Demonstration of non-linear detection in ELISA resulting in up to 1000-fold too high affinities of fibrinogen binding to integrin αIIbβ3

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Abstract To clarify the question as to why different solid-phase assays yield different results in terms of interaction strength, we used fibrinogen binding to immobilized αIIbβ3 integrin as a test system. A classical 'three step' enzyme-linked (ELISA), a 'two step' biotin enzyme-linked streptavidin and a 'one step' radioligand assay were compared under otherwise identical conditions. Only the last assay yielded binding constants comparable to earlier data by total internal reflection fluorescence microscopy while the other assays yielded apparent binding constants 5- to 1000-fold too high. These effects are explained by non-linearity of detection signals.

Key words: Solid phase assay; Detection; Integrin αIIbβ3; Fibrinogen

1. Introduction

Enzyme-linked immunosorbent assays (ELISA) developed by Engvall et al. in 1971 [1] and related solid-phase assays are very popular for monitoring interactions between extracellular matrix proteins and between these proteins and their receptors [2–6]. Their decisive advantages are the high sensitivity, the low amounts of materials needed, and the speed of analysis, but problems exist concerning quantitation of binding strength. In enzyme-linked assays very early saturations at low ligand concentrations are frequently observed, suggesting high apparent affinities.

In the present work an ELISA and a biotinylated ligand assay were compared with a radioligand assay. The same system, namely binding of soluble fibrinogen to coated integrin αIIbβ3, was applied, the binding constants for which were known from total internal fluorescence microscopy (TIRFM) [7]. Experimental conditions, including surface adsorption, were kept constant in order to exclude differences in coating or surface densities as possible reasons for differences in affinity.

2. Materials and methods

Integrin αIIbβ3 was purified from detergent extracts of human platelets by anion-exchange and molecular sieve chromatography [7]. Human fibrinogen, grade L, was obtained from Kabi. The proteins were iodinated with Iodo beads (Pierce Chemical Co.) according to [8] which yielded specific activities of $4 \cdot 10^{17}$ cpm/mol for fibrinogen and $1.6 \cdot 10^{18}$ cpm/mol for αIIbβ3. Fibrinogen was biotinylated with a biotinylation kit following the suggestions of the manufacturer.

To determine the amount of protein absorption to Nunc Maxisorb Breakapart plates, iodinated proteins were diluted 1:20 in 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ (buffer A) and 100 μl/well were used for coating for 17 h at 4°C. After removal of unbound ligand by washing with buffer A with 0.04% Tween 20 added, binding was analyzed in a γ-counter (Packard).

100 μl/well of 21 nM and 4 nM αIIbβ3 in buffer A was adsorbed for 17 h at 4°C. The wells were then blocked for 1 h at room temperature with 3% bovine serum albumin in buffer A. All subsequent washing and binding steps were performed in buffer A with 0.04% Tween 20 added. Fibrinogen was incubated in the coated wells for 2 h at room temperature. For ELISA, primary rabbit anti-human fibrinogen immunoglobulins (Dako) and secondary goat anti-rabbit immunoglobulins coupled to horseradish peroxidase (Bio-Rad) were then applied for 1–2 h, and their binding was visualized with 2,2'-azino-di-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS; Bio-Rad). The color reaction was stopped with 2% oxalic acid and the plates were evaluated in an ELISA reader at 414 nm. Biotinylated fibrinogen was detected with biotinylated horseradish peroxidase–streptavidin complex (Bio-Rad). Iodinated fibrinogen was assayed by counting its radioactivity.

The signals of all assays were normalized to 1 at the experimentally observed saturations. For data evaluation the CHEMFIT 2.0 (Technosoft, Graz) program was employed. Curves were fitted by the following relationship, assuming a single class of equivalent and independent binding sites: $Y = [L]/(K_D + [L])$ in which Y represents the degree of saturation, K_D is the equilibrium dissociation constant (reciprocal binding constant), and $[L]$ the concentration of free ligand.

3. Results

Radiolabelled integrin αIIbβ3 was coated on to a microtitre plate for 17 h at 4°C and bound protein was determined by radioactivity analysis (Fig. 1). The percentage of occupied area per well was calculated assuming areas per molecule of 8×12 (head) + 8×18 (stalks) = 240 nm^2 for αIIbβ3 and $47 \times 8 = 376$ nm^2 for fibrinogen [9]. A value of 100% indicates a monolayer. With increasing coating concentration, αIIbβ3 saturated approximately 80% of the available well surface. The coating buffer did not contain any detergents which would keep the integrin in a monomeric state. In solution without detergent, αIIbβ3 molecules aggregate to multimers [9] which may persist in the coat. In contrast to integrin, fibrinogen aggregated without saturation and formed multilayers (Fig. 1). Therefore, because of the much better defined adsorption, all the following experiments were performed with integrin coats to which soluble fibrinogen was added; in the literature experiments with adsorbed fibrinogen are also described [5,10,11].

The assays were performed with integrin coated at 21 and 4 nM (Fig. 2A and B) which correspond to surface saturations of 60 and 12% (Fig. 1), respectively. Iodinated fibrinogen was bound to αIIbβ3. This assay consists of only a single step. On the same microtitre plate [^{125}I]fibrinogen was detected by an antibody directed against fibrinogen and a secondary antibody conjugated with horseradish peroxidase. The reaction of horseradish peroxidase with the substrate, ABTS, resulted in an absorbance value. This classical ELISA may be considered a three-step assay. The signal in the ELISA already saturated at

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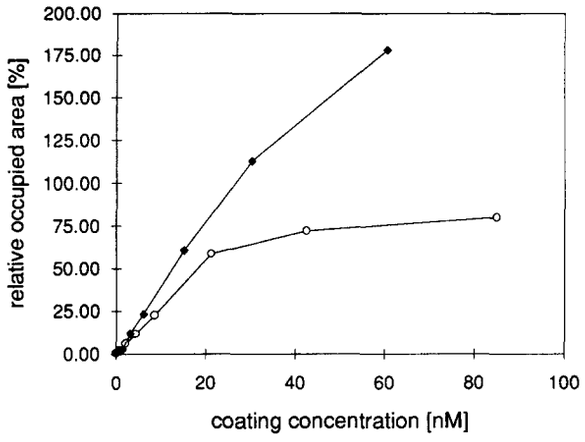

Fig. 1. Coating efficiency of the microtitre plates for integrin αIIbβ3 (○) and fibrinogen (◆).

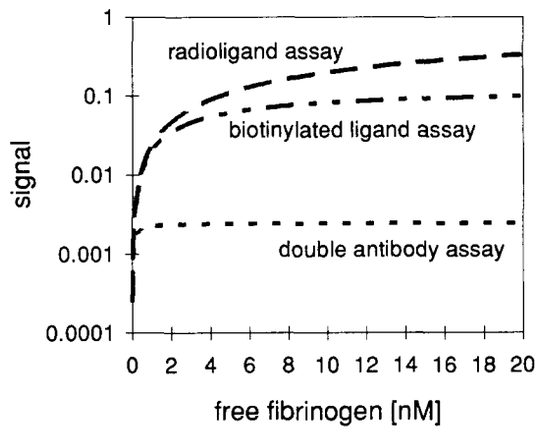

Fig. 3. Calculated dependence of the signals on free fibrinogen concentration for different detection assays. Data were taken from Fig. 2A and the same initial slope was assumed for all three assays.

a concentration of free fibrinogen of about 0.3 nM in Fig. 2A and at about 1 nM in Fig. 2B. These plateau values are in striking contrast to the results of the radioligand assay in which saturations were observed only at about 1000 nM, independent of the surface density of the integrin. When apparent dissociation constants were evaluated from the curves of Fig. 2, the values summarized in Table 1 were obtained. Only those values determined by the radioligand assay agreed with a previously determined value from total internal reflection fluorescence microscopy of K_D = 50 nM [7]. The value determined by ELISA was 1000-fold lower. To a lesser extent too low dissociation constants were also found for a two step assay with biotinylated fibrinogen using a horseradish peroxidase–streptavidin complex for detection. The deviations from the radioligand assay were significantly reduced when the surface density of the integrin was lowered. The curve shifted by a factor of 10 in the biotin–streptavidin assay and by a factor of 2 in the ELISA.

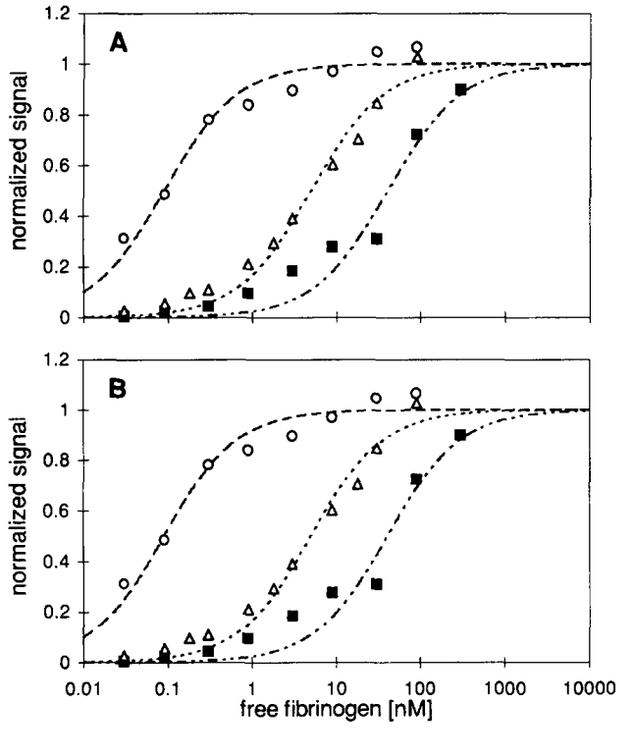

Fig. 2. Binding curves of fibrinogen to αIIbβ3. All three assays were performed with identical integrin coats of 21 nM (A) and 4 nM (B). Bound fibrinogen was detected with a primary and a secondary antibody in ELISA (○), biotinylated fibrinogen was detected with horseradish peroxidase–streptavidin complex (△), and iodinated fibrinogen was assayed by counting its radioactivity (■).

4. Discussion

Various surface effects have been considered as possible explanations for differences in affinities in solid-phase assays [12–14]. Coating of protein on microtitre plate wells may change conformation or even lead to denaturation [15]. Because of the usually high surface density, several adsorbed molecules may be recognized simultaneously by a multivalent ligand, thus leading to an increase in affinity [16]. Also, the effect of non-idealities which disturb equilibration of the binding reaction [17], and kinetic complications associated with the high surface concentrations, have been discussed [18,19].

In the present work, coating conditions and surface densities were kept constant and only the assay systems were changed. It is therefore reasonable to assume that the differences originate from non-linearities of the detection signals. With the assumption that signals are proportional to the amount of bound ligand only at very low binding ratios, we normalized all binding curves to identical initial slopes (Fig. 3). It can be seen that the curves reach a plateau at very low ligand concentrations in ELISA, and to a lesser extent in the biotinylated

Table 1
Apparent dissociation constants, K_D (nM), determined by the different assays

Integrin coating concentration	ELISA	Biotin–streptavidin	Radioligand
5 nM	0.09	5	47
21 nM	0.045	0.43	47

ligand assay. In agreement with the suggestion of other authors [14], we think that the non-linearities of the signals are due to steric hindrances between the large antibodies at higher concentrations. Unfortunately, in all ELISA-type assays it is essentially impossible to check the extent of saturation in an independent way because of the unknown amount of active binding sites on the solid support.

Non-linearity appears to be smaller for the biotin–streptavidin assay than in ELISA, in agreement with the smaller size of the streptavidin conjugate used for detection. The steric hindrance and therefore non-linearity appear to increase with the number of steps in the assay. The steric restrictions should be smaller at lower surface densities of the integrin and this was qualitatively confirmed.

In conclusion, our data demonstrate that enzyme-linked solid-phase assays are not a priori suitable for determination of binding constants, most likely because of the high non-linearity of the detection signals.

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