Binding of Soluble Type I Collagen to Fibroblasts: Specificities for Native Collagen Types, Triple Helical Structure, Telopeptides, Propeptides, and Cyanogen Bromide-derived Peptides

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ABSTRACT Unlabeled collagenous proteins were quantified as inhibitors of binding of native, soluble, radiiodinated type I collagen to the fibroblast surface. Collagen types IV, V, a minor cartilage isotype (1α2α3α), and the collagenlike tail of acetylcholinesterase did not inhibit binding. Collagen types II and III behaved as competitive inhibitors of type I binding. Denaturation of native collagenous molecules exposed cryptic inhibitory determinants in the separated constituent α chains. Inhibition of binding by unlabeled type I collagen was not changed by enzymatic removal of the telopeptides. Inhibitory determinants were detected in cyanogen bromide-derived peptides from various regions of helical α1(I) and α1(III) chains. The aminoterminal propeptide of chick proα1(I) was inhibitory for binding, whereas the carboxyterminal three-chain propeptide fragment of human type I procollagen was not. The data are discussed in terms of the proposal that binding to surface receptors initiates the assembly of periodic collagen fibrils in vivo.

MATERIALS AND METHODS

Binding Inhibition Assays

Swiss mouse 3T3 fibroblast cultures were used in standard binding assays as described (1, 2). Unlabeled lathyritic rat skin (LRSC) and other unlabeled collagenous proteins were dissolved in 0.05 M Tris HCl-0.15 M NaCl pH 7.5 buffer at 1-2 mg/ml. Protein concentrations of the stock solutions were computed from the difference in absorbances at 215 and 225 nm of appropriate dilutions of the stock (3). For each experiment, increasing amounts (0-100 μg) of unlabeled LRSC and of putative binding proteins were each premixed with a fixed amount (130,000 cpm) of ~125I-LRSC in 2 ml of binding buffer (Dulbecco's modification of Eagle's medium with half bicarbonate concentration, 0.05 M HEPES, pH 7.2 and 2.5 mg/ml bovine serum albumin). The solutions were warmed at 37°C for 30 min, cooled to room temperature (26°C) and added to washed, replicate confluent cultures of 3T3 fibroblasts. After a 2-h incubation at 26°C, the cell layers were rapidly washed and lysed, and the radioactivity specifically bound to the cells was measured. Percentage inhibition of binding at each protein concentration was computed by:

\[
\left(1 - \frac{\text{cpm bound with unlabeled protein}}{\text{cpm bound without unlabeled protein}}\right) \times 10^2
\]

Limited Digestion of LRSC with Pepsin and α-Chymotrypsin

Pepsin was purchased from Sigma Chemical Co., St. Louis, MO and α-chymotrypsin from Worthington Biochemical Corp., Freehold, NJ. Pepsin digestion was done according to the procedures of Leibovich and Weiss (4) and Helseth and Veis (5). Digestion with α-chymotrypsin was done by the procedure of Bornstein et al. (6). Control samples without enzyme additions were carried through all the procedures. Samples were finally dissolved in Tris buffer and tested as inhibitors of binding as described above.

SDS PAGE

Samples were dissolvd in sample buffer containing 2% SDS, 10% glycerol, 0.001% bromphenol blue in 0.0625 M Tris HCl, pH 6.8. After heating at 100°C.
for 2 min, the samples were applied to a 3% stacking/6% separating slab gel (7) and, after development, the gels were stained with Coomassie Blue.

**Materials**

Collagenous proteins were generously donated by the following investigators: Dr. Terrence L. Rosenberry, Case Western Reserve University, Cleveland, OH, the collagenlike tail of acetylcholinesterase; Dr. Jerome Seyer, Veterans Administration Medical Center, Memphis, TN, cyanogen bromide peptides of collagen; Dr. George Martin, National Institute of Dental Research, Bethesda, MD, LRSC and rodent collagen types II and IV; Dr. Bjorn R. Olsen, Department of Biochemistry, College of Medicine and Dentistry of New Jersey-Rutgers Medical School, Piscataway, NJ, the aminoterminal propeptide of chick pro-α1(I).

The synthetic peptides DNP-Pro-Gly-Gly-Ele-Ala-Gly-Gln-D-Arg and DNP-Pro-Leu-Gly-Ile-Ala-Gly-Arg-NH2 were purchased from the Peptide Institute, Protein Research Foundation, Osaka, Japan.

**RESULTS**

**Specificities: Collagen Structure, Collagen Types, and Native Helicity**

Potential competitors for 125I-LRSC binding sites were compared to the unlabeled LRSC in inhibition assays as described in Materials and Methods. That general collagen structure does not ensure binding to or blocking of type I sites is shown in Fig. 1. In this instance the putative competitor was the collagenlike tail of acetylcholinesterase (8, 9). Additions of up to 20 μg of this unlabeled protein did not significantly inhibit binding of the radioactive ligand, whereas 20 μg of unlabeled LRSC produced 78% inhibition of binding. C1q, the complement component containing some collagen structure, also fails to inhibit type I binding (1).

Native Collagen types II, III, IV, V, and a minor isotype present in cartilage but distinct from type II collagen (10) were tested in binding inhibition assays (Fig. 2). These data show that unlabeled type II and type I collagens cause equivalent inhibition whereas unlabeled type III collagen is a somewhat less efficient inhibitor of binding. The much lower levels of inhibition produced by the other collagen types are considered to be of doubtful significance.

Graphical analyses of binding data were performed to determine whether collagen types II and III acted as competitive or noncompetitive inhibitors of type I binding. Fig. 3 presents Scatchard plots of type I binding with and without the addition of 5 μg (17.5 pmol) of type II collagen. Calculation of the slopes showed that inhibition was accompanied by an increase in the binding dissociation constant (Kd) from 7.5 × 10^-9 M to 1.7 × 10^-8 M. The common abscissal intercept for the two lines indicates that the number of cell binding sites was not changed by the inhibition. Fig. 4 is a Hanes-Woolf plot (11, 12) of the same data and it, like the Scatchard plot, is typical for a form of competitive inhibition. Qualitatively similar plots were obtained for inhibition by type III collagen.

The collagen types tested in the experiments of Fig. 2 were native triple helical molecules. It is of interest to know whether native helicity critically determines binding specificity. If triple helicity is critical for binding, the separated (denatured) α chains should yield different binding inhibition data than the assembled parent molecules. In the experiments presented in Fig. 5, the three denatured α chains from the minor cartilage isotype and the denatured α1 and α2 chains of type V collagen were individually tested in binding inhibition assays. Whereas the parent triple helical molecules did not inhibit binding (see Fig. 2), each chain class of the cartilage isotype and the α2(V) chain gave equivalent or significant inhibition relative to the LRSC standard. Denatured reduced and alkylated type III chains were better inhibitors of type I binding than native III, and non-denatured reduced and alkylated type IV collagen did not inhibit binding (data not shown). We conclude that triple helicity can confer binding specificity and that denaturation of α chains can expose additional binding determinants.

**Figure 1** Acetylcholinesterase as a competitor of 125I-LRSC binding. Increasing amounts of unlabeled LRSC and the collagenlike tail of acetylcholinesterase were each mixed with 1.36 × 10^5 cpm of 125I-LRSC in binding buffer. Binding studies were performed in replicate cultures and the percentage inhibitions produced by the unlabeled proteins were computed. LRSC, O, acetylcholinesterase.

**Figure 2** Native collagen types as inhibitors of 125I-LRSC binding. Composite of binding inhibition experiments performed with the following native collagen types: type I, LRSC; type II from a rat chondrosarcoma; type III, from human placenta; type IV, from the mouse EHS sarcoma; 1α2α3α cartilage isotype, from human cartilage, and type V, from human placenta.

**Figure 3** Type II inhibition: scatchard plot. Increasing amounts (0–100 μg) of unlabeled LRSC were premixed with a fixed amount of 125I-LRSC (0.16 pmol, 118,000 cpm) in the presence (C) and absence (O) of 5 μg (17.5 pmol) of unlabeled type II collagen. The respective mixtures were presented to replicate plates of 3T3 fibroblasts and specific binding measured after 2.5 h at 26°C. Picomoles of type I collagen (radiolabeled and unlabeled) bound (B) and free (F) were calculated.

**Figure 4** Type II inhibition: Hanes-Woolf plot. Data from experiment of Fig. 3. (L) Picomoles of type I collagen ligand (labeled and unlabeled) added. (B) Picomoles of type I collagen ligand (labeled and unlabeled) bound. (O) 5 μg (17.5 pmol) of unlabeled type II collagen added as inhibitor (D), in absence of type II inhibitor.
Are Telopeptides Required for Binding

The telopeptides are short (10-25 residues) nonhelical extensions at the ends of the collagen α chains. Collagen molecules in solution can self-assemble to form native fibrils (14) and the initiation ("nucleation") of such self-assembly seems to depend upon a thermally induced conformational change in the amino terminal telopeptides (5). Collagen binding to the fibroblast also requires thermal activation of the ligand, and such binding has been proposed as an initiating event for fibrillogenesis (1, 2). Accordingly, experiments were performed to determine whether the telopeptides were necessary for binding of collagen to the fibroblast. The telopeptides were first removed from unlabeled LRSC by limited digestion with α-chymotrypsin or pepsin. Fig. 6 shows the SDS-polyacrylamide gel patterns given by α-chymotrypsin-digested LRSC and the control preparation. The enzyme-digested collagen contains somewhat fewer covalently cross-linked molecules (γ and β forms) and has a major population of α1 and α2 chains with slightly greater mobilities than the control preparation. This result shows that the enzyme digested the telopeptides and did not alter the triple helix in most of the molecules. Binding inhibition assays were performed with enzyme-digested and control LRSC, with and without prior thermal activation (37°C, 30 min) of the samples. Fig. 7 shows that, with heat activation, molecules lacking telopeptides bound as well as intact collagen. In the absence of heat activation, neither preparation displayed significant binding. The latter result shows that the requirement for heat activation is independent of radioiodination of the ligand. Similar data were obtained when the telopeptides were removed by limited digestion with pepsin. We conclude that the telopeptides are not necessary for thermal activation and binding of type I collagen to fibroblasts.

Potential Binding Determinants in Cyanogen Bromide-derived Helical Peptides

Peptides generated by cyanogen bromide cleavage of helical

![Figure 5](image)

**Figure 5** Denatured α chains as competitors of 125I-LRSC binding. Cartilage isotype chains α1, α2, α3, and α1 and α2 chains of type V collagen were resolved by carboxymethyl cellulose chromatography under denaturing conditions (13). The individual chain classes and native LRSC (I) were tested as inhibitors of 125I-LRSC binding.

![Figure 6](image)

**Figure 6** Gel electrophoresis of LRSC and chymotrypsin-modified LRSC. Lane (A): LRSC digested with chymotrypsin at pH 7.5, 15°C, 24 h, at enzyme/substrate ratio of 1/10. Reaction stopped by cooling to 4°C, addition of protease inhibitors and acidification. Collagen separated by three cycles of precipitation with NaCl to 7.5%. Lane (B): Control LRSC. Same protocol, enzyme omitted. 5 µg of collagen applied to both lanes.

regions of α1 type I and III chains were evaluated as inhibitors of type I binding. Fig. 8 depicts the positions in the α chains of the peptides tested and quantifies inhibition by each peptide relative to the type I LRSC standard. The values given below the lines are the ratio of LRSC (µg)/peptide (µg) which produced 30% inhibition of 125I-LRSC binding in the same experiment. For all of the experiments, 1-2 µg of LRSC regularly produced 30% inhibition of binding. Inhibition ratios <0.1 are not considered as significant, and a ratio value of zero means that addition of 100 µg of the peptide produced <30% inhibition of binding.

By the test of inhibition, binding determinants were identified in more than one peptide in each species and type of α chain and these peptides were variably distributed along the chains from amino to carboxy termini. Peptides of similar size from the same regions of type I α chains of different species varied in binding capacities, for example, human α1(I)-CB8 as compared to the homologous peptides of rat and chick.

The rodent α1(I)-CB7 peptide which includes residues 552-822 of the α1 chain is notable for being equivalent on a concentration basis to LRSC as an inhibitor of binding. The same peptide has been reported to contain the determinants for attachment of fibronectin to α1(I) but the following observations indicate that 125I-LRSC is not binding to fibronectin on the fibroblast surface (α) α1(I)-CB7 has been claimed to contain the sole binding site for fibronectin (15), but the data of Fig. 8 show that peptides positioned elsewhere in α1(I) chains can significantly inhibit binding of 125I-LRSC. (b) Critical determinants for binding of α1(I)-CB7 to fibronectin have been localized to the cleavage site for animal collagenase (residues 775-776) and a chymotrypsin-sensitive site (residues 779-780) (15). However, two synthetic peptides which have identical sequences to residues 773-780 and 775-778 of α1(I), respectively, and which serve as substrates for animal collagenase (16), did not inhibit 125I-LRSC binding (binding inhibition

![Figure 7](image)

**Figure 7** Binding inhibition by LRSC and chymotrypsin-modified LRSC. Chymotrypsin-modified LRSC (C) and control LRSC (O), activated at 37°C, 30 min. Chymotrypsin-modified LRSC (C) and control LRSC (O), without thermal activation.

![Figure 8](image)

**Figure 8** Cyanogen bromide-derived helical peptides as inhibitors of binding. Linear schematic of helical α chains of collagen types I and III indicating positions, standard numbering (above lines), and relative sizes of peptides produced by cyanogen bromide cleavages. Only the peptides tested in binding assays are numbered. Numbers below lines are the ratio of µg LRSC/µg peptide that produced 30% inhibition of 125I-LRSC binding. N and C, amino and carboxytermini of the chains, respectively.
Potential Binding Determinants in Propeptides

Collagen is secreted as a precursor molecule (procollagen) that has large polypeptides (propeptides) covalently linked to the terminal telopeptides of the three helical α chains of collagen. Type I procollagen could bind to the cell surface through the demonstrated determinants in its central triple helical region but it is possible that propeptide residues could also contribute to procollagen binding. A reasonable candidate for a propeptide binding region is the short, collagenlike segment (“Col 3”) in the aminoterminal propeptides of type I procollagen (see reference 17 for review). When the aminopropeptide isolated from the chick pro α1(I) chain (18) was tested as an inhibitor of 125I-LRSC binding, it gave a binding inhibition ratio of 0.19, indicating some affinity for the cell binding site.

The carboxyterminal propeptides of type I procollagen do not contain any collagenlike structure and they failed to inhibit 125I-LRSC binding in the standard assay system. The protein tested was a three-chain, disulfide-linked fragment of 100,000 daltons isolated from human type I procollagen (19).

DISCUSSION

Quantitative inhibition assays have further defined the specificity of binding of native, soluble radiiodinated type I collagen to the fibroblast surface. The binding is not inhibited by every collagenous molecule; thus, the collagenlike tail of acetylcholinesterase and native collagen types IV, V and cartilage Iα2α3α do not inhibit binding. Only native collagen types II and III inhibit type I binding, and graphical analyses indicate that they act as competitive inhibitors. These inhibitions can be ascribed to interactions with type I cell binding sites or to complexes formed directly with type I collagen. Radiiodinated type II collagen binds to the fibroblast (unpublished data), so there is independent evidence for a cell binding site for this collagen type. In terms of receptor models, competitive inhibition can mean that collagens I, II, and III bind to the same cell receptor site, to different sites that interact allosterically, or to both shared and independent sites. Interactions of collagens I, II, and III with a common receptor on the fibroblast can be interpreted in the following context. Collagen types I and III are synthesized by the fibroblast and the mesenchymal precursor of the differentiated chondrocyte. Although the latter cell type synthesizes only type II collagen, it is prone to dedifferentiate and to switch to the synthesis of collagen types I and III (20). The data suggest, therefore, that the same cell binding complex is used in the course of these phenotype conversions.

Previously published and less complete binding inhibition data supported the view that the primary structure of collagen α chains determined the binding specificity of the assembled molecule (1). We currently report that α chains separated from collagen types V and cartilage Iα2α3α inhibit type I binding but that the triple helical molecules formed from such chains are not inhibitory in the assays. We now conclude, therefore, that tertiary or quartenary molecular structure can critically modify the binding specificities of the collagens.

Enzymatic removal of telopeptides from native unlabeled type I collagen did not alter its capacity for inhibiting 125I-LRSC binding nor obviate the requirement for thermal activation of the ligand.

Potential binding sites in helical α1(I) and α1(III) chains from different species were partially mapped by testing a limited number of cyanogen bromide-derived peptides as inhibitors. Inhibitory and noninhibitory peptides were thus identified, and inhibitory activity was shown to be subject to species variation. As all of the peptides shared the repeating collagen triplet Gly-X-Y and were not much different in their content of proline and hydroxyproline, these elements of collagen primary structure must be insufficient as determinants of binding specificity. Every α1(I) and α1(III) chain was shown to contain two or more inhibitory peptides and, as considered as a group, such peptides were variously positioned in the chains. It seems reasonable to suppose that some or all of these regions contribute to binding determinants expressed by the assembled triple helical molecules.

Fibronectin is a reasonable candidate as the molecule responsible for the binding of collagens to the fibroblast surface. However, the ligand-binding specificities described above do not correspond to those reported for collagen-fibronectin interactions. In contrast to our data, fibronectin binds to the collagenlike tail of acetylcholinesterase (21), has different binding affinities for the various collagen types (22-24), and has been reported to bind exclusively to a single region of the α1(I) chain (15). Because of these discordancies and other data (1), we do not favor the view that fibronectin is responsible for the binding of native, soluble 125I-LRSC to the fibroblast surface.

The reported data are interpreted to mean that ligand determinants distributed over the central collagen triple helix are sufficient to bind the interstitial procollagens and collagens (types I, II and III) to the fibroblast plasma membrane. Such binding is proposed as an in vivo mechanism for initiating fibril assembly and for specifying the spatial order of that assembly (1, 2). The essential element of the hypothesis is that binding to a membrane component is determine for fibrillogenesis, and so the model can include those cases where procollagen fibrils apparently assemble within intracellular vacuoles (25, 26). The binding model allows for modulations of fibril assembly by cell receptor metabolism as well as by ligand-ligand interactions and, if there are independent binding sites for each collagen type, the model provides a mechanism for segregation of type-specific fibrils. The model assumes that, upon achieving a limiting size, the soluble surface-bound aggregates detach from the cell and that they then combine to form insoluble fibrils. Perhaps it is at this later stage of fibrillogenesis that collagen-fibronectin and proteoglycan interactions become important. The binding model as formulated is quite compatible with proposals by others that type I collagen fibrils form by stepwise additions of molecular aggregates (see reference 27, for review).

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