The Tandemly Repeated Sequences of Cartilage Link Protein Contain the Sites for Interaction with Hyaluronic Acid

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Abstract. The ternary complex involving link protein (LP), proteoglycan monomer, and hyaluronic acid (HA) is an important component of the extracellular matrix of cartilage. LP contains tandemly repeated sequences that were tested for their ability to interact with HA. A solid-phase assay was developed in which LP could specifically bind to immobilized HA. Detection of LP was by means of an antiserum directed against a peptide from the NH_2-terminal half of LP. LP binding to HA could be inhibited with mAb 8A4 (Caterson, B., J. R. Baker, J. E. Christner, Y. Lee, and M. Lentz. 1985. *J. Biol. Chem.* 260:11348–11356). Using synthetic peptides that correspond to specific amino acid sequences of chicken LP (Deák, F., I. Kiss, K. J. Sparks, W. S. Argraves, G. Hampikian, and P. F. Goetinck. 1986. *Proc. Natl. Acad. Sci. USA.* 83:3766–3770) the epitopes for mAb 8A4 were determined to reside in peptides Gly^{217}-Pro^{226} and Arg^{316}-Arg^{325}. These two peptides were also capable of inhibiting the interaction between LP and HA. The peptide Trp^{242}-Val^{251} and Pro^{339}-Val^{348} could also inhibit the interaction between LP and HA. All four peptides reside in the tandemly repeated domains of LP and they contain clusters of positively charged amino acids. Polylysine could not inhibit the interaction of LP with HA. The results indicate that the sites for interaction with HA are in the tandemly repeated sequences of LP and that there are four potential sites available for that interaction.

THE extracellular matrix of cartilage consists of a number of interacting macromolecules that are unique to that tissue. The most studied group of these molecules are those that make up the ternary complex involving proteoglycan monomer, link protein (LP)[1], and hyaluronic acid (HA) (10). As many as 100 proteoglycan monomers and LPs can be found to interact with a single HA polymer (8). Although the molecular basis for the formation of the ternary complex is not known, it is thought that LP and proteoglycan monomer interact with HA in a similar manner and there is evidence that this type of interaction is different from that observed between LP and proteoglycan monomer (12). Chemical modification of either LP (14) or proteoglycan (9) have implicated arginine and lysine residues in the binding of these macromolecules to HA. Also, proteoglycan monomers that have been synthesized in the presence of the arginine analog, canavanine, have a reduced ability to interact with HA (Bowik, C. C., and P. F. Goetinck, manuscript in preparation). When uronic acid residues in HA are modified, the interaction with proteoglycan monomers is also eliminated (4). Taken together these observations suggest an ionic type of interaction between positively charged amino acid residues in LP and proteoglycan monomer and the negatively charged uronic acid residues in HA. The recent elucidation of the complete amino acid sequence of rat chondrosarcoma (16) and chicken (5) LP has revealed that these molecules are composed of three discrete domains. The first domain (Domain I) consists of the NH_2-terminal half of the molecule and it possesses homologies with immunoglobulin-like proteins (2). The second half of the molecule contains tandemly repeated sequences (5, 6, 16) (Domains IIa and IIb) that have homologies with an amino acid sequence of the HA-binding region of cartilage proteoglycan monomer (17). Based on the sequence homology between LP and proteoglycan monomer it has been suggested (5, 17) that the tandemly repeated sequences contain the sites for interaction with HA. Indeed, the tandem repeats contain four clusters of positively charged amino acids that could be involved in the interaction of link protein with HA. This communication reports on the identification of the amino acid sequences in LP that are involved in the binding of this molecule to HA.

1. *Abbreviations used in this paper*: GAG, glycosaminoglycan; HA, hyaluronic acid; KLH, Keyhole Limpet Hemocyanin; LP, link protein.

Materials and Methods

Purification of LP and Proteoglycan Monomer

Cartilaginous xyphoid processes of 4–6-wk-old White Leghorn chickens were extracted using dissociative procedures. LP and proteoglycan monomer were purified by sequential associative and dissociative CsCl centrifugation of the extracts according to the method of Hascall and Sajdera (11). All extractions and centrifugations were done in the presence of the protease inhibitors introduced by Oegema et al. (18). LP obtained from the second CsCl gradient was separated from any residual proteoglycan by chromatography on DEAE-cellulose in the presence of 8 M urea (1). LP was recovered

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in the unbound fraction. Upon analysis of the unbound fraction in 10% SDS-PAGE (13), two bands were revealed when stained for protein with Coomassie Blue. These had relative molecular masses of 48,000 and 42,000 D as previously reported (15). Upon transfer of the proteins (which were subjected to electrophoresis) to nitrocellulose (21), both bands bound to a polyclonal antiserum directed against purified avian LP (15) and with the antiserum directed against peptide Gly^{100}-Arg^{112} described in this paper.

Synthetic LP Peptides

Peptides corresponding to specific amino acid sequences of LP were synthesized using an automated peptide synthesizer (model No. 430A; Applied Biosystems, Inc., Hauppague, NY) equipped with the chemistry provided by the manufacturer. After cleavage from the resin with hydrogen fluoride, the peptides were washed with cold ethyl ether, redissolved in water, and lyophilized. Coupling efficiency was monitored after the addition of each residue. Peptide purity was tested by HPLC on a C-18 column (3.9 mm internal diameter × 300 mm; Waters Chromatography Division, Millipore Corp. Milford, MA; microBondapark). In every instance the chromatograms gave a single major peak indicating an estimated purity of 90% or greater. The numbering system used in the identification of the peptides is based on that introduced by Deák et al. (5) in which the initiation methionine of the 15-amino acid signal peptide is considered as the first residue. The following synthetic peptides were used in this study:

Peptide from Domain I:

Gly^{100}-His-His-Arg-Lys-Ser-Tyr-Gly-Lys-Tyr-Gln-Gly-Arg^{112} (Peptide Gly^{100}-Arg^{112});

Peptides from Domain IIa:

Asn^{207}-Ala-Gly-Trp-Leu-Ser-Asp-Gly-Ser-Val-Gln-Tyr-Pro-Ile-Thr-Lys-Pro-Arg-Glu-Pro^{226} (Peptide Asn^{207}-Pro^{226});

Gln^{217}-Tyr-Pro-Ile-Thr-Lys-Pro-Arg-Glu-Pro^{226} (Peptide Gln^{217}-Pro^{226});

Trp^{242}-Asp-Lys-Glu-Arg-Ser-Arg-Tyr-Asp-Val^{251} (Peptide Trp^{242}Val^{251});

Peptides from Domain IIb:

Asp^{306}-Ala-Gly-Trp-Leu-Ala-Asp-Gly-Ser-Val-Arg-Tyr-Pro-Ile-Ser-Arg-Pro-Arg-Lys-Arg^{325} (Peptide Asp^{306}-Arg^{325});

Arg^{316}-Tyr-Pro-Ile-Ser-Arg-Pro-Arg-Lys-Arg^{325} (Peptide Arg^{316}-Arg^{325}); and

Pro^{339}-Asp-Lys-Lys-His-Lys-Leu-Tyr-Gly-Val^{348} (Peptide Pro^{339}-Val^{348}).

Polylysine (8-12) was purchased from IC Immunobiologicals (Lisle, IL).

Antibodies

A polyclonal antiserum was generated in rabbits against peptide Gly^{100}-Arg^{112} coupled to Keyhole Limpet Hemocyanin (KLH) by means of *N*-succinimidyl-3-(pyridylithio)propionate (SPDP) to a cysteine residue introduced at the amino group of Gly^{100} during the synthesis of the peptide. The conditions were based on those described by Pierschbacher et al. (19). For each immunization 3 mg of the peptide was conjugated to 1 mg of KLH with 0.5 mg of SPDP in 1.5 ml of PBS. For the first immunization the mixture was combined with an equal volume of Freund's complete adjuvant, and for booster immunizations with Freund's incomplete adjuvant. The anti-LP monoclonal antibody 8A4 (3) was obtained as a culture supernatant from the Developmental Studies Hybridoma Bank maintained by contract NO-HD-6-2915 from National Institute of Child Health and Human Development (NICHD, Bethesda, MD). Goat anti-rabbit and goat anti-mouse IgG alkaline phosphatase conjugates were obtained from BioRad Laboratories (Richmond, CA).

Binding Assays

All macromolecular interactions were studied using ELISAs (7). Immobilization of macromolecules to microtiter plates (EIA, Lindbro; Flow Laboratories, McLean, VA) was carried out in 0.05 M sodium carbonate buffer, pH 9.6, overnight at room temperature. All subsequent additions in the binding assays were done for 1 h in PBS containing 0.05% Tween (PBS-T). After each addition the plates were washed with PBS-T. HA was immobilized by applying in each well 60 µl of a solution containing 50 mg/ml of HA. For each determination of the binding of LP to HA, 180 ng of LP was first allowed to react with antibodies against peptide Gly^{100}-Arg^{112} and then added as a complex to an HA-coated well. Detection of bound LP was with a goat anti-rabbit IgG alkaline phosphatase conjugate (1:400). The substrate was *p*-nitrophenyl phosphate (0.1%) in diethanolamine (1 M), pH 9.8, containing 1.0 mM $MgCl_2$. LP was immobilized by applying in each well 10 µl of a solution containing 9 mg/ml of LP. Binding of mAb 8A4 was monitored with goat anti-mouse IgG alkaline phosphatase conjugate (1:400) followed by the addition of substrate as indicated above. The optical density developed in the ELISA was determined at 405 nm in the linear portion of the colorimetric assay using a plate reader (Artek Systems Corp., Farmingdale, NY). Glycosaminoglycans (GAGs) were obtained through the courtesy of Drs. M. B. Mathews and J. A. Cifonelli of The University of Chicago (Chicago, IL). Protein concentrations were determined with the BioRad Laboratories reagent using BSA as a standard.

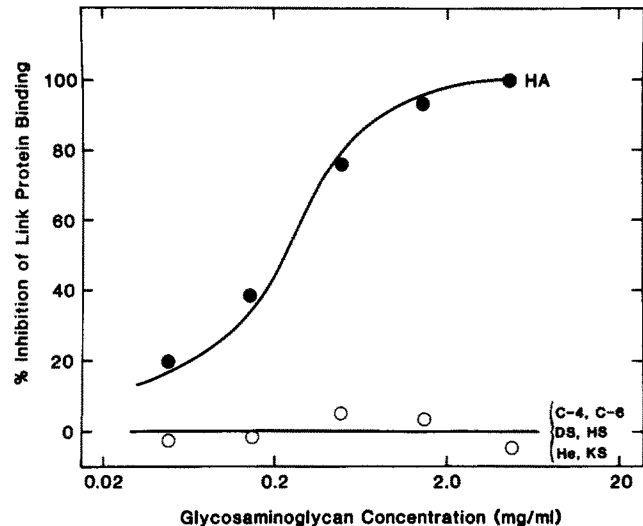

Figure 1. Effect of soluble GAGs on the binding of LP to immobilized HA. *HA*, hyaluronic acid; *C-4*, chondroitin-4-sulfate; *C-6*, chondroitin-6-sulfate; *DS*, dermatan sulfate; *HS*, heparan sulfate; *He*, heparin; *KS*, keratan sulfate. The open circles represent the average of the individual values for each of the six GAGs used at each concentration indicated.

Results

Specificity of the Binding Assay

Peptide Gly^{100}-Arg^{112} was selected for the generation of antibodies to measure the interactions between LP and HA because this peptide is situated in domain I of LP and this domain was hypothesized not to be involved in the interaction with HA. Thus, the detecting antibody would not interfere with the measurement of the LP-HA interactions.

The specificity of the solid-phase assay for measuring LP-HA interactions was determined in competition experiments involving soluble GAGs. The binding of LP to immobilized HA could be inhibited only with soluble HA and not with soluble chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratan sulfate, heparin, or heparan sulfate (Fig. 1). Furthermore, when these GAGs were immobilized onto microtiter plates, binding of LP could be demonstrated only to HA (data not shown).

The effect of ionic strength on both the maintenance and the establishment of the LP-HA interactions was investigated to determine if the observed interactions are ionic in nature. To test the maintenance of the interactions, LP was allowed to bind to immobilized HA in PBS-T and was subsequently washed with solutions of increasing concentration of NaCl.

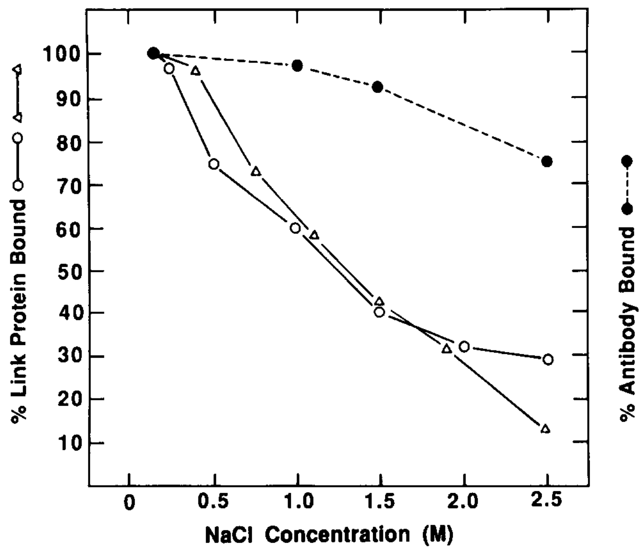

Figure 2. Effect of NaCl concentration on the establishment and maintenance of LP binding to immobilized HA. (○——○) LP was added to the wells of microtiter plates in solutions of increasing levels of NaCl concentration. (△——△) LP was added to the wells in 0.15 M NaCl and then washed with solutions of increasing NaCl concentrations. (●- - -●) LP was immobilized to microtiter plates, challenged with antipeptide Gly^{100}-Arg^{112} antibodies and then washed with solutions of increasing NaCl concentrations.

The binding of LP to HA decreased linearly as the NaCl concentrations were increased. Similar results were obtained when the establishment of binding was tested at increasing concentrations of NaCl (Fig. 2). These results indicate that ionic interactions play a large role in the binding of LP to HA in the assays used. To ascertain that the salt effect observed was not occurring at the level of binding of the anti-peptide antibody to LP, antibody was allowed to bind to immobilized LP and then washed with solutions of increasing salt concentrations. Only a minor reduction in binding is observed at the higher salt concentrations used (Fig. 2).

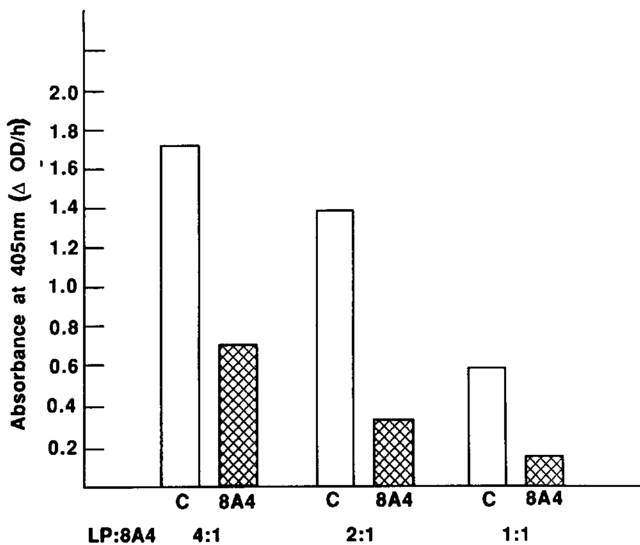

Figure 3. Inhibition of binding of LP to immobilized HA with mAb 8A4. Decreasing quantities of LP-antipeptide antibody complex were mixed with the same amount of the mAb 8A4 before the addition to HA-coated microtiter plates. *C*, control; *8A4*, mAb 8A4.

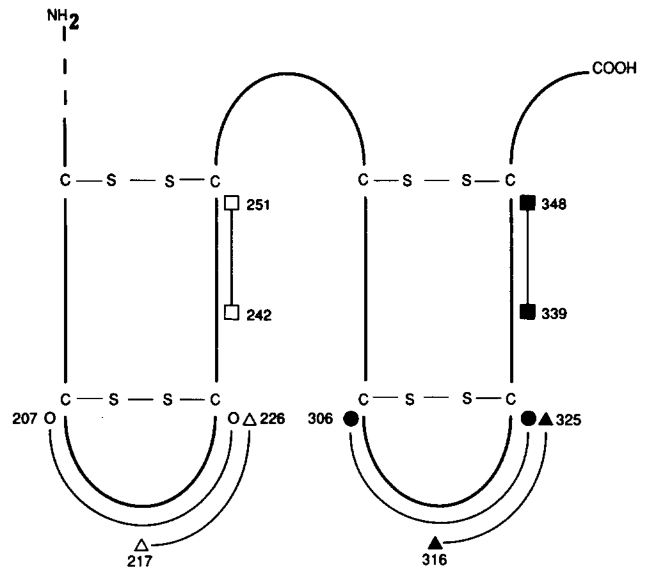

Figure 4. Schematic representation of the tandemly repeated sequences of chicken LP representing domains IIa and IIb. The location of the synthetic peptides used in the study are indicated. The dotted line representing the NH_2-terminal half of LP corresponds to domain I.

Monoclonal Antibody (mAb) 8A4 Inhibits the Binding of LP to HA

mAb 8A4 is directed against epitopes on LP from a large number of species (3). In the present study it was found that mAb 8A4 could inhibit the binding of LP to immobilized HA (Fig. 3). Solutions containing 180, 90, and 45 ng of LP were mixed with an equal quantity of mAb 8A4 culture supernatant and added to HA-coated wells of microtiter plates. The amount of LP bound in control groups decreased in proportion to the amount of LP added. The inhibitory effect of mAb 8A4 for each of the LP concentrations was 63, 74, and 87%, respectively. Lowering the quantity of LP even further did not increase the inhibitory effect of the mAb 8A4 used.

Identification of the Epitopes for mAb 8A4

The epitopes recognized by mAb 8A4 have been localized on tryptic peptides that map between the two central cysteine residues located in domains IIa and IIb of LP (17). In an effort to map precisely the epitopes for mAb 8A4 a series of synthetic peptides were used in inhibition assays involving LP and mAb 8A4. The peptides used are designated on the diagram of the tandemly repeated sequences of LP in Fig. 4. The symbols, used in Fig. 4 to designate the peptides, are also used in later figures. The results of the addition of the various peptides on the binding of mAb 8A4 to LP are shown in Fig. 5. The two 20-amino acid peptides (Asn^{207}-Pro^{226} and Asp^{306}-Arg^{325}) situated between the two centrally located cysteine residues of domains IIa and IIb, respectively, reduce the binding of mAb 8A4 to LP at a concentration of 10^{-6}–10^{-5} M. The two 10-amino acid sequences (Gly^{217}-Pro^{226} and Arg^{316}-Arg^{325}) inhibit the binding of mAb 8A4 to LP to the same extent. These two peptides contain the positively charged amino acid sequences between the two centrally located cysteine residues of domains IIa and IIb. In

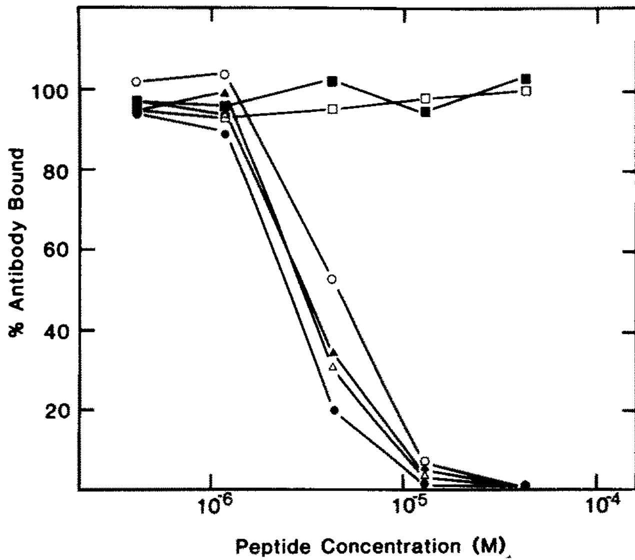

Figure 5. Effect of synthetic LP peptides on the binding of mAb 8A4 to immobilized LP. The symbols used are those given in Fig. 4.

contrast, two other sequences within domains IIa and IIb that contain clusters of positively charged amino acids (Trp[242]-Val[251] and Pro[339]-Val[348]) fail to inhibit the binding of mAb 8A4 to LP in that concentration range. Inhibition by these peptides could be demonstrated only at concentrations of 10^{-2} M (data not shown).

Peptide Inhibition of LP Binding to HA

The ability of each of the peptides to inhibit the binding of LP to HA was investigated next. Fig. 6 *A* shows the effects of the three peptides from domain IIa. All three peptides inhibit the interaction of LP with HA in a similar dosage-dependent manner. The three peptides from domain IIb inhibit the interaction between LP and HA to the same degree (Fig. 6 *B*). To test the possibility of cooperativity between the peptides, equimolar mixtures of the two 10-amino acid peptides from domain IIa (Fig. 5 *C*) and of the four 10-amino acid peptides from domains IIa and IIb (Fig. 6 *D*) were tested. Both mixtures inhibited the LP-HA interaction at a total molar concentration that was identical to the effective concentrations of any one of the individual peptides. That the inhibitions observed are a specific result of the LP peptides rather than from a nonspecific effect of the interaction of positively charged peptides with HA is evident from the inability of lysine peptides to show inhibition (Fig. 6 *D*).

Discussion

The data presented in this paper provide experimental evidence that supports the hypothesis that the HA-binding sites of LP are located in the tandemly repeated domains of this matrix molecule. The method for testing the interaction between LP and HA was a solid-phase ELISA in which the detecting antibody was directed against a peptide sequence in a domain of LP predicted not to be in the HA-binding domains. The test, as developed, was specific for the interactions between LP and HA. The binding of LP to immobilized HA could be inhibited only by soluble HA and not by any other soluble GAGs tried. Furthermore, when GAGs were immobilized, LP binding could be demonstrated only to immobilized HA. The LP-HA interactions measured were largely of an ionic nature and they could be reduced with solutions of increasing ionic strength. At an NaCl concentration of 1.0 M the binding of LP to HA was reduced by 40%. This value is very close to the 37% inhibition observed by Tengblad (20) for the same NaCl concentration.

The HA-binding sites of LP were identified in two ways.

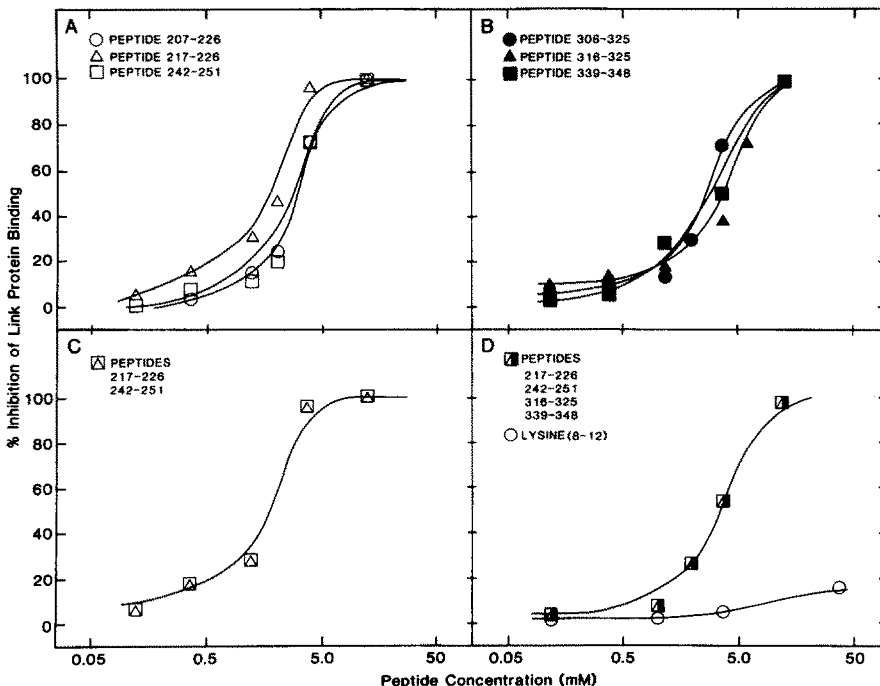

Figure 6. Effect of synthetic LP peptides on the binding of LP to immobilized HA. (*A*) Peptides from domain IIa tested individually. (*B*) Peptides from domain IIb tested individually. (*C*) Equimolar mixture of two peptides from domain IIA. (*D*) Equimolar mixture of four peptides, two from domain IIa and two from domain IIb. *D* also demonstrates the lack of effect of lysine peptide on the LP-HA interaction. The symbols used are the same as those shown in the legend of Fig. 4.

It was shown first that the binding of LP to HA could be inhibited by mAb 8A4 and second that the two 10-amino acid peptides Gln^{217}-Pro^{226} and Arg^{316}-Arg^{325} could block the binding of mAb 8A4 to LP. The identification of these two peptides as epitopes for mAb 8A4 extend the results of Neame et al. (16) who had shown that the antibody bound to LP in the 20-amino acid regions that are situated between the two centrally located cysteine residues of each of the two repeats. The combination of the results of the mAb 8A4 blocking experiments and the identification of the epitopes for that antibody permitted the identification of peptides Gln^{217}-Pro^{226} and Arg^{316}-Arg^{325} as two sites on LP that are involved in the binding to HA. The fact that these two peptides are situated in the regions that show the highest degree of homology between domains IIa and IIb is consistent with the view that conserved sequences reflect a conservation of function. The results of the experiments in which the LP-HA interaction was blocked with mAb 8A4 were supported and extended with the results of tests in which peptides were used to inhibit the binding between LP and HA. All the peptides that contain the epitopes for mAb 8A4 could compete with LP for binding to HA and thus inhibit the interaction between LP and HA. In addition, peptide Trp^{242}-Val^{251} of domain IIa and peptide Pro^{339}-Val^{348} of domain IIb could also inhibit the interaction of LP with HA. These two peptides also contain clusters of positively charged residues. Therefore, domains IIa and IIb each contain two peptides with clusters of positively charged amino acids that can inhibit the interaction of LP with HA. Thus, the binding of LP to HA may involve four or more subsites of the tandemly repeated domains.

All the available evidence indicates that the interactions between LP and HA are largely of an ionic nature (12, 14, 20, the present studies). The inhibitory effect of the LP peptides, therefore, could be the result of nonspecific blocking of binding sites on HA by the positively charged peptides. That this is not the case is evident from the results obtained with the polylysine peptide of 8-12 residues. This peptide was not able to inhibit the binding of LP to HA. Therefore, we conclude that the inhibitory effect of the LP peptides is specific. When peptides that could inhibit the LP-HA interaction singly were mixed in equimolar combinations, there was no evidence of any cooperativity in the inhibition. These results are interpreted to mean that each peptide is probably recognizing the same site on HA and that it is the quantity of any one of the four peptides that recognize HA that is important. Together the results indicate that LP can bind to HA through four different sites. It is not possible to conclude from the present studies if there are primary and secondary sites of interaction. The observation that mAb 8A4 can inhibit the binding of LP to HA by 87% can be interpreted to mean that the two sites recognized by the antibody are primary sites of interaction. Alternatively, the mAb blocking experiments can also be interpreted to mean that all four sites are needed for the binding of LP to HA. Indeed, in view of the highly repetitive structure of HA, multiple-binding sites may be necessary to provide sufficient affinity for LP to interact detectably with the GAG. The four sites on LP may be spaced for optimum binding to HA through the two disulfide bonds in each of the two repeated domains. This mechanism would explain why we have not been able to detect binding of the peptides directly to HA.

Each of the tandemly repeated domains, IIa and IIb, contain four cysteine residues that form disulfide bonds as shown in Fig. 4. A comparison of the amino acid sequence between the most NH_2- and COOH-terminally located cysteine residues of each domain reveals that the homology between similar domains of different species is greater than the homology between the two domains within a species. For example, the chicken-rat homologies are 90 and 94% for domains IIa and IIb, respectively. In contrast, the homology between the sequences of domains IIa and IIb of both chicken and rat are 46%. From an evolutionary point these calculations would suggest that the duplications of the two domains occurred before the separation of birds and mammals. The homology between domains IIa and IIb within a species is highest for the sequences of 22 amino acids involving the two centrally located cysteine residues of each repeat. The homology value for these regions is 68% for the chicken LP and 63% for the rat LP. This relatively high degree of homology between these two regions could be interpreted to mean that they contain conserved functional domains. Indeed, these sequences contain the epitope for mAb 8A4 and, therefore, the conclusion based on homology between domains is in agreement with the experimental evidence presented in this study. However, the homology analysis does not address the question of possible primary and secondary sites of interaction with HA. Experiments in which specific sites are modified by site-directed mutagenesis will help clarify this point.

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