LOCALIZATION OF THE CELL ATTACHMENT REGION IN TYPES I AND II COLLAGENS

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Summary

The sites on type I and II collagen which bind to the cell attachment protein (CAP) have been identified using an inhibition assay. Both chains of type I collagen contain binding sites but α1(I) is more active than the α2 chain. The most active peptide obtained from the α1(I) chain corresponds to residues 568 through 835. A comparable region was found to be the only active site on the α1 (II) chain.

INTRODUCTION

Collagen has been demonstrated to be a mediator of mammalian cell attachment (1,2), platelet aggregation (3), and an activator of Factor XII (Hageman Factor) of the blood clotting series (4). In addition, collagen has been shown to play a role in the differentiation of myoblasts (5).

Although four genetically different collagen molecules have been described (6), the specific roles of these molecules in cell attachment have not been determined. Type I collagen is the most abundant collagen found in skin, bone and tendon. It exists as a trimer composed of two α1(I) molecules and one α2 molecule. Type II collagen, composed of three α1(II) chains, is the cartilage specific collagen. Type III collagen which is composed of α1(III) chains is found in young skin and blood vessels and type IV is basement membrane collagen. In this report the binding region of type I and type II collagens to a serum-derived cell attachment protein (CAP) is determined.

It has been reported that two CNBr peptides, α1(I)-CB5 of α1(I) (7) and α2-CB5 of α2 (8), are active in platelet aggregation and that two CNBr peptides, α1 (I)-CB7 and α1(I)-CB8, of α1(I) are active in myoblast differentiation

(9). We demonstrate here that α1(I)-CB7 and α1(I)-CB8 of α1(I) and α1(II)-CB10 of α1(II) are active in cell attachment.

MATERIALS AND METHODS

Collagen Preparations. Type I collagen was prepared from lathrytic rat skins according to the method of Bornstein and Piez (10). The constituent chains of the type I collagen, α1(I) and α2, were separated by CM-cellulose chromatography under denaturing conditions (11). After cyanogen bromide cleavage (10) the peptides were isolated on a CM-cellulose column using 0.02M sodium formate buffer, containing 0.04 NaCl, pH 3.8 and a linear salt gradient from 0 to 0.10 M NaCl over a total volume of 1600 ml of the formate buffer at 45°C. Resolution of the low molecular weight peptides α1(I)-CB1, α1(I)-CB2 and α1(I)-CB3, not bound to the column was achieved by chromatography on phosphocellulose (10). The larger peptides were purified further by 10% agarose sieve (11) chromatography.

Type II collagen was prepared from the Swarm chondrosarcoma (12) grown in lathrytic rats. Type II collagen CNBr peptides were prepared from the purified α1(II) tumor collagen chains as well as from bovine nasal cartilage (a gift from Dr. Vincent C. Hascall), from which about 85% of the proteoglycans had been extracted with 4M guanidine HCl (13,14). Separation and purification of the CNBr peptides were achieved with CM-cellulose (14) column chromatography. The identification and the assessment of purity of the collagens and various peptides were judged by their appearance on chromatography and by their size and homogeneity on 5% and 7.5% acrylamide gels (15).

Assay for Collagen and Peptides that Bind Cell Attachment Protein (CAP)
Due to the fact that CNBr peptides cannot be firmly bound to a petri dish, it is not technically feasible to assay for active peptides by assessing the amount of CAP that binds to peptide-coated petri plates. For this reason, an inhibition assay has been devised to detect active peptides.

The principle of the inhibition assay is to (a) pre-incubate collagen peptides with excess CAP (1) in order to form a mixture of soluble CAP-peptide complexes and free CAP, (b) to incubate the mixture, prepared above, on a collagen-coated petri plate in order to permit free CAP to bind to the collagen substratum, and (c) to assess the amount of CAP bound to the collagen substratum by determining the number of CHO cells which will now bind to the CAP-collagen substratum. In this assay system, an active peptide will result in fewer cells bound.

Technical details are as follows: (step a) Peptides are dissolved at 1 mg/ml in Eagles medium and added to 12 x 75 mm borosilicate glass test tubes containing 2 ml of Eagles medium plus 2% bovine serum. After a pre-incubation of 1 hour at 37°C which permits binding of CAP to soluble peptides, (step b) the contents of the tubes are added to collagen-coated petri plates. After a second pre-incubation of 1 hour at 37°C which permits free CAP to bind to the collagen-coated petri plate, (step c) CHO cells are added and the cells attached after 1.5 hour are trypsinized and counted with an electronic cell counter. The assay for cell attachment to collagen has been described in detail (1). All assays were performed in a double blind fashion.

Calculation of CAP Binding Activities of Peptides.
Figure 1 shows sample data obtained from one experiment. The data presented in Figure 1 indicate the number of cells that bound to the CAP-collagen substratum after prior incubation of CAP with collagen peptides (step a). The percent of CAP bound to the collagen substratum can be

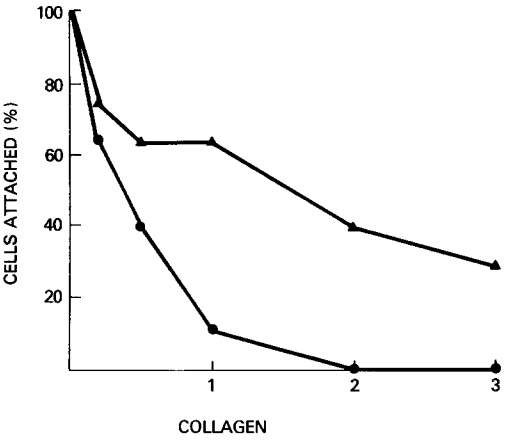

Figure 1, Percent of attached cells in the presence of α1(I) (●——●) and α2 (▲——▲) collagen α chains (mg).

Assay conditions are described in the Methods. In the absence of an active collagen peptide, cells were attached and spread. In the presence of an active peptide, microscopic observation indicates that cells become rounded and finally do not attach as the concentration of active peptide increases. Differences in the slopes of the curves are probably due to dislodgment of rounded cells during the washing of the plates prior to trypsinization and counting.

calculated by correcting the data for background (number of cells in the minus serum controls) and using the equation below.

$$\left[\frac{\text{number of cells attached}}{\text{control (number of cells) attached in the presence of 2\% serum.}}\right] \times 100 = \%\text{ of Cells Bound to CAP-Collagen substratum on the Petri Plate}$$

From the profiles of Figure 1 the amount of each collagen (or peptide) required for 50% inhibition of cell attachment to collagen can be estimated. This 50% inhibitory amount of a collagen (or peptide) is then divided by its molecular weight to obtain molar activities.

RESULTS

Pre-incubation of increasing amounts of collagen, chains or certain peptides derived from the α chains with serum decreased the number of cells that would attach to the collagen-coated plates. This observation as seen in Figure 1 allowed a calculation of the relative activities of these compounds.

As shown in Table I, the isolated α1(I) chain is a more active inhibitor of cell attachment than type I collagen itself. In contrast, the α2 chain

TABLE I

CAP BINDING ACTIVITIES OF TYPE I AND TYPE II COLLAGEN AND CNBr PEPTIDES

		Amount (in mg) of collagen or peptide required to inhibit cell attachment by 50%	Molar Activity ($\times 10^{-8}$)
TYPE I	$\alpha 1(I)_2\alpha 2$	1.15	1.22
	$\alpha 1(I)^2$	0.40	0.42
	$\alpha 1$(I)CNBr	0.75	0.79
	$\alpha 1$(I)-CB1	Inactive[a]	--
	$\alpha 1$(I)-CB2	Inactive[a]	--
	$\alpha 1$(I)-CB3	>>4.00	--
	$\alpha 1$(I)-CB4+5	>>4.00	--
	$\alpha 1$(I)-CB6	>>4.00	--
	$\alpha 1$(I)-CB7	0.50	2.08
	$\alpha 1$(I)-CB8	2.00	8.26
	$\alpha 2$	1.60	1.68
	$\alpha 2$ CNBr	1.60	1.68
TYPE II	$\alpha 1(II)^b$	1.15	1.22
	$\alpha 1(II)^{3c}$	0.65	0.68
	$\alpha 1$(II)CNBr[c]	2.00	2.11
	$\alpha 1$(II)-CB3	Inactive[a]	--
	$\alpha 1$(II)-CB4+6	>>4.00	--
	$\alpha 1$(II)-CB8	>>4.00	--
	$\alpha 1$(II)-CB9,7	>>4.00	--
	$\alpha 1$(II)-CB10	0.90	3.13
	$\alpha 1$(II)-CB11+12	>>4.00	--

a. No inhibition was observed.
b. Obtained from rat chondrosarcoma.
c. Obtained from bovine nasal cartilage

is less active than the original collagen. This would suggest that the major binding site lies in the $\alpha 1$(I) chain. Since $\alpha 1$(I) was more active than the original collagen, further attention was directed toward evaluating the activity of peptides derived from this chain. CNBr digests of the $\alpha 1$ (I) chain were found less active than $\alpha 1$(I) presumably because some active portions were altered. The total digest, however, was still more active than type I collagen. In contrast, digestions of $\alpha 2$ with CNBr had no effect on the activity of this chain.

Of the various peptides obtained from the CNBr digest, some such as $\alpha 1$(I)-CB1 and $\alpha 1$(I)-CB2 showed no activity while others such as $\alpha 1$(I)-CB3,

α1(I)-CB4+5, and α1(I)-CB6 showed low but detectable activity. α1(I)-CB7 was the most active peptide, while α1(I)-CB8 had about 20% of the activity. On a weight basis α1(I)-CB7 was more active than the original collagen and nearly as active as the α1(I) chain. On a molar basis, the peptide was considerably less active than the original α chain or collagen.

Type II collagen was also found to inhibit the attachment of CHO cells. Considerable variation in the activity of the native collagen was observed. The value shown in Table I is the most active obtained. Variation in the activity seemed to be related to the aggregation of this collagen into fibers. Type I collagen appeared to be much more soluble. The isolated α1(II) chain was considerably more active than the native protein. Of the various CNBr peptides obtained from this collagen, α1(II)-CB10 was the most active.

The activity of a variety of synthetic peptides was assayed. These included poly-L-hydroxyproline, poly-O-acetyl-L-hydroxyproline and polyproline. No activity was observed with these substances.

DISCUSSION:

This study indicated that the binding of collagen to CAP involved discrete sites on the collagen molecule. The α1(I) chain inhibited more strongly than α2. α1(I)-CB7 corresponding to the residues 568 to 835 in the 1052 residues that comprise the α1(I) chain was the most active peptide obtained. In molar amounts the activity of this peptide was considerably less than that of the α1(I) chain from which it was derived. The CNBr digestion may have altered the reaction site through cleavage or other modifications.

The most active peptide, α1(II)-CB10, obtained from a CNBr digest of type II collagen occupies a position in the protein equivalent to α1(I)-CB7. Considerable homology exists between the α1(I) and α1(II) chains and may include a binding site for CAP in the same region. This portion of the α1(I) chain contains the site of cleavage of the animal collagenases but is devoid of any carbohydrate.

It is interesting to note that the CNBr peptides of collagen that were found to be active in the cell attachment assay have also been found to be active in promoting myoblast differentiation (9). In comparing amounts (in mg) of collagen and peptides in regard to their ability to promote myogenesis Hauschka and White found the entire α1(I) chain slightly more active than α1(I)-CB7,and α1(I)-CB7 two to six times more active than α1(I)-CB8. The results presented here concerning cell attachment indicate α1(I)-CB7 is slightly less active than α1(I) and four times as active as α1(I)-CB8. This difference in activity between α1(I)-CB7 and α1(I)-CB8 does not reside in physical size since α1(I)-CB7 and α1(I)-CB8 are nearly identical in molecular weight (approximately 24,000). In addition we have isolated and assayed peptides produced from α1(I)-CB7 by enzymatic cleavage and have found a peptide 2/3 the size of the original α1(I)-CB7 with most of the activity (unpublished observations).

In summary, this study indicates that types I and II collagen both bind CAP and that certain homologous regions of the collagen chain appear to contain the specific attachment site. The results presented also indicate that the binding site of cells on α1(I) collagen chains differs from that of platelets which recognize α1(I)-CB5 (7).

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