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Tenascin: cDNA cloning and induction by TGF-β

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cDNA clones coding for tenascin, an extracellular matrix glycoprotein with a restricted tissue distribution, were isolated from a chicken fibroblast cDNA expression library using a specific tenascin antiserum. Antibodies eluted from the cDNA-encoded fusion proteins reacted exclusively with tenascin. Limited trypsin treatment of purified tenascin resulted in a peptide which confirmed the deduced protein sequences. The largest clone encoding 632 amino acids showed a cysteine-rich region containing 13 consecutive epidermal growth factor-like repeats of unusual uniformity. Northern blot analysis revealed 8- to 9-kb messages. Tenascin is shown to be induced *in vitro* by fetal calf serum as well as by transforming growth factor β (TGF-β). A 4-fold increase in tenascin secretion by chick embryo fibroblasts was seen after TGF-β treatment. The induction of tenascin protein synthesis was preceded by an increase of tenascin mRNA as determined by Northern blot analysis. The induction of tenascin was compared with fibronectin. The accumulation of the two extracellular matrix proteins in the medium was differentially affected by fetal calf serum and TGF-β and the increase was in both cases higher for tenascin.

Key words: EGF-like repeats/extracellular matrix/fibronectin/tenascin/TGF-β

Introduction

Tenascin is an extracellular matrix protein with a spatially and temporally restricted tissue distribution. It is a hexameric, multidomain protein with disulphide-linked subunits of 190–240 kd originally characterized as myotendinous antigen (Chiquet and Fambrough, 1984b). In the embryo, it is present in the dense mesenchyme surrounding developing epithelia in the mammary gland, vibrissa, lung, tooth and kidney (Chiquet and Fambrough, 1984a; Chiquet-Ehrismann *et al.*, 1986; Aufderheide *et al.*, 1987; Thesleff *et al.*, 1987). It is also found in the tendon anlagen, as well as in developing cartilage and bone (Chiquet and Fambrough, 1984a; Mackie *et al.*, 1987; Vaughan *et al.*, 1987). In the adult tenascin remains present in tendons and myotendinous junctions in the perichondrium and periosteum as well as in smooth muscle (Chiquet and Fambrough, 1984a).

Tenascin-like molecules have also been discovered in embryonic brain and have been designated J1 (Kruse *et al.*, 1985; Faissner *et al.*, 1988), cytotactin (Grumet *et al.*, 1985) or GMEM in gliomas (Bourdon *et al.*, 1983, 1985). The six-armed structure of tenascin, as it is seen in the electron microscope, was first described as the hexabrachions present in cell surface fibronectin preparations (Erickson and Iglesias, 1984; Erickson and Taylor, 1987).

Because of the dynamic and often transient occurrence of tenascin, the study of the regulation of tenascin expression is challenging. Transforming growth factor-β (TGF-β) causes a multitude of biological effects in a large number of cell types (for recent reviews see Sporn *et al.*, 1987; Mercola and Stiles, 1988). One particularly important action of TGF-β is believed to be its stimulating effect on extracellular matrix protein production (Ignotz and Massagué, 1986). Previous studies have focused mainly on fibronectin and collagens (Ignotz *et al.*, 1987; Penttinen *et al.*, 1988; Rossi *et al.*, 1988). We have, however, recently shown how tenascin is able to interfere with the function of fibronectin (Chiquet-Ehrismann *et al.*, 1988). We believe, therefore, that any changes in fibronectin expression need to be judged in comparison with the expression of tenascin.

Here, we report on the molecular cloning and sequencing of cDNA clones of tenascin that yielded part of the primary structure of the protein. One of the cDNA clones was used to study the regulation of expression of tenascin by TGF-β and fetal calf serum (FCS) at the mRNA level.

Results

Identification of tenascin cDNA clones

A primary chick embryo fibroblast (CEF) cDNA library in λgt11 was screened using a polyclonal antiserum raised against tenascin. In the screening of 1.6×10^5 phage plaques for immunoreactive tenascin fusion proteins, seven positives were identified and purified for subsequent analysis. The largest subcloned fragment cTn10 (817 bp) was used to rescreen the cDNA library, which led to the isolation of cTn8 (1899 bp). Since cTn8 was in the wrong orientation for detection of an immunoreactive fusion protein, the *Eco*RI insert of cTn8 was recloned into λgt11. The fusion proteins in phage plaques of both cTn10 and cTn8 (fp10, fp8) were now recognized by tenascin antiserum. In addition, the phage plaques of cTn10 were recognized by a monoclonal tenascin antibody, anti-Tn60 (not shown). Anti-Tn60 reacts on immunoblots with purified intact tenascin, but fails to react with tenascin after limited trypsin digestion which initially reduces each subunit by ~15 kd (Figure 1A).

Immunoblots of fusion proteins from crude lysates of cTn8 and a λgt11 control phage are shown in Figure 1B. The antiserum reacts with the IPTG-induced β-galactosidase fusion protein of cTn8. To confirm that the antibodies reacting with the fusion proteins recognized tenascin

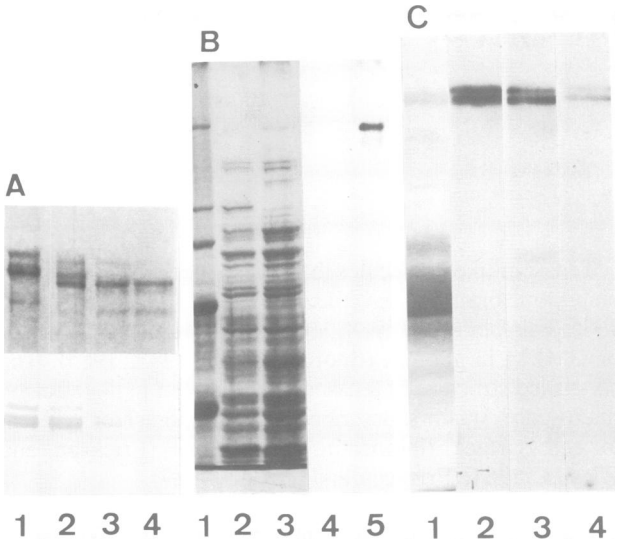

Fig. 1. Immunoblots. (**A**) Tenascin digested with trypsin at varying concentrations for 1 h at 37°C; undigested (**lane 1**); 1.0 μg/ml (**lane 2**); 5.0 μg/ml **lane 3**); 10 μg/ml (**lane 4**). Samples stained with Coomassie Blue (**upper panel**). Immunoblot of samples in parallel lanes using monoclonal anti-Tn60 shows a gradual loss of reactivity, and only intact chains are recognized (**lower panel**). (**B**) Mol. wt markers showing 205, 116, 94, 67 and 45 kd (**lane 1**). Crude lysates containing fusion proteins of a λgt11 control (**lanes 2** and **4**) and clone cTn8 (**lanes 3** and **5**) stained with Coomassie Blue (**lanes 2** and **3**) and immunoblots with anti-tenascin (**lanes 4** and **5**). (**C**) Conditioned medium of CEFs stained with Coomassie Blue (**lane 1**). Immunoblots of samples in parallel lanes treated with tenascin antiserum (**lane 2**); affinity-purified anti-fp8 (**lane 3**); and anti-fp10 (**lane 4**).

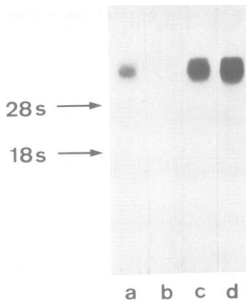

Fig. 2. Northern blot of CEF RNA (10 μg/lane) shows hybridization to 8- to 9-kb mRNAs: probing with 5′ end cTn10 (**lane a**); 3′ end of cTn10 (**lane b**); all of cTn8 (**lane c**); 3′ end of cTn8 (**lane d**). Probes used are shown in Figure 3.

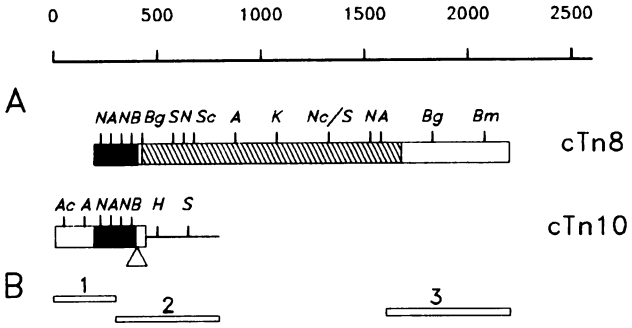

Fig. 3. Chicken tenascin cDNA clones and restriction map. (**A**) *Eco*RI inserts of clones cTn8 and cTn10 with respective open reading frames (boxed) and restriction map of each, not all sites are indicated. Ac = *Acc*I; N = *Nci*I; A = *Ava*II; B = *Bgl*I; Bg = *Bgl*II; H = *Hin*cII; S = *sty*I; Sc = *Sac*I; K = *Kpn*/Asp718; Nc = *Nco*I; Bm = *Bam*HI. The overlap region (filled), the potential 5′ splice site (open triangle), the cysteine-rich repeats (hatched) are indicated. (**B**) The lines indicate probes used for Northern blot analysis. 1 = 5′ end cTn10; 2 = 3′ end cTn10; 3 = 3′ end cTn8.

exclusively, we performed an immunoblot with anti-tenascin antiserum which was affinity purified using crude phage lysates containing fp8 or fp10. The only protein recognized in total fibroblast conditioned medium was tenascin (Figure 1C).

Both cDNAs hybridized to the same mRNA species of 8−9 kb, which is of sufficient size to encode a subunit of tenascin with a mol. wt of 190−240 kd (Figure 2). The DNA sequence of all clones was determined on both strands and across all restriction sites used to initiate sequence determination. A schematic representation of the cDNAs and their restriction maps as well as the probes used for Northern blot analysis is shown in Figure 3. cTn10 and cTn8 are identical over 194 bp at the 5′ end of cTn8, but then diverge. On Northern blots, there was no detectable hybridization when probed with the 3′ end of cTn10 (Figure 2). Sequence analysis of cTn10 revealed that immediately after the 194-bp overlap there is an exon−intron splice junction consensus sequence (Padgett *et al.*, 1986) followed by stop codons in all three reading frames. Therefore, the 3′ sequences of cTn10 seem to represent part of an intron.

Further evidence that these cDNA clones encode tenascin was obtained by sequencing a peptide which corresponds to part of the deduced protein sequence. A limited tryptic digest of tenascin initially purified over an anti-TnM1 column was performed. The large tenascin fragments that are not recognized by anti-Tn60 (compare Figure 1) were removed by passing the digest over the anti-TnM1 column. The flow-through was collected, concentrated and further purified by gel filtration and reverse-phase chromatography. One of the peptides had the following partial sequence: AEIEPVKNYEEXTVNEGN>. It overlaps completely with the deduced amino acid sequence from cTn10, and with the first five amino acids from the deduced protein sequence of cTn8. The amino acid residue at cycle 12 (histidine) could not be identified due to the broadness of the peak of the phenylthiohydantoin derivative of this residue in the HPLC system used. The DNA sequence and the deduced protein sequence are shown in Figure 4 with the isolated peptide sequence boxed.

Primary structure of tenascin reveals the presence of EGF-like cysteine repeats, homology with fibronectin and short neurotoxins

Sequence analyses of cTn8 revealed a region of strong homology to epidermal growth factor (EGF) (Carpenter and Cohen, 1979) and other proteins containing EGF-like repeats (Hursh *et al.*, 1987; Appella *et al.*, 1988). The homology spans amino acids 85−499 of cTn8, and consists of a set of 13 cysteine-rich domains which are highly homologous among each other. These repeats consist of six invariant cysteine residues in the pattern $X_4CX_3CX_5CX_4CX_1CX_8C$ (Figure 5). Homology was also detected in cTn8 at amino acids 500−588 with the fibronectin type III domain (Kornblihtt *et al.*, 1985).

Analysis of the primary structure of cTn10 showed 58% homology of amino acids 76−88 with short neurotoxins.

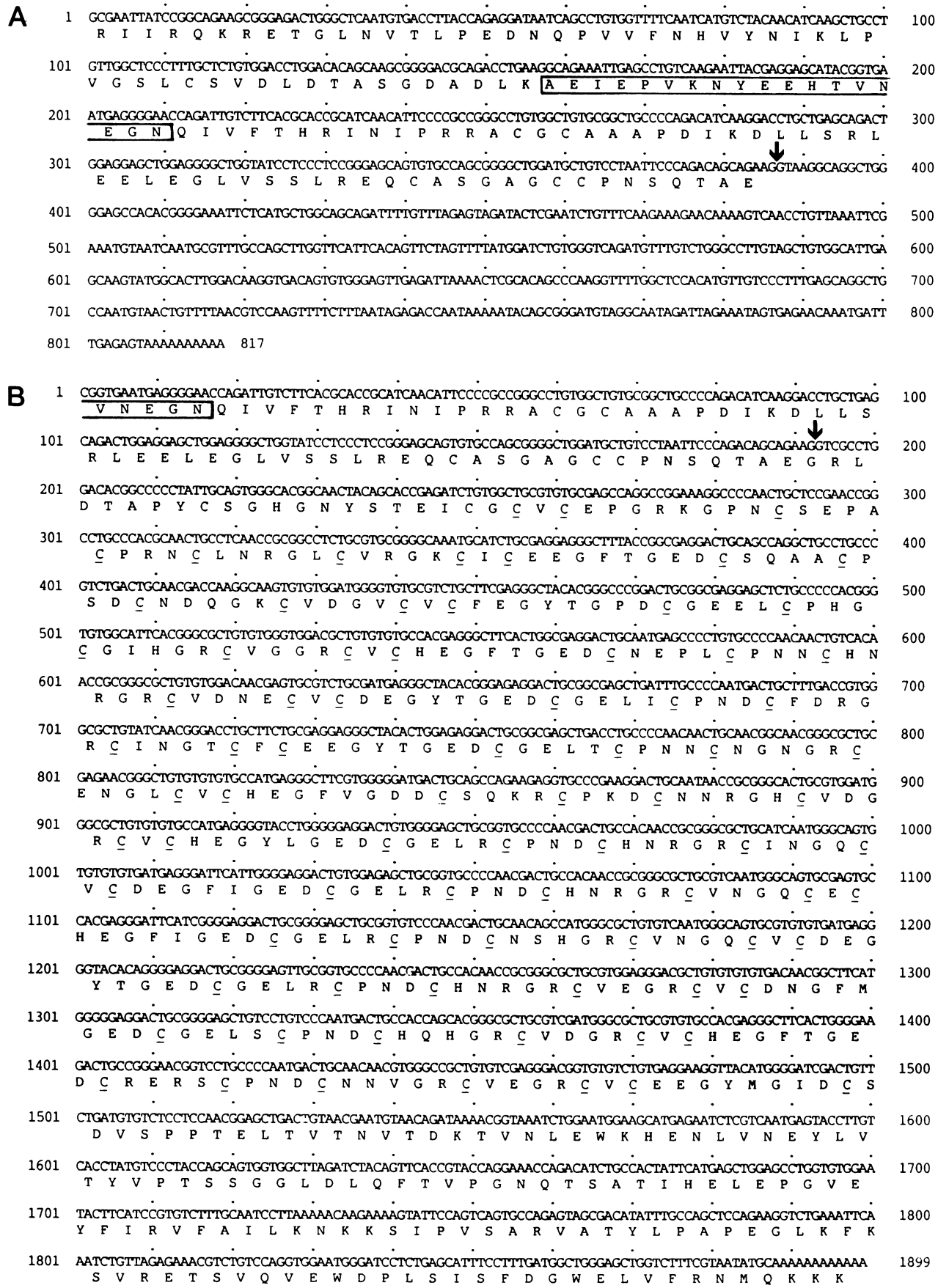

Fig. 4. Sequence of cDNA clones and the deduced amino acid sequence. (**A**) Clone cTn10. (**B**) Clone cTn8. The isolated peptide is boxed. The end of the overlap between the two clones at the putative exon−intron junction is marked by an arrow. The 13 repeating patterns of cysteines are shown with every cysteine underlined.

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                        *       *           *         *   *                 *
85-96                                                     C V C E P G R K G P N C
97-127      S E P A C P R N C L N R G L C V R G K C I C E E G F T G E D C
128-158     S Q A A C P S D C N D Q G K C V D G V C V C F E G Y T G P D C
159-189     G E E L C P H G C G I H G R C V G G R C V C H E G F T G E D C
190-220     N E P L C P N N C H N R G R C V D N E C V C D E G Y T G E D C
221-251     G E L I C P N D C F D R G R C I N G T C F C E E G Y T G E D C
252-282     G E L T C P N N C N G N G R C E N G L C V C H E G F V G D D C
283-313     S Q K R C P K D C N N R G H C V D G R C V C H E G Y L G E D C
314-344     G E L R C P N D C H N R G R C I N G Q C V C D E G F I G E D C
345-375     G E L R C P N D C H N R G R C V N G Q C E C H E G F I G E D C
376-406     G E L R C P N D C N S H G R C V N G Q C V C D E G Y T G E D C
407-437     G E L R C P N D C H N R G R C V E G R C V C D N G F M G E D C
438-468     G E L S C P N D C H Q H G R C V D G R C V C H E G F T G E D C
469-499     R E R S C P N D C N N V G R C V E G R C V C E E G Y M G I D C
                    *       *           *         *   *                 *
Consensus:
            G E L X C P N D C X N R G R C V X G R C V C X E G F T G E D C
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Fig. 5. Alignment of EGF-like repeats. The asterisks (*) mark the invariant cysteines. The consensus sequence is presented at the bottom and is based on the presence of an amino acid in >50% of the sequenced repeats. The amino acids that are 100% conserved are underlined.

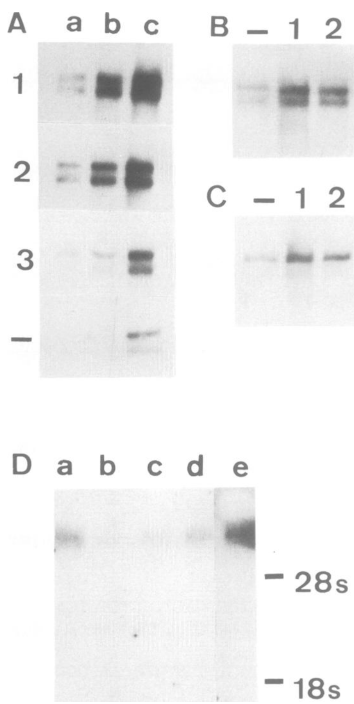

Fig. 6. Tenascin induced by TGF-β *in vitro*. Samples were run on a 7.5% PAGE. TGF-β was added at the following concentrations. 20 ng/ml (**1**); 6 ng/ml (**2**); 2 ng/ml (**3**); no addition (–). Immunoblots of CEF conditioned media (10 μl/lane) cultured in 0.3% FCS for 2 days and treated with tenascin antiserum (**A**, **B**) or anti-fibronectin anti-FnM6 (**C**). In (**A**) media were collected 12 h (**a**), 24 h (**b**), 40 h (**c**) after the addition of TGF-β. (**B**, **C**) Media collected after 40 h. (**D**) Northern blot of CEF RNA (2.5 μg/lane) shows hybridization to RNA from cultures initially in 0.3% FCS and then changed to 10% FCS for 3 h (**a**); RNA isolated from cells grown in 0.3% FCS after addition of 20 ng/ml TGF-β for 0, 3, 7 and 14 h (**b**–**e**). cTn8 was used as probe to detect tenascin mRNA.

This corresponding region in short neurotoxins found in several snake venoms is known to be functionally important for receptor binding (Endo *et al.*, 1986).

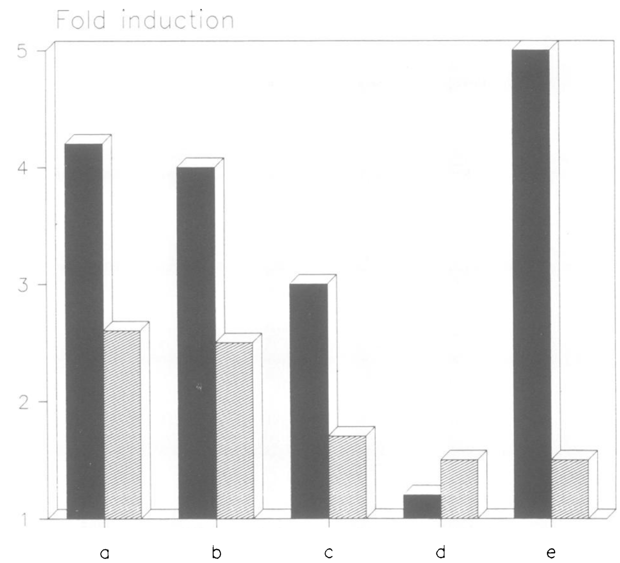

Fig. 7. Quantitation of tenascin (filled) and fibronectin (hatched) induction by TGF-β and serum from media of CEFs as measured by ELISA. Control cultures with 0.3% FCS and without TGF-β addition were taken as a value of 1. Samples were analysed 24 h after the addition of TGF-β at 60 ng/ml (**a**); 20 ng/ml (**b**); 6 ng/ml (**c**); 2 ng/ml (**d**); or 10% FCS (**e**).

Induction of tenascin expression by FCS and TGF-β in vitro

Primary CEFs secrete large quantities of tenascin when cultured in 10% FCS. We noticed that cells grown in 1% or even 0.3% FCS secreted very little tenascin. The possibility of culturing cells in low serum permitted us to study the induction of tenascin synthesis. The addition of TGF-β to the culture medium of CEFs is known to stimulate the secretion of fibronectin, the incorporation of fibronectin into the extracellular matrix as well as the accumulation of fibronectin mRNA (Ignotz and Massagué, 1986). TGF-β addition to our CEF cultures rapidly induced the secretion of tenascin as well as fibronectin in a dose-dependent manner (Figure 6). Twelve hours after the addition of TGF-β a drastic increase of tenascin present in the conditioned medium was detected by immunoblot analysis (Figure 6A). Tenascin and fibronectin responded similarly to the respective TGF-β dose given, although the fold-induction was greater for tenascin than for fibronectin. Half maximal activity of TGF-β for the induction of both proteins was between 2 and 6 ng/ml of TGF-β (Figure 7). The incorporation of tenascin and fibronectin into the extracellular matrix also increased as judged by immunofluorescence staining of the cell layers, paralleling the effect seen on secretion into the medium (not shown).

TGF-β treatment led to a rapid increase in tenascin mRNA levels. After 3 h the effect of tenascin mRNA accumulation became visible and tenascin mRNA levels increased up to 14 h (Figure 6D).

One major difference between fibronectin and tenascin was their response to FCS. Whereas fibronectin secretion was hardly affected by FCS addition, the secretion of tenascin was induced 5-fold (Figure 7).

Discussion

The cDNA cloning of tenascin has revealed the presence of a domain consisting of 13 EGF-like repeats. Among the other proteins containing EGF-like repeats are those involved in blood coagulation, cell surface proteins, growth factors as well as the extracellular matrix protein laminin (Hursh *et al.*, 1987; Sasaki *et al.*, 1987). None of the previously described repeats are identical to the ones found in tenascin. The function of the EGF-like domains in these proteins is in many cases unknown; however, in some instances they were implicated in receptor binding (see Appella *et al.*, 1988). The repeats in tenascin may simply form a scaffolding structure which provides stability to the protein, or in addition may be the domain causing the growth stimulating effect seen by tenascin on carcinoma cells (Chiquet-Ehrismann *et al.*, 1986).

Tenascin and cytotactin are identical molecules since they have the same appearance in the electron microscope and are recognized by the same antibodies (Hoffman *et al.*, 1988). Recently a partial cDNA sequence of cytotactin has been reported (Jones *et al.*, 1988). Within this sequence EGF-like repeats, fibronectin-homologous type III repeats and an area of fibrinogen homology was found. The cytotactin and tenascin cDNAs partially overlap by 781 bases. The overlap extends from the tenth EGF-like repeat to the second fibronectin type III repeat. The sequences are identical except for some inserted or missing bases in the cytotactin sequence, which caused shifts into wrong reading frames in two areas of cytotactin, one of which resulted in the partial loss of the last EGF-like repeat. Our cTn8 extends the reported cytotactin sequence by 1118 bases in the 5′ direction. Since cTn8 and cTn10 are overlapping and the isolated peptide spans both clones, the first 194 bases of cTn10 can be joined to cTn8 to code for a total of 697 amino acids. Combining the data from the clones of tenascin and cytotactin results in ~80% of the tenascin primary structure.

It was striking to see that the tissue distribution of TGF-β in mouse embryos (Heine *et al.*, 1987) was similar to the reported distribution of tenascin. We therefore tested whether TGF-β was able to stimulate the production of tenascin. Such a stimulation has already been described for fibronectin and collagens (see Introduction for references), as well as for cell adhesion receptors (Ignotz and Massagué, 1987).

Tenascin was not only found to be induced by TGF-β but also by FCS. Serum is known to contain TGF-β (Childs *et al.*, 1982). However, there seems to be at least one other factor present in FCS which can induce tenascin synthesis, since the FCS effect was even higher than the TGF-β effect and at the same time fibronectin secretion was only marginally affected.

Our work indicates that the induction of tenascin by TGF-β is more pronounced than for fibronectin. Thus, the ratio of tenascin to fibronectin in the extracellular matrix could be increased by TGF-β. This may explain the cartilage-inducing effect of TGF-β (Seyedin *et al.*, 1987), since tenascin has been shown to promote cartilage formation, whereas fibronectin is known to inhibit it (Mackie *et al.*, 1987). We have demonstrated that tenascin interferes with fibronectin action (Chiquet-Ehrismann *et al.*, 1988), and it is, therefore, conceivable that the result of induction of fibronectin by TGF-β is largely counteracted by the abundance of tenascin. The possible local induction of tenascin *in vivo* upon release or application of TGF-β at the time of wounding (Mustoe *et al.*, 1987) could be more important for repair and regeneration than the increase of a protein which is constitutively abundant like fibronectin.

Materials and methods

Cloning and characterization of cDNA encoding chicken tenascin

Total RNA was extracted from primary cultures of the skin of 11-day chick embryos by the method of Chirgwin *et al.* (1979). Poly(A)$^+$ RNA was isolated by oligo(dT)−cellulose chromatography. A cDNA library in λgt11 was constructed from 15 μg of CEF poly(A)$^+$ RNA and screened with a polyclonal antibody (see below) according to Shibahara *et al.* (1985). The *Eco*RI inserts of positive phage clones cTn10 and cTn8, schematically drawn in Figure 3, were purified, ligated into *Eco*RI-restricted pUC18 plasmid and used to transform *Escherichia coli* HB101. The subcloned DNA fragments were used for further analysis and their nucleotide sequences were determined by the method of Maxam and Gilbert (1980).

RNA isolation and Northern blots

We cultured 2.5−5.0 × 10^4 CEFs/ml for 2 days in Dulbecco's medium (Gibco) with 0.3% FCS (Gibco) and then added either TGF-β (from human platelets; Calbiochem, Behring Diagnostics, La Jolla, CA) or FCS for varying time periods. Total RNA was isolated according to the method of Chomczynski and Sacchi (1987). The RNA was denatured according to Lizardi and Engelberg (1979), electrophoresed on a 1.1% agarose gel containing 1 M formaldehyde, transferred by capillary action overnight to a nitrocellulose filter using 10 × SSC, and hybridized with gel purified ^{32}P-labelled *Eco*RI fragments. All DNA probes used in this study were labelled with [α-^{32}P]dCTP (Amersham; 3000 Ci/mmol) by the method of Feinberg and Vogelstein (1983). The rRNA bands were visualized by staining with 0.5 μg/ml acridine orange prior to the transfer.

Antibodies

Polyclonal antibodies to tenascin and fibronectin were characterized previously (Chiquet-Ehrismann *et al.*, 1986; Ehrismann *et al.*, 1981). Anti-Tn60 was selected from a collection of monoclonal tenascin antibodies (Chiquet-Ehrismann *et al.*, 1988) by its reactivity with the positive phage clone cTn10. Anti-Tn60 is an IgG and was purified from ascites fluid by ammonium sulphate precipitation and chromatography on DE52 (Whatman, Clifton, NJ) as earlier described (Chiquet and Fambrough, 1984a).

A monoclonal anti-chick fibronectin antibody, anti-FnM6 (gift from Dr M.Chiquet, Biozentrum, Basel), was obtained from the same fusion as the original anti-tenascin antibody, anti-TnM1 (Chiquet and Fambrough, 1984a). Anti-FnM6 is an IgG and reacted with fibronectin specifically as verified by immunoprecipitation and radioimmune assays. Preclearing of conditioned medium with fibronectin antiserum prevented subsequent precipitation of a protein band by anti-FnM6.

Affinity-purified antibodies to the fusion proteins expressed in bacteriophage λgt11 were prepared essentially according to Lewis *et al.* (1986), except that our TBST solution consisted of 50 mM Tris−HCl (pH 7.5), 150 mM NaCl and 0.1% Triton X-100 (w/v).

Immunoblots and ELISA

Crude phage lysates containing fusion proteins of cTn10 and cTn8 were produced as described by Huynh *et al.* (1985), and samples were separated on 7.5% SDS−polyacrylamide gels according to Laemmli (1970). After electrophoresis of the gel, a section was cut and the proteins were visualized by staining with Coomassie Brilliant Blue R (Merck, GmbH), while the rest of the gel was transferred electrophoretically to nitrocellulose by the method of Towbin *et al.* (1979). The nitrocellulose-bound proteins were then stained with 2% Ponceau S (Merck, GmbH) in 3% perchloric acid. This reversible stain allows confirmation of a successful transfer. The blots were incubated with the appropriate antibodies and the bound antibodies visualized by [^{125}I]fusion protein A (Amersham) as described previously (Shibahara *et al.*, 1985) or by using the peroxidase anti-peroxidase system (Cappel) and staining with 4-chlornaphthol (Merck).

Solid-phase binding assays were performed with 96-well cluster plates (Falcon, Lincoln Park, NJ) that were coated with serial dilutions of the conditioned media to be tested. After washing the wells, bound tenascin and fibronectin were detected by anti-TnM1 or anti-FnM6 using the ELISA procedure described by Engvall (1980). Data obtained in the linear range of absorbance (i.e. a 2-fold dilution of the coating solution resulted in one-half of the absorbance value) are presented in Results.

Protein sequence determination

Tenascin was purified from conditioned medium of CEF cultures as described

(Chiquet and Fambrough, 1984b). Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Labs, Richmond, CA). Purified tenascin (700 μg) at 100 μg/ml, was dialyzed against 50 mM $(NH_4)HCO_3$ overnight, and cleaved with 5 μg/ml bovine trypsin (Worthington, Freehold, NJ) for 1 h at 37°C. To inhibit trypsin, Leu-Ala-Arg Ch_2Cl (gift from Dr E.Shaw, FMI, Basel) was added to a final concentration of 10^{-5} M and incubation was continued for 10 min at room temperature. The sample was passed over a 10-ml column containing anti-TnM1 (Chiquet and Fambrough, 1984a). The flow-through was lyophilized and subsequently passed over a gel filtration column (Superose 12, Pharmacia, Uppsala, Sweden). Fragments were further purified by reverse phase chromatography on a C_8 column (Aquapore, RP-300, Brownlee Labs, Santa Clara, CA) using 0.1% trifluoroacetic acid as the eluent. Peptides were eluted with a linear gradient of acetonitrile. Protein sequencing was performed as described previously (Hofsteenge and Stone, 1987).

Computer analysis

DNA sequences were compiled with the UWGCG software package (Devereux *et al.*, 1984). Homologies were found with the Protein Information Resource package according to Sidman *et al.* (1986) as well as by the FASTP and FASTN algorithms of Pearson and Lipman (1985).

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These sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession numbers X08030 and X08031.