The small splice variant of tenascin-C (TN) has eight fibronectin type III (FN3) domains. The major large splice variant has three (in chicken) or seven (in human) additional FN3 domains inserted between domains five and six. Chiquet-Ehrismann et al. (Chiquet-Ehrismann, R., Matsuoka, Y., Hofer, U., Spring, J., Bernasconi, C., and Chiquet, M. (1991, Cell Regul. 2, 927-938) demonstrated that the small variant bound preferentially to fibronectin in enzyme-linked immunosorbent assay, and only the small variant was incorporated into the matrix by cultures of chicken fibroblasts. Here we have studied human TN, and confirmed that the small variant binds preferentially to purified fibronectin and to fibronectin-containing extracellular matrix. Thus this differential binding appears to be conserved across vertebrate species. Using bacterial expression proteins, we mapped the major binding site to the third FN3 domain of TN. Consistent with this mapping, a monoclonal antibody against an epitope in this domain did not stain TN segments bound to cell culture matrix fibrils. The enhanced binding of the small TN variant suggests the existence of another, weak binding site probably in FN3 domains 6-8, which is only positioned to bind fibronectin in the small splice variant. This binding of domains 6-8 may involve a third molecule present in matrix fibrils, as the enhanced binding of small TN was much more prominent to matrix fibrils than to purified fibronectin.

Tenascin-C (TN) is a large extracellular matrix (ECM) protein localized in many embryonic and some adult tissues (reviewed in Refs. 1 and 2). The distribution of TN is determined primarily by the differential expression of the cells that secrete it, but probably also involves binding to specific ECM molecules, including collagens, proteoglycans, and fibronectin. Binding of TN to collagens and proteoglycans appears to be important in several functions, but the present study will focus on the interaction of TN with fibronectin (FN). Several studies have examined the binding of TN to FN. Chiquet-Ehrismann et al. (3, 4) demonstrated binding of soluble TN, in the range of 5-50 μg/ml, to FN-coated plastic. Faisstner et al. (5) and Lightner and Erickson (6) reported no binding in a similar ELISA assay, but their maximum concentrations of soluble TN were less than 2 μg/ml. Because ELISA assays have one component partially denatured on plastic, and the binding is usually limited to a very small fraction of the soluble component, it is important to have independent assays of binding. Lightner and Erickson (6) developed a sedimentation assay that demonstrated binding of TN to FN with both molecules native and soluble. The interaction was obvious at 50 μg/ml FN and stronger at 200 μg/ml, indicating a dissociation constant in the range of 0.2 μM relative to the FN dimer.

Complementing the biochemical demonstration of TN binding to FN, co-distribution of TN with FN fibrils has been demonstrated clearly both in cell cultures and embryos. Chiquet and Fambrough (7) demonstrated substantial overlap of TN with FN immunostaining in primary fibroblast culture. Riou et al. (8) provided convincing evidence that TN was distributed together with FN in the dorsal region of the amphibian embryo. Subsequently, they showed that when TN was injected into the blastocoel cavity of living embryos at the late blastula stage, the injected TN bound to fine FN-containing fibrils assembled at the midblastula stage as well as to the complex FN-rich ECM observed at late gastrula stage (9). Therefore, it is evident that TN can bind to FN fibrils assembled in cell culture and embryonic tissues.

Vertebrate tenascins have several splice variants, in which a number of FN3 domains, indicated by letters A-D, are inserted between FN3 domains 5 and 6 (Fig. 1). The number of alternative splice domains varies according to the species. In chicken TN the most common splice variants have 0, 1, or 3 added domains, while in human they have 0, 1, or 7 (1). We will refer to the form with 0 added domains as small TN, and the forms with 3 or 7 added domains as large TN. It is reasonable to expect that the alternative splice domains might bind to unique ligands, and indeed we have recently demonstrated that the human large TN binds to a cell-surface receptor, annexin II (10), through its alternative splice segment, TNfnA-D. More surprising are reports that small TN binds to some ligands more avidly than large TN. Zisch et al. (11) observed that only small TN bound to the cell surface molecule F11/contactin.

We decided to pursue this interesting finding to determine first if it held for species other than chicken, and to begin mapping the interaction sites. We employed the recombinant human TN proteins HxβL and HxβS, which correspond to the large and small splice variants of human TN. These are produced by transfected BHK cells and are fully assembled into native hexabrachions (12). In addition we used bacterial expression proteins corresponding to defined small segments of FN3 domains to map the binding sites in TN. Binding measured in solution, in ELISA, and in cell culture gave consistent demonstration of the preferential binding of the small splice variant, and mapping of the primary binding site.

*This work was supported by National Institutes of Health Grant R37 CA47056 (to H. P. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Cell Biology, Rm. 365, Sands Bldg., Duke University Medical Center, Research Dr., Durham, NC 27710. Tel.: 919-684-6385; Fax: 919-684-6387; E-mail: Harold_Erickson@cellbio.duke.edu.

1 The abbreviations used are: TN, tenascin-C; Ab, antibody; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; FN, fibronectin (FN3, fibronectin type-III (domains)); PBS, phosphate-buffered saline; BHK, baby hamster kidney; EGF, epidermal growth factor.

†From the Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710 and the ‡Laboratory of Cell Biology, Istituto Nazionale per la Ricerca sul Cancro, Viale Benedetto XV, 10, 16132 Genoa, Italy.

‡Chung Y. Chung, Luciano Zardi, and Harold P. Erickson

From the Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710 and the Laboratory of Cell Biology, Istituto Nazionale per la Ricerca sul Cancro, Viale Benedetto XV, 10, 16132 Genoa, Italy.

Binding of Tenascin-C to Soluble Fibronectin and Matrix Fibrils* (Received for publication, July 5, 1995, and in revised form, August 23, 1995)
Tensacin Binding to Fibronectin

Material Methods

Cells and Cell Cultures— A human glioma cell line U-251MG (clone 3, obtained from Dr. Darell Bigner, Duke University), and the BHK cells transfected with the small and large splice variants of TN (12) were grown in Dulbecco's modified Eagle's medium, high glucose, supplemented with 10% heat inactivated fetal calf serum.

Proteins and Abs—Splice variants of human TN were produced in transfected BHK cells (12). Native TN was purified from culture supernatant of the BHK cells or U-251MG human glioma cells by gel filtration and Mono Q ion exchange chromatography (13). FN was purified from human plasma or horse serum by gelatin-agarose affinity chromatography (14). Bacterial expression proteins were purified as described (12). A rabbit polyclonal antibody (Ab), Hxb-9172, was produced by injecting recombinant TNfn1–5; and Hxb-9504 by injecting TNfn6–8 plus TNfbg. Rat monoclonal Abs BC-4 (against an epitope in the EGF or central knob region) and 7–13 (against an epitope in TNfn1–5) were provided by Dr. Mioriaki Kusakabe, RIKEN, J. apam. Mouse mononclonal Abs TN-190 and BC-4 are against epitopes in TNfn3 and the EGF region (15, 16).

Glycerol Gradient Sedimentation Assay— A glycerol gradient sedimentation assay was done as described by Lightner and Erickson (6). A rabbit polyclonal antibody (Ab), Hxb-9172, was produced by injecting recombinant TNfn1–5; and Hxb-9504 by injecting TNfn6–8 plus TNfbg. Rat monoclonal Abs BC-4 (against an epitope in the EGF or central knob region) and 7–13 (against an epitope in TNfn1–5) were provided by Dr. Mioriaki Kusakabe, RIKEN, J. apam. Mouse monoclonal Abs TN-190 and BC-4 are against epitopes in TNfn3 and the EGF region (15, 16).

FN-III domains of HxB.S and TNfn1–8 contain only the 8 domain of FN. Thus, the major binding site for FN binding appears to be the FN-III domain of TNfn3.

Solid-phase Binding Assay— A solid-phase binding assay was used to study the interaction between TN and FN. In this assay, 96-well plates (Falcon 3912) were coated overnight at 4°C or 2 h at 37°C with FN solution (25 μg/ml FN in TBS containing 1 mM CaCl2 and 1 mM MgCl2) and washed, followed by blocking for 1–2 h at 37°C with 5% non-fat dry milk in PBS. Plates were then incubated with native TN or recombinant domains of TN in the TBS + magnesium/calcium for 1 h at 37°C. After washing the wells, bound proteins were detected by conventional ELISA technique using polyclonal or monoclonal anti-TN Ab and horseradish peroxidase-conjugated secondary Ab.

Results

Binding of TN to FN in Solution—Our previous study (6) demonstrated accelerated sedimentation of TN when FN was present throughout the glycerol gradient. In these gradients the FN was added only to the 15% glycerol, so its concentration was initially 100 μg/ml at the top of the gradient and 50 μg/ml in the middle. However, FN sediments at about 8 S, compared to 13 S for TN, so its concentration at the position of TN should be continuously in the range of 75–100 μg/ml. The accelerated sedimentation of TN indicates the formation of larger complexes resulting from the association of TN with FN. Our previous study used human TN purified from glioma cell culture, which is ~90% large TN. Here we tested individually HxB.L and HxB.S, obtained from our transfected BHK cell lines. Both TN variants showed accelerated sedimentation of about one or two fractions in the presence of FN (Fig. 2). HxB.S gave a somewhat larger shift (two fractions) than HxB.L (one fraction).

In order to map the domain(s) of TN that bind to FN, recombinant TN segments were added to the gradient along with FN to test for competition. These experiments were done in TBS buffer, in which the shift in TN sedimentation with FN is somewhat larger than in ammonium bicarbonate. As shown in Fig. 3, TNfn6–8 and TNfnA-D produced a small displacement of the TN-FN complex, while TNfn3–5 and TNfn3 produced a large displacement, shifting the TN peak almost back to its position in the absence of FN. A strong disruption of the TN-FN complex by TNfn3, TNfn3–5, and TNfn1–5 was consistently observed in several experiments, in both the TBS and ammonium bicarbonate gradients. TNfn6–8, TNfbg, and HxB.S, egf did not show significant displacement of the acceleration of TN in ammonium bicarbonate (not shown). TNfnA-D showed weak displacement activity in some experiments in both TBS and ammonium bicarbonate, but no activity in other experiments. Thus, the major binding site for FN binding appears to be the domain TNfn3.

Fig. 1. The domain structure of human TN subunit is shown above, and the bacterial expression proteins used in this study shown below (see Ref. 12 for more details). The large TN splice variant, HxB.L, contains the shaded FN domains lettered A–D; HxB.S is missing these seven domains. Bacterial protein TNfnALL contains all 15 FN3 domains of HxB.L, and TNfn1–8 contains only the 8 domains of HxB.S.
centrations of soluble HxB.L or HxB.S proteins. Bound TN was detected by ELISA using a polyclonal Ab against the TNfn1–5 region of TN, which should bind equally to both splice variants (Fig. 4). HxB.S achieved a higher level of binding and reached saturation at lower concentrations than HxB.L.

The bacterial expression proteins were again used as competitors to map the binding domain (Fig. 5). All fragments containing TNfn3 gave strong competition, consistent with mapping in the solution-phase assay. TNfn1–5, TNfn1–8, and TNfn3–5 showed the strongest competition at similar molar concentrations. The single domain TNfn3 required a 3-fold higher molar concentration for similar competition. Thus, TNfn3 appears to be the major FN binding site in TN, but additional binding sites in TNfn4–5 may enhance binding.

Based on the results with native TN, we expected TNfn1–8, which corresponds to the small splice variant, to bind to FN more avidly than TNfnALL. This was confirmed, especially at lower concentrations of TN proteins, where TNfn1–8 showed significantly higher binding (Fig. 6). At higher concentrations both proteins bound equally, probably saturating the FN substrate. The apparent biphasic nature of TNfn1–8 binding seen in Fig. 6, in particular the weak binding at higher concentrations, was not seen consistently; however, the enhanced bind-
Incorporation of TN into FN Matrix Fibrils in Cell Culture—Chiquet-Ehrismann et al. (4) used Abs specific for alternative splice domains to show that cultures of chicken fibroblasts, which secrete both small and large splice variants, incorporate only small TN into FN matrix fibers. We used our transfected BHK cells (12) to test this in separate cultures secreting HxB.egf, HxB.S, or HxB.L. Cells were grown to be fully confluent, and FN and TN were localized by immunostaining using anti-FN polyclonal Ab and rat monoclonal Ab 8C9, which binds to HxB.egf and therefore recognizes all three forms (Fig. 7). All four BHK cell lines deposited prominent FN fibrils in the ECM (not shown). Immunostaining of BHK-HxB.egf showed weak cellular staining, but no TN staining in the ECM. BHK-HxB.L likewise showed weak staining inside or on cells but little fibrillar staining. In contrast, BHK-HxB.S showed a prominent fibrillar pattern of staining. Most HxB.S was co-localized with FN fibrils and was associated especially with thick, well-developed FN fibrils.

The co-localization of TN and FN was not universal in the ECM, as reported previously by others (7). Fine FN fibrils without any staining of TN were often observed, and HxB.S was found in some patches that did not stain for FN (data not shown). Thus, HxB.S may bind to other ECM molecules such as collagen or proteoglycans, in addition to FN.

Binding of Purified TN Splice Variants to FN Matrix Fibrils—Binding of the two TN splice variants to the ECM was also tested by adding purified HxB.L and HxB.S separately to NIH 3T3 cell cultures. These cells make a prominent FN matrix, but no detectable TN. After a 24-h incubation with added human TN, the cultures were washed and the bound human TN was visualized using the species-specific monoclonal Ab BC-4. Cultures treated with HxB.L did not show any staining, while cells treated with HxB.S showed the typical fibrillar pattern of staining (Fig. 8).

Differential Binding of TNfn1–8 and TNfnALL to FN Matrix Fibers—We finally tested whether bacterial expression proteins containing only FN3 domains could bind to FN fibrils in cell culture and whether TNfn1–8, which contains only the FN3 domains of small TN, bound better than TNfnALL, which contains all the FN3 domains of large TN. BHK cells were grown to be fully confluent, and TNfn1–8 or TNfnALL (30 μg/ml) were added to the medium. After 24 h the cultures were washed and bound protein was detected by immunostaining using four different Abs: HxB-9172, a rabbit polyclonal Ab recognizing TNfn1–5; HxB-9504, a rabbit polyclonal Ab recog-
nizing TNfn6–8 plus TNfbg; TN-190, a mouse monoclonal Ab recognizing TNfn3; and 7–13, a rat monoclonal Ab recognizing TNfn4–5. Cells not treated with recombinant protein did not show any staining, demonstrating no cross-reaction of Abs with hamster TN, if any. With all four Abs, TNfnALL showed punctate staining at pericellular spaces and over cell bodies, but no fibrillar staining (Fig. 9). In contrast, TNfn1–8 showed virtually no specific staining with either monoclonal Ab, but both polyclonal Abs demonstrated prominent fibrillar staining. The staining of TNfn1–8 to ECM was not as prominent as that of native small TN, but the localization pattern was similar. The enhanced binding of TNfn1–8 to these ECM fibers appeared to be absolute (TNfnALL did not bind), and certainly much greater than to purified FN in the solid-phase ELISA. This suggests that the preferential binding is due to the arrangement of FN3 domains, and does not require the other domains nor the hexabrachion structure. However, binding of expression proteins containing only FN3 domains was always much weaker than that binding of native TN, suggesting that the other domains and/or the hexabrachion structure contribute significantly to overall binding.

How can the absence of the splice segment enhance the binding for FN? The simplest model would postulate two binding sites, one in TNfn1–5 and another in TNfn6–8 (4), which can bind simultaneously to FN only when they are brought together in HxB.S. We have now mapped the primary binding site to TNfn3, which is 10 nm distant from TNfn6, but this does not pose a serious problem, because FN itself is elongated and could have two complementary binding sites separated by 10–15 nm.

If there are two sites, one in TNfn3 and another somewhere in TNfn6–8, we have to address the question of why the binding of HxB.S to FN could be completely blocked by 1 μM concentration of segments containing TNfn3, while TNfn6–8 had no effect. The most likely explanation lies in the nature of cooperative binding (18). If the site in TNfn3 produces binding at a K_D near 1 μM, a second site in TNfn6–8 could enhance the binding by several orders of magnitude, even if it were far too weak to produce an observable binding of TNfn6–8 by itself. The very modest 3–10-fold enhancement we observe in ELISA, or even a 100-fold enhancement that seems to occur in tissue culture, is fully consistent with a modest affinity binding site in TNfn3, enhanced by a very weak binding of a site in TNfn6–8.

We have now confirmed that the preferential binding of HxB.S is conserved in both chickens and humans. The conservation of this activity over the 300 million years that separate chickens and humans suggests that it is biologically important for the functioning of TN in tissues.

REFERENCES
1. Erickson, H. P., and Bourdon, M. A. (1989) Annu. Rev. Cell Biol. 5, 71–92
2. Erickson, H. P. (1993) Curr. Opin. Cell Biol. 5, 869–876
3. Chiquet-Ehrismann, R., Mackie, E. J., Pearson, C. A., and Sakakura, T. (1986) Annu. Rev. Cell Biol. 5, 71–92
Tenascin Binding to Fibronectin

4. Chiquet-Ehrismann, R., Matsuoka, Y., Hofer, U., Spring, J., Bernasconi, C., and Chiquet, M. (1991) Cell Regul. 2, 927–938
5. Faissner, A., Kruse, J., Kühn, K., and Schachner, M. (1990) J. Neurochem. 54, 1004–1015
6. Lightner, V. A., and Erickson, H. P. (1990) J. Cell Sci. 95, 263–277
7. Chiquet, M., and Fambrough, D. M. (1984) J. Cell Biol. 98, 1926–1936
8. Riou, J.-F., Shi, D.-L., Chiquet, M., and Boucaut, J.-C. (1988) Development 104, 511–524
9. Riou, J.-F., Shi, D.-L., Chiquet, M., and Boucaut, J.-C. (1990) Dev. Biol. 137, 305–317
10. Chung, C. Y., and Erickson, H. P. (1994) J. Cell Biol. 126, 539–548
11. Zisch, A. H., D’Alessandri, L., Ranscht, B., Falchetto, R., Winterhalter, K. H., and Vaughan, L. (1992) J. Cell Biol. 119, 203–213
12. Aukhil, I., Joshi, P., Yan, Y., and Erickson, H. P. (1993) J. Biol. Chem. 268, 2542–2553
13. Aukhil, I., Siemp, C. A., Lightner, V. A., Nishimura, K., Briscoe, G., and Erickson, H. P. (1990) Matrix 10, 98–111
14. Engvall, E., and Ruoslahti, E. (1977) Int. J. Cancer 20, 1–5
15. Murphy-Ullrich, J. E., Lightner, V. A., Aukhil, I., Yan, Y. Z., Erickson, H. P., and Höök, M. (1991) J. Cell Biol. 115, 1127–1136
16. Siri, A., Carnemolla, B., Saginati, M., Leprini, A., Casari, G., Baralle, F., and Zardi, L. (1991) Nucleic Acids Res. 19, 525–531
17. Lightner, V. A., Siemp, C. A., and Erickson, H. P. (1990) Ann. N. Y. Acad. Sci. 580, 260–275
18. Erickson, H. P. (1989) J. Mol. Biol. 206, 465–474