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# PROCOLLAGEN TYPE I C-PROTEINASE ENHANCER IS A NATURALLY OCCURRING CONNECTIVE TISSUE GLYCOPROTEIN

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SUMMARY. Using antibodies to the procollagen C-proteinase enhancer of mouse fibroblast culture medium, we have screened by immunoblotting extracts of several post natal mouse and rat tissues for the presence of the enhancer antigen. All rodent connective tissues were relatively rich in enhancer; lower amounts were found in skeletal muscle and heart and essentially no enhancer was detected in kidney, liver or brain. The amounts of enhancer in mouse tendon and calvaria extracts were age related, with highest amounts in 11 and 19 d tendons and in 1 d calvaria - the times of rapid growth of these organs. The results suggest that procollagen C-proteinase enhancer is a specific connective tissue glycoprotein that is likely to regulate procollagen processing in vivo. 

Conversion of procollagen type I to collagen requires the enzymic removal of amino- and carboxyterminal propeptides by specific N- and C- procollagen proteinases (1,2). Type I procollagen N-proteinase, a metalloproteinase, was purified from chick embryo tendons (3,4) and from whole chick embryos (5) and shown to be a tetramer of about 500 kDa molecular weight, consisting of 4 distinct subunits (4). Type I procollagen C-proteinase, also a metalloproteinase but a monomer of about 100 kDa molecular weight, was purified from the media of cultured chick embryo tendons (6) and from the media of cultured mouse fibroblasts (7,8). In mouse fibroblast culture media we also found a glycoprotein which, although devoid of procollagen processing activity, enhanced the activity of the C-proteinase by approximately one order of magnitude (8). Enhancing activity was associated with three proteins of molecular weights 55, 36, and 34 kDa, all of which were glycosylated. All three proteins bound to the carboxyl propeptide of type I procollagen (a property we utilized for their purification (8,9)), and antibodies to the 36

Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SBTI, soy bean trypsin inhibitor, EDTA, ethylenediamine tetraacetate.

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kDa enhancer protein cross-reacted with the 55 and 34 kDa enhancer forms, suggesting a possible precursor- product relationship. Here we demonstrate that procolloagen C-proteinase enhancer is a naturally occuring protein, found in various mouse and rat connective tissues but practically undetectable in other, non-collagen producing tissues.

## MATERIALS AND METHODS

Affinity purified anti-enhancer antibodies. Antibodies to the mouse fibroblast 36 kDa enhancer protein were raised in rabbits and purified by ammonium sulfate precipitation (8) and enhancer - Sepharose affinity chromatography. For the latter, the antigen (a mixture containing 300 and 140 μg of the affinity purified 36 and 34 kDa enhancer proteins, respectively (9)) was coupled to CNBr activated Sepharose 4B (6 ml; Pharmacia) and the substituted resin equilibrated in a column with phosphate buffered saline adjusted to 1M NaCl. The immunoglobulin fraction in the same buffer was applied to the column by recirculation for 16 to 20 h at a flow rate of 20 ml/h. After initial washing with equilibration buffer, bound antibodies were released with 6M urea. About 90% of the total specific antibodies (fraction I) were recovered under these conditions. The remaining antibodies (fraction II) were released from the column with 4.9M $MgCl_2$ (Sigma). SDS-PAGE showed fraction I to contain a mixture of IgM and IgG at an approximately 1:1 ratio whereas fraction II consisted of IgG only. IgG in fraction I were further purified by Protein-A Sepharose chromatography (10).

Tissue extracts. Unless otherwise stated, tissues (including tail tendons, calvaria, sterna, skin, cornea, brain, heart, liver and kidneys) were excised from 21 to 25d old ICR mice or Charles River rats, killed with ether. After weighing, each tissue was cut into small pieces, suspended in ice cold 0.05M Tris.HCl, 0.15M NaCl pH 7.5 containing protease inhibitors (20 mM EDTA, 10 mM N-ethylmaleimide, 10 mM benzamidine, 0.4 mM phenyl methyl sulfonyl fluoride and 1 μg/ml leupeptin) and extracted by boiling for 10 min after addition of SDS to 2%. Insoluble material remaining after standing at room temperature for additional 20 h was removed by centrifugation. For trypsin treatment, rat and mouse tendons were extracted under native conditions as described in the legend to Figure 2.

Electrophoretic Methods. SDS-PAGE was performed according to Laemmli (11) with 4% stacking gels and 10% separating gels. Electrophoretic transfer of proteins from SDS-gels to nitrocellulose was achieved with 25 mM ethanolamine, 38 mM glycine, 20% methanol, 0.01% SDS pH 9.4 (12) and the paper blocked with 5% skimmed milk (Difco). After incubation (2 h, room temperature) with the specific antibody or, as a control, with antibody against *Pseudomonas aeruginosa* elastase (13) (0.5 μg/ml IgG) enhancer related proteins were detected with alkaline phosphatase conjugated goat anti rabbit IgG (14). In all experiments, control blots (incubated with antibody to the bacterial elastase), were negative (data not shown).

## RESULTS AND DISCUSSION

Our earlier finding of the C-proteinase enhancer in mouse fibroblast culture media (8,9) was followed by searching for the enhancer antigen in rodent and embryonic chick tissues. Tissue extracts were analyzed by immunoblotting with antibodies specific to the mouse fibroblast 36 kDa enhancer. These antibodies also recognize the 34 and 55 kDa enhancer proteins (9). Figure 1 shows that tail tendons from both mouse and rat contain several

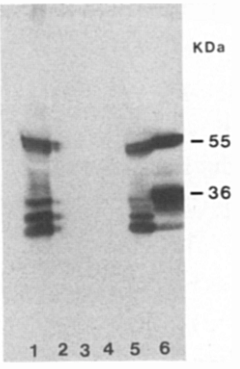


Figure 1. Immunoblotting analysis of C-proteinase enhancer in rat and mouse tail tendon extracts (lanes 1 and 5, respectively) and in extracts of 17d chick embryo tendons, calvaria, and sterna (lanes 2 to 4, respectively). Lane 6, enhancer standard (0.4 μg) purified from mouse fibroblasts culture medium (8). Equal volumes of extracts (all at 200 mg wet weight per ml except for the mouse tendon extract which was at 133 mg/ml) were loaded on to each lane.

immunologically reactive proteins. These include a doublet migrating almost identically with the 55 kDa enhancer protein and 3 to 4 smaller proteins with molecular weights around 36 kDa or lower. The smaller proteins may be degradation products resulting from non-specific proteolysis of the 55 kDa proteins. In support of this interpretation, a mild trypsin treatment of native tendon extracts fully converted the 55 kDa protein(s) to the smaller species (Figure 2A). Furthermore, a similar treatment of the mouse fibroblast 55 kDa enhancer converted it to a protein migrating identically with the 36

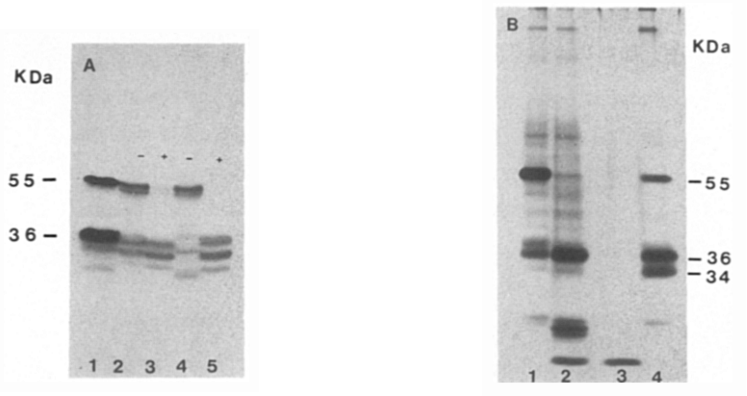


Figure 2. Effect of trypsin on the enhancer proteins. A. Immunoblot of mouse and rat tendon extracts. Tendons were extracted (60 min, 4°C) with 0.05M Tris.HCl, 0.15M NaCl, 5mM $CaCl_2$, pH 7.5, incubated (30 min, 25°C) with trypsin at a protein - trypsin ratio of 50 to 1, and the reaction stopped by adding 5 fold excess SBTI. Lane 1, enhancer standard (0.2 μg); lanes 2 and 3, mouse tendons; lanes 4 and 5, rat tendons. (+) and (-), with and without trypsin, respectively. B. Silver stained (14) gel of purified mouse fibroblast enhancer. Lane 1, without trypsin; lane 2, with trypsin; lane 3, trypsin and SBTI alone; lane 4, standard enhancer, containing the 55, 36 and 34 kDa enhancer forms, as indicated at right. Incubations with trypsin were as described for the tendon extracts in A.

kDa enhancer form (Figure 2B, compare lanes 1,2 and 4). Immunoblots of 17d chick embryo tendons, calvaria, and sterna extracts were all negative (Figure 1). Though this is most likely due to a lack of cross-reactivity with the antibody (directed to the mouse antigen), we cannot exclude the possibility that enhancer is not expressed during embryonic development.

Analysis of other mouse and rat tissues revealed that the enhancer was predominantly present in the "classical" interstitial connective tissues, including tendons, calvaria, skin, cornea, and sterna, with the highest amounts seen in tendons (Figure 3). The enhancer was also present in tissues containing some interstitial connective tissue such as skeletal muscle and heart, though enhancer levels in these tissues were relatively low. Practically no enhancer was detectable in kidney, liver and brain; these organs contain negligible amounts of interstitial connective tissue. The results clearly establish a correlation between the collagen synthesizing potential of the tissue and enhancer expression.

The amount of enhancer in mouse tail tendons seems to be age related. Extracts of 5d and 39d old (adult) mouse tendons contained less enhancer than those of 11 and 19 d old mice. The levels of enhancer in the latter two extracts were comparable (Figure 4). The pattern seen with mouse calvaria was somewhat different. Here, a maximum was observed on d 1 (no tendons could be obtained from 1 d mice). From 5 to 19 d, the amount of enhancer was slightly lower than that seen on d 1, although, through the entire period, the enhancer levels were about the same. A further drop in the enhancer level was seen in

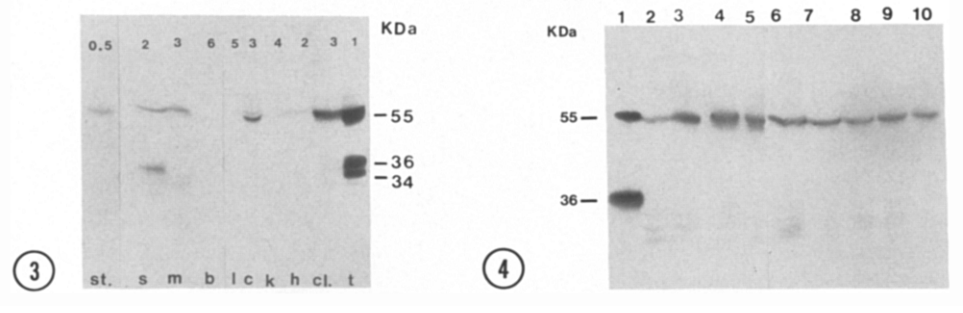


Figure 3. Tissue distribution of the C-proteinase enhancer, analyzed by immunoblotting of SDS-extracts. st, sterna; s, skin; m, muscle; b, brain; l, liver; c, cornea; k, kidney, h, heart; cl, calvaria; t, tendons. All tissues were from mice except cornea and sterna which were from rats. The numbers at the top of each lane represent relative extract concentrations (multiples of 133 mg wet weight per ml); equal volumes of extracts were applied to each lane.

Figure 4. Age dependence of C-proteinase enhancer in mouse tendon and calvaria. SDS- extracts, all at 133 mg wet weight per ml, were prepared and immunoblotted as in Methods, with equal volumes applied to lanes 2 to 10. Lane 1, mouse fibroblast enhancer (0.2 μg); lanes 2 to 5: 5,11,19 and 39 d tendons, respectively; lanes 6 to 10: 1,5,11,19, and 39 d calvaria, respectively. Left, migration positions of the 55 and 36 kDa enhancer forms.

the adult calvaria (39 d). Also noticable is that almost all of the immunologically reactive material in the calvaria samples, especially from 5 d onwards, migrated as a single band at the 55 kDa position. This result is consistent with our assumption that the smaller enhancer proteins are non-specific degradation products of the 55 kDa protein. Thus, although the low molecular weight proteins also exhibit C-proteinase enhancing activity (8,9), the 55 kDa protein is most likely the native, physiologically functional form of the enhancer. The age dependence of enhancer amounts in both tendon and calvaria suggests a correlation between enhancer expression and collagen deposition. Maximum enhancer was observed in the 11 and 19 d tendons, and in 1 d calvaria, i.e., stages of development at which rapid growth of these tissues takes place (16).

This is the first documentation of procollagen C-proteinase enhancer in connective tissues. The finding that enhancer is not only present in all connective tissues examined, but virtually restricted to these tissues, along with the apparent correlation between enhancer expression and extent of collagen deposition, indicate that procollagen C-proteinase enhancer is a specific connective tissue glycoprotein, that is likely to regulate procollagen processing *in vivo*. The presence of enhancer in sterna and muscle suggests that it also functions in the maturation of types II and III procollagens.

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