actin	2.2	4.0
α-Actinin	2.5	3.0
Vinculin/Talin (1:1)	2.8	5.2

Note. The dissociation constant, K_d (μM) for integrin or integrin complexed with fibronectin at a molar ratio of 1:1 and α-actinin, filamin, F-actin, vinculin complexed with talin at a molar ratio of 1:1, talin, and fibronectin. Experimental conditions were as described in the legend to Fig. 2.

complexed in lipid bilayers a K_d of ~0.050 μM, which is a factor ten times larger than measured here. High K_d values for talin and paxillin binding to vinculin have also been reported. The affinities measured by LS (unpublished results) were about an order of magnitude lower than 10^{-8} M and 6×10^{-8} M (2, 33).

Despite the reported differences in K_d, which are probably based on the techniques, detection methods, and buffer conditions, i.e., temperature, ionic strength, and pH used, this detailed study using one method and identical experimental conditions throughout opens up the possibility of a more comprehensive understanding of the role of focal adhesion complex (FAC) formation. Applying steady state kinetics to highly purified FAC proteins has provided insights into the mechanism and order of how these species associate *in vitro* and has given some clues about its dynamic nature in cells. For instance, where focal adhesion proteins interact with modest affinity, cells normally gain a high degree of versatility and velocity in the assembly and disassembly of focal adhesion sites as reported for thrombocytes (34).

DISCUSSION

Cell adhesion to ECM is necessary for the maintenance of cell growth and function. Cells attach to ECM molecules, such as fibronectin, through the binding of cell-surface integrin receptors that cluster in localized attachment domains called focal adhesions. Originally, focal adhesions were defined morphologically, as those regions of the ventral cell membrane that came in closest contact with the underlying ECM and in which actin stress fibers terminated. However, more recent studies demonstrate that cell adhesion is mediated through the formation of the FAC, which contains actin-associated proteins similar to the ones used here. It has been shown that these FAC proteins interact with the cytoplasmic portion of integrins and thus physically interconnect ECM with the CSK. Hence this transmembrane path for transfer of CSK tension to the ECM is critical for cell spreading, cell migration, and tissue remodeling. Structural interconnections between integrins and FAC proteins also mediate mechanonsensations, the process by which cell sense and respond to external mechanical signals. In addition, the FAC appears to function as a site for local chemical signaling by integrins (35).

Thus, so far, the mechanism of FAC assembly and organization is only poorly understood. This study demonstrates that all focal adhesions proteins except vinculin bind directly to integrin ($\alpha_{IIb}\beta_3$) with modest affinity. Although it is known that large numbers of intracellular proteins and transmembrane proteins can associate indirectly with integrins, only a few proteins have been shown to bind directly. The identification of proteins interacting directly with integrin is the first step towards understanding the FAC (36, 37), and results presented in the present study provides further insight into how the focal adhesion proteins may potentially interact with integrin ($\alpha_{IIb}\beta_3$) in thrombocytes. The order with which these proteins associate with integrin—judged by their affinity—and, possibly, are recruited in the signaling pathway is intriguing and now needs to be tested *in vivo.*

Previous studies using a mouse embryonic F9 cell line showed that transmembrane force transfer across integrins correlates with the recruitment of the FAC proteins, vinculin, talin, and α-actinin and thus, physical linkage of integrins to the actin cytoskeleton (38, 39). F9 cells that lacked vinculin retained the ability to form filopodia and contained normal levels of total

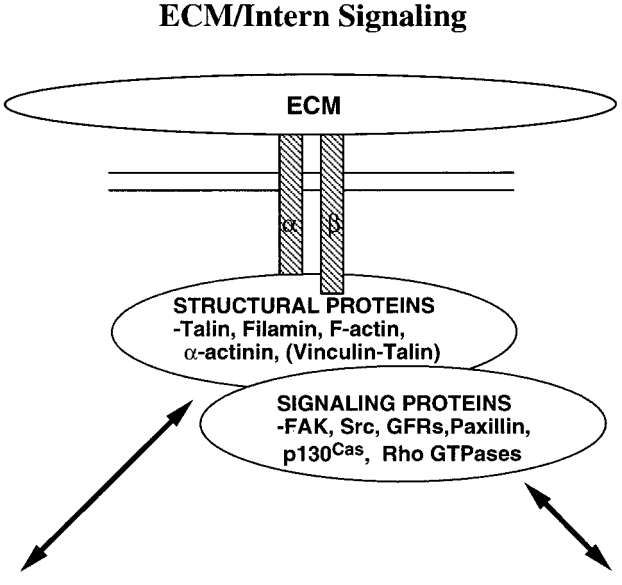

FIG. 4. Schematic representation of ECM ↔ Intern signaling.

polymerized and cross-linked actin, yet they could not form lamellipodia, assemble stress fibers, or efficiently spread when plated on fibronectin. However, when vinculin was replaced by transfection, the efficiency of transmembrane coupling, stress fiber formation, and cell spreading were all restored to near wild-type levels. This demonstrates that vinculin and probably other focal adhesion proteins present downstream molecules that mediate the transfer of mechanical and/or biochemical signals from integrins to the cytoskeleton (Fig. 4).

ACKNOWLEDGMENTS

The gifts of the focal adhesion proteins from Drs. S. Kaufmann, C. P. Sharma, and Ms. H. Kirpal are gratefully acknowledged. The author thanks Dr. Ben Fabry, Harvard School of Public Health, Boston, for helpful discussions, and Judith Feldmann, Ph.D., for copyediting and proofreading the manuscript. This work was supported by a grant from the North Atlantic Treaty Organization.

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