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Tenascin variants: differential binding to fibronectin and distinct distribution in cell cultures and tissues

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In the chicken, three tenascin variants have been characterized that are generated by alternative splicing of 3 of its 11 fibronectin type III repeats. Using monoclonal antibodies that react with common regions versus extra repeats of tenascin, we could distinguish and separate tenascin variants and investigate their interaction with fibronectin using multiple experimental procedures. Interestingly, in all assays used the smallest tenascin variant bound more strongly to fibronectin than the larger ones. These biochemical data were paralleled by the observation that in chick embryo fibroblast cultures only the smallest form of tenascin could be detected in the fibronectin-rich extracellular matrix network laid down by the cells. Furthermore, each tissue present in adult chicken gizzard contained a distinct set of tenascin variants. Those tissues particularly rich in extracellular matrix, such as the tendon, contained the smallest tenascin only. Intermediate-sized tenascin was present in smooth muscle, whereas the largest form was exclusively detectable underneath the epithelial lining of the villi. Thus it appears that cell type-specific forms of tenascin exist that are appropriate for the functional requirements of the respective extracellular matrices.

Introduction

Alternative splicing is found to occur for an increasingly large number of genes resulting in the generation of several distinct protein products from one single gene. In many cases, the consequences of alternative splicing concern the targeting and localization of a protein. Often also the function of a protein is affected; activities are gained, others are lost, or in many cases subtle changes in the function of protein variants can be observed (for review see Smith *et al.*, 1989).

Many extracellular matrix proteins occur as different splicing variants. Often the biological significance is unknown, but in several cases the alternatively spliced regions were postulated to be important for the interaction with other extracellular matrix components as in elastin (Pollock *et al.*, 1990), type IX collagen (Svoboda *et al.*, 1988), or type VI collagen (Chu *et al.*, 1989, 1990). In the case of type VI collagen, experimental evidence was presented for the interaction of its so-called A-domains with collagen type I (Bonaldo *et al.*, 1990). Because some of these A-domains are subject to alternative splicing, it was postulated that the type VI collagen variants could differ by their affinity for type I collagen.

In fibronectin one of the alternatively spliced exons, called IIICS, encodes a cell type-specific cell binding site, whereas no functions for the other two so-called fibronectin type III domains, which are encoded by alternatively spliced exons, are known. No functional differences could so far be detected between fibronectin with or without these extra domains; however, differential expression of these variants in different tissues have been observed (for review see Hynes, 1990).

Tenascin is also one of the extracellular matrix proteins encoded by several splicing variants (for reviews see Erickson and Bourdon, 1989; Chiquet-Ehrismann, 1990). In the chicken three variants have been characterized. These tenascin variants differ by the insertion of one or three additional fibronectin type III repeats into the minimal version of tenascin (Spring *et al.*, 1989). The different types of subunits are disulfide-linked to homo-oligomers (Chiquet *et al.*, 1991). Mouse and human tenascin contain even more of these alternatively spliced extra repeats (Gulcher *et al.*, 1989; Siri *et al.*, 1991; Weller *et*

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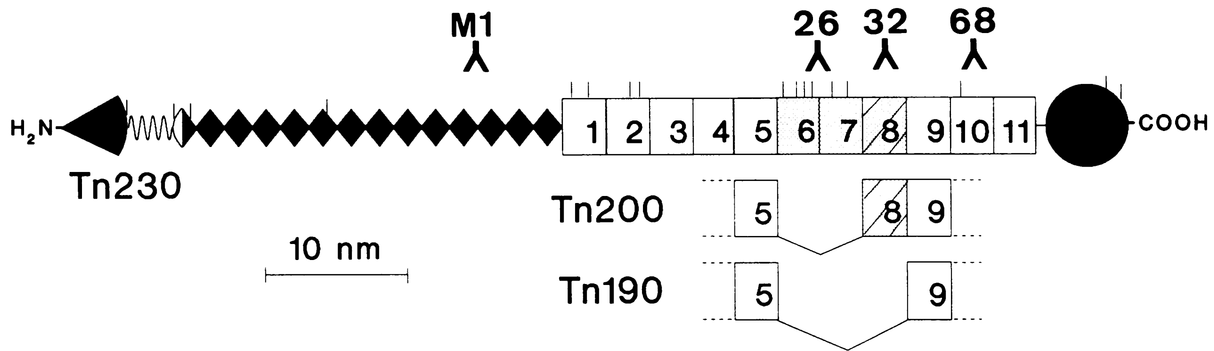

Figure 1. **Localization of the epitopes of the anti-tenascin antibodies.** This model of chicken tenascin was derived from the primary sequence (Spring *et al.*, 1989). It is drawn to scale and includes the three major splicing variants found in chick embryos. The different symbols represent the various structural features building up one tenascin subunit. Sector of a circle, N-terminal 1/6 of central globule; wavy line, heptad repeats; diamonds, EGF-like repeats; boxes, fibronectin type III repeats; shaded boxes, alternatively spliced repeats; C-terminal circle, fibrinogen homology; dashes, potential N-linked glycosylation sites. The epitopes of the monoclonal anti-tenascin antibodies anti-TnM1, anti-Tn32, anti-Tn26, and anti-Tn68 used in this study are indicated above the model.

al., 1991). The expression of tenascin-splicing variants can vary in different tissues, cell types, or cell lines (Chiquet and Fambrough, 1984b; Matsuoka *et al.,* 1990; Kaplony *et al.,* 1991). By in situ hybridization using probes common to all tenascin (cytotactin) variants or specific to the extra domains, respectively, Prieto *et al.* (1990) could demonstrate differential expression of splicing variants in developing chick embryos. In addition, the analysis of extracts from gut or cerebellum revealed shifts in molecular forms of tenascin subunits in the course of development (Aufderheide and Ekblom, 1988; Hoffman *et al.,* 1988; Weller *et al.,* 1991).

Clearly the tissue distribution of tenascin variants changes during development, but whether the different variants serve different functions is not known. The only difference reported so far concerns their susceptibility to proteolytic cleavage. We have shown that the extra repeats contain preferential proteolytic cleavage sites and postulated that the large tenascin variants could be used at places where transient expression of tenascin is required, permitting a faster turnover than for the more stable smaller tenascin variants (Chiquet *et al.,* 1991).

Tenascin has been shown to interact with proteoglycans (Chiquet and Fambrough, 1984b; Hoffman and Edelman, 1987; Salmivirta *et al.,* 1991), but contradictory results have been obtained concerning the interaction with other extracellular matrix proteins. Lightner and Erickson (1990) did not find significant interaction of tenascin neither with several collagen types tested nor with fibronectin. Faissner *et al.* (1990) reported on the binding of tenascin to various collagens but not to fibronectin, and finally Chiquet-Ehrismann *et al.* (1988) and Hoffman *et al.* (1988) presented evidence for the interaction of tenascin with fibronectin.

In this paper we reinvestigated the binding between tenascin and fibronectin using several experimental procedures. Interestingly, we could detect a differential binding capacity of the tenascin variants to fibronectin. This finding may in part explain the contradictory results obtained previously in different labs. Furthermore, our biochemical binding data are corroborated by the differential incorporation of tenascin variants into the fibronectin-rich matrix of fibroblast cultures, implying that the binding data reflect a property of tenascin of biological relevance. Furthermore, we demonstrate differential distribution of tenascin variants in the adult chick gizzard as an example for an organ built up from several different tissues, each containing a distinct set of tenascin variants.

Results

Differential distribution of tenascin variants in the chick gizzard

The splicing variants of chick tenascin differ in the number of fibronectin type III repeats included in their subunits (Spring *et al.,* 1989). The smallest tenascin subunit contains eight such repeats and has a M_r of 190 000 (Tn190). The intermediate-sized variant contains one additional repeat and has a M_r of 200 000 (Tn200). The largest tenascin subunit contains two more such extra repeats resulting in a M_r of 230 000 (Tn230). In this study we used four different monoclonal antibodies against tenascin to distinguish between these

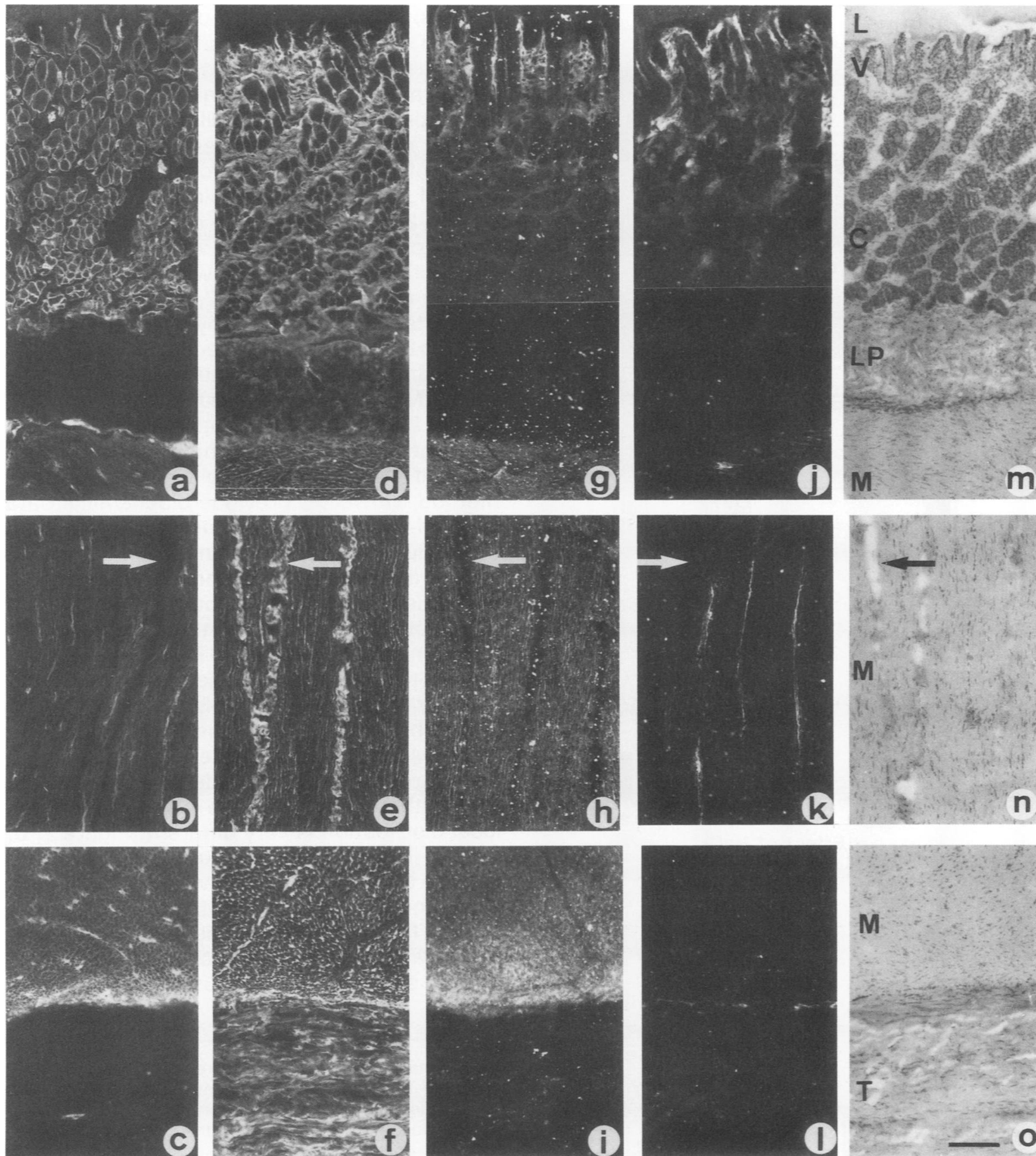

Figure 2. **Differential distribution of tenascin variants in adult chick gizzard.** Sequential cryosections of adult chick gizzard were stained by anti-laminin (a–c), anti-TnM1 (d–f), anti-Tn32 (g–i), anti-Tn26 (j–l), or hematoxilin and eosin (m–o). The top row shows the lumen of the gizzard (L), the villi (V), the crypts (C), the lamina propria (LP) between the epithelium, and the smooth muscle (M). The middle row shows smooth muscle cut longitudinally. The arrows point to the elastic tissue bands. The bottom row shows smooth muscle in cross section and the tendon (T). Bar, 100 μm.

variants. Anti-TnM1 and anti-Tn68 recognize all tenascin variants and anti-Tn32 recognizes the extra repeat of the intermediate subunit and thus reacts with the two larger subunit types, whereas anti-Tn26 only recognizes the largest tenascin variant. A schematic representation of the locations of the epitopes of these monoclonal antibodies is shown in Figure 1.

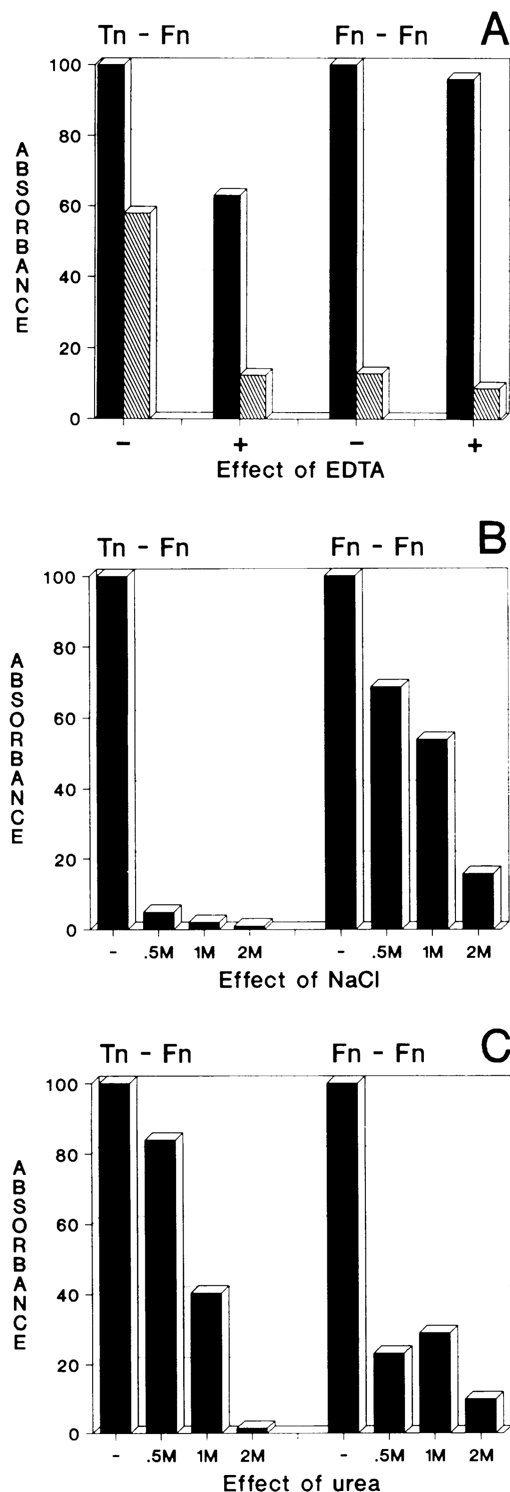

Figure 3. **Effect of EDTA, NaCl, and urea on the binding of tenascin and fibronectin to fibronectin.** (A) Wells coated with bovine fibronectin (■) or plain wells (□) were blocked with skim milk solution before incubating them with 5 μg/ml of either tenascin (left side) or chick fibronectin (right side) in the presence (+) or absence (−) of 5 mM EDTA. The bound tenascin and fibronectin were analyzed by an ELISA using anti-TnM1 for tenascin and anti-TnM6 for fibronectin. The addition of EDTA did not influence the specific binding (filled minus hatched bars), but in the case of tenascin reduced the unspecific binding to the control well. (B) and (C) Wells were coated with bovine fibronectin and incubated with either tenascin (left side) or fibronectin (right side) with increasing concentrations of either NaCl or urea as indicated. Bound tenascin and fibronectin were quantitated as in (A) except that the values obtained on skim milk-blocked control wells were subtracted to obtain the specific binding only. The interaction of tenascin with fibronectin was highly sensitive to the addition of NaCl and less sensitive to urea, whereas the opposite was true for the fibronectin-fibronectin interaction. Because in these experiments two different antibodies, resulting in different intensities of the color reactions, were used, data are shown in percent of the maximal response obtained for each antibody, which was 0.65 for anti-TnM1 and 0.15 for anti-FnM6, respectively.

When sections of adult chick gizzard were labeled with anti-laminin antiserum and different anti-tenascin antibodies, each antibody revealed a very different staining pattern (Figure 2). Anti-laminin labeled all basement membranes, namely around the epithelium lining the lumen of the gizzard, around the blood vessels, as well as around the smooth muscle cells (Figure 2, a–c). The tendon of the gizzard (Figure 2, c, f, i, l, and o) as well as the elastic connective tissue bands within the smooth muscle (arrows in Figure 2, b, e, h, k, and n) were stained exclusively by anti-TnM1. Thus only Tn190 can be detected in these two tissues. Smooth muscle tissue was stained by anti-TnM1 and anti-Tn32 but not by anti-Tn26 (Figure 2, e, h, and k). Smooth muscle therefore contains Tn200, whereas the concurrent presence of Tn190 cannot be excluded by this method. However, previous biochemical data showed that the major tenascin band isolated from adult gizzard smooth muscle migrates at 200 kd and contains the epitope of anti-Tn32, and thus the gizzard smooth muscle may indeed contain mainly this intermediate form of tenascin (Chiquet *et al.,* 1991). The smooth muscle tissue was not stained by anti-Tn26 except for some blood vessels, and thus Tn230 cannot be found in gizzard smooth muscle. The only tissue stained by all tenascin antibodies was the stromal tissue underlying the epithelium, although even in this compartment the local distribution is very different (Figure 2, d, g, and j). Anti-TnM1 stained throughout the stroma of the crypts and villi, whereas anti-Tn32 and anti-Tn26 only stained the area of the villi. None of the antibodies stained the area of the lamina propria between the epithelium and the smooth muscle. Thus in the adult chick

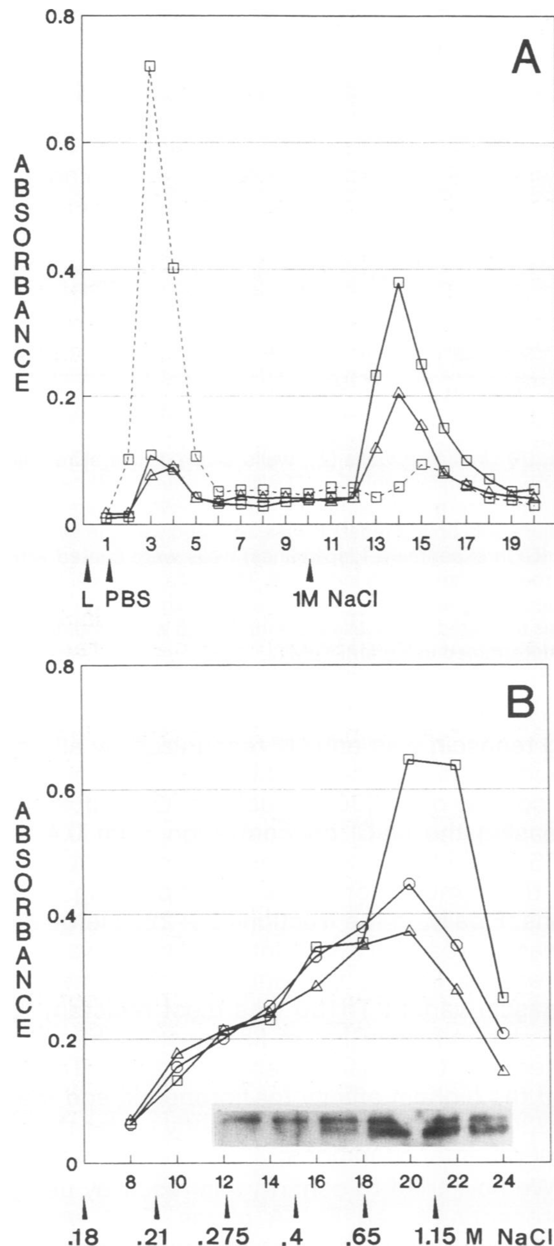

Figure 4. **Differential binding of tenascin variants to a fibronectin affinity column.** (A) Conditioned medium was applied to either a fibronectin-Sepharose column or a control column containing glycine-Sepharose (L). The columns were washed with PBS and eluted using 1 M NaCl added to the PBS. All fractions were collected and assayed for the presence of tenascin using anti-TnM1 (□) and anti-Tn26 (△). The elution profile of the control column (stipled) shows all tenascin in the flow through fractions, whereas the fibronectin column (solid lines) quantitatively retained all tenascin variants. (B) Conditioned medium was applied to the fibronectin column. After washing the column with PBS, bound proteins were eluted with a NaCl step gradient as indicated. The fractions were tested for the presence of tenascin using anti-TnM1 (□), anti-Tn32 (○), and anti-Tn26 (△), respectively. Every second fraction was also analyzed on a gel and the tenascin subunit pattern determined by immunoblotting using anti-Tn68. The resulting immunoblot is positioned according to the fractions applied to the gel. The early fractions contain Tn230 exclusively, whereas the major peak of Tn190 recognized by anti-TnM1 is in fractions 19–22.

gizzard Tn230 is exclusively detected in the stroma beneath the villous epithelium. Tendon, elastic connective tissue, and the stroma surrounding the crypts contain Tn190 only, whereas in smooth muscle Tn200 appears to be prominent.

Specificity of tenascin-binding to fibronectin

We have previously demonstrated that chick fibroblast tenascin was binding to fibronectin in solid phase binding assays (Chiquet-Ehrismann *et al.,* 1988). We now decided to extend these studies by characterizing the binding conditions and by investigating whether the binding to fibronectin was different for the different tenascin variants. In a first series of experiments we investigated the binding of purified tenascin to fibronectin-coated wells and compared it with fibronectin–fibronectin binding (Figure 3). All binding assays were performed in physiological salt solution, i.e., at 150 mM NaCl. Wells coated with bovine fibronectin were incubated with 5 μg/ml of either tenascin or chicken fibronectin. Bound tenascin was detected with anti-TnM1 and bound fibronectin with the chick-specific monoclonal antibody anti-FnM6. Uncoated wells, i.e., wells blocked with skim milk solution, were used as controls. Figure 3A shows that in the case of adding tenascin quite a high background was obtained on uncoated wells. However, this unspecific binding was largely abolished in the presence of 5 mM EDTA without reducing the specific binding (value obtained on fibronectin-coated minus value from control wells) of either tenascin to fibronectin or fibronectin to fibronectin (Figure 3A). Thus divalent cations are not required for these protein–protein interactions. The binding of tenascin to fibronectin was, however, inhibited by the addition of NaCl, more efficiently than binding of fibronectin to itself. The addition of 0.5 M NaCl almost completely abolished the interaction of tenascin with fibronectin, whereas 2 M NaCl was required to inhibit the fibronectin–fibronectin interaction to a similar extent (Figure 3B). In contrast, the fibronectin–fibronectin binding was more sensitive to urea than the interaction of tenascin with fibronectin (Figure 3C).

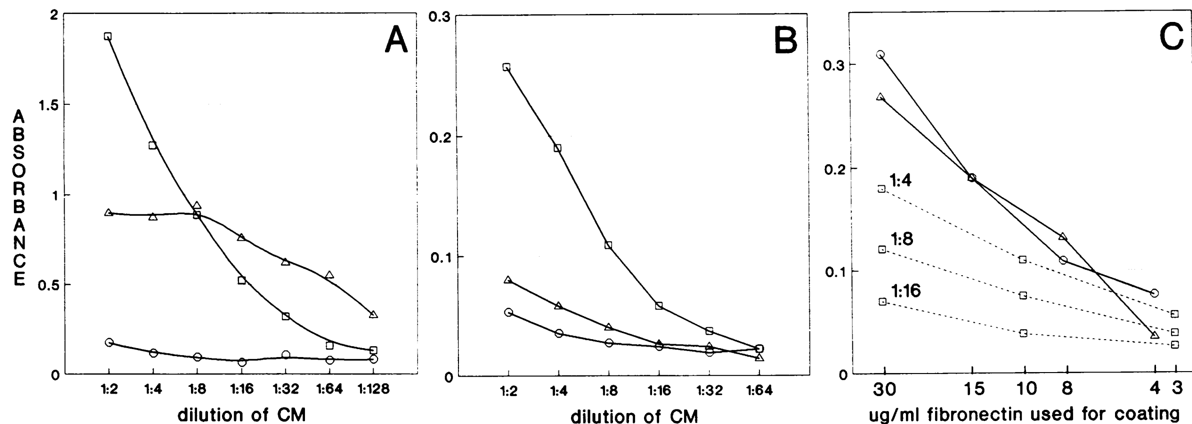

Figure 5. **Binding of tenascin to fibronectin-coated ELISA plates.** (A) Uncoated wells (△), wells blocked with skim milk solution (○), or wells coated with fibronectin and subsequently blocked with skim milk solution (□) were incubated with twofold dilutions of conditioned medium, and the tenascin bound to the wells was determined using anti-TnM1. (B) Wells coated with fibronectin were incubated with twofold dilutions of conditioned medium, and bound tenascin was detected with the variant-specific antibodies anti-TnM1 (□), anti-Tn32 (△), and anti-Tn26 (○). (C) In experiment I (solid lines) wells were coated with either chick serum fibronectin (○) or chick cellular fibronectin (△) at the concentrations indicated. The wells were incubated with conditioned medium diluted 1:2. Bound tenascin was quantitated using anti-TnM1. In the second experiment (stipled lines), wells were coated with bovine fibronectin at the concentrations indicated. The wells were incubated with conditioned medium diluted 1:4, 1:8, or 1:16, as indicated. Bound tenascin was determined using anti-TnM1.

Differential binding of tenascin variants to fibronectin

Because tenascin exists as homo-oligomers of only one type of subunits, it was possible to test whether different tenascin variants show differential affinity for fibronectin. We first analyzed the binding of tenascin present in fibroblast-conditioned medium to a fibronectin affinity column. A 200-μl aliquot of conditioned medium was applied to a fibronectin-coupled Sepharose 4B column with a bed volume of 0.5 ml. The column was washed with phosphate-buffered saline (PBS) and subsequently eluted with a step gradient of increasing NaCl concentration. During the whole experiment, 200-μl fractions were collected and equal aliquots of each fraction were analyzed for the presence of tenascin variants. This was done by an enzyme-linked immunosorbent assay (ELISA) procedure using the variant-specific anti-tenascin antibodies as well as by immunoblots of the fractions with anti-Tn68, an anti-tenascin antibody that reacts with all tenascin variants (Figure 4). In a first control experiment we determined that the binding of tenascin to the affinity column was dependent on the presence of coupled fibronectin and that no tenascin was retained on a control column (Figure 4A). Analysis of the fractions eluted from the fibronectin column showed that after increasing the NaCl concentration from 0.15 to 0.21 M, tenascin started to be eluted (Figure 4B). In the ELISA, this early-eluting tenascin was equally recognized by all antibodies, and by immunoblotting it was shown to consist of Tn230 exclusively. Only after increasing the NaCl concentration from 0.4 to 0.65 M did the lower tenascin variants appear on the immunoblot of the corresponding fractions. Finally, in the fractions 20–22, a large increase in reactivity with anti-TnM1 only was apparent by ELISA, showing that the smallest tenascin variant Tn190 was most resistent to the elution by NaCl from fibronectin. This indicated that the smallest tenascin variant Tn190 had the highest affinity for fibronectin and that Tn230 was binding less efficiently under these experimental conditions.

We now tried to confirm this result by using fibronectin-coated ELISA plates. First we investigated the binding of tenascin present in the conditioned medium of chick embryo fibroblasts. Fibronectin-coated as well as uncoated wells were blocked with 1% casein solution before incubation with twofold dilutions of the conditioned medium. Tenascin was bound only by plates coated with fibronectin and not by the control wells, as determined by the anti-TnM1 reactivity measured in the wells. Direct coating of plain wells with the conditioned medium resulted in a binding curve reaching a plateau at a dilution of the conditioned medium of 1:8, whereas on fibronectin-coated wells the binding increased up to a dilution of 1:2, reaching much higher values than on the plain wells (Figure 5A).

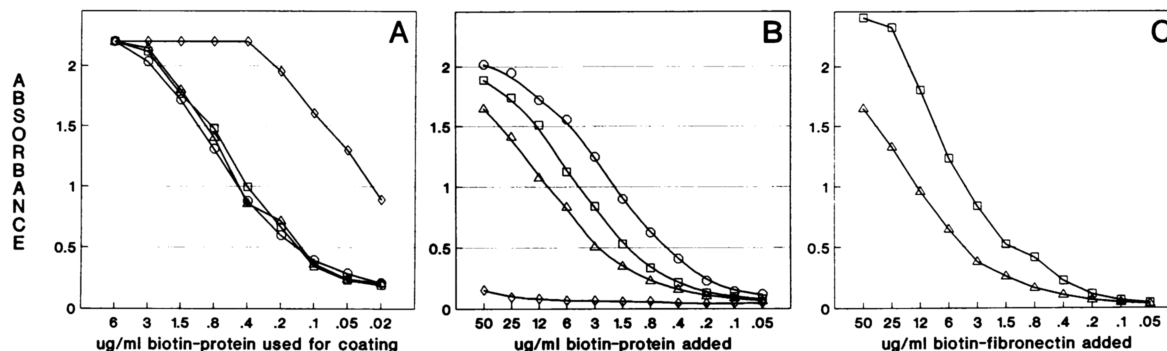

Figure 6. **Binding assays using biotinylated proteins.** (A) Biotinylated BSA (◇), Tn190 (□), Tn230 (△), or fibronectin (○) were coated on ELISA plates at the concentrations indicated. The amount of biotin contained in the coated proteins was quantitated using peroxidase-labeled streptavidin. (B) Wells were coated with fibronectin and incubated with biotinylated BSA (◇), Tn230 (△), Tn190 (□), or fibronectin (○) at the concentrations indicated. Bound biotinylated proteins were detected as in (A). (C) Wells were coated with Tn230 (△) or Tn190 (□) and incubated with biotinylated fibronectin at the concentrations indicated. Bound fibronectin was detected as in (A). This figure shows the results from one representative experiment. Repeating this experiment with different batches of biotinylated proteins reproducibly gave twofold differences in the binding of tenascin variants to fibronectin.

This shows that the binding of tenascin was dependent on the coating with fibronectin and does not merely reflect its high binding to plastic as was claimed previously by Lightner and Erickson (1990). Using conditioned medium instead of purified tenascin, in contrast to the first set of experiments, had the advantage that no binding of tenascin to skim milk-blocked control wells was detectable (Figure 5A). The conditioned medium of chick embryo fibroblasts contains about equal quantities of each type of tenascin variant. When plain plates were coated with dilutions of conditioned medium and the bound tenascin was detected with the variant-specific antibodies, all antibodies gave equal binding curves (Matsuoka *et al.,* 1990). In contrast, when fibronectin-coated wells were used, only anti-TnM1 but not anti-Tn32 and anti-Tn26 revealed a substantial binding of tenascin, again demonstrating that Tn190 was binding significantly better to fibronectin than Tn200 and Tn230 (Figure 5B). The same results were obtained when using purified tenascin instead of conditioned medium, except that about a 5- to 10-fold higher concentration of the purified tenascin had to be used than is contained in the conditioned medium (not shown).

Because there appears to be a significant difference between tenascin variants in their binding to fibronectin, we wondered whether this interaction also depended on the type of fibronectin variant used. We therefore purified fibronectin from either chick serum or the cellular fibronectin from the cell surfaces of chick embryo fibroblasts and used both types of fibronectins to coat wells. However, we could not detect any difference in the binding of tenascin to the different fibronectin preparations (Figure 5C), but the amount of tenascin bound to fibronectin-coated wells was dependent on the concentration of fibronectin used to coat the wells (Figure 5C).

To show that the differential fibronectin binding by tenascin variants was not due to an artefact caused by using monoclonal antibodies to detect the bound tenascin, we purified and separated Tn230 and Tn190 as described (Chiquet *et al.,* 1991). Each of these preparations as well as fibronectin and bovine serum albumin (BSA) as control were biotinylated. Streptavidin-peroxidase was used to detect bound biotinylated protein. In a control experiment, wells were directly coated with serial dilutions of all four proteins. Fibronectin and the two tenascin variants yielded overlapping response curves, indicating that their amount of biotinylation and their binding to plastic were indistinguishable and that the obtained signal was proportional to the amount of bound biotinylated protein (Figure 6A). In contrast, when wells were first coated with unlabeled fibronectin and then incubated with the same dilutions of biotinylated tenascin variants or fibronectin itself or BSA, a shift of the curves was observed (Figure 6B). The binding of fibronectin was roughly twofold higher than the binding of Tn190, and Tn190 bound again about twofold better than Tn230 because to obtain equal absorbance values of bound proteins twofold higher concentrations of Tn190 had to be used than of fibronectin and again twofold higher concentrations of Tn230 than of Tn190 (Figure 6B). Biotin-BSA gave a

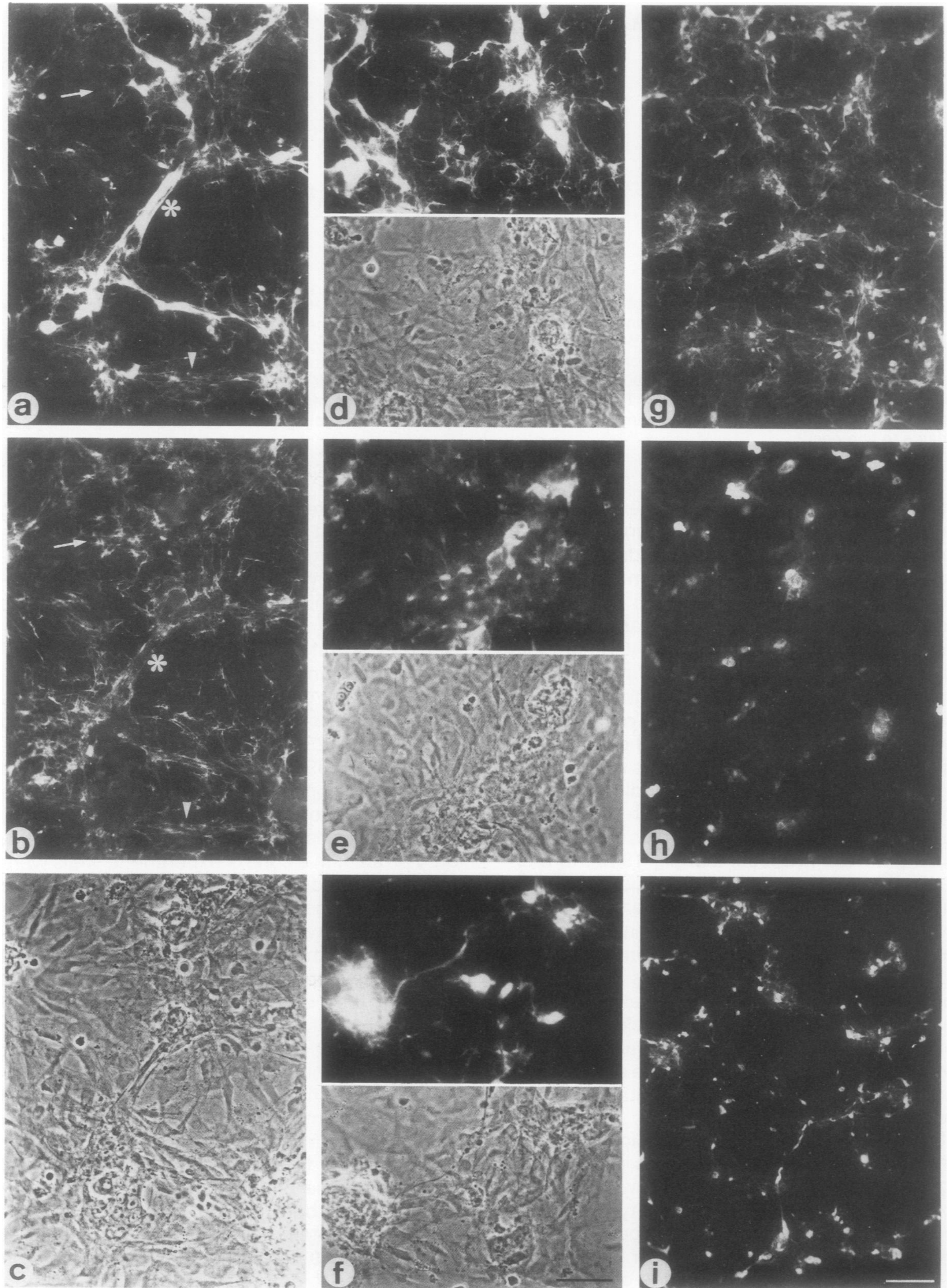

Figure 7. **Distribution of tenascin variants in chick embryo fibroblast cultures.** Immunofluorescence of chick embryo fibroblast cultures 1 d after plating. The cells were fixed and permeabilized before staining. Double-labeling using anti-TnM1 (a) and anti-fibronectin antiserum (b) of the cells shown in phase contrast (c) reveals a partial codistribution. The arrowhead marks

higher response than the other proteins on plain plastic wells but did not bind to fibronectin-coated wells. In a reciprocal experiment, wells were coated with either unlabeled Tn190 or Tn230 and then incubated with biotinylated fibronectin. In this case, the binding curve on Tn190 was steeper and reached a plateau at 25 μg/ml of fibronectin, whereas on Tn230-coated wells even 50 μg/ml of fibronectin were below the plateau value (Figure 6C). Thus, also in this last experimental setting, Tn190 showed stronger interaction with fibronectin than Tn230, and hence the previous results were confirmed.

Differential incorporation of tenascin variants in the extracellular matrix of chick embryo fibroblasts

Could the differential affinity of tenascin variants for fibronectin be correlated with their incorporation into the fibronectin-rich matrix of chick embryo fibroblasts? To answer this question, fibroblast cultures were stained with the variant-specific anti-tenascin antibodies as well as with anti-fibronectin. Double-labeling immunofluorescence using anti-fibronectin and anti-TnM1 revealed a partial codistribution of tenascin with fibronectin fibrils, but not all fibronectin fibers contained tenascin (Figure 7, a–c). When parallel cultures were stained with either anti-TnM1, anti-Tn32, or anti-Tn26, only anti-TnM1 revealed a matrix-associated fibrillar staining, whereas both other antibodies stained some cells very brightly, however only intracellularly (Figure 7, d–i). From these data it appears that only Tn190 is incorporated into the matrix of cultured chick embryo fibroblasts, whereas Tn200 and Tn230 are secreted and accumulate in the conditioned medium. This observation provides evidence that tenascin variants interact differently, not only with isolated fibronectin, but also with the fibronectin-rich extracellular matrix assembled by fibroblasts.

Discussion

In this study we analyzed the binding of tenascin variants, isolated from chick embryo fibroblast cultures, to fibronectin and investigated their distribution in cell cultures and tissues. One of the most interesting results from our studies was the finding that different tenascin splicing variants showed differential affinity to fibronectin. The shortest tenascin variant bound better to fibronectin than the larger ones. This may at first sight seem surprising because any binding site for fibronectin that is present on the minimal tenascin variant should also be present on the larger ones. However, several possibilities exist to explain our findings. The binding site(s) for fibronectin could encompass sequences present in the fibronectin type III repeats 5 and 9, which are contiguous in the smallest tenascin variants, but not in the others. If more than one fibronectin-binding site existed, the spacing between the sites could be important, which obviously could differ between the variants. Furthermore, the carbohydrate side chains, particularly enriched in the extra repeats of the larger tenascin variants (Spring *et al.,* 1989), could be inhibitory for the tenascin-fibronectin interaction. Whatever the reason for the diminished fibronectin-binding, this fact could explain the unsuccessful binding studies by Lightner and Erickson (1990). Their tenascin was isolated from a human glioma cell line that, judging from its size, is likely to be one of the larger tenascin variants with extra repeats and may indeed be a form of tenascin not binding to fibronectin.

Our binding studies have been performed using purified fibronectin and either purified tenascin or conditioned medium. The results were always qualitatively identical. However, when purified tenascin was used for the binding to fibronectin-coated wells, a 5- to 10-fold higher concentration of tenascin was necessary than is contained in conditioned medium to get significant binding. Furthermore, although tenascin from conditioned medium bound quantitatively to a fibronectin affinity column, only about 10% of purified tenascin was retained (not shown). The reason for this difference is not clear. Either the conditioned medium contains additional components that support the interaction or the purification procedure partly destroys the binding function of tenascin.

The presumption that the small tenascin variant is most efficiently bound by fibronectin is corroborated by the fact that in chick embryo fibroblast cultures we could easily detect the small tenascin variant within the extracellular

an area of codistribution, the arrow a region positive for fibronectin but not for tenascin, and the star is placed adjacent to cells very brightly stained with anti-TnM1 and much less with anti-fibronectin. Parallel cultures were stained with anti-TnM1 (d), anti-Tn32 (e), and anti-Tn26 (f) and also photographed at lower magnification for anti-TnM1 (g), anti-Tn32 (h), and anti-Tn26 (i), showing that only anti-TnM1 stains the extracellular matrix and anti-Tn32 and anti-Tn26 show bright intracellular staining of some cells only. Bar (a–f), 50 μm; bar (g–i), 100 μm.

matrix, but the larger forms mainly intracellularly and in the conditioned medium. Furthermore, it is intriguing to note that in the chicken gizzard those tissues particularly rich in extracellular matrix, namely the tendon and the dense elastic connective tissue bands within the smooth muscle, were the ones apparently containing the smallest tenascin variant exclusively. We speculate that the smallest tenascin variant could have a typical structural function within these extracellular matrices. Also the previously reported higher stability of this variant against proteolysis would be favorable for such a function (Chiquet *et al.*, 1991).

The differential binding to fibronectin may be a physiologically important difference between tenascin variants. It has been shown by in situ hybridization that in early lung development the epithelium at the tips of the growing bronchi is the source of the tenascin transcripts, but the protein is found quite distantly from the site of its synthesis (Koch *et al.*, 1991). Interestingly, Prieto *et al.* (1990) have found that the embryonic lung epithelium contains tenascin mRNA, including the extra repeats, and thus should secrete the large tenascin variants. It can be speculated that in this situation of the developing lung, tenascin is able to diffuse away from its site of synthesis because it is not bound efficiently by fibronectin in the extracellular matrix. A similar observation of initial epithelial synthesis of tenascin followed by subsequent mesenchymal tenascin production has also been made in the case of feather morphogenesis (Tucker, 1991).

The segregation of tenascin variants within the different tissue compartments of the gizzard develops gradually. In a previous study we had analyzed the distribution of tenascin variants in embryonic gizzard (Matsuoka *et al.*, 1990). In 7-d embryos all tenascin antibodies gave an identical staining pattern. They all labeled the developing smooth muscle tissue. In the gizzard of 11-d embryos tendon has formed. The tendon tissue was only stained by anti-TnM1 as in the adult gizzard. The smooth muscle tissue was still stained by both anti-Tn32 and anti-Tn26 and no staining below the epithelium was visible yet. In 19-d embryos, we still found Tn230 present in the smooth muscle (Matsuoka *et al.*, 1990). Thus embryonic gizzard smooth muscle contains Tn230, which is lost late during development. This variant is not detectable anymore in adult gizzard smooth muscle but is instead expressed underneath the differentiating epithelium. These shifts in molecular forms of tenascin variants during gizzard development are reminiscent of the situation reported for gut development in the mouse (Aufderheide and Ekblom, 1988). It is possible that the late appearance of the largest form of tenascin in the gut of newborn mice could correlate with the final differentiation of the epithelial cells of the crypts where tenascin has been postulated to promote cell shedding (Probstmeier *et al.*, 1990).

There was only one location in the adult chicken gizzard where the largest tenascin variant could be detected, namely the mesenchyme underneath the villous epithelium. Because in the embryonic lung the epithelium has been shown to produce tenascin, it is tempting to speculate that also in the gizzard the epithelium could be a cellular source of tenascin. Could it be that the occurrence of the large tenascin variant is an indication for epithelial synthesis? Clearly more data are necessary to decide these questions, but the very distinctive distribution of tenascin variants in different tissues may indicate that cell type-specific forms of tenascin could exist, appropriate for the requirements of the respective extracellular matrices.

Materials and methods

Antibodies, immunofluorescence, chick embryo fibroblast cultures, and metabolic labeling

The monoclonal anti-tenascin antibodies anti-TnM1 (Chiquet and Fambrough, 1984a), anti-Tn68 (Chiquet-Ehrismann *et al.*, 1988), anti-Tn26 and anti-Tn32 (Spring *et al.*, 1989), the monoclonal anti-fibronectin anti-FnM6 (Pearson *et al.*, 1988), and the anti-fibronectin antiserum (Ehrismann *et al.*, 1981) have been described previously. The locations of the epitopes of the monoclonal anti-tenascin antibodies are shown in the schematic model in Figure 1. Immunofluorescence of cell cultures and cryosections was performed as described in Mackie *et al.* (1987).

Isolation of tenascin and fibronectin and their variants

Tenascin and tenascin variants were isolated using affinity chromatography to different immobilized monoclonal antibodies as described by Chiquet *et al.* (1991). Chicken and bovine plasma fibronectin were purified using affinity chromatography to gelatin-agarose. Cellular fibronectin was isolated from 1 M urea extracts of chick embryo fibroblast cultures as described by Yamada *et al.* (1975). Tenascin contaminating this fibronectin preparation was removed by passing the dialysed extract over an anti-TnM1 column before using the cellular fibronectin for binding studies.

Affinity chromatography to fibronectin-Sepharose

Bovine fibronectin was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. A fibronectin column of 0.5 ml and a control column without cross-linked fibronectin but

blocked with glycine were used for the experiments. Two hundred microliters of conditioned medium from nearly confluent chick embryo fibroblast cultures were applied to the columns. The columns were washed and eluted using aliquots of 200 μl of PBS followed by PBS containing increased concentrations of NaCl as indicated. During the course of the entire column runs, fractions of 200 μl were collected. From the column run using metabolically labeled medium, equal aliquots were analyzed on a 6% sodium dodecyl sulfate (SDS)-polyacrylamide gel and fluorographed. Aliquots of the unlabeled medium were analyzed for the presence of tenascin variants by ELISA. Aliquots of some fractions were used for immunoblotting with anti-Tn68 of the samples separated on a 6% SDS-polyacrylamide gel (Pearson *et al.*, 1988). For the ELISA, 50-μl aliquots of each fraction were used to coat the wells of the cluster plates (Falcon 3072, Falcon, Lincoln Park, NJ). Bound tenascin was detected using the variant-specific anti-tenascin antibodies as described previously (Pearson *et al.*, 1988).

Solid-phase binding assays using antibodies

The cluster plates (Falcon 3072) were coated overnight at 4°C with a solution of 50 μg/ml of fibronectin, unless indicated otherwise. All wells were washed and blocked for 1 h at 37°C with PBS containing 1% skim milk powder. All further washes and incubations were done with the reagents diluted in PBS containing 1% skim milk. Precoated wells were incubated with tenascin, fibronectin, conditioned medium, or blocking solution for 1 h at 37°C. After washing the wells three times, bound proteins were detected using the antibodies indicated in an ELISA performed as described previously (Chiquet-Ehrismann *et al.*, 1988). In most experiments raw data are shown except for some indicated cases where background values obtained on uncoated, blocked wells were subtracted.

Solid-phase binding assays using biotinylated proteins

Fibronectin, tenascin variants, and BSA were biotinylated according to Gretch *et al.* (1987). Briefly, proteins were dialyzed against 100 mM $NaHCO_3$, pH 8.2, and biotin amidocaproate-succinimide ester (Sigma, St. Louis, MO) dissolved at 1 mg/ml in dimethylsulfoxide was added (300 nmol/mg protein). After incubation for 1 h at room temperature, samples were dialyzed for 72–96 h in the cold against PBS with daily changes. Solid-phase binding assays with the biotinylated proteins were done essentially as described above for ELISA, except that streptavidin-peroxidase (5 μg/ml in PBS containing 5 mg/ml BSA) was used to detect bound protein.

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