Vinculin Binding Site Mapped on Talin with an Anti-idiotypic Antibody

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Vinculin and talin are major adhesion plaque components which interact in vitro and presumably in vivo. The amino acid sequence of talin is now known so details of its domain structure can be mapped. We localized vinculin binding sites in the talin sequence by overlaying peptide maps of talin with an anti-idiotypic vinculin antibody that recognizes talin and with [125I]vinculin. A rabbit injected only twice with vinculin and producing anti-vinculin antibodies spontaneously generated a second antibody that recognizes talin. Vinculin and anti-vinculin antibodies specifically compete with this second antibody for binding to talin as determined by solid-phase binding and overlay assays. The antibody is thus most likely an anti-idiotypic antibody which mimics a region of vinculin that interacts with talin. The binding site of the anti-idiotypic antibody on talin was mapped to the 196 amino acids spanning residues 1653 to 1848. A second vinculin binding site identified with an 125I-vinculin blot overlay technique was located between residues 483 and 1652. The observation that talin has two immunologically distinct vinculin binding sites suggests that vinculin may have two different talin binding sites or one "complex" site with two interacting regions.

Cell-cell and cell-matrix adherens junctions are sites of tight association between cells or between cells and the extracellular matrix. Adherens junctions contain three regions: an extracellular domain, a transmembrane domain, and a cytoplasmic domain (reviewed in Ref. 1). The cytoplasmic domain includes actin as the major component along with an assemblage of proteins that presumably function to link actin to the plasma membrane and to mediate signal transduction pathways (1).

Two major components of the cytoplasmic domain are vinculin and talin (reviewed in Refs. 2 and 3). These two proteins interact with each other (4–6). Studies with purified fragments of vinculin have localized the binding domain of talin on vinculin to the 90-kDa amino-terminal head piece (7, 8). Further studies with small peptides of vinculin have narrowed this binding region to the amino-terminal 225 residues (9), and deletion analysis of cDNAs encoding vinculin mutant protein localize the binding region to residues 167–207 (10, 11).

However, comparable studies have not been done to define the binding domain(s) for vinculin on talin, although it is known that the 190-kDa major proteolytic fragment of talin binds vinculin (12). Recently, a mouse talin cDNA was sequenced (13) so details of its domain structure can be studied. In this paper, we report the localization of a vinculin-binding site on talin that was mapped with an anti-idiotypic antibody against talin. This antibody appeared spontaneously in a rabbit injected only with vinculin. This binding site is compared to one localized with 125I-vinculin overlays.

EXPERIMENTAL PROCEDURES

Protein Preparation—Vinculin and talin were purified from turkey gizzard and quantified according to Groesch and Otto (7).

Antibody Preparation—A New Zealand White male rabbit was immunized subcutaneously with 0.3 mg of vinculin in complete Freund’s adjuvant. It was boosted once with the same amount of antigen in incomplete Freund’s adjuvant. After the boost, the rabbit was bled approximately every other week. The rabbit produced anti-vinculin antibody immediately after the boost. The second antibody (anti-talin antibody) could be detected in the sera after the 14th bleed (7 months after the first bleed); these sera are referred to as “late sera.”

Purification of Antibodies—IgG fractions from rabbit preimmune serum and immune sera were purified on an Affigel Blue column (Bio-Rad). The IgG was precipitated with 33% ammonium sulfate and then dialyzed against phosphate-buffered saline (PBS): 0.14 M NaCl, 2.6 mM KCl, 8 mM NaHPO4, 1.5 mM KH2PO4, pH 7.4. For the affinity purification of anti-vinculin antibody, vinculin (1.3 mg/ml) was coupled to Affigel 10 (Bio-Rad) according to the manufacturer’s instructions, and the IgG fraction of the early immune sera was loaded. After extensive washing with PBS, the anti-vinculin antibody was eluted with 0.2 M glycine-HCl, pH 2, neutralized immediately with 2 M Tris base, and dialyzed overnight against PBS.

For affinity purification of the anti-vinculin and anti-talin-antibodies from the late sera, talin or vinculin were separated on sodium dodecyl sulfate (SDS) gels (14) and electroblotted onto nitrocellulose with a semi-dry blotting apparatus (7). After incubation in blocking solution (0.25% gelatin, 3% bovine serum albumin in Tris saline buffer (0.15 M NaCl, 50 mM Tris-HCl, pH 7.5)), the nitrocellulose was incubated overnight with the late sera. After extensive washing with Tris saline buffer, the bound antibodies were eluted with 0.2 M glycine-HCl, pH 2.0, and immediately neutralized with 2 M Tris base. The antibodies were then diluted in blocking solution.

Enzyme-linked Immunosorbent Assay—Poly styrene microtiter wells were incubated overnight at 4°C with 2.2 µg of talin in 50 µl of carbonate buffer (0.01 M Na2CO3, 0.056 M NaHCO3, pH 9.6). Wells were blocked with 200 µl of blocking solution. After washing the wells, the affinity-purified spontaneous anti-talin antibody (5 µl) and various competitors (50 µl) were incubated for 2 h at room temperature. After the incubation, the wells were washed with PBS. The bound antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit antibody (Kirkegaard and Perry, Inc., Gaithersburg, MD) in a 1-h incubation at room temperature. After extensive washes, color development substrate (0.1% p-nitrophenyl phosphate, 10% diethanolamine, 0.05% Na2HPO4, pH 9.8 with HCl) was added. The

* The abbreviations used are: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; AIA, anti-idiotypic antibody.
optical density at 405 nm was read after 15 min with a Molecular Device plate reader (Menlo Park, CA).

Peptide Mapping—Talin and its major proteolytic fragment (11 μg) were digested with *Staphylococcus aureus* V8 protease (550 units/ml; Miles Laboratories, Elkhart, IN) at a concentration of 10 μg/ml, chymotrypsin A₉ (90 units/mg; Boehringer Mannheim) at 1.2 μg/ml, or trypsin (110 units/mg; Boehringer Mannheim) at 0.6 μg/ml for 50 min at 37°C. For double digestions, talin was pretreated with V8 protease for 15 min at 37°C then digested with chymotrypsin A₉ or trypsin for 50 min at 37°C. The peptides were separated on 7% SDS gels (14) and blotted onto a polyvinylidene difluoride membrane (15). Selected peptides were sequenced for their amino terminus with an Applied Biosystems Model 473A sequenator (pulsed-liquid system) equipped with the 610A data module.

RESULTS AND DISCUSSION

A rabbit was immunized only twice with highly purified (7) native chicken vinculin and immediately began producing high titer anti-vinculin antibody. The rabbit was bled periodically. In immunoblots of chick embryonic fibroblast proteins with later sera, we noticed that the rabbit had begun to produce a second antibody against talin (230 kDa) (Fig. 1, a and b).

The presence of the second antibody to talin suggests several possible hypotheses. The first is that the vinculin used as antigen was contaminated with talin. This seems unlikely since the second antibody did not appear until seven months after the first (and only) boost. The second is that the secondary antibody recognizes an epitope common to vinculin and talin. In order to test this hypothesis, the anti-vinculin and anti-talin antibodies in the late sera were affinity-purified and used for immunoblotting against vinculin and talin (Fig. 1c). Affinity-purified anti-vinculin antibody binds only to vinculin, and affinity-purified anti-talin antibody binds only to talin. This indicates that the presence of spontaneous anti-talin antibody is not due to cross-reaction with vinculin. The third hypothesis is that the anti-talin antibody is a spontaneously generated anti-idiotypic antibody (16) that reacts with a site on talin that binds vinculin (4–6). This hypothesis assumes that a subpopulation of anti-vinculin polyclonal antibodies in the early sera recognized a talin binding site on vinculin, and later the idiotope of that subpopulation of anti-vinculin antibodies functioned as an immunogen, resulting in an anti-idiotypic antibody that recognizes talin.

In order to test if the spontaneous anti-talin antibody is indeed an anti-idiotypic antibody, we conducted two sets of competition assays (Fig. 2). If the idiotope of the second antibody mimics a talin binding site on vinculin, then vinculin should compete with the spontaneous anti-talin antibody for this vinculin binding site on talin. In addition, the rabbit’s early sera should contain a subpopulation of anti-vinculin antibody to which the anti-idiotypic antibody reacts. Thus, the anti-vinculin antibody from the early sera should prevent the anti-talin antibody from binding to talin. In Fig. 2a, talin

![Fig. 1. Spontaneous generation of an anti-idiotypic antibody](image-url)

![Fig. 2. Competition assays to characterize the spontaneous antibody that recognizes talin](image-url)
was blotted onto nitrocellulose after SDS-gel electrophoresis. Each lane contained the same amount of talin and was cut out for the assay. Each piece of nitrocellulose was incubated with a constant amount of affinity purified second anti-talin antibody and various amounts of different competitors. Antibody binding to talin was detected with IZ5I-protein A. In contrast, other cytoskeletal proteins such as α-actinin and tubulin which are not known to interact with talin in vitro do not compete with the putative anti-idiotypic antibody for binding to talin. In the blot assay, affinity-purified anti-vinculin antibody from the early sera prevented the putative anti-idiotypic antibody from binding to talin, while the preimmune IgG did not (Fig. 2a). This suggests that an anti-vinculin antibody from the early sera contains the epitope that elicited the second antibody. We conclude from these experiments that the secondary antibody which recognizes talin is an anti-idiotypic antibody.

Because the idiotope of the anti-idiotypic antibody (AIA) mimics the conformation of a talin binding site on vinculin, it should recognize this vinculin binding site on talin and can be used to map its location. The vinculin binding site on talin was identified from immunoblots with the AIA on peptide maps of talin. Intact talin was partially digested with V8 protease at a concentration of 10 µg/ml (lanes 2), chymotrypsin A, at 1.2 µg/ml (lanes 3), or trypsin at 0.6 µg/ml (lanes 4) for 50 min at 37 °C. Talin was pretreated with V8 protease for 15 min at 37 °C then digested with chymotrypsin A (lanes 5) or trypsin (lanes 6) for 50 min at 37 °C. Lane M contains standard molecular mass markers of 200, 116.3, 92.5, 66.2, and 45 kDa. The peptides were resolved in 7% SDS-polyacrylamide gels. A, Coomassie Blue-stained gel of peptide maps of talin. B, immunoblots of talin peptide maps. The blots were incubated with affinity-purified spontaneous anti-talin antibody from later sera (lanes 1a, 2a, 3a, 4a, 5a, and 6a). Similar immunoblots were performed with an anti-talin polyclonal antibody from a different rabbit (lanes 1b, 2b, 3b, 4b, 5b, and 6b). The blots were then incubated with 125I-protein A and autoradiographed. The peptides which were sequenced are numbered on the side of each lane for identification (see Fig. 4). Peptides 2, 3, 5, 8, and 9 do not have the epitope for the anti-idiotypic antibody, while peptides 1, 4, 6, and 7 contain the epitope. In peptide 5, the lower band was sequenced. For peptide 8, the doublet was subjected to sequencing. A peptide (114 kDa) just below peptide 2 (120 kDa) in a also interacted with AIA. However, because the sequencing data show too many unmatched amino acids (FXXVPX), this peptide is excluded from consideration. Like peptides 6 and 7, this peptide did not interact with 125I-vinculin. For reference, the amino terminus of this peptide probably resides at residue 1581. C, a blot of a similar peptide map was overlaid with 125I-vinculin. Iodinated vinculin binds to peptides 2, 3, and 5 which do not contain the epitope for anti-idiotypic antibody as well as those peptides (1 and 4) containing the epitope. 125I-Vinculin does not interact with peptides 6 and 7; the positions of these peptides are marked with a small square.

Because one vinculin binding site on talin is not recognized by the AIA and 125I-vinculin did not interact with peptides 6 and 7 which possess the epitope for the AIA, vinculin may have at least two immunologically distinct talin binding sites. The lack of interaction between 125I-vinculin and the AIA binding site could be due to a low affinity interaction. Alternatively, binding of vinculin to the AIA epitope may require a conformational change in vinculin conferred by vinculin binding to its binding site on talin located between residues 483 and 1652. Such an intermolecular interaction which uses two distinct binding domains should generate a very stable protein complex, possibly in a cross-linked structure if each binding domain is occupied by different individuals. It is also possible that talin may possess only one vinculin binding site, (Fig. 3b). Selected peptides were sequenced to map the epitope for AIA. After sequencing, these peptides were placed within the talin sequence (13). The epitope for the AIA, and thus a vinculin binding site, localized to the 196 amino acids spanning residues 1583 to 1848 (Fig. 4).

Similar blots of talin peptides were overlaid with 125I-vinculin (7). Unexpectedly, the binding pattern of vinculin on the peptide map was different from that of the AIA (Fig. 3c). Peptides 1–5 interacted with 125I-vinculin. Among these peptides, only peptides 1 and 4 contain the epitope for the AIA. This indicates that there is another vinculin binding site on talin which maps between residues 483 and 1652. A previous report indicated that talin possesses two vinculin binding sites with different affinities and that vinculin binds talin in a 1:3 molar ratio (6).

Because one vinculin binding site on talin is not recognized by the AIA and 125I-vinculin did not interact with peptides 6 and 7 which possess the epitope for the AIA, vinculin may have at least two immunologically distinct talin binding sites. The lack of interaction between 125I-vinculin and the AIA binding site could be due to a low affinity interaction. Alternatively, binding of vinculin to the AIA epitope may require a conformational change in vinculin conferred by vinculin binding to its binding site on talin located between residues 483 and 1652. Such an intermolecular interaction which uses two distinct binding domains should generate a very stable protein complex, possibly in a cross-linked structure if each binding domain is occupied by different individuals. It is also possible that talin may possess only one vinculin binding site,
FIG. 4. Localization of epitope recognized by the anti-idiotypic antibody. The peptide maps in Fig. 3A were blotted onto polyvinylidene difluoride membrane, and selected peptides were sequenced. The peptides numbered 1–9 correspond to the same numbered bands in Fig. 3. The enzymes used to generate the peptides were V8 protease (V), trypsin (T), chymotrypsin (CT), or a combination of two of these proteases as detailed in the legend of Fig. 3. The sequenced peptides were placed within the mouse talin sequence. Turkey talin was used in this study and nonmatched amino acids between the turkey and mouse talin are indicated by X. The amino-terminal residues are: peptides 1, 2, and 3, NFQVGX; peptides 4 and 5, GHMPXL; peptides 6 and 7, DKAPGQXE; peptide 8, GSFVXY; peptide 9, VDYQTX. Peptides which contain the epitope for anti-idiotypic antibody (AIA binding domain) are shaded, and the region containing the epitope for anti-idiotypic antibody is black. Peptides which interacted with 125I-vinculin are heavily outlined. A second vinculin binding domain identified by 125I-vinculin overlay is indicated as a dotted area.

but it is composed of two interacting regions (residues 483–1652 plus 1653–1848). In this case, although the AIA would only recognize the region of residues 1653–1848, it could be displaced by vinculin binding to the one site.

It will be interesting to determine where the epitope, or talin binding site, identified by the anti-vinculin antibody that served as the idiotope localizes on vinculin. The talin binding site on vinculin has been deduced to residues 167 to 207 by deletion analysis (10, 11) and to the amino-terminal 325 amino acids by 125I-talin overlays (9). The use of idiotypic or anti-idiotypic antibodies to map binding sites may avoid the potential problem of conformational distortion that can occur with deleted proteins.

The information presented in this paper will be useful for the site-directed mutagenesis of talin cDNAs. Such mutant cDNAs can then be used to examine the function of the vinculin and talin interaction in vivo. Furthermore, the anti-idiotypic and idiotype antibodies themselves can be used in microinjection experiments to examine the function of the proteins in vivo.

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