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Identification and Characterization

of Type II Collagen Mutations

by

Raymond Bogaert

A dissertation submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Washington

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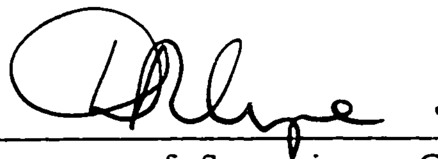

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University of Washington

Abstract

IDENTIFICATION AND CHARACTERIZATION OF TYPE II COLLAGEN MUTATIONS

by Raymond Bogaert

Chairperson of the supervisory committee
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Collagen type II is a prototypic fibrillar collagen. It is the main structural protein of articular cartilage and the cartilaginous enlage of endochondral bone development. One way to study the normal structure and function of type II collagen is to study the structure of abnormal type II collagen in inherited diseases of the skeleton.

Within the skeletal dysplasias a small subset of chondrodysplasias are emerging that are produced by mutations in the gene for type II collagen, COL2A1. This dissertation presents thru protein biochemistry the identification and characterization of structurally defective type II collagen in cases of hypochondrogenesis, spondyloepimetaphyseal dysplasia (SEMD), and Kniest dysplasia.

In a perinatal lethal form of hypochondrogenesis a glutamate for glycine(853) substitution was identified. In the SEMD case a serine for glycine(841) was identified. And in two unrelated probands with Kniest dysplasia a deletion of a seven amino acids(102-108) was identified.

The type II collagen from the cartilage of these affected probands and from additional cases of hypochondrogenesis and premature osteoarthritis were post-translationally overmodified. Amino acid sequencing of specific lysines revealed that the overmodification occurred primarily amino terminal to the mutation site and the degree of overmodification was proportional to the severity of the disease phenotype. The exception was the two Kniest dysplasia probands. The seven amino acid deletion was unique for it disrupted the Gly-x-y repeat of the triple helix. Type II collagen from these probands was overmodified amino and carboxy terminal to the mutation site.

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INTRODUCTION

This dissertation contains the identification and characterization of structurally defective type II collagen at the protein level from inherited diseases of skeletal development. In three cases the defects were substitutions for glycine. In two other cases the defect was an identical seven amino acid deletion. These findings contribute to our understanding of the pathogenesis of inheritable diseases.

A brief review of collagen structure and function is presented first followed by a review of inherited disorders of collagen. Much of the experimental studies have been previously presented (Bogaert et al., 1992 and 1994).

CHAPTER I

A BRIEF REVIEW OF COLLAGEN STRUCTURE

Collagen Family of Proteins

Collagen molecules are the building blocks that form the framework of connective tissues. Their main molecular feature is the collagen triple-helical domain.

The collagen triple helix is composed of three polypeptide chains in a coiled-coil structure. Each polypeptide chain is coiled into a left-handed helix. The primary structure of each left-handed helix consists of repeating units of Gly-X-Y, such that glycine is stacked upon each preceding glycine with every turn of the helix staggered about 30 degrees. Three left-handed helices are coiled or twisted around each other to form a right-handed triple helix. In order to form this structure each left-handed helix must have glycine , the smallest amino acid facing towards the center of the triple helix. Mutations that substitute a bulkier amino acid for glycine or that disrupt the Gly-X-Y repeat alter the structure of the collagen triple helix (Byers 1993).

The remaining amino acids in the collagen triple helix are frequently the imino acids proline and hydroxyproline in the X and Y positions respectfully. The presence of hydroxyproline serves to stabilize the triple helix with additional hydrogen bonds. Since the amino acids in the X and Y positions face outwards from the helix, they probably serve to laterally interact with other collagen molecules.

The collagen triple helical domain is also found in other proteins. In these proteins and collagen as well, the triple helical domain serves as a semi-rigid rod to physically separate domains on either end of the helix, while the amino acid side chains in the X and Y positions have the potential to interact with adjacent helices, other proteins, or cells. Proteins such as C1q, lung surfactant protein, macrophage scavenger receptor, conglutinin and acetylcholine esterase contain collagen triple helical domains but do not contribute to the structure of the extracellular matrix and are therefore not considered as members of the collagens(van der Rest and Garrone, 1991).

Currently there are at least 19 collagen types (Prockop and Kivirikko, 1995). The numbering system of collagen uses Arabic

numerals for individual polypeptide chains or alpha chains and Roman numerals for each collagen type. Each individual alpha chain is the product of a distinct gene, except for the $\alpha 3$(XI) chain which is a product of the gene for collagen type II, COL2A1(Vuorio and de Crombrugghe, 1990). Collagen molecules exist as homotrimers or heterotrimers of α chains. These molecules usually contain alpha chains from the same collagen type, although hybrids or isoforms may be formed from alpha chains of different collagen types such as the hybrid formed from the alpha chains of collagen types V and XI (Niyibizi and Eyre, 1989).

The collagen family of proteins can be divided into fibril forming, fibril associated, and nonfibrillar collagens. The fibrillar collagens form quarter-staggered collagen fibrils. The FACIT (Fibril-Associated Collagen with Interrupted Triple helices) collagens as proposed by Olsen (Gordon and Olsen, 1990), do not form fibrillar collagen themselves, but are associated with the surfaces of fibrillar collagen. The nonfibrillar collagens are a heterogeneous group that participate in the formation of specific microstructures, for example beaded fibrils, anchoring fibrils, and membranes (van der Rest and Garrone, 1991).

Fibrillar Collagen

Collagen type I is the prototype fibrillar collagen. It is a heterotrimer composed of two $\alpha1(I)$ chains and one α 2(I). The main feature of this collagen type is the uninterrupted triple helical domain. The molecule also contains two noncollagenous domains or globular domains, the amino terminal or N-propeptide and the carboxyl terminal or C-propeptide.

The fibrils formed from type I collagen have a characteristic banding pattern or striations seen with the electron microscope. The banding pattern is attributed to the molecules being staggered by one D or 67 nm in the fibrils. The molecules are 4.4 D in length, thus they are quarter-staggered.

Collagen types II, III, V, and XI also form D-staggered fibrils. These collagen types along with type I collagen are structurally and evolutionarily related, and hence belong to the subfamily or group of fibrillar collagens. All of these collagens contain an uninterrupted triple helical domain of about 1014 amino acids bordered on both ends with N and C-propeptides. The C-propeptides contain disulfide bonds and are highly homologous to each other (Dion and Myers, 1987). The N-propeptides are variable in size and sequence, and

may reflect unique functions of this domain that are as yet unidentified. The N and C-propeptides are cleaved from the molecule during extracellular processing, except for collagen types V and XI which retain their N-propeptides (Eyre and Wu, 1987; Fessler et al., 1985).

The structural similarities of these collagens are also seen at the gene level. The intron-exon structures are similar with a predominance of 54 base pairs in each exon. This conservation in gene structure can be traced back to sponges and sea urchins (Esposito and Garrone, 1990). It is proposed that these collagens evolved from a single ancestral gene.

Fibril Associated Collagen

The FACIT collagens includes collagen types IX, XII, and XIV (Gordon and Olsen, 1990). These collagen types do not form fibrils, but are intimately associated with the surface of fibrillar collagen (Vaughan et al., 1988; Sugrue et al., 1989). The FACIT collagens are composed of NC (noncollagenous) domains separated by COL (triple helical) domains. These domains are numbered from the carboxyl to the amino terminus. The COL domains perform at least two functions. The COL domain near the carboxyl terminus serves to

provide lateral interactions with the fibrillar collagens. The COL domain near the amino terminus serves as a rod to extend the amino terminal NC domain away from the fibrils.

The NC domains are mainly short noncollagenous sequences that interrupt the COL domains providing flexibility to the molecules. The amino terminal NC domain is unique to the other NC domains. It is variable in size and shape, and its' extension out into the matrix may allow for interactions with cells or extracellular matrix molecules (van der Rest and Garrone, 1990).

The prototype FACIT molecule is collagen type IX. It is a heterotrimer composed of three different α chains; α1(IX), α2(IX), α3(IX). It is found mainly in cartilage and in tissues of the eye, associated with type II collagen. Type IX collagen contains four NC domains and three COL domains. The COL1 and COL2 domains function to provide lateral interactions with fibrils of collagen type II. These interactions are stabilized by intermolecular cross-links (Eyre et al. 1987).

The size of the NC4 domain varies depending on the use of an alternate promoter which may be tissue specific (Nishimura et al., 1989). The NC3 domain acts like a hinge allowing the COL3 domain

to extend away from the fibril. The NC3 domain of the $\alpha 2(IX)$ chain also contains an attachment site for the proteoglycan chondroitin sulfate. The size of this glycosaminoglycan also appears to be tissue specific (Yada et al., 1990).

Collagen type XII is a homotrimer of $[\alpha 1(XII)]_3$, and is found in tissues containing type I collagen. It has three NC domains and two COL domains. The COL1 domains of collagens type IX and XII are homologous and may allow for the interaction of type XII collagen with type I collagen in the same manner as type IX collagen interacts with type II collagen (van der Rest and Garrone, 1991). The NC3 domain of type XII collagen is very large and has a cruciform shape in rotary shadowing experiments (Dublet et al., 1989).

Collagen type XIV is similar to type XII. It also contains a COL1 domain that may function similar to the COL1 domains of collagen types IX and XII (van der Rest et al., 1993).

Nonfibrillar Collagen

The nonfibrillar collagens form a variety of structures. These include basement membranes, nonstaggered fibrils, and sheet-like structures. The collagens in this group are types IV, VI, VII, VIII, and X.

Collagen type IV, also known as basement membrane collagen, exists as a heterotrimer of $[\alpha 1(IV)]_2,[\alpha 2(IV)]_1$ in most basement membranes, but there are also three additional α chains (α3,α4,and α5) whose molecular assemblies are unknown. These α chains have been found in basement membranes of the kidney and the ear (Mochizuki, T. et al. 1994).

Type IV collagen is a long interrupted triple helical molecule. These interruptions (non gly-x-y) give the molecule the flexibility required for the formation of a network of interlaced cords(Timpl, R. 1989). The formation of this network in basement membranes is facilitated by the interactions of two domains. The 7S domain, a triple helical region near the amino terminus, is responsible for laterally interacting with three 7S domains from other type IV collagen molecules in an anti-parallel fashion with the remaining helical portions extending out like legs of a spider(Yurchenco, P.D. et al., 1986). This tetrameric arrangement is the basic structural unit of type IV collagen. The carboxyl terminal domain, NC1, dimerizes with NC1 domains of other molecules for tail-to-tail binding of tetramers (Yurchenco, P.D. et al., 1986).

Type VIII collagen, initially called EC collagen(Sage, H. et al., 1980), is found in Decemet's membrane(Labermeier and Kenney, 1983) and blood vessel membranes. The molecule is probably a heterotrimer of $[\alpha 1(VIII)]_2$,$[\alpha 2(VIII)]_1$. Type VIII collagen is a short triple helical molecule with interruptions. It retains it's amino and carboxyl terminal globular domains thus resembling a dumbbell(Sawada et al., 1984). The sheet-like supramolecular aggregate formed by type VIII collagen is best illustrated in Decemet's membrane. This membrane supports the endothelial cells of the cornea and consists of hexagonal lattices of type VIII collagen(Sawada et al., 1984). The microstructure supporting the endothelial cells of blood vessels may be similar, but is unknown at present.

The primary structure of type X collagen is very similar to type VIII. It is a short triple helical molecule with similar interruptions and retained globular domains. Type X collagen is a homotrimer of $[\alpha 1(X)]_3$. It is found in the hypertrophic zone of growth plate cartilage(Schmid and Linsenmayer, 1990). In this region the supramolecular aggregate formed is similar to the hexagonal lattice

of Decemet's membrane(Kwan et al., 1991). This is not surprising given the similarities between collagens type VIII and type X.

Collagen type VI forms beaded microfibrils(Mayne and Burgeson, 1987). It is a heterotrimer of [α1(VI), α2(VI), α3(VI)], and is found in most connective tissues. The triple helix is very short(105nm) containing two interruptions and 11 arginine-glycine-aspartate(RGD) sequences. These are probably involved in integrin mediated interactions. The amino and carboxy terminal globular domains are large compared to the triple helix especially the α3(VI) chain(Furthmayr et al., 1983). Interestingly these domains, whose functions are unknown, contain sequences similar to the collagen-binding A domains of Von Willebrand factor, fibronectin type III repeats, salivary proteins, and Kunitz type protease inhibitors(Chu et al., 1990).

Analysis of rotary shadowing experiments has provided insights into the molecular aggregation of type VI collagen(Mayne and Burgeson, 1987). It appears that two molecules of type VI overlap about 75nm in an anti-parallel manner to form dimers. The dimers then aggregate in a parallel manner to form a tetramer, the basic unit of type VI collagen. The formation of the tetramers occurs

intracellularly(Wu et al., 1987), while end-to-end aggregation of the tetramers into microfibrils occurs extracellularly. The microfibrils produced are not D-staggered as is the case for the fibril forming collagens, but are beaded filaments of 3-5nm in diameter with a 100nm periodicity(Engvall et al., 1986).

Type VII collagen forms anchoring fibrils. It is synthesized by keratinocytes and connects the connective tissue stroma to basement membranes of skin and mucosa. Type VII collagen is a large probably homotrimeric molecule with a molecular weight of about 1,000,000. It has a long(420nm) interrupted triple helical domain and two NC domains on it's ends. The NC2 domain is small and appears as a small globule. In contrast, the NC1 domain is large(Mr 450,000) and appears in rotary shadowing experiments as a three-armed structure with small globules on the ends(Mayne and Burgeson, 1987).

The extracellular processing begins with the formation of dimers by a 60nm anti-parallel overlapping of two molecules at the amino terminal end. This produces a molecule with a length of about 795nm thought to be stabilized by intermolecular disulfide bonds(Mayne and Burgeson, 1987). The molecules then aggregate in

a parallel manner through lateral helical interactions to form non-staggered anchoring fibrils. The NC1 domains on both ends of these long fibrils can then interact or bind with type IV collagen on one end and the lamina densa on the other(Bachinger et al., 1990).

Collagen Biosynthesis

The biosynthesis of collagen is a complex process. Much of what we know about this process comes from studies of fibril forming collagens in particular type I collagen. These were the first collagens discovered in part due to their relative abundance compared to the other collagen groups. In fact the fibril forming collagens are probably the most abundant proteins found in the vertebrates. Only the biosynthesis of the fibril forming collagens will be discussed, since this dissertation concerns the structure of type II collagen, and it is relevant to the synthesis of all the collagen types discovered so far.

The biosynthesis of collagen begins with the translation of pre-procollagen mRNAs on free ribosomes and the synthesis of the signal peptide. This signal peptide is used for the selective targeting of nascent protein chains to the endoplasmic reticulum(Wiedmann et al., 1987). Once inserted into the ER, the signal peptide is cleaved

with a signal peptidase during or shortly after translocation(Evans et al,. 1986).

During translation specific amino acids of the triple helical domain are co-translationaly modified by specific enzymes. These enzymes are also involved in post-translational modifications required for the stability of the triple helix and will be discussed later.

The sequence of events involved in collagen biosynthesis after translation entails chain selection, chain association, nucleation, and propagation. Little is known about the selection process and will not be discussed.

Chain association is thought to be mediated through the C-propeptide. The precise details of this event are also unclear, but is believed to begin with non-covalent interactions between C-propeptides that are stabilized through disulfide bonds. These bonds are catalyzed by the ER resident enzyme protein disulfide isomerase(PDI)(Pihlajaniemi et al., 1987).

The folding of the collagen triple helix occurs by nucleation and propagation after stable chain association. The formation of collagen triple helices requires repeating units of gly-x-y, and also requires a

high proportion of 4-hydroxyproline in the y position for stability. The presence of hydroxyproline in the y position increases the stability by the addition of hydrogen bonds. Thermal stability experiments have shown that gly-pro-hypro triplets form the most stable triple helices. The carboxy terminus of the triple helical domain contains a stretch of gly-pro-hypro triplets considered the nucleation site. After nucleation, the propagation of the triple helix occurs in a carboxy to amino direction.

As stated previously, enzymes involved in modifying specific amino acids for proper helix assembly act co- and post-translationaly. These enzymes act only on unfolded collagen molecules, and are responsible for the hydroxylation of prolyl and lysyl residues and the glycosylation of certain hydroxylysyl residues.

The hydroxylation of prolyl and lysyl residues is catalyzed by members of the 2-oxoglutarate dioxygenase family, prolyl 3-hydroxylase, prolyl 4-hydroxylase, and lysyl hydroxylase(Kivirikko and Myllyla, 1980). They each require Fe^{2+}, α-ketoglutarate, molecular O^2, and ascorbate as cosubstrates. In the reaction α-ketoglutarate is decarboxylated, with one atom of the O^2 molecule

incorporated into succinate and the other into the hydroxyl group of the prolyl or lysyl residue.

Prolyl 4-hydroxylase is a tetramer ($\alpha_2\beta_2$), has a minimum substrate requirement of x-pro-gly, and is required for the formation of stable triple helical molecules at physiologic temperatures. The β subunit is identical to the enzyme PDI.

Prolyl 3-hydroxylase catalyzes the hydroxylation of prolyl residues in the x position only when the y position is 4-hydroxyproline (Tryggvason et al., 1979). Little is known about the structure of this enzyme or the function of 3-hydroxyproline.

Lysyl hydroxylase has a minimum substrate requirement of x-lys-gly. The hydroxylysine residues produced are involved in at least two functions. They serve as attachment sites for carbohydrate residues and play a role in the formation of collagen cross-links. This enzyme only acts on unfolded triple helical lysine residues. This suggests that a separate enzyme may be responsible for the hydroxylation of telopeptide lysine residues involved in cross-linking. Telopeptides are nonhelical and located on both ends of the molecule between the propeptides and the helix.

The glycosylation of certain hydroxylysine residues within the triple helical domain is catalyzed by two enzymes, hydroxylysyl galactosyl transferase and galactosyl hydroxylysyl glucosyl transferase. These enzymes require Mn^{2+} and transfer galactose to hydroxylysine and glucose to galactosylhydroxylysine respectively (Prockop et al., 1976). The function of these modifications is unknown at present and varies for different collagen types and for the same collagen type in different tissues and at different ages (Kivirikko and Myllyla 1980).

There are also modifications that occur in the propeptide domains. Type I and II collagens contain asparagine-linked oligosaccharides. These N-linked oligosaccharides are attached to the sequence asn-x-thr(ser) and therefore do not occur in the triple helical domain (Clark and Kefalides, 1976). The function of these modifications is also unknown.

The transport of procollagen molecules from the cell to the extracellular matrix follows the classical secretion route for proteins. After stable helix formation within the ER, the procollagen molecule is transported to the golgi apparatus, where it is packaged and exported from the cell.

Fibrillogenesis and Cross-Linking

The presence of the propeptides confers solubility to the molecule and prevents intracellular aggregation. Once outside the cell, the procollagen molecules are processed to collagen molecules by cleavage of their N- and C-propeptides. This is accomplished by the actions of two specific proteases, N- and C-propeptidases. Why this occurs for some collagen types and not others is not known. Also it is not known if this occurs prior to, during, or shortly after fibrillogenesis.

The aggregation of collagen molecules into fibrils is an entropy driven process. The energy required is provided by the formation of hydrophobic and charged interactions between molecules. The staggering of the molecules or microfibrils(Kajava, 1991) is thought to be facilitated by the presence of four regions with internal homology 67nm in length(234 amino acids) or 1D (Hofmann et al., 1980). The fibrils are stabilized by intermolecular covalent cross-links after the initial aggregation.

The formation of cross-links for the fibril forming collagens and some of the other collagen types requires only one enzyme, lysyl

oxidase. It is a copper metaloenzyme requiring pyridoxal phosphate and molecular O_2 as a cofactors(Bird and Levene, 1982). Specific lysines or hydroxylysines in the telopeptides are oxidatively-deaminated by the enzyme producing aldehydes, allysine and hydroxyallysine. The remaining reactions are spontaneous and are probably catalyzed by active sites near the triple-helical cross-linking site(Eyre, 1984).

There are two routes for cross-link formation based upon the aldehyde formed. In skin the allysine route prevails, while in bone and cartilage the hydroxyallysine route is used. Since this dissertation is concerned with type II collagen and disorders of skeletal development, only the hydroxyallysine route will be described.

On the hydroxyallysine route, hydroxyallysine can form divalent cross-links with lysine or hydroxylysine yielding aldimines(Schiff bases), hydroxylysinonorleucine(HLN) and dihydroxylysinonorleucine(DHLN) respectively. These Schiff bases then undergo spontaneous rearrangement to ketoamines, lysino-5-ketonorleucine and hydroxylysino-5-ketonorleucine. Two divalent cross-links can then condense to form the mature cross-linking

residue 3-hydroxypyridinium. There are two forms of this cross-link, hydroxylysyl pyridinoline(HP) and lysyl pyridinoline(LP). HP contains three residues of hydroxylysine and LP contains two residues of hydroxylysine and one lysine. The maturation of HP in cartilage is almost complete and is the predominant cross-link formed(Eyre, 1984).

The fibril forming collagens have four sites on which cross-linking occurs. These sites, two in the triple helical domain at positions 87 and 930 of the triple helix and two in the telopeptides(Wu and Eyre, 1984), are conserved among the fibrillar collagens. The trivalent cross-link joins two telopeptide aldehydes to a helical site. The sites at 87 and 930 are 0.4D from the end of the molecule. This is in agreement with the quarter-staggered fibril model where the distance of the overlap zone is 0.4D

CHAPTER II

A BRIEF REVIEW OF COLLAGEN AND DISEASE

Collagenopathies

Heritable diseases of connective tissue are caused by defects in the biosynthesis of various connective tissue components. Collagen is the major structural component in many connective tissues, and defects in it's biosynthesis result in a wide range of connective tissue disorders. The term collagenopathies describes a hetergeneous group of diseases in which mutations in collagen genes have been identified or genetically linked with these diseases.

Defects in type I collagen biosynthesis were first identified about 18 years ago in the studies of osteogenesis imperfecta(OI) or brittle-bone disease(Byers et al., 1991). This heritable disease of the skeleton in at least 90% of the cases studied so far has been shown to be the result of mutations in the genes for type I collagen, COL1A1 and COL1A2. These mutations: insertions, deletions, and single base substitutions result in the reduction of the amount of collagen secreted and/or the production of structurally abnormal collagen.

In general, those mutations that reduce the amount of collagen secreted produce the mildest phenotypes (Sykes, 1983). The decreased secretion may be the result of a null allele, failure of defective α chains to incorporate into molecules, or the incorporation of defective and normal α chains into molecules which are subsequently degraded in a process coined "procollagen suicide" (Prockop, 1990).

Mutations involving the COL1A1 allele that result in decreased secretion should be more deleterious than mutations involving the COL1A2 allele, if the reduction of collagen secretion directly correlates with disease phenotype. For example, with mutations that affect COL1A1 75% of the intracellular trimers should contain one or two α1(I) chains from the mutant allele. If none of the mutant containing trimers is secreted and all are degraded intracellularly, then only 25% of the total collagen produced will be secreted. Mutations affecting the COL1A2 allele should reduce collagen secretion to 50% of normal since 50% of trimers would contain the product of the mutant allele. Frame shift mutations and point mutations within the C-propeptide have been identified in this

group, although the null allele has yet to be identified (Byers et al., 1991).

Of the hundred or so mutations identified in OI the vast majority are single base substitutions. The majority of these result in substitutions of bulkier amino acids for glycine when they occur in codons for glycine and exon splicing mutations when they occur in consensus donor or acceptor sequences. These mutations probably alter the structure of the triple helix by introducing phase shifts in chain registration that may reduce the stability of molecules containing structurally abnormal α chains (Willing, et al., 1990). These types of mutations along with deletions and insertions that are tolerated intracellularly usually result in a more severe phenotype presumably due to the secretion of structurally abnormal collagen molecules into the extracellular matrix.

Precisely how these mutations affect the phenotypic outcome is unknown. There is however a trend that mutations affecting the carboxy terminal end of the molecule produces a more severe phenotype (Byers, 1993). This appears to be a gradient effect such that the closer the defect is to the amino terminus the milder the disease phenotype. The phenotype is also influenced by the type of

mutation, the amino acid substituting for glycine, the α chain affected, and the amount of defective protein secreted into the matrix. There are exceptions to this trend that may eventually be explained by the presence of certain domains more critical to the stability of the molecule or critical to other as yet discovered functions of these proposed domains(Kuivaniemi, et al., 1991). It is also unknown what effects these mutations have on the cell, other collagen types, or other matrix molecules. What is known is that intracellular collagen biosynthesis and extracellular fibrillogenesis is altered.

Intracellularly, molecules containing α chains with mutations in the triple helical domain are processed slower than normal molecules and leads to delayed secretion. The delay in folding of the molecules begins at the site of the mutation and is propagated towards the amino terminus since this is the direction of folding. This delay, since the molecules are still unfolded, allows for ER resident enzymes to post-translationaly overmodify prolyl and lysyl residues. Evidence of this delay is seen in the pulse-chase experiments of OI skin fibroblasts in vitro where mutant α chains identified by their delayed mobilities on gel electrophoresis or other identifying

features of the mutation such as shortened α chains from deletion mutations do not appear in the extracellular matrix at the same time as normal α chains but are delayed(Byers, 1993). Evidence for post-translational overmodification occurring at and amino terminal to the mutation is seen in the delayed mobility of the cyanogen bromide(CNBr) digested peptides of mutant type I collagen compared to control. In OI only the cyanogen bromide(CB) peptides containing the mutation and those amino terminal to it are electrophoretically delayed(Byers, 1993).

Evidence that the delayed electrophoretic mobility is an effect of post-translational overmodification comes from amino acid analysis (Bateman et al., 1987), and the increase or restoration of electrophoretic mobility upon inhibition of the modifying enzymes with an iron chelator α,α dipyridyl in vitro (Barsh and Byers, 1981).

Alternatively, the increased post-translational overmodification may be the result of an altered triple helical structure occurring at and amino terminal to the mutation site. In this scenario, lysyl and prolyl residues at and amino terminal to the mutation site should all be equally modified. In the delayed folding model, those prolyl and lysyl residues nearest to the amino terminus should be the most

modified, i.e. glucosylgalactosylhydroxylysine versus galactosylhydroxylysine or hydroxylysine. Evidence to support either model would have to come from direct amino acid sequencing of these residues from the diseased tissues. Currently there is little evidence to favor one model over the other.

In general molecules containing mutant α chains are less stable than normal molecules(Byers et al., 1991). The decrease in stability is seen in melting point experiments with trypsin and chymotrypsin. In these experiments the nondenatured molecules are incubated with these enzymes as the temperature is slowly increased from physiologic temperatures(37°) to the melting point(42°) of normal collagen molecules. Aliquots are taken at increasing temperatures and analyzed by gel electrophoresis. These enzymes will not degrade the molecule in the triple helical conformation, but completely degrade them when the helix begins to unfold or denature.

Not all mutations show a decrease in thermal stability. This may be an effect of the increased post-translational overmodification occurring in the molecules, since an increase in prolyl hydroxylation has been shown to increase the melting point of collagen molecules. The increase in glycosylation of hydroxylysine may also be an

attempt of the cell to further stabilize the triple helix or may just be a secondary consequence of the delayed secretion or the altered triple helical structure formed. There is no evidence for this speculated role of glycosylated hydroxylysines, nor is the function of these residues known.

The formation of fibrils is altered in OI. There are no rules to guide us in predicting how mutations produce altered fibrils other than for glycine substitutions to cysteine. In vitro studies of fibril formation using cysteine substituted collagen molecules produced dendritic fibrils versus the straight and pointed fibrils from controls (Vogel, et al., 1987). This probably occurs in cysteine substitutions due to the formation of intramolecular disulfide bonds which as seen by rotary shadowing experiments produces a kink or bend in the molecule at the site of the mutation (Vogel, et al., 1988).

The proteolytic processing of procollagen to collagen appears to be hindered in OI. Again in vitro studies using the cysteine substituted protein showed a slower rate of cleavage from procollagen to collagen (Vogel, et al., 1988). This is interpreted as the glycine substitution producing a phase shift in the molecule that extends to the propeptide cleavage sites. This shift alters the

conformation of the cleavage site thereby decreasing the rate of cleavage, but not totally inhibiting it.

Not all mutations in the genes for type I collagen produce OI. Mutations in COL1A1 and COL1A2 that remove the N-propeptidase cleavage site produces the Ehlers-Danlos syndrome (EDS) type VII phenotype(Kuivaniemi, et al., 1991). EDS type VII is characterized by extreme joint laxity and skin abnormalities (McKusick, 1983). The mutations identified thus far have been exon 6 splicing mutations. Exon 6 codes for the N-propeptidase cleavage site. Retention of the N-propeptide interferes with fibrillogenesis such that only thin and irregular fibrils are formed and decreases the amount of intermolecular cross-links formed (Eyre, et al., 1985).

Familial forms of osteoporosis may also be the result of mutations in the gene for type I collagen. Parents of a patient with a recessive form of OI showed radiographic evidence of osteoporosis while still in their third decade. The parents were third cousins and carried the same four base pair deletion in the C-propeptide of the COL1A2 gene (Pihlajaniemi, et al., 1984). Although the parents showed no evidence of the OI phenotype, they may represent very

mild forms of OI where the clinical features are either subclinical or are not manifested until later in life.

Indeed other patients with osteoporosis and type I collagen gene mutations appear to have some of the features of a mild OI phenotype and other connective tissue abnormalities. For example, one patient with severe post-menopausal osteoporosis and slightly blue sclera had a serine substitution for glycine at position 619 of the triple helix in the $\alpha 2(I)$ chain (Spotila, et al., 1991). In another patient diagnosed with premenopausal osteoporosis, a cysteine for glycine substitution at position 43 in the $\alpha 1(I)$ chain was identified(Shapiro, et al., 1992). This patient belonged to a family considered to have an osteopenic non-fracture syndrome with mild OI features. The emergence of features of the OI phenotype later in life is seen in a family with joint hypermobility, premature osteoporosis, and late-onset fractures. A deletion mutation leading to the skipping of exon 9 in the $\alpha 2(I)$ chain was found in this family (Nichols et al., 1992).

Mutations in the genes for type I collagen produce a spectrum of disease phenotypes. The spectrum includes familial osteoporosis with or without features of a mild OI phenotype at one end and

severe perinatal lethal forms of OI at the other. The EDS type VII phenotype may be included in the type I collagenopathies, but clinically represents a distinct disease phenotype.

The studies of OI have prompted the search for mutations in other collagen types. Thus over the past few years the numbers of diseases found to be associated with defective collagen biosynthesis has mushroomed. The remainder of this section briefly summarizes those identified so far.

Mutations in the gene for type III collagen, COL3A1 produces the EDS type IV phenotype. EDS type IV is a severe disease with affected persons prone to sudden death from the rupture of large arteries or other hollow organs. So far the mutations identified include glycine substitutions, splicing mutations, and large deletions(Kuivaniemi, et al., 1991).

Alport syndrome or hereditary glomerulonephritis is characterized by progressive loss of kidney function and hearing. The $\alpha 5(IV)$ protein has been immunolocalized in the basement membrane of the glomerulus and is suspected to be a component of the inner ear basement membrane. Defects in the gene for $\alpha 5(IV)$,

COL4A5 have been identified in the X-linked form of Alport syndrome (Tryggvason et al., 1993).

Epidermolysis Bullosa (EB) is a blistering disease of the skin. In the severe dystrophic form of EB (recessive and dominant), blistering at the dermis/epidermis junction is caused by minor trauma to the skin(Uitto and Christiano 1992). The basement membrane of the skin contains type IV collagen and anchoring fibrils of type VII collagen. Genetic linkage of EB with COL7A1 has been demonstrated (Ryynanen et al., 1991) and mutations identified in both dominant and recessive forms of the disease (Uitto and Christiano, 1994).

Several mutations in the gene for type X collagen have recently been reported in the study of spondylometaphyseal dysplasia (Warman et al., 1993, McIntosh et al., 1994). In transgenic mice, mutations in the genes for collagen types IX and X have produced phenotypes very similar to the human disorders of osteoarthrosis with mild chondrodysplasia and spondylometaphyseal dysplasia respectively (Nakata et al., 1993, Jacenko et al., 1993). These data suggest the possibility that defects in the genes of these collagen types may be responsible for these human disorders. As there are at

least 27 collagen genes, there is no doubt that more mutations will be found that are responsible for human diseases.

Type II Collagenopathies

Analogous to the mutations in the genes for type I collagen that produce the OI phenotype, mutations in the gene for type II collagen are proposed to produce some of the phenotypes in the chondrodysplasias.

There are well over 100 distinct human chondrodysplasias. These inherited connective tissue disorders are characterized by disturbed skeletal development and linear bone growth. Characterized clinically by dwarfism usually with abnormal body proportions, they are commonly accompanied by skeletal deformities and other connective tissue defects (Horton, and Dayton, 1988).

Spranger has proposed grouping families of bone dysplasias based on patterns of bone changes (Spranger J, and Maroteaux P, 1990). His concept of bone dysplasia families includes the idea that similar bone dysplasias are pathogenetically related.

Among the chondrodysplasias, the spondyloepiphyseal dysplasia (SED) congenita family is a heterogeneous group of

disorders characterized by abnormal epiphyses, varying degrees of metaphyseal irregularity, flattened vertebral bodies, myopia and vitreous degeneration (Murray et al., 1989). Type II collagen is present in all affected sites. According to Spranger, the SED congenita family includes: achondrogenesis type II (Langer-Saldino and Parenti-Fraccaro), hypochondrogenesis, and SED congenita.

Evidence is emerging for type II collagen defects in the SED congenita spectrum of disease (Godfrey et al., 1988, Anderson et al., 1990). The severe end of the spectrum includes achondrogenesis and hypochondrogenesis (Bogaert et al., 1992, Bonaventure et al., 1995, Chan et al., 1995, Mortier et al., 1995, Williams et al., 1995). Langer-Saldino achondrogenesis has shown a total block of type II collagen expression (Eyre et al., 1986). In spondyloepiphyseal dysplasia there appears to be an overmodification of type II collagen expressed extracellularly (Murray LW and Rimoin DL. 1988, Murray et al., 1989). COL2A1 mutations have been defined for spondyloepiphyseal dysplasia (Lee et al., 1989, Vissing et al., 1989, Tiller et al., 1990, Vikkula et al., 1993, Bleasel et al., 1995, Chan et al., 1995, Tiller et al., 1995,).

Kniest dysplasia and Stickler/Wagner syndrome may also belong with the SED spectrum of disease in the family of type II collagenopathies. Kniest dysplasia is a severe chondrodysplasia characterized clinically by disproportionate dwarfism, cleft palate, myopia, progressive conductive hearing loss, arthropathy, and scoliosis(Taybi H, Lachman RS. 1990). So far, COL2A1 mutations in Kniest dysplasia have been exon splicing mutations (Winterpacht et al., 1993, Bogaert et al., 1994, Spranger et al., 1994 Mortier et al., 1995, Fernandes et al., 1998, Weis et al., 1998).

Stickler syndrome or hereditary arthro-ophthalmopathy is characterized by ocular defects including myopia and retinal detachment, and features such as cleft palate, craniofacial abnormalities, deafness, and progressive arthropathy (Stickler et al., 1965). It is a relatively mild chondrodysplasia with affected persons achieving the height of normal individuals. In at least some kindreds, the defect has been identified as missense mutations in COL2A1 that result in chain termination and presumably a reduction in type II collagen production (Ahmad et al., 1991, Brown et al., 1992, Ahmad et al., 1993, Ritvaniemi et al., 1993). A single case of Wagner syndrome, a mild variant of Stickler syndrome without

skeletal involvement, has been shown to be a mutation in COL2A1 as well (Körkkö et al., 1993).

There is evidence of familial precocious osteoarthritis linked to defects in type II collagen biosynthesis. In two large kindreds, a Finnish and an American family, early onset osteoarthrosis has been linked to COL2A1 (Knowlton et al., 1990, Vikkula et al., 1993, Williams et al., 1995). The defect in the American family has been identified as an arginine to cysteine 519 substitution that is highly expressed in the type II collagen found in the matrix (Eyre et al., 1991), but this kindred also shows radiographic signs of a mild SED phenotype. The defect in the Finnish family has yet to be identified and apparently shows no signs of altered skeletal growth.

From the published studies available thus far, defects in type II collagen biosynthesis are probably responsible for a wide range of disease phenotypes similar to the range of disease phenotypes found to be the result of defects in type I collagen biosynthesis. The spectrum of phenotypes in the type II collagenopathies is proposed to include familial osteoarthrosis and Stickler/Wagner syndrome at the mildest end and achondrogenesis/hypochondrogenesis at the severe end.

CHAPTER III

STRUCTURALLY ABNORMAL TYPE II COLLAGEN IN THE SPONDYLOEPIPHYSEAL DYSPLASIA FAMILY

Summary to Chapter III

The spondyloepiphyseal dysplasia (SED) sub-classification of bone dysplasias includes achondrogenesis, hypochondrogenesis, spondyloepimetaphyseal dysplasia (SEMD), and spondyloepiphyseal dysplasia congenita (SEDc). The phenotypic expression of these disorders ranges from mild to perinatal lethal forms. We report the detection and partial characterization of a defect in type II collagen in a perinatal lethal form of hypochondrogenesis and a lethal form of SEMD. Electrophoresis in SDS-polyacrylamide of CB-peptides from type II collagen of the diseased cartilages showed a doublet band for peptide $\alpha 1$(II)CB10 and evidence for post-translational overmodification of the major peptides, CB8, 10 and 11, seen as a retarded electrophoretic mobility. Peptide CB10 was digested by sequence grade enzymes and on reverse phase HPLC fragments of abnormal mobility were noted. Amino acid sequence analysis of peaks unique to the diseased tissue revealed a glutamate

substitution for glycine at position 853 of the triple helical domain in the case of hypochondrogenesis and a serine substitution for glycine at position 841 of the triple helical domain in the case of SEMD. Electron micrographs of the diseased cartilage showed sparse extracellular matrix and chondrocytes containing dilated rough endoplasmic reticulum, which suggested impaired assembly and secretion of the mutant protein. These results indicate that structurally defective type II collagen present in the extracellular matrix underlies the pathogenesis in these disorders.

Introduction to Chapter III

The chondrodysplasias are a heterogeneous group of disorders characterized by abnormal formation and growth of cartilage (Rimoin and Lachman, 1990). The spondyloepiphyseal dysplasia congenita (SEDc) sub-classification includes a spectrum of heritable disorders characterized clinically by abnormal epiphyses, flattened vertebral bodies, varying degrees of metaphyseal irregularity, myopia, and vitreous degeneration (Spranger and Langer,1970, Spranger, 1975, Lachman et al., 1975). At one end of the spectrum, the perinatal lethal form achondrogenesis II is usually characterized by an

absence of type II collagen in cartilage matrix and presence of type I collagen (Eyre et al., 1986, Eyre, 1988). In the radiographically less severe but still lethal form, hypochondrogenesis, matrix typically contains post-translationally overmodified type II collagen, with variable amounts of type I collagen (Murray and Rimoin, 1988, Godfrey and Hollister, 1988). Cartilage from the moderately severe SEDc patients contains mostly type II collagen, which can be structurally abnormal (Murray et al., 1989).

Dominant mutations in the type II collagen gene, COL2Al, have been identified within the SEDc spectrum of disease (Lee et al., 1989, Vissing et al., 1989, Tiller et al., 1987, Bleasel et al., 1995, Chan et al., 1995, Mortier et al., 1995, Williams et al., 1995, Bonaventure et al., 1995). These findings provide genetic support for the concept of a family of type II collagenopathies with a phenotypic continuum ranging from mild to perinatal lethal, similar to the osteogenesis imperfecta spectrum of clinical phenotypes that results from a diversity of structural mutations in the genes for type I collagen (Kuivaniemi, 1991; Byers et al., 1988; Cohn et al., 1988; Starman et al., 1989). For the type II collagen mutations, it is not yet clear how different molecular defects relate to the clinical phenotype or

whether the abnormal protein chains are incorporated into the extracellular matrix and so directly affect its structure and function.

The present study identifies the protein defect in the type II collagen of cartilage in a case of hypochondrogenesis and SEMD.

Materials and Methods

Clinical summary. The hypochondrogenesis proband was born at 33 weeks gestation to clinically normal parents, with a birth length of 34.5 cm (50th percentile for a 26 week fetus). Short limbs and a small chest were noted on prenatal ultrasound examination at 19 weeks gestation. In addition to these findings, unilateral polydactyly was noted at birth. Early radiographs were consistent with a severe form of SEDc, while skeletal films at 3 months of age more closely resembled hypochondrogenesis (Taybi and Lachman, 1990). The infant required continuous respiratory support until his death at 3 months of age.

The SEMD (unclassified) proband was born to clinically normal parents. Disproportionate short stature and bilateral clubfoot was noted at birth. The proband was hospitalized at eight months of age with pneumonia. Evaluation at that time revealed biventricular

cardiac hypertrophy and pulmonary hypertension, thought to be secondary to small chest circumference. Mild cerebral atrophy and a hypoplastic first cervical vertebra were also noted. The proband was developmentally delayed and remained oxygen dependent until his death at 22 months of age. At autopsy, the patient was 55cm in length (<5th percentile). Physical features included disproportionate short stature, narrow chest with pectus carinatum, brachydactyly, and protuberant abdomen.

Protein analysis

Pepsin-solubilized collagen. Cartilage was extracted in 4M guanidine HCl, 0.05M Tris/HCl, pH 7.0 at 4°C for 48 hours. The washed residue was digested with pepsin (Miller, 1972). Human articular cartilage collagen from a 25-year-old normal male was used as a control. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (Laemmli, 1970) in 6% and 12.5% gels.

Peptide analysis. Cartilage was digested with cyanogen bromide in 70% (w/v) formic acid for 24 hours at room temperature (Eyre and Muir, 1975). The CB peptides were fractionated by

sequential cation exchange HPLC (Mono-S HR 5/5, Pharmacia Fine Chemicals) and reverse phase HPLC (C8, 25 cm x 4.6 mm, Brownlee Labs RP-300) (Bateman et al., 1986; Eyre, 1987). Pooled fractions containing α1(II)CB10 (identified by SDS-PAGE) were dried and the peptide was digested with endoproteinase asp-N (sequencing grade, Boehringer-Mannheim Biochemicals). Pooled fractions from the endoproteinase digestion were further digested with trypsin in the SEMD case. The resulting peptides were fractionated by reverse phase HPLC using a gradient (0-30% in 60 min) of acetonitrile: n-propanol (3:1 v/v) in 0.1% (v/v) trifluoroacetic acid at 1 ml/min (Eyre et al., 1984). Peptide yields were estimated by areas under absorbance peaks in the chromatography profiles and by recoveries of PTH-amino acids on sequence analysis.

Protein microsequencing Edman amino-terminal sequencing of individual peptides was carried out on a Porton 2090E machine equipped with on-line HPLC analysis of PTH-amino acids using the manufacturer's standard program.

Collagen cross-link analysis. An acid hydrolysate (6M HCl, 24 hours, 110°C) of cartilage was analyzed for the hydroxypyridinium (pyridinoline) cross-links of collagen by reverse phase HPLC as

previously described (Eyre et al., 1984). Hydroxyproline was measured in the hydrolysate by colorimetric analysis (Stegemann, 1958). The cross-link concentration was expressed as moles per mole of collagen relative to normal type II collagen.

Results

The αl(II) chain of pepsin-solubilized collagen extracted from the hypochondrogenesis cartilage migrated as a doublet with most of the protein in the more slowly migrating component, and the α1(II) chain from the SEMD cartilage migrated as a smeared band on SDS-PAGE, (Figure 1). The CB peptides of the abnormal type II collagen also migrated more slowly than their counterparts from control human cartilage, with α1(II)CB10 running as a doublet (Figure 1).

Analysis of the CB digest by cation exchange HPLC and SDS- polyacrylamide electrophoresis showed that the upper (slower) component of the CB10 doublets was more acidic for the hypochondrogenesis and SEMD cases (Figure 2). Fractions containing α1(II) CB10 were pooled. The peptide was further purified by reverse phase HPLC, and digested with endoproteinase asp-N. On the basis of the elution profile on reverse phase HPLC, individual asp-N peptides unique to the

Figure 1. SDS-polyacrylamide electrophoresis of pepsin-solubilized collagen and CB-derived peptides from C) control, 1) hypochondrogenesis, and 2) SEMD tissues. The pepsin-solubilized αl(II) chains (6% gel) shows a doublet with retarded mobility for (1), and a broad band with retarded mobility for (2) in comparison with (C) normal human α1(II). The CB-digest of both (1) and (2) shows a doublet for α1(II)CB10 and retarded mobilities of CB-peptides 10, 11, and 8 compared with CB-peptides from (C) control human cartilage.

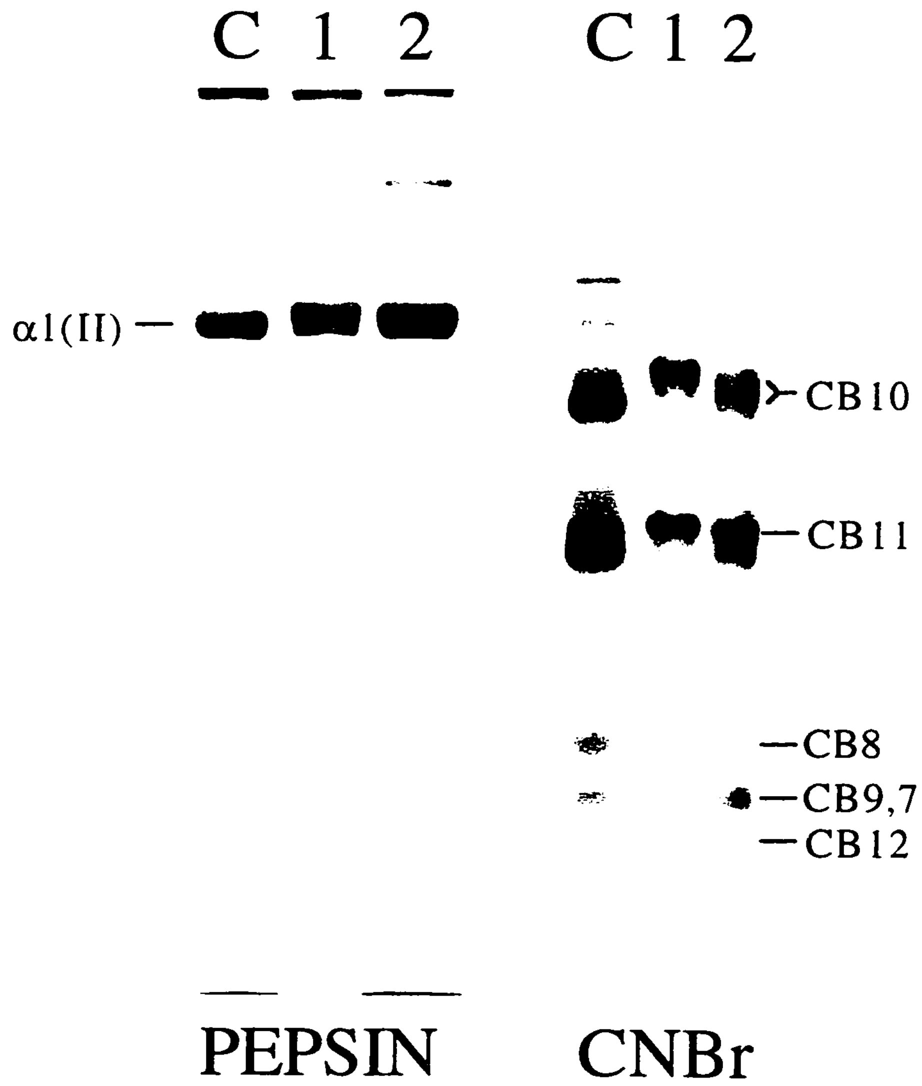
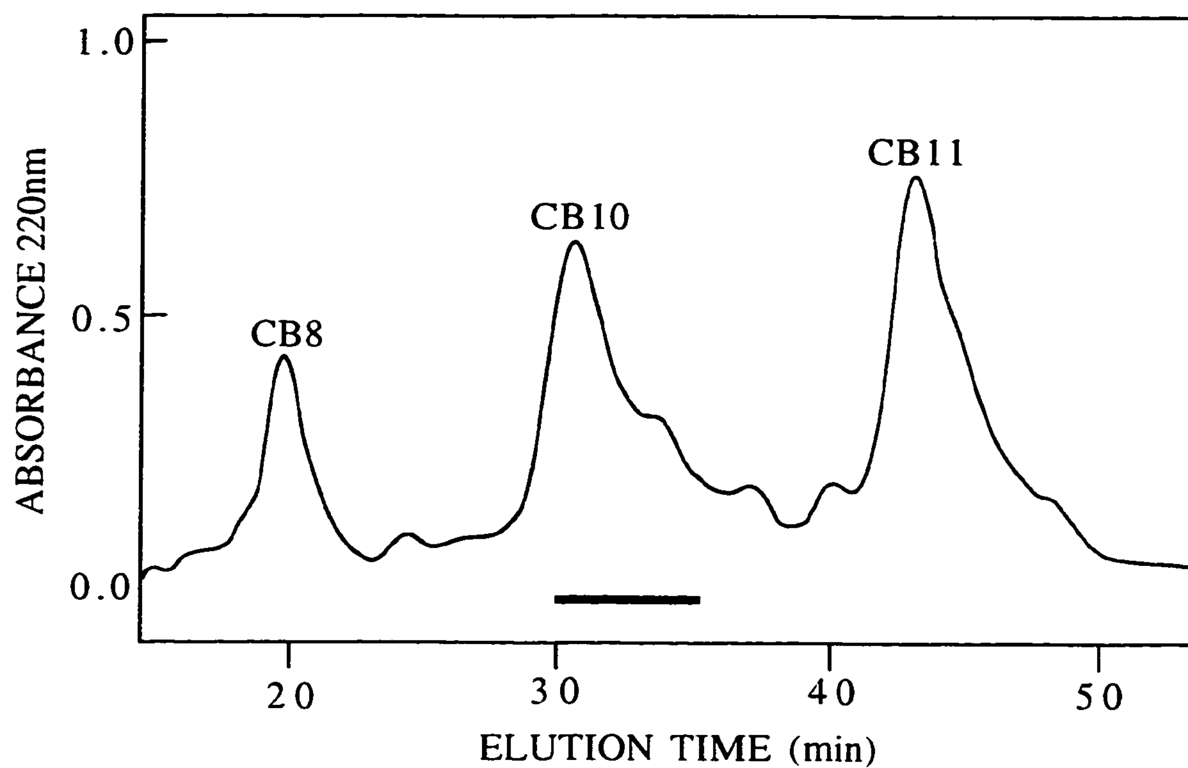
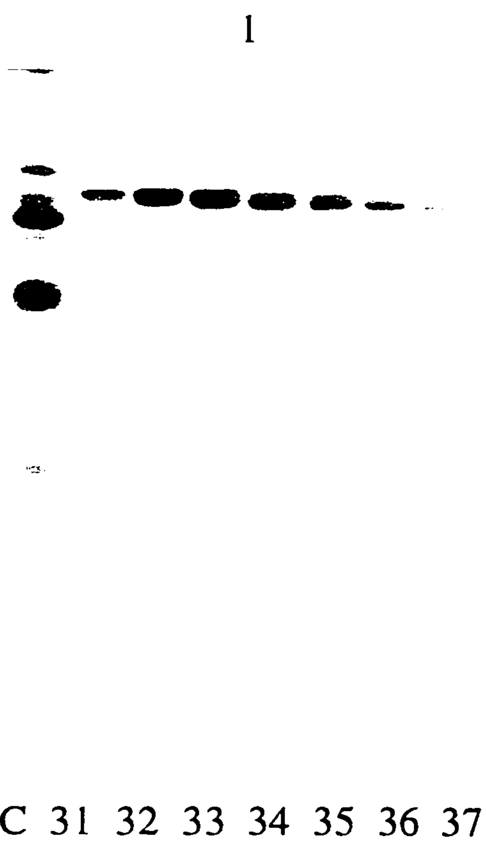
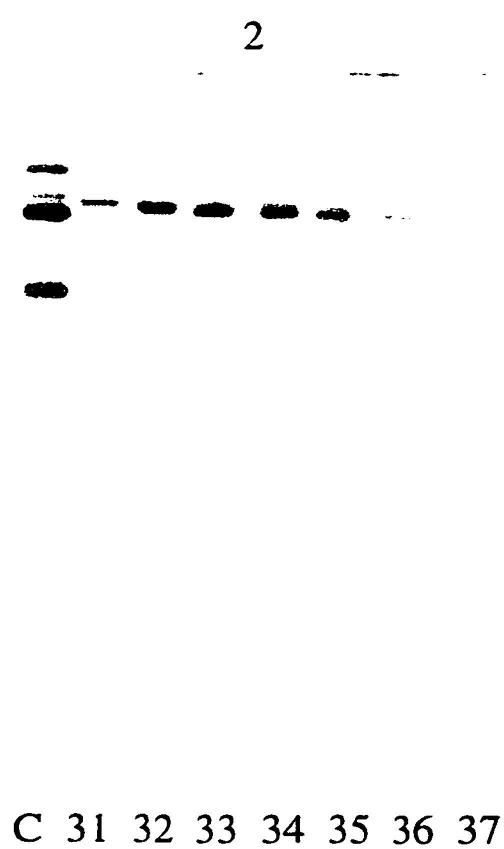

C 1 2
C 1 2
α1(II)
CB10
CB11
CB8
CB9,7
CB12
PEPSIN
CNBr

Figure 2. Upper, elution profile from cation-exchange HPLC of CB-peptides of type II collagen from the hypochondrogenesis cartilage. The fractions marked by the bar were pooled for reverse phase HPLC. 1) SDS-12.5% polyacrylamide electrophoresis of fractions sampled across the αl(II)CB10 peak as indicated by the bar (upper). 2) SDS-12.5% polyacrylamide electrophoresis of fractions sampled across the αl(II)CB10 peak from the SEMD tissue (HPLC data not shown). Peptide CB10 from both cases ran as a doublet on the gel. The upper bands eluted slightly earlier in the chromatogram.


1.0
0.5
0.0
ABSORBANCE 220nm
CB8
CB10
CB11
20
30
40
50
ELUTION TIME (min)


1
C 31 32 33 34 35 36 37


2
C 31 32 33 34 35 36 37

hypochondrogenesis (Figure 3) and SEMD (Figure 4) cartilages were selected for aminoterminal microsequence analysis.

Sequence analysis of peptides in the fraction 39 region of the CB-10 asp-n digest from the SEMD tissue suggested a possible glycine to serine substitution at position 841 of the triple helical domain (Figure 4). CB-10 endoproteinase asp-N fractions indicated by the bar in figure 4 from control and SEMD were pooled, digested with trypsin, and peptide maps generated by RP-HPLC (Figure 5). Sequence of a novel peptide in the SEMD profile confirmed the serine substitution (Figure 5).

The sequence of a novel form of peptide D12 (residues 849-856 of the $\alpha 1$(II) chain triple helix) from the hypochondrogenesis tissue identified glutamate at cycle 5 (corresponding to position 853 of the triple helical domain), instead of glycine seen in the normal version of D12 at this position (Figure 3). The ratio of glutamate to glycine peptide forms was about 1:1. The ratio of galactosylhydroxylysine:hydroxylysine:lysine at cycle 7 was the same in the glutamate containing peptide as in the normal peptide from the hypochondrogenesis tissue (data not shown).

Two versions of peptide D3 (residues 569-581 of $\alpha 1$(II)

Figure 3. Reverse phase HPLC separation of peptides generated by endoproteinase asp-N digestion of the purified CB fragment α1(II)CB10 from (a) hypochondrogenesis cartilage and (b) human control cartilage.

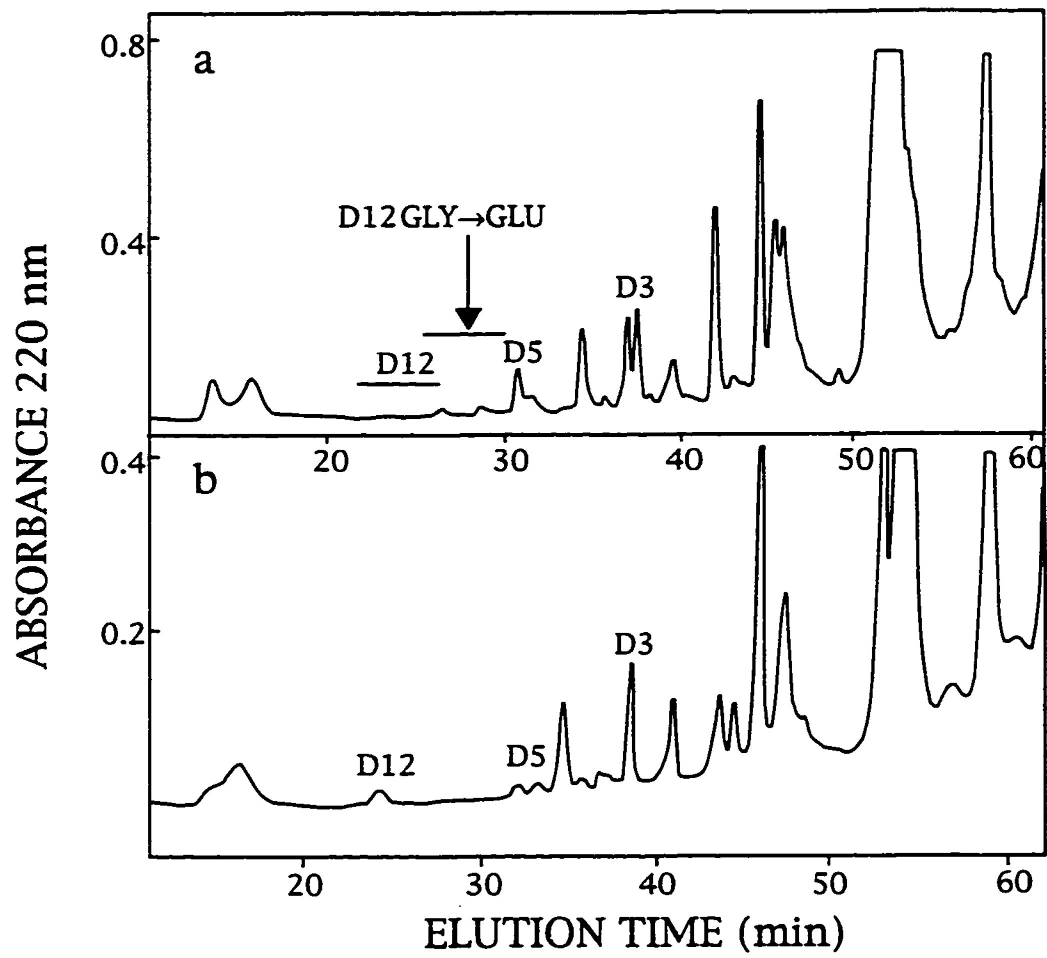

a
b
0.8
0.4
0.4
0.2
D12GLY→GLU
D12
D5
D3
D12
D5
D3
20
30
40
50
60
20
30
40
50
60
ABSORBANCE 220 nm
ELUTION TIME (min)

Figure 4. Reverse phase HPLC separation of peptides generated by endoproteinase asp-N digestion of the purified CB fragment α1(II)CB10 from SEMD cartilage (lower) and human control cartilage (upper). The fractions marked by the bar were pooled for trypsin digestion.

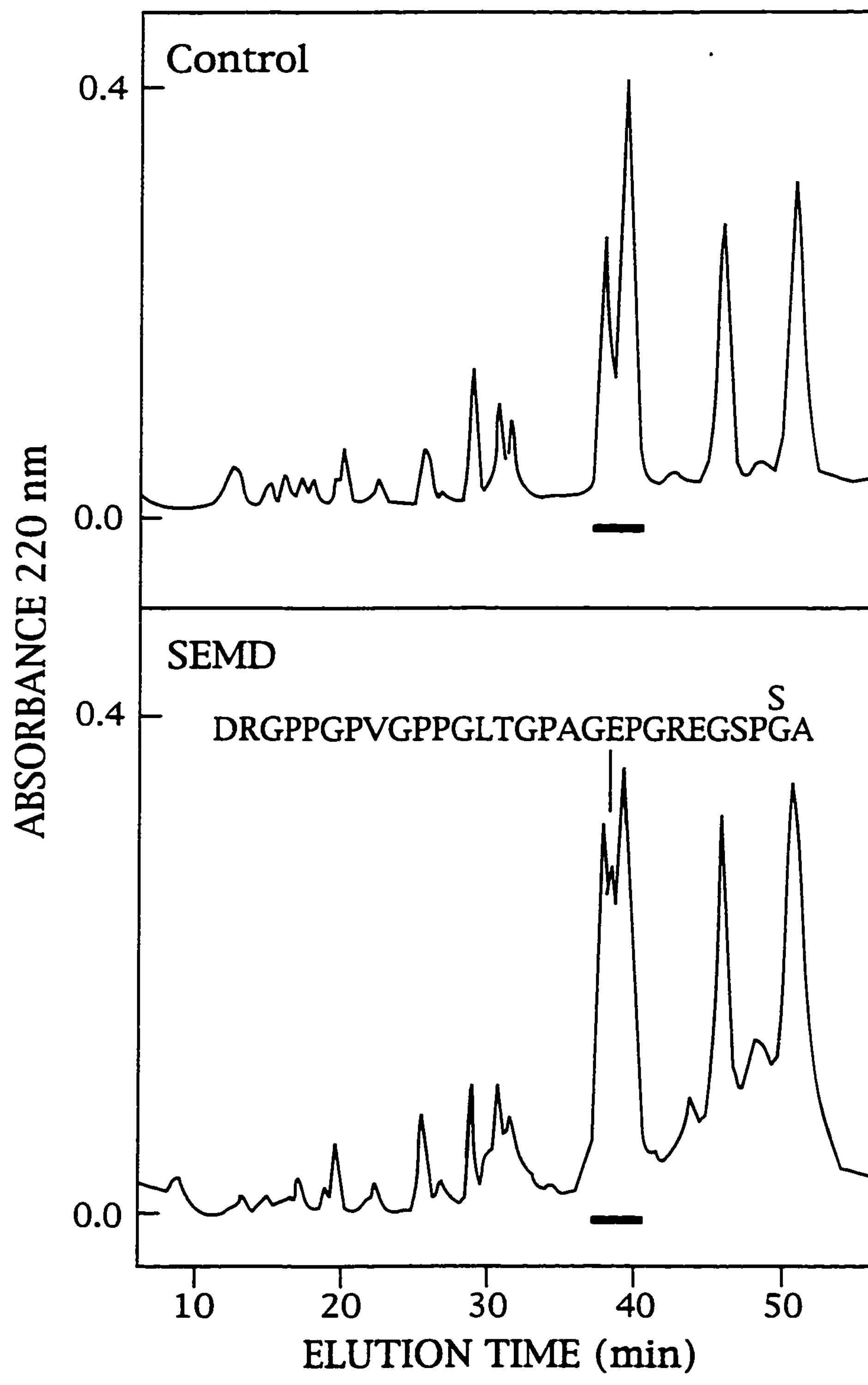
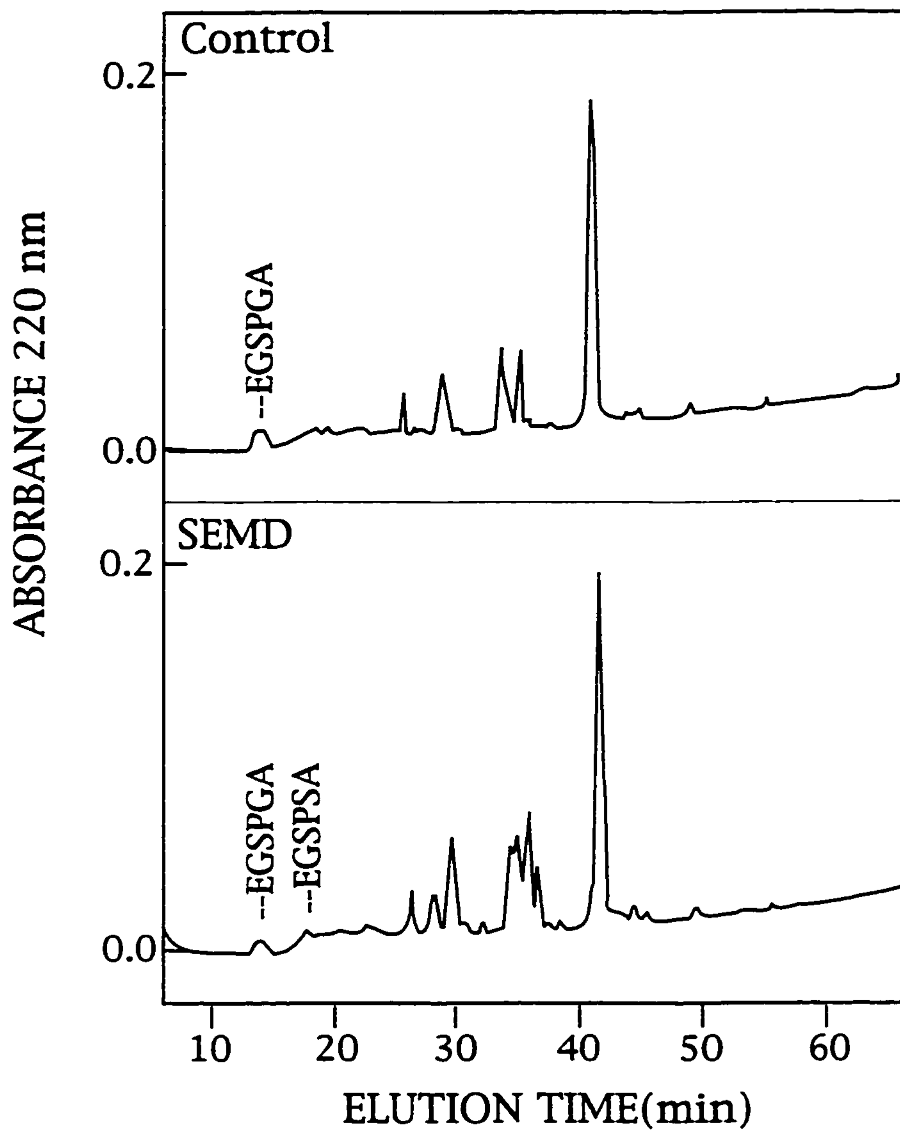

Control
0.4
0.0
SEMD
0.4
S
DRGPPGPVGPPGLTGPAGEPGREGSPGA
0.0
ABSORBANCE 220 nm
10
20
30
40
50
ELUTION TIME (min)

Figure 5. Reverse phase HPLC separation of peptides generated by trypsin digestion of the asp-N peptides (as indicated in figure 4) from SEMD cartilage (lower) and human control cartilage (upper).



Control
0.2
0.0
EGSPGA
SEMD
0.2
0.0
EGSPGA
EGSPSA
ABSORBANCE 220 nm
10
20
30
40
50
60
ELUTION TIME(min)

Figure 6. The amino acid sequence of human $\alpha 1(II)CB10$ from published cDNA data (Baldwin et al., 1989). Endoproteinase asp-N cleavage sites are indicated by arrows. The asp-N peptides subjected to microsequence analysis are indicated. The sequence of the mutation-containing peptides, D12 and D10, are underlined. The amino terminal end of the tryptic peptide within peptide D10 is marked with a star.

552	P	G	E	R	G	A	A	G	I	A
562	G	P	K	G	↓ D	R	G	↓D3 D	V	G
572	E	K	G	P	E	G	A	P	G	K
582	↓ D	G	G	R	G	L	T	G	P	I
592	G	P	P	G	P	A	G	A	N	G
602	E	K	G	E	V	G	P	P	G	P
612	A	G	S	A	G	A	R	G	A	P
622	G	E	R	G	E	T	G	P	P	G
632	P	A	G	F	A	G	P	P	G	A
642	↓D5 D	G	Q	P	G	A	K	G	E	Q
652	G	E	A	G	Q	K	G	↓ D	A	G
662	A	P	G	P	Q	G	P	S	G	A
672	P	G	P	Q	G	P	T	G	V	T
682	G	P	K	G	A	R	G	A	Q	G
692	P	P	G	A	T	G	F	P	G	A
702	A	G	R	V	G	P	P	G	S	N
712	G	N	P	G	P	P	G	P	P	G
722	P	S	G	K	↓ D	G	P	K	G	A
732	R	G	D	S	G	P	P	G	R	A
742	G	E	P	G	L	Q	G	P	A	G
752	P	P	G	E	K	G	E	P	G	↓ D
762	↓ D	G	P	S	G	A	E	G	P	P
772	G	P	Q	G	L	A	G	Q	R	G
782	I	V	G	L	P	G	Q	R	G	E
792	R	G	F	P	G	L	P	G	P	S
802	G	E	P	G	K	Q	G	A	P	G
812	A	S	G	↓D10 D	R	G	P	P	G	P
822	V	G	P	P	G	L	T	G	P	A
832	G	E	P	G	R	★ Q	G	S	P	G/**S**
842	A	↓ D	G	P	P	G	R	↓D12 D	G	A
852	A	G/**E**	V	K	G	↓ D	R	G	E	T
862	G	A	V	G	A	P	G	T	P	G
872	P	P	G	S	P	G	P	A	G	P
882	T	G	K	Q	G	↓ D	R	G	E	A
892	G	A	Q	G	P	M				

Figure 6) were derived from the hypochondrogenesis tissue in equal yield, an earlier-eluting peak which yielded hydroxylysine at cycle 5 (residue 573 of the triple helix) and a second peak which gave lysine at cycle 5. Control D3 gave only lysine at this position. Similarly, peptide D5 (residues 642-658 Figure 6) was also resolved as a doublet, with the glucosylgalactosylhydroxylysyl form at cycle 7 eluting first. The later-eluting version of peptide D5 gave two peaks at cycle 7, indicating a mixture of galactosylhydroxylysine and hydroxylysine. Our experience with glycosylated hydroxylysines on microsequence analysis is a blank cycle for glucosylgalactosylhydroxylysine and a distinctive peak of a PTH derivative for galactosylhydroxylysine. Hence, the earlier-eluting version of peptide D5 gave a blank at cycle 7. Peptide D5 from control tissue gave a mixture of hydroxylysine and lysine at cycle 7 but no galactosylhydroxylysine.

The concentration of hydroxylysyl pyridinoline cross-links in the hypochondrogenesis cartilage collagen was 0.55 residues per mole of collagen and 1.34 residues per mole of collagen in the SEMD cartilage, compared with a mean of about 0.5 and 1.5 residues per mole for control neonatal cartilage and control adult cartilage

respectively (Eyre et al., 1988). Ultrastructural examination of the hypochondrogenesis cartilage revealed a normal cell density within a sparse extracellular matrix (Bogaert et al., 1992). Chondrocytes contained inclusion bodies of dilated rough endoplasmic reticulum filled with a granular material.

Discussion

To date, specific mutations in type II collagen reported for patients with clinical phenotypes within the spondyloepiphyseal dysplasia family of chondrodysplasia were identified by analysis of genomic DNA or cDNA clones (Lee et al., 1989, Vissing et al., 1989, Tiller et al., 1987). The present study has detected and defined 2 new mutations at the protein level. The mutations were a glutamate for glycine substitution at residue 853 of the $\alpha 1(II)$ triple helix which produced a hypochondrogenesis phenotype and a serine for glycine substitution at residue 841 of the $\alpha 1(II)$ triple helix which produced an SEMD phenotype.

Sequence analysis of cDNA clones derived from RNA isolated from the patient's cartilage performed by our collaborators in the hypochondrogenesis case showed that the glutamate for glycine substitution at residue 853 of the helix was the result of a point

mutation in the second position of the glycine codon (GGA to GAA). They demonstrated that the mutation arose in the paternally-inherited allele, but that there was no evidence of the abnormal allele in sperm from the father. We conclude that this hypochondrogenesis phenotype resulted from a new dominant mutation in the type II collagen gene, COL2A1.

Our collaborators also confirmed the serine substitution for glycine841 in the SEMD proband by SSCP analysis of cDNA followed by direct sequencing. This substitution was the result of a point mutation in the first position of the glycine codon (GGT to AGT).

The amino acid substitutions produced a more slowly migrating population of α1(II) collagen chains suggesting an increased degree of lysyl hydroxylation and hydroxylysyl glycosylation. Judging by the glutamate/glycine ratio in D12 peptide and the serine/glycine ratio in the tryptic D10 peptide, which contains the mutated residues, there were roughly equal amounts of the mutant α1(II) and normal α1(II) chains in the cartilage matrix. Thus both normal and mutant COL2A1 alleles had contributed equally to matrix protein.

The amino acid sequencing results on peptides D3 and D5 from the hypochondrogenesis tissue at cycles where lysine occurs at the Y

position of the Gly-X-Y repeat are consistent with post-translational overmodification, but this appears to be variable in extent, dependent on the actual sequence. In theory, for the homotrimeric type II collagen molecule, three-quarters of the molecules would contain 1 or 2 mutant chains, one-eighth would contain 3 normal chains and one-eighth 3 mutant chains. If all molecules with at least one abnormal chain became post-translationally overmodified, seven- eighths of the chains should have an increased hydroxylysine content, assuming that overmodification occurs equally in both normal and mutant chains of molecules that contain a mutant chain. The observations that only a small proportion of protein corresponding to normally migrating α1(II) chains was present in a pepsin digest, and similarly for the CB peptides, support these theoretical considerations.

The lysine at residue 573 in peptide D3 appears not to be hydroxylated in normal cartilage, since the peptide elutes as a single peak on reverse phase HPLC (Figure 3) and gave only lysine on sequence analysis. From the hypochondrogenesis tissue, about equal amounts of hydroxylysine-containing and lysine-containing peptides were resolved (Figure 3). Our interpretation is that the lysine at

residue 573 normally resists hydroxylation (probably because it follows glutamate), and the extent of overmodification in the hypochondrogenesis case is therefore limited at this residue to partial hydroxylation. It may be that this lysine residue escapes hydroxylation even in some chains of molecules that contain one or more mutant α1(II) chains. At other lysine sites (the lysine at residues 648 of D5 for example), each residue may normally be largely hydroxylated and so the overmodification is in degree of glycosylation. In short, the degree of post-translational modification of lysine residues clearly varies dependent on the local sequence in normal α1(II) chains thus resulting in site-dependent variability in the overmodification of molecules bearing mutant α1(II) chains. These results and the electrophoretic evidence show post-translational overmodification in peptides amino-terminal to the mutation site, consistent with theory.

How does expression of this mutant protein molecule affect the organization of the collagen fibril and associated matrix? The measured content of mature cross-links in the cartilage collagen implies that there is no dramatic effect on intermolecular cross-linking of the mutant protein. The ultrastructural observation of

grossly dilated endoplasmic reticulum, which presumably contains abnormal type II collagen molecules (Godfrey et al., 1988), suggests that there is a reduced amount of type II collagen in the extracellular matrix and therefore a reduced ratio of type II collagen to other matrix molecules. It is also possible that the intracellular retention secondarily affected the function of chondrocytes at the growth plate. The overmodification and/or the direct effect on protein conformation of the amino acid substitution, may interfere with specific associations between the type II collagen fibrils and other matrix molecules, such as types IX and XI collagens, decorin, biglycan, fibromodulin or aggrecan (Heinegard and Oldberg, 1989). Finally, fibrils containing the mutant molecules may not be turned over normally within the matrix.

These cases of hypochondrogenesis and SEMD add support to the concept that dominant mutations in the COL2A1 gene characterize at the molecular level those disorders that have been clinically grouped into the spondyloepiphyseal dysplasia family of chondrodysplasias. Analysis of additional cases of type II collagenopathies at the histologic, protein, and nucleic acid levels will likely lead to the identification of functional domains within the type

II collagen molecule, and provide a better understanding of how a molecular defect translates into a clinical phenotype and of the relationships between the various macromolecules of the cartilage matrix.

CHAPTER IV

STRUCTURALLY ABNORMAL TYPE II COLLAGEN PRODUCES THE KNIEST DYSPLASIA PHENOTYPE

Summary to Chapter IV

Kniest dysplasia is a heritable chondrodysplasia that severely affects skeletal growth. Recent evidence suggests that the etiology is based on mutations in COL2A1, the gene for collagen type II(Winterpacht et al. 1993; Spranger et al. 1994). We report the detection and partial characterization of an identical defect in type II collagen in two unrelated patients (probands 1&2) with Kniest dysplasia and the confirmation of another defect in type II collagen identified by our collaborators at the gene level in a third case (proband 3) of Kniest dysplasia. Analysis of cyanogen bromide(CB) digested cartilage samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis from the three probands revealed an abnormal band for peptide $\alpha 1$(II)CB12. The peptide was purified and digested with endoproteinase Asp-N. Fragments unique to the Kniest tissues were identified on reverse-phase high pressure liquid chromatography and by sequence analysis. For probands 1 and 2 the

results established a deletion of amino acids 102-108 of the α1(II) triple helical domain, which disrupted the (gly-X-Y)n repeat needed for helix formation. Our collaborators confirmed this by sequence analysis of DNA amplified from both probands, revealing the molecular basis to be a single nucleotide mutation at a CpG dinucleotide (GCG→ GTG) in the codon for alanine102. The mutation created a new splice donor site which would account for the absence of the last seven amino acids from the 3' end of exon 12 in α1(II)CB12. For proband 3, the protein data confirmed an aspartate for glycine103 substitution identified by our collaborators as a G to A transition in the codon for glycine103. Light and electron micrographs of proband 1's cartilage showed the perilacunar foamy matrix ("Swiss cheese") characteristic of Kniest dysplasia, and chondrocytes containing dilated rough endoplasmic reticulum which earlier studies (Poole et al. 1988) had shown were filled with type II procollagen. These three cases strengthen the concept that Kniest dysplasia is based on mutations of COL2A1, and belongs within the broad spectrum of chondrodysplasias caused by type II collagenopathies.

Introduction to Chapter IV

Type II collagen is a homotrimeric molecule found in hyaline cartilages, intervertebral disc, and tissues of the eye. Mutations in the gene for type II collagen, COL2A1, have been identified in certain chondrodysplasias. These heritable disorders of skeletal growth show a wide range of clinical phenotypes (Rimoin and Lachman 1990). Those based on COL2A1 mutations commonly express skeletal and ocular anomalies, but individual characteristics and clinical severity presumably depend on the degree of disruption of collagen structure, assembly, secretion, and fibrillogenesis. The current list of clinical forms includes: achondrogenesis, (Eyre et al., 1986; Vissing et al., 1989) hypochondrogenesis, (Bogaert et al., 1992; Horton et al., 1992; Bonaventure et al., 1995; Chan et al., 1995; Mortier et al., 1995; Williams et al., 1995), Kniest dysplasia, (Wilkin et al., 1993; Winterpacht et al., 1993a; Spranger et al., 1994; Mortier et al., 1995; Fernandes et al., 1998; Weis et al., 1998), spondyloepiphyseal dysplasia congenita (SEDc), (Lee et al., 1989; Tiller et al., 1990; Bleasel et al., 1995; Chan et al., 1995; Tiller et al., 1995), spondyloepimetaphyseal dysplasia (SEMD), (Vikkula et al., 1993; Tiller et al., 1993) familial osteoarthritis/late onset SED (Ala-

Kokko et al., 1990; Katzenstein et al., 1992; Williams et al., 1993; Winterpacht et al., 1993b), Stickler syndrome, (Ahmad et al., 1991; Ahmad et al., 1993; Ritvaniemi et al., 1993); and Wagner syndrome, (Korkko et al., 1993). These diseases span an extremely wide range of clinical severity, from the Stickler and Wagner syndromes at the mild end, which are characterized by ocular pathology and arthropathy, to the perinatal lethal forms of achondrogenesis/hypochondrogenesis.

Kniest dysplasia is characterized clinically by disproportionate dwarfism, cleft palate, myopia, progressive conductive hearing loss, arthropathy, and scoliosis. Radiographically, Kniest dysplasia exhibits progressive skeletal changes, including splayed epiphyses and metaphyses, platyspondyly, and narrowed joint spaces (Taybi and Lachman, 1990). Histologically, cartilage from Kniest dysplasia patients demonstrates a unique extracellular matrix of sparse fibrils with a vacuolar appearance, referred to as "Swiss-cheese" cartilage (Horton and Rimoin, 1979). Recent reports suggest that Kniest dysplasia may be the result of deletions or point mutations in COL2A1, which result in abnormal mRNA splicing and deletions in

type II collagen (Winterpacht et al., 1993a; Spranger et al., 1994; Fernandes et al., 1998; Weis et al., 1998).

In three sporadic cases of Kniest dysplasia, we have identified in two unrelated patients a deletion of seven amino acids , and in another patient an aspartate substitution for glycine within a proportion of the α-chains of type II collagen present in cartilage. The deletion in the first two probands resulted from the same mutation, which occurred at a CpG dinucleotide in exon 12. The mutation created a preferred splice donor, effectively truncating the exon. The substitution in the third proband resulted from a G to A transition in the codon for glycine 103 also in exon 12.

Materials and Methods

Clinical Summary. Each of the first two patients studied was the only affected individual within their respective families. The two families were of different ethnic backgrounds and from different parts of the United States. We therefore presume that they were unrelated.

Proband 1: This patient was initially seen when she was 23 years old. Physical examination showed her trunk to be quite short

with a high dorsal lumbar lordosis. Her elbows were held in a fixed position with extension limited to almost 90°. Shoulder movement was also limited to about 90°. Her fingers were long with prominent interphalangeal joints. There was genu valgum, some knobbiness around the knees, and her feet were held in a varus position. She had a repaired cleft palate, severe myopia and hearing loss. Examination of x-rays defined her diagnosis as Kniest dysplasia. Neither parent was affected.

Proband 2: This patient was initially seen at 12 years of age. Her medical history revealed that at birth she had a cleft palate, bilateral varus deformity of the tibias, bilateral hip dislocation and a heart murmur. During early childhood, deformities of the arms, legs, and spine developed, as did high frequency hearing loss and myopia. Physical examination revealed significant short stature, a flattened face with prominent eyes, and maxillary hypoplasia. There was a repaired cleft palate and speech was markedly palatal. There was thoracolumbar lordosis, with a prominence at the lumbar sacral junction. All joints of the fingers were prominent and had limited flexibility. There was also limited extension of the elbows and external rotation of the hips. Her knees appeared knobby with

severe genu valgum, and varus deformity of the feet. The radiographic phenotype was compatible with the diagnosis of Kniest dysplasia. Neither parent was affected.

Proband 3: This patient was the second child of clinically normal parents and was initially seen at 2 years of age. Moderate bowing of the tibiae, mild myopia, and a flat midface was noted at that time. Her height was 77 cm (< 5th percentile), equivalent to a normal height:age ratio for a 14 month old. Her gait was slightly wide, with external rotation of the femurs and genu varum. The internal rotation of her hips was limited to 20°. She had prominent wrist and ankle joints with rhizomelic shortening. Pectus excavatum with moderate recession on inspiration was observed. Her history was significant for a cleft palate at birth. The diagnosis of Kniest dysplasia was made based on radiographic findings at birth; short "dumbbell" shaped femora and tibiae were observed, as well as mild platyspondyly and coronal clefts. Subsequent films taken at two years of age confirmed the diagnosis.

Protein Analysis

Pepsin-solubilized collagen Samples of articular and costal cartilages were extracted in 4M guanidine HCl, 50mM Tris/HCl, pH 7.0 at 4°C for 48hr. The washed residue was digested with pepsin (Miller 1972). Articular cartilage from a 24 year old male was processed as a control. SDS-PAGE was carried out by the method of Laemmli (1970) on 6 and 15% gels.

Peptide analyses Articular and costal cartilage samples were digested with CNBr in 70% (w/v) formic acid for 24hr at room temperature (Eyre and Muir 1975). Peptides were fractionated by molecular sieve HPLC (TosoHaas G3000SW, 7.5 mm x 60 cm, two columns in tandem; eluent, 100 mM Na phosphate, pH 6.8, 30% (v/v) acetonitrile), then reverse phase HPLC (Bateman et al., 1986; Eyre 1987). The pool containing $\alpha 1$(II)CB12 (identified by SDS-PAGE) was dried and digested with endoproteinase Asp-N (sequencing grade, Boehringer Mannheim), and the peptides were fractionated by reverse phase HPLC (Eyre 1987). Individual peptide yields were estimated by integrated 220nm absorbance of peaks and PTH-amino acid recovery on Edman sequence analysis.

Protein microsequencing. Peptides were covalently bound to Sequelon-AA membranes (Millipore) activated by carbodiimide.

Sequencing was carried out on a Porton 2090E instrument equipped with on-line HPLC analysis of PTH-amino acids using a modified manufacturer's program that resolved derivative peaks for 4-hydroxyproline, hydroxylysine and hydroxylysine glycosides.

Collagen cross-linking analysis. Cartilage was acid hydrolyzed (6M HCL, 110°C, 24 hr.) and pyridinoline cross-linking residues were quantified by reverse-phase HPLC and fluorometry (Eyre et al., 1984). Hydroxyproline was assayed as an aliquot of the hydrolysate colorimetrically (Stegemann 1958). Pyridinoline content was expressed as moles per mole of collagen.

Protease Susceptibility. Pepsin solubilized collagen molecules were digested with elastase (porcine pancreatic) in 50 mM Tris/HCl, pH 6.8 for 4 hours at 20°C. Digestion products were separated by SDS-PAGE and electroblotted to PVDF membranes for direct sequence analysis.

Cross-linked Peptide Analysis. Pepsin solubilized cartilage collagen molecules were digested with CNBr in 70% (w/v) formic acid for 24hr at room temperature (Eyre and Muir 1975). Peptides were fractionated by molecular sieve HPLC (TosoHaas G3000SW, 7.5 mm x 60 cm, two columns in tandem; eluent, 100 mM Na phosphate, pH

6.8, 30% (v/v) acetonitrile). The pool containing α1(II)CB12 cross-linked to α1(II) C-telopeptides (identified by fluorescence (excitation 330 nm emission 398 nm) and SDS-PAGE) was then further separated by reverse phase HPLC (Bateman et al., 1986; Eyre 1987), dried and digested with endoproteinase Asp-N (sequencing grade, Boehringer Mannheim), and the peptides were fractionated by reverse phase HPLC (Eyre 1987).

Results

Probands 1&2

Electrophoresis (SDS-PAGE) of pepsin-extracted type II collagen from cartilage of proband 1 consistently showed marginally slower α1(II) chains compared with controls (Figure 7). Similarly peptides α1(II) CB10 and CB11 from both Kniest dysplasia patients tended to be marginally slower than control CB10 and CB11 on SDS-PAGE (Figure 7). Close inspection of the CB12 peptide from the Kniest dysplasia tissue showed a broad smear with a portion running faster than the control CB12 peptide, suggesting an abnormality in this domain (Figure 7). Sufficient CB12 for peptide mapping and sequence analysis was isolated by sequential molecular sieve (Figures 8&9) and reverse-phase HPLC (Figure 10). SDS-PAGE of

Figure 7. SDS-polyacrylamide electrophoresis of pepsin-solubilized collagen (lanes 1&2) and CNBr-derived peptides (lanes 3,4&5) from human control and Kniest dysplasia cartilage. Pepsin-solubilized α1(II) chains are slightly retarded in the Kniest sample(lane 2; proband 1) compared with the control (lane 1). Peptide α1(II)CB12 is abnormally broad in the Kniest (lanes 4&5; probands 1 & 2) compared with control tissue (lane 3).

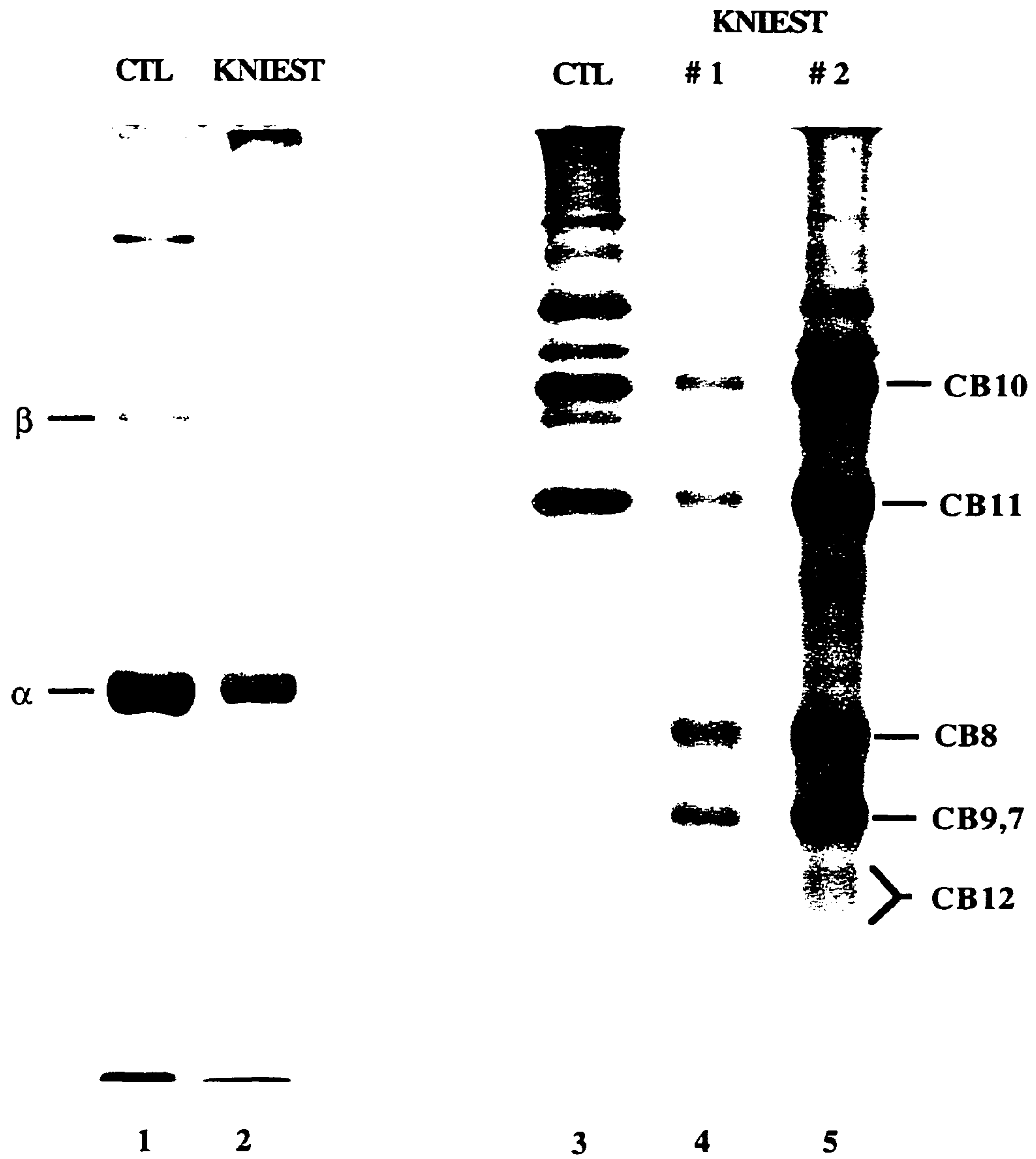

CTL
KNIEST
β
α
1
2
KNIEST
CTL
1
2
CB10
CB11
CB8
CB9,7
CB12
3
4
5

Figure 8. Elution profile from molecular-sieve HPLC of CB-peptides of type II collagen from the Kniest cartilage (proband 1). The fractions marked by the bar were pooled for reverse phase HPLC.

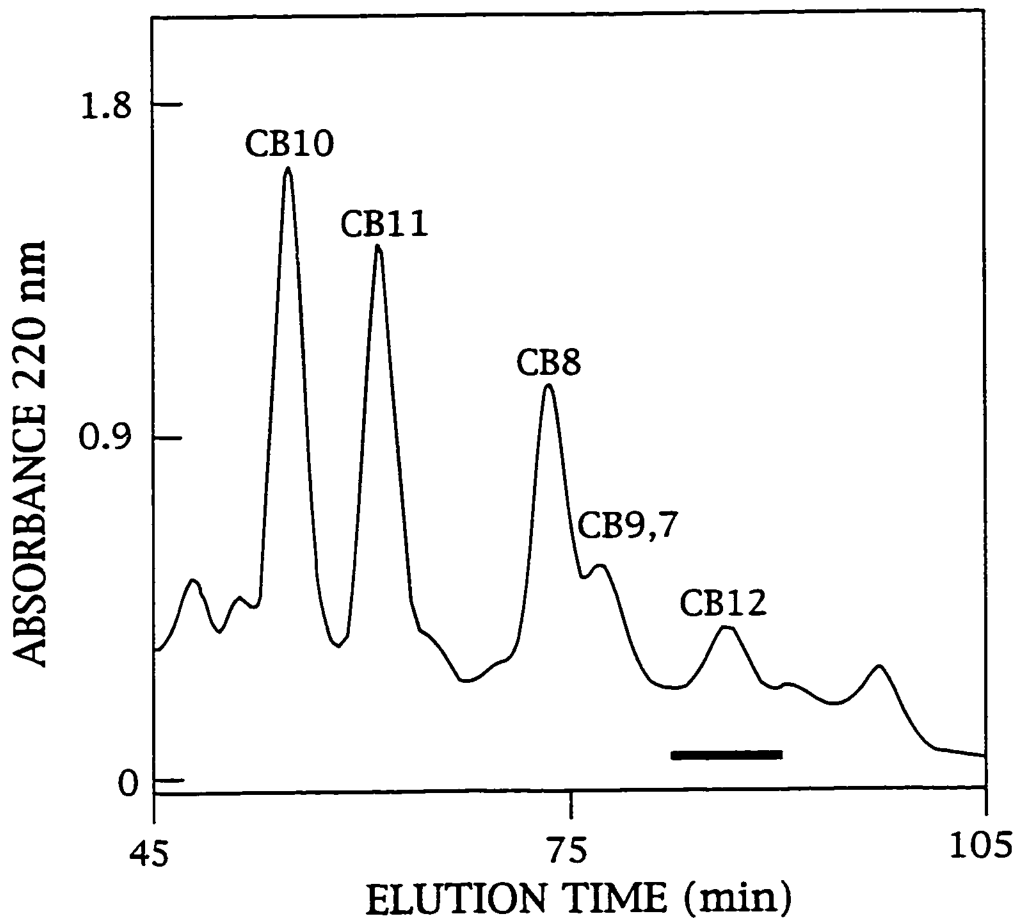

CB10
CB11
CB8
CB9,7
CB12
1.8
0.9
0
45
75
105
ABSORBANCE 220 nm
ELUTION TIME (min)

Figure 9. SDS-12.5% polyacrylamide electrophoresis of fractions sampled across the molecular-sieve chromatogram in figure 8. The fractions marked by the bar were pooled for reverse phase HPLC. The first lane contains cyanogen bromide digested $\alpha 1(II)$ used as a molecular weight standard.

CB10-
CB11-
CB8-
CB9,7-
CB12-

reverse-phase HPLC fractions revealed a doublet, with the lower component eluting slightly earlier than the upper component (Figure 10), suggesting a peptide with a deletion.

A pool containing total α1(II)CB12 was digested with endoproteinase Asp-N and the resulting peptide profile on reverse-phase HPLC revealed features unique to the Kniest dysplasia cartilages. Reverse-phase HPLC of Asp-N digested peptides demonstrated a single peptide peak for control cartilages, D3 (Figure 11a). The Kniest dysplasia samples gave two versions of peptide D3 (D3 and D3'), and a new peptide ΔD3 (Figure 11b). Microsequencing of peptide D3 from control and Kniest dysplasia tissues yielded mostly galactosylhydroxylysine at cycle 4 and a mixture of galactosylhydroxylysine and hydroxylysine at cycle 13. Peptide D3' proved to be an overmodified version of D3, with predominantly glucosylgalactosylhydroxylysine at both cycles 4 and 13. The new peptide, ΔD3, revealed a 7-amino acid deletion (α1(II) residues 102-108; Figure 11), and exclusively glucosylgalactosylhydroxylysine at cycle 4. The ratio of peptides D3, D3', and ΔD3 was about 1:1:1. In yield, therefore, mutant α1(II) chains accounted for about one-third of total type II collagen in the cartilage. The results of peptide

Figure 10. Reverse-phase HPLC of peptide CB12 (from Fig. 9). The inset shows the results of SDS-15% PAGE on fractions across the peak as indicated by the bar. A doublet is evident, the lower band being partially resolved slightly earlier in the chromatogram. Fractions 38-41 were pooled for proteolysis (endoproteinase Asp-N) and peptide mapping. Lane K: total CB-peptides from the Kniest tissue.

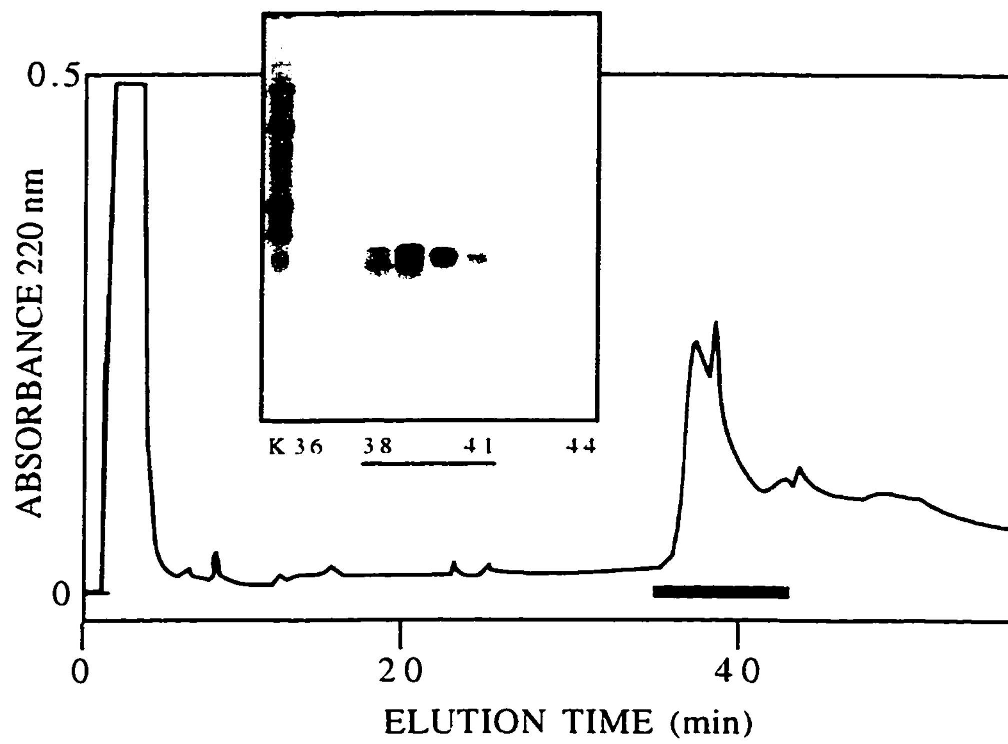
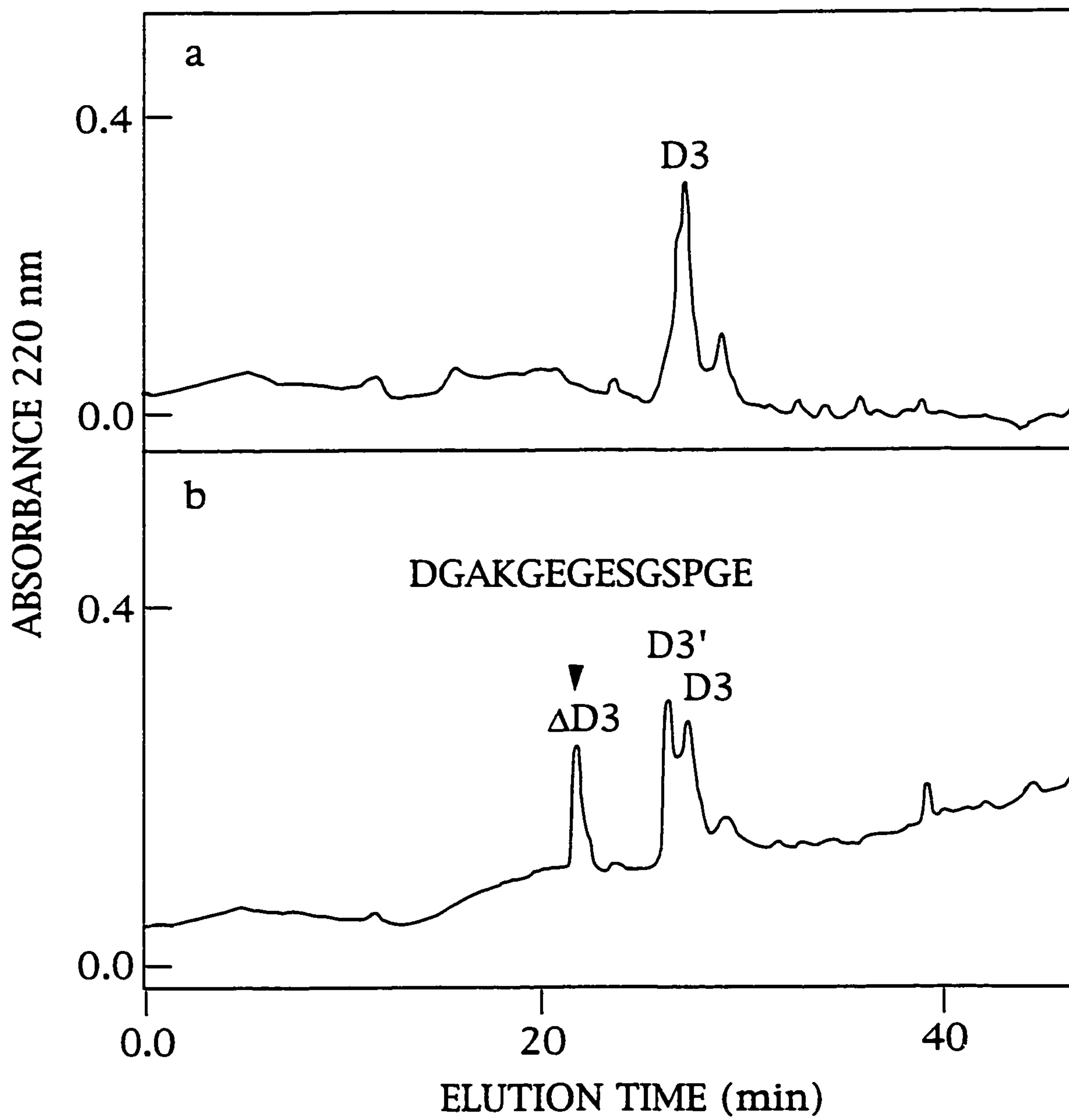

0.5
ABSORBANCE 220 nm
0
K 36
38
41
44
0
20
40
ELUTION TIME (min)

Figure 11. Elution profile on reverse-phase HPLC of endoproteinase Asp-N peptides from the CB12 peptide fragment of αl(II) from (a) human control cartilage and (b) Kniest cartilage (proband 1). Peptide ΔD3 harbored the seven-amino acid deletion.

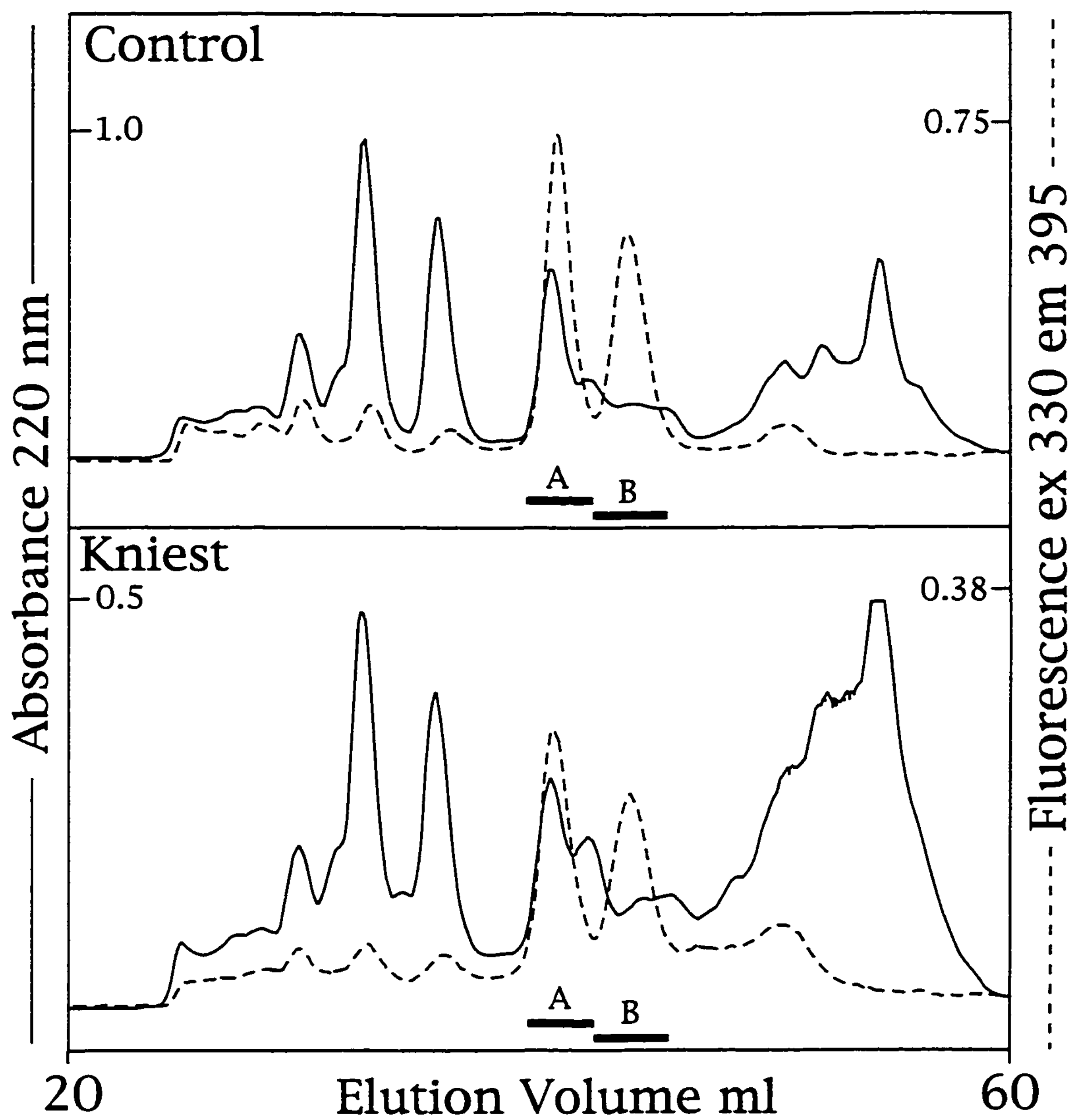

a
D3
b
DGAKGEGESGSPGE
ΔD3
D3'
D3
0.4
0.0
0.4
0.0
ABSORBANCE 220 nm
0.0
20
40
ELUTION TIME (min)

mapping and microsequencing of peptide CB12 were essentially identical for both Kniest dysplasia cases.

The measured concentration of hydroxylysylpyridinoline crosslinks in collagen of the Kniest dysplasia cartilages was 1.2 mole/mole for proband 1 and 1.3 mole/mole for proband 2,compared with a mean of 1.5 ± 0.2 (S.D.) mole/mole of collagen for control adult human cartilage (Eyre et al, 1984).

The molecular sieve profile of CNBr digested pepsin solubilized type II collagen gives two main fluorescent peaks (figure 12). Peak A corresponds to α1(II)CB9,7 cross-linked to two N-telopeptides, and peak B corresponds to α1(II)CB12 cross-linked to two C-telopeptides. Peak B from control and Kniest were pooled and further purified by RP-HPLC (figure 13). SDS-PAGE analysis of the fluorescent peptidesrevealed a lack of noncross-linked α1(II)CB12 peptide in the fluorescent peaks and showed that noncross-linked α1(II)CB12 eluted earlier in the profile. The fluorescent peptides were pooled and digested with endoproteinase asp-n. The resulting profile (figure 14) and sequence analysis revealed the presence of α1(II)CB12 cross-linked to C-telopeptides and peptide D3, but not the deletion containing peptide ΔD3. This suggests that the shortened

Figure 12. Molecular seive HPLC of CB-peptides of pepsin solubilized type II collagen from the control (upper panel) and the Kniest (lower panel). Fluorescence as indicated by the dashed lines was monitored as an excitation of 330 nm and an emission of 395 nm. The fluorescent peptides indicated by the bars were pooled for reverse phase HPLC.



Control
1.0
0.75
A
B
Kniest
0.5
0.38
A
B
Absorbance 220 nm
Fluorescence ex 330 em 395
20
Elution Volume ml
60

Figure 13. Reverse phase HPLC of fluorescent peptides CB12 crosslinked to two C-telo peptides from control and Kniest cartilages. The insets are 15% SDS-PAGE sampled around the fluorescent peaks as indicated by the double lines. The first lanes of the insets are the total CB peptides from control (C) and Kniest (K) used as molecular weight markers. The inset shows noncross-linked CB12 eluting eluting before cross-linked CB12 and noncross-liked CB9,7. Fluorescence as indicated by the bold lines was monitored as an excitation of 297nm and an emission of 395nm. The fluorescent peptides as indicated by the solid bars were pooled for enzyme digestion.

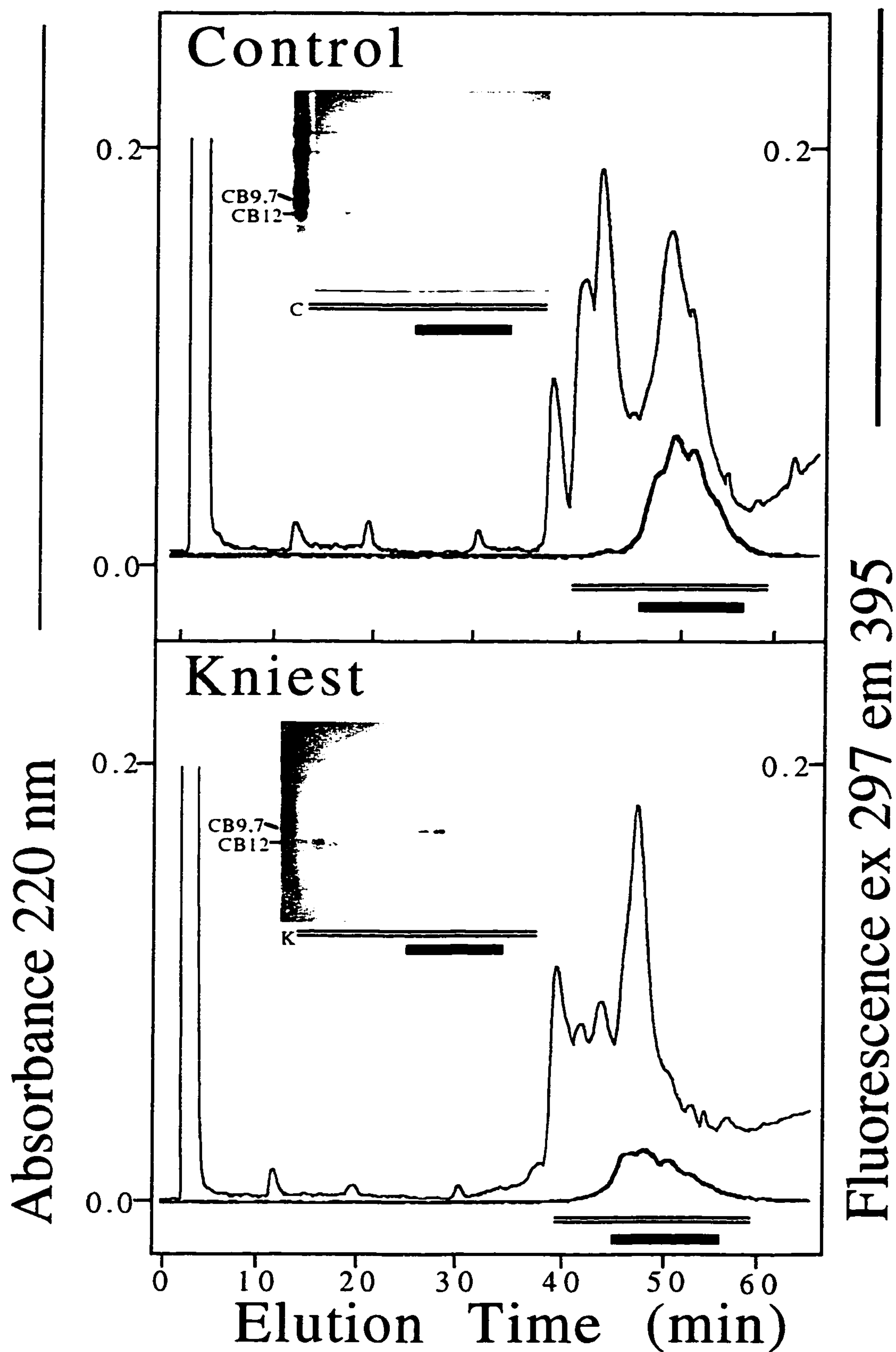
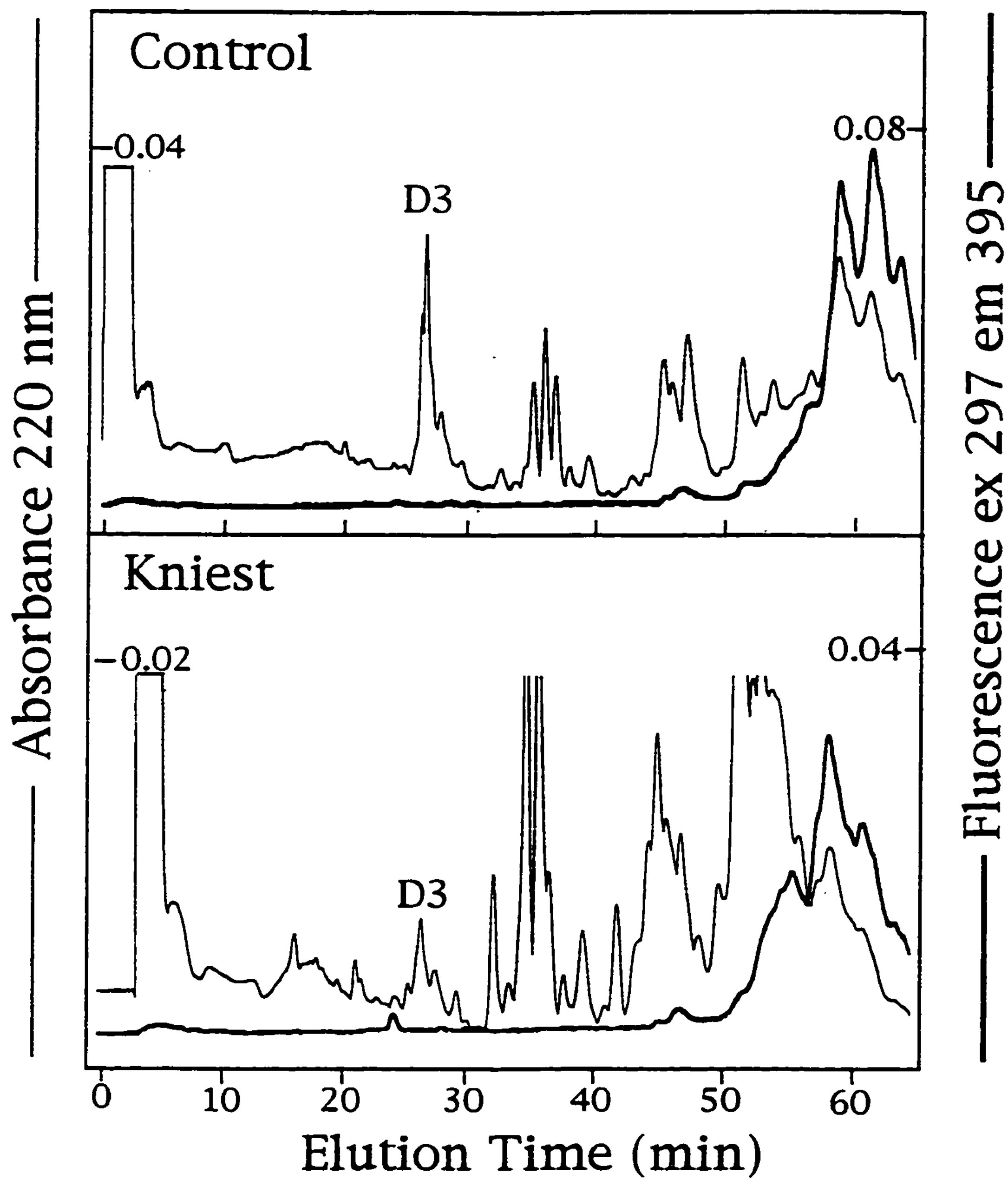

Control
CB9.7
CB12
C
0.2
0.0
Kniest
K
10
20
30
40
50
60
Elution Time (min)
Absorbance 220 nm
Fluorescence ex 297 em 395

Figure 14. Elution profile on reverse phase HPLC of endoproteinase Asp-N peptides of cross-linked CB12 from control (upper) and Kniest (lower). Peptide D3 was identified by amino acid sequencing. The peptide ΔD3 containing the seven amino acid deletion was not present. Fluorescence as indicated by the bold lines was monitored as an excitation of 297 nm and an emission of 395 nm.


Control
Kniest
D3
D3
0.04
0.08
0.02
0.04
Absorbance 220 nm
Fluorescence ex 297 em 395
0
10
20
30
40
50
60
Elution Time (min)

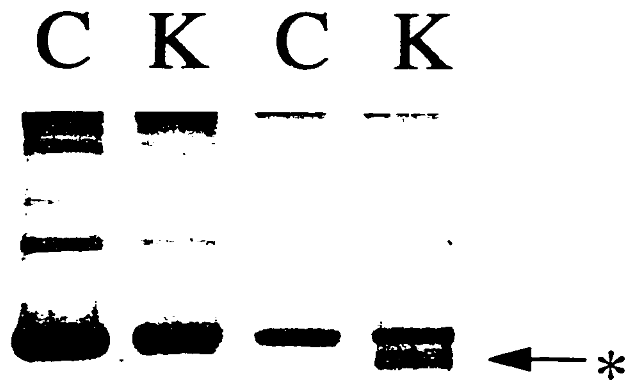
$\alpha 1(II)$ chain containing the seven amino acid deletion did not participate in the formation of mature trivalent cross-links.

Elastase digestion of collagen molecules from proband 2 under nondenaturing conditions revealed a shorter peptide not found in the control digest (figure 15). Direct sequence analysis of a PVDF electroblot of this peptide gave the sequence RGFPGTPGLP, which is the amino end of $\alpha 1(II)$ beginning at position 75 of the triple helical domain. This data indicates that a site at least 27 residues amino terminal to the mutation site is susceptible to proteolysis, whereas the control molecules were not susceptible nor was the mutation site in the Kniest molecules.

Proband 3

Electrophoretic analysis of pepsin-solubilized cartilage revealed no differences in the mobility of the control and Kniest $\alpha 1(II)$ chains (Figure 16). The CB-peptides from the Kniest tissue showed a decreased electrophoretic mobility of $\alpha 1(II)$CB-12(Figure 16), confirmed from chromatographically purified CB-12. Microsequencing of the asp-N peptide D3B from the Kniest tissue identified aspartate at cycle 1 (residue 103 of the triple helical domain) in approximately one-third of the peptide pool sequenced

Figure 15. SDS-polyacrylamide gel electrophoresis of control (C) and Kniest (K) pepsin solubilized type II collagen digested with elastase (+) at room temperature for 4 hours. The sequence shown below is the result of direct amino acid sequencing off a PVDF electroblot of the new peptide indicated by the arrow.



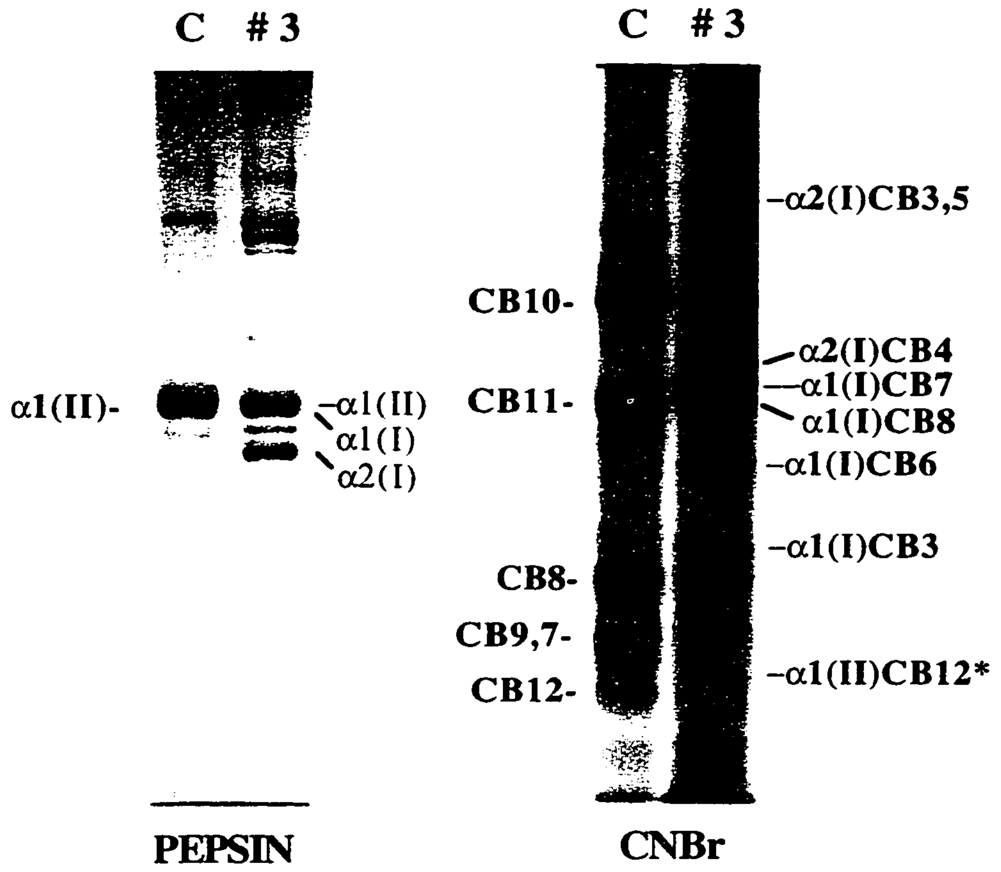
- - + +

* * *

*RGFPGTPGLP

75 84

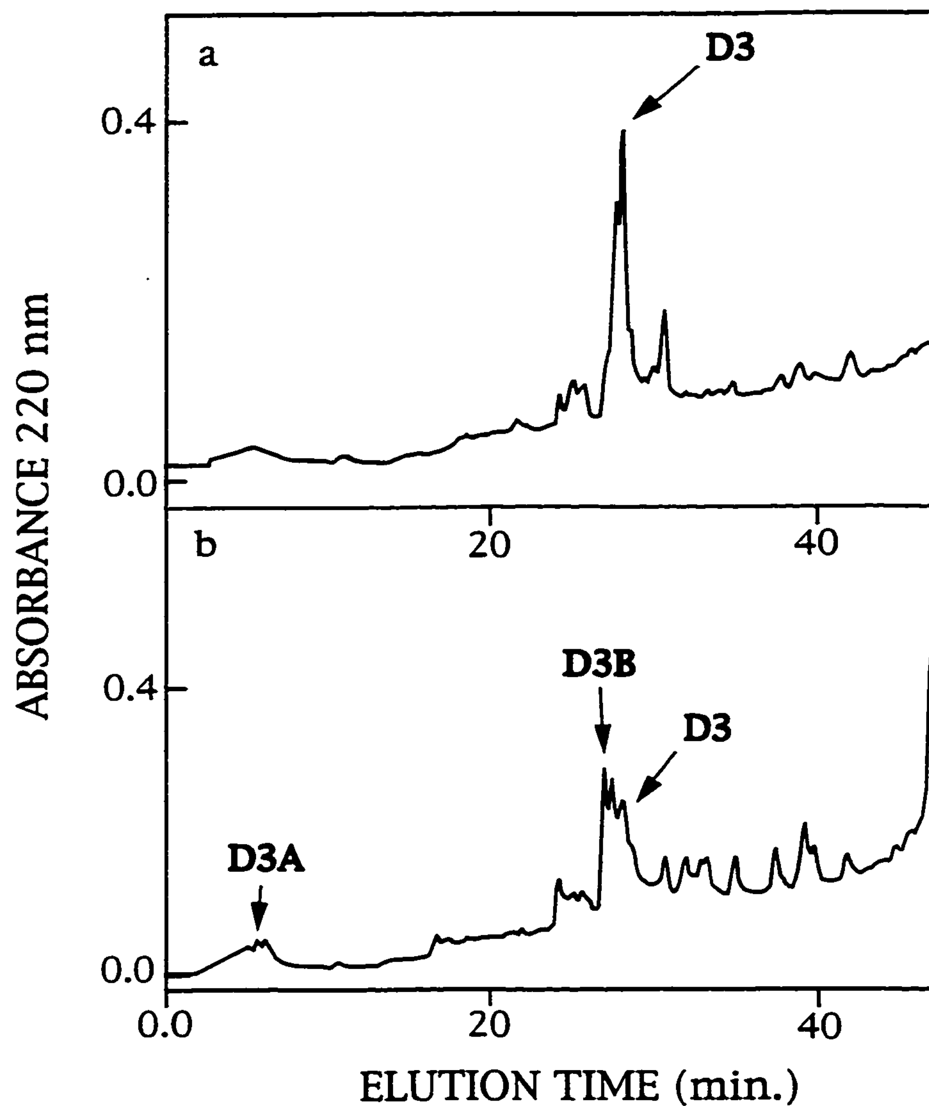
Figure 16. SDS-polyacrylamide gel electrophoresis of pepsin and CNBr-derived peptides from human control (C) and Kniest dysplasia cartilage (#3, proband 3). The proband's tissue shows a mixture of types I and II collagens with essentially normal mobility of α1(II), and for the major α1(II)CB-peptides (#3) compared with control tissue peptides(C). Peptide α1(II)CB-12* gave a broad, retarded band (confirmed on purification) suggesting overmodification.


C
3
α1(II)-
-α1(II)
α1(I)
α2(I)
PEPSIN
C
3
-α2(I)CB3,5
CB10-
α2(I)CB4
α1(I)CB7
CB11-
α1(I)CB8
-α1(I)CB6
-α1(I)CB3
CB8-
CB9,7-
-α1(II)CB12*
CB12-
CNBr

(Figure 17). This confirmed the mutation identified in COL2A1 by our collaborators and the presence of abnormal collagen in the proband's cartilage.

Post-translational overmodification of α1(II)CB-12 was revealed by sequence analysis. Peptide D3 from control and a fraction of D3 from the Kniest dysplasia tissue yielded mostly galactosylhydroxylysine at cycle 4 and a mixture of galactosylhydroxylysine and hydroxylysine at cycle 13. Peptide D3A, the product of the mutant allele, and an early eluting version of D3 from the Kniest tissue, at cycle 4 gave approximately equal amounts of glucosylgalactosylhydroxylysine and galactosylhydroxylysine. Therefore, this lysine residue was post-translationally overmodified in α-chains containing the Gly->Asp substitution and in a portion ofthe normal α-chains, presumably the latter being incorporated with abnormal chains into heterotrimeric molecules. Post-translational undermodification was also seen on sequence analysis. Peptide D3B, gave mostly proline in cycle 3, and equal amounts of lysine and hydroxylysine in cycle 6 (corresponds to cycle 13 of peptide D3). Apparently, therefore, proline hydroxylation was inhibited by the presence of the mutation near the hydroxylation

Figure 17. Reverse phase HPLC of endoproteinase Asp-N peptides from the $\alpha 1$(II)CB-12 (a) control human cartilage, (b) Kniest cartilage (proband 3). Determined N-terminal sequences (single-letter amino acid code) for peptides D3, D3A, and D3B are shown below.



<table>
<tr><td colspan="2">103
D3 DGAKGEAGAPGVKGESGSPGEN</td></tr>
<tr><td>DGAKGEA
D3A</td><td>**D**APGVKGESGSPGEN
D3B</td></tr>
</table>

site, though the proposed minimum substrate, X-Pro-Gly, was preserved (Kivirikko KI, Myllyla R (1980). The decrease in hydroxylation and glycosylation of the lysine residue at cycle 6 suggests that lysyl hydroxylase and the glycosyl transferases were inhibited by the neighboring Gly->Asp substitution.

Histology

Our collaborators examined the cartilage from proband 2 by light microscopy. As seen in other individuals with Kniest dysplasia (Horton and Rimoin, 1979), a perilacunar foamy appearance was observed in the matrix surrounding the chondrocytes (Wilken et al., 1994). On transmission electron microscopy, chondrocytes contained swollen rough endoplasmic reticulum filled with a granular material (data not shown). At the light microscopic level, earlier studies (Poole et al., 1988) showed that the chondrocytes from this same patient stained with antibodies to type II procollagen. Similar histological results were found for proband 1.

Discussion

The results established that two unrelated individuals with typical Kniest dysplasia were heterozygous for the same mutation in

the type II collagen gene. The mutation, a C to T transition, created a splice donor identical to the splice donor of COL2A1 intron 27 (Baldwin et al., 1989) that resulted in truncation of exon 12. Although the mutation also changed the codon for alanine^{102} (GCG) to valine (GTG), protein analysis showed absence of a valine-containing peptide. The mutation thus resulted exclusively in use of the new splice donor in the product of the mutant allele. However, we cannot rule out the possibilities that either a normally spliced mRNA containing the point mutation or an $\alpha 1$(II) chain containing the A102V substitution are unstable. This appears to be the first example of a splicing mutation that produces a deletion in the collagen triple helix and also disrupts the gly-X-Y repeat.

Specific effects on the post-translational chemistry of the cartilage type II collagen molecule were also evident. Peptide sequencing clearly showed that the lysines at residues 99 and 108 were overmodified in the normal $\alpha 1$(II) chain, and at residue 99 in the mutant chain of the Kniest dysplasia cartilages, compared with the control tissue $\alpha 1$(II) chain. There was also slight retardation on SDS-PAGE of the $\alpha 1$(II) CB10 and CB11 peptides, suggesting a minor degree of overmodification (Figure 7), but much less marked than

observed, for example, with the gly853→glu substitution which resulted in a hypochondrogenesis phenotype (Figure 1).

In interpreting these findings, current concepts of triple-helix formation and the known effects of mutations that disrupt (gly-X-Y)n need to be considered. The triple-helix is believed to fold from the carboxyl-terminal end of class 1 collagen molecules (types I, II, and III). Substitutions for glycine in the triple helix of type I collagen result in marked post-translational overmodification of those molecules containing one or more abnormal chains, amino-terminal to the mutation site (Byers 1993). Both, the mutated and the normal chains are believed to be overmodified because the mutation results in retarded helix formation, which affects all three chains equally. On this basis, the 1:1:1 ratio of the three forms of peptide D3 (Figure 11) from Kniest dysplasia cartilage can be interpreted as follows. One-third of the type II collagen molecules in the matrix were normal homotrimers (no overmodification). Two-thirds of the molecules contained one or more mutant chains (both mutant and normal being overmodified), with an overall ratio of mutant to normal chains of about 1:1 in this pool. For this to occur, there would have to be preferential retention in the cell and/or degradation of

molecules containing mutant chains. For example, failure of all mutant homotrimers and two-thirds of heterotrimers to leave the cell would account for the observed peptide ratios, and would result in only three-eighths of theoretically possible molecules being deposited in the matrix.

The observation of type II procollagen in dilated, rough endoplasmic reticulum of chondrocytes in cartilage of proband 2 (Poole et al., 1988), and sparsely distributed collagen staining in the matrix (Horton and Rimoin, 1979), are consistent with the above interpretation of poorly secreted type II collagen bearing mutant chains. In addition to poor secretion, and presumably some degradation in the cell, of molecules that contain mutant chains, abnormal molecules that are secreted and incorporated into the extracellular fabric may be preferentially degraded during normal matrix remodeling. Nevertheless, it is clear that the phenotype results in part from the deposition of a significant amount of mutant protein into the cross-linked fabric of the extracellular matrix.

The deletion disrupted the gly-X-Y triple-helical repeat of the type II collagen molecule. In considering how heterotrimeric molecules may assemble from shortened and normal chains, it is

assumed that deletions which preserve the (gly-X-Y)n structure would result in out-of-register chains amino-terminal to the deletion site (Willing et al., 1988; Chessler and Byers, 1992; Wallis et al., 1992). The mutation described here is unusual in that it deletes a peptide segment and disrupts the (gly-X-Y)n triple-helical repeat, so this theory of helix assembly may not apply. Figure 18 illustrates two possible consequences of the deletion described in this study. In model (a), assuming the triple-helix forms from the carboxyl-terminus to the amino-terminus in a zipper-like fashion (Kuivaniemi et al. 1991), α-chains resume helix formation out-of-register from a hiatus at the mutation site. This potentially could interfere with amino-propeptide removal by the propeptidase (Vogel et al., 1988). However, the lack of pN-α-chains in denaturant extracts of the Kniest dysplasia cartilage (not shown) is evidence against this model. Yet in our protease susceptibility assay, assuming that elastase does not cleave triple helical molecules under these conditions, the data does suggest an altered triple helical conformation amino terminal to the mutation site. In model (b), mutant and normal chains resume an in-register triple-helix from the mutation site, accommodated by a looping out of the normal α-chain sequence. This would result in

Figure 18. Divergent molecular concepts of heterotrimeric molecules containing one mutant α1(II) (Δ102-108) chain and two normal chains. In proposal a) chains are out of register amino-terminal to the mutation site; in b) chains are in register in both directions away from the disrupted mutation site.

a) Chains out of register N-terminal to the mutation site

100 115

N GLDGAKGEAGAPGVKGESGSPGEN C

GLDGAKGEAGAPGVKGESGSPGEN

GLDGAKGESGSPGEN

GE

b) Chains in register from mutation site

A P G G A V E K

97 115

GLDGAKG GESGSPGEN

N GLDGAKGEGESGSPGEN C

GLDGAKG GESGSPGEN

E K A V G G A P

N C

6 12 11 8 10 9,7

Type II procollagen molecule

shorter molecules. Evidense for the in-register model with looping out of the normal sequence has recently been presented for two cases of Kniest dysplasia (Fernandes et al., 1998, Weis et al., 1998). However, both of these were exon deletions that did not interupt the Gly-x-y repeat. Though the results from this report do not allow conclusions on which model is correct, the deletion is close to one of the two helical cross-linking sites in α1(II) at residue 87, so an out-of-register molecule or an in-register shorter molecule might be expected to inhibit normal cross-linking. Analysis of cross-linked α1(II)CB12 indicate α1(II) chains containing the deletion did not form trivalent cross-links at residue 87. However, cross-linking of the matrix collagen was essentially normal, albeit at the low end of the range.

In another case of Kniest dysplasia, Winterpacht et al. (1993a) identified a 28bp deletion across the exon12/intron12 boundary in a two year-old girl. This mutation completely removed the splice donor site in intron 12, which probably resulted in the splicing out of exon 12 (Winterpacht et al., 1994). Kniest dysplasia is not exclusively produced, however, by mutations in the exon 12 region of COL2A1. We have identified a mutation in a splice donor

consensus sequence which resulted in the skipping of exon 24 and produced typical Kniest dysplasia (Wilkin et al.,1993). A 6-amino acid deletion at the carboxyl-terminus of $\alpha 1$(II) and a 6-amino acid deletion in exon 21 have been identified in additional Kniest dysplasia patients (Spranger et al., 1994). A skipping of exon 15 (Fernandes et al., 1998) and another skipping of exon 24 (Weis et al., 1998) has also recently been identified. While the evidence indicates that most cases of Kniest dysplasia may result from deletion mutations, deletions within the type II collagen triple-helix do not exclusively produce Kniest dysplasia. Deletion of exon 48 from genomic DNA (Lee et al., 1989) and an exon skipping mutation that resulted in deletion of exon 20 (Tiller et al., 1992) both produced mild forms of SED. However, inheritance of the latter mutation resulted in Kniest-like radiographic findings in an affected fetus (Tiller et al., submitted).

Furthermore, the data from proband 3 demonstrates that substitution for a triple helical glycine residue can also produce the Kniest dysplasia phenotype. Proband 3 was heterozygous for a new dominant mutation (Wilkin et al., 1994). The predicted substitution of aspartate for glycine103 was collaborated by our protein

sequencing analysis of the patients cartilage. One other aspartate for glycine substitution has been characterized in the type II collagen helix. Substitution for residue 67 leads to a Wagner/Stickler phenotype (Korkko et al., 1993), the mildest type II collagen disorder. While there are insufficient data available at this time to correlate genotype with phenotype, it is clear that both the type of mutation and the location within the molecule will be important in determining the clinical outcome of each mutation. Furthermore, since the $\alpha 3$ chain of type XI collagen is the product of the C0L2A1 gene (Vuorio and deCrombrugghe, 1990), effects on the structure and function of this minor cartilage-specific fibrillar collagen are also implied by type II collagen gene mutations. Beyond abnormalities in type II collagen function that are likely to be common to all phenotypes produced by structural changes in the molecule, how abnormal collagen chains result in specific phenotypes within the type II collagenopathy spectrum may be related to the disruption of specific domains within the triple helix.

As has been shown for the type I collagen gene, mutations that produce osteogenesis imperfecta, the relationship between mutation and phenotype is unlikely to be simple, and the recognition of

patterns will emerge only when a far greater number of mutations have been characterized.

CHAPTER V

POST-TRANSLATIONAL MODIFICATIONS OF TYPE II COLLAGEN

Summary to Chapter V

The chondrodysplasias are a large and hetergeneous group of cartilage disorders. Defects in type II collagen biosynthesis are responsible for a small subset of these disorders and range from mild cases of familial osteoarthrosis to severe perinatal lethal forms of hypochondrogenesis/achondrogenesis (Spranger et. al. 1994). The cartilage from these individuals contains post-translationally overmodified type II collagen (Bogaert et. al. 1992). In this report we compare the overmodification of collagen molecules from cartilages containing known type II collagen defects.

Analysis of cyanogen bromide (CB) digested cartilage samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed delayed mobilities of $\alpha 1(II)$ CB peptides compared to controls, suggesting an increase in post-translational overmodification of these CB peptides. $\alpha 1(II)$ CB peptides 10 and 12 were purified and digested with endoproteinase ASP-N. Peptides

containing Y-position lysines were identified by reverse phase HPLC and sequence analysis.

The results revealed that for glycine substitutions overmodification occurred amino terminal to the mutation site and the extent of the overmodification was proportional to the severity of the disease. However, type II collagen containing a unique seven amino acid deletion gave a different result. In this case overmodification occurred amino and carboxy terminal to the mutation site. Since collagen folds in a carboxy to amijno direction this was an unexpected finding.

Introduction to Chapter V

Collagen polypeptides are enzymaticaly modified within the endoplasmic reticulum. This occurs prior to and during the folding of the triple helix. Once a stable triple helical conformation is achieved the enzymes are no longer able to modify proline and lysine in the collagen molecule (Kivirikko and Myllyla 1980).

The modifications that occur within the triple helical domain of the molecule are hydroxylation of proline and lysine and glycosylation of certain hydroxylysines to galactosylhydroxylysine

and glucosylgalactosylhydroxylysine. The extent of the modifications varies among collagen types and for the same collagen type in different tissues and at different ages of the same tissue (Kivirikko and Myllyla 1980). The extent of the modifications can be increased in collagen molecules that are expressed with a structural defect. In osteogenesis imperfecta type I collagen molecules containing defects are post-translationaly overmodified amino terminal to the defect (Byers 1989).

The triple helix folds in a carboxy to amino direction, therefore it is hypothesized that a structural defect would cause a delay in folding and more time for enzymatic modification of the molecule. This would predict greater overmodification of lysine towards the amino terminus. It has also been hypothesized that the defect could create a conformational change in the molecule permitting enzyme modification (Chessler and Byers 1992). This would predict no difference in the amount of lysine overmodification from one end of the molecule to the other. In either situation the molecule is overmodified amino terminal to the defect.

COL2A1 mutations produce a spectrum of diseases within the chondrodysplasias (Spranger et. al. 1994). In these diseases type II

collagen is post-translationally overmodified. The extent of lysine overmodification is not known. The comparison of modified lysines from a range of disease phenotypes may give us insights into how normal and defective molecules fold.

Materials and Methods

Clinical Summary Articular cartilages from a 2 year old, a 24 year old, and a 45 year old were processed as controls. Cartilages from patients with known protein defects in type II collagen were obtained from previous studies. The phenotypic outcome from these defects represent a spectrum of diseases within the chondrodysplasias. The mildest of these was a case of familial osteoarthrosis with mild features of SED. A case of Kniest dysplasia and spondyloepimetaphyseal dysplasia (SEMD) represented the middle of the spectrum. The most severe cases were lethal forms of hypochondrogenesis and achondrogenesis. The protein defects were: a glycine substitution to serine at position 493, a deletion of seven amino acids 102 to 108, a glycine substitution to serine at position 841, a glycine substitution to glutamate at position 853, and a glycine

substitution to argenine at position 691, of the triple helical domain, respectively.

Peptide analyses Articular and costal cartilage samples were digested with CNBr in 70% (w/v) formic acid for 24hr at room temperature (Eyre and Muir 1975). SDS-PAGE was carried out by the method of Laemmli (1970) on 15% gels. Peptides were fractionated by molecular sieve HPLC (TosoHaas G3000SW, 7.5 mm x 60 cm, two columns in tandem; eluent, 100 mM Na phosphate, pH 6.8, 30% (v/v) acetonitrile), then reverse phase HPLC (Bateman et al., 1986; Eyre 1987). The pools containing α1(II)CB12 and α1(II)CB10 (identified by SDS-PAGE) were dried and digested with endoproteinase Asp-N (sequencing grade, Boehringer Mannheim), and the peptides were fractionated by reverse phase HPLC (Eyre 1987). Individual peptide yields were estimated by integrated 220nm absorbance of peaks and PTH-amino acid recovery on Edman sequence analysis.

Protein microsequencing. Edman amino-terminal sequencing was carried out on a Porton 2090E instrument equipped with on-line HPLC analysis of PTH-amino acids using a modified manufacturer's program that resolved derivative peaks for 4-hydroxyproline, hydroxylysine and hydroxylysine glycosides.

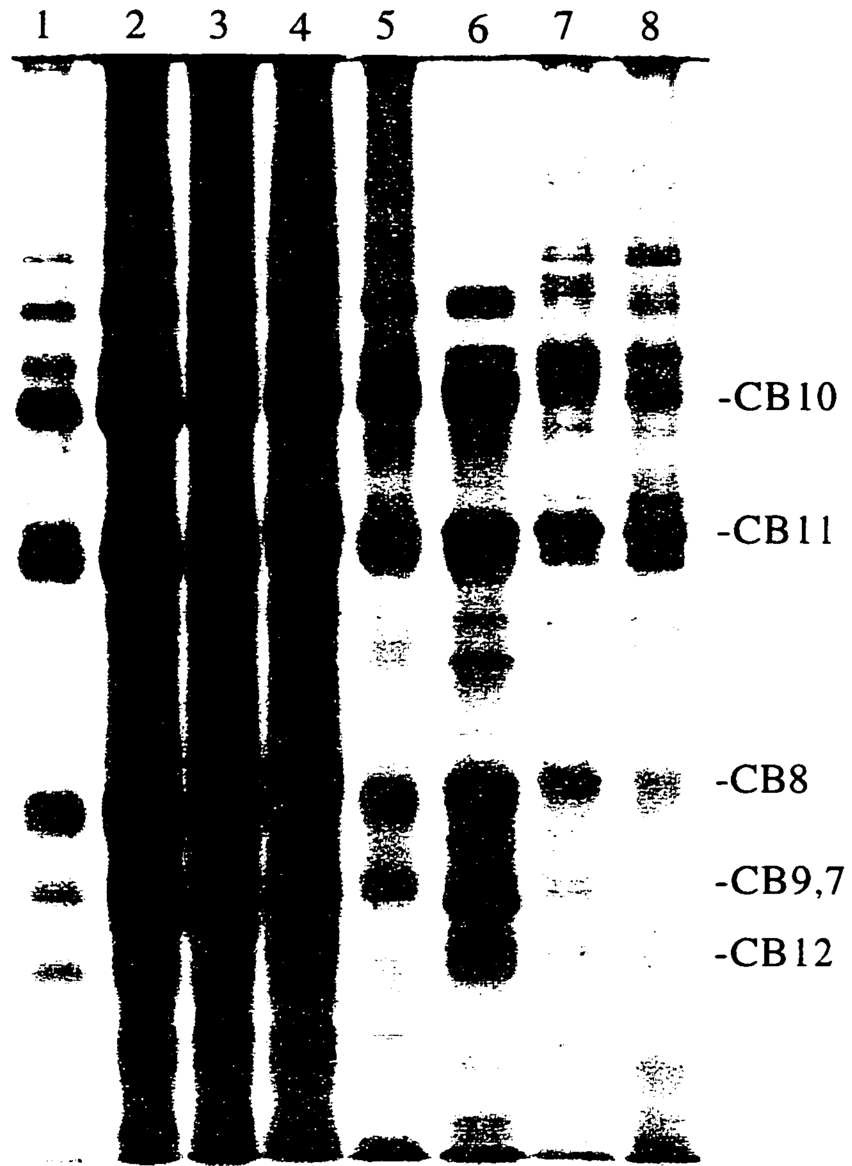
Results

Electrophoresis (SDS-PAGE) of cyanogen bromide (CB) digested cartilage from the three controls (lanes 1-3, figure 19) showed no difference in the mobilities of $\alpha 1$(II) CB peptides, except for CB12. This peptide from the 2 year old control cartilage (lane 1) ran slightly faster than the other controls. The $\alpha 1$(II) CB12 peptides from the diseased cartilages (lanes 4-8) all exhibited a delayed electrophoretic mobility compared to the controls. The CB12 peptide is located near the amino terminal end of the triple helix. Post-translational overmodification of certain residues within this peptide due to delays in folding or an altered triple helical conformation would account for the delayed mobility of this peptide.

In the control and diseased cartilages there are essentially no detectable differences in the mobility of the $\alpha 1$(II) CB9,7 peptide. CB9,7 is at the carboxyl end of the triple helical domain and contains few modifiable lysines. In this study all of the structural defects in type II collagen were amino terminal to this peptide.

The mobilities of $\alpha 1$(II) CB8 and $\alpha 1$(II) CB11 from the diseased cartilages (lanes 4-8) were delayed compared to the controls. The

Figure 19. SDS-polyacrylamide gel electrophoresis of cyanogen bromide digested cartilages from: 2 year old control (1), 24 year old control(2), 45 year old control(3), osteoarthrosis(4), Kniest dysplasia(5), SEMD(6), hypochondrogenesis(7), achondrogenesis(8).



CNBr

extent of delayed mobility was essentially the same, except for the Kniest dysplasia peptides (lane 5). CB8 and CB11 from the Kniest proband was only slightly delayed compared to the corresponding control peptides.

The α1(II) CB10 peptide from the diseased cartilages exhibited varying degrees of retarded mobility compared to control α1(II) CB10. A gradient of decreasing mobility was seen with CB10 from the osteoarthrosis proband (lane 4) being the least delayed and CB10 from the hypochondrogenesis proband (lane 7) the most delayed of the diseased peptides. The mobility of CB10 from the achondrogenesis proband (lane 8) was equivalent to that of the SEMD proband (lane 6).

Endoproteinase asp-n peptide maps of α1(II) CB12 and α1(II) CB10 from the control and diseased cartilages were compared. Figure 20 shows the early portion of reverse phase HPLC elution profiles of endoproteinase asp-n digested α1(II) CB12. Peptide D3 contains two y-position lysines, one at residue 99 and the other at residue 108 of the triple helical domain. Microsequence analysis of this peptide yielded galactosylhydroxylysine at cycle 4 (position 99) and an equal mixture of hydroxylysine and galactosylhydroxylysine

Figure 20. Early portion of the elution profile on reverse phase HPLC of endoproteinase digested CB12 from: 2 year old control (A), 24 year old control(B), 45 year old control(C), osteoarthrosis(D), Kniest dysplasia(E), SEMD(F), hypochondrogenesis(G), achondrogenesis(H). Peptide D3 is identified as is the overmodified version of it, peptide D3'. ΔD3 in panel E identifies the seven amino acid deletion in the Kniest dysplasia proband. The asterisk in panel A identifies an undermodified version of peptide D3.

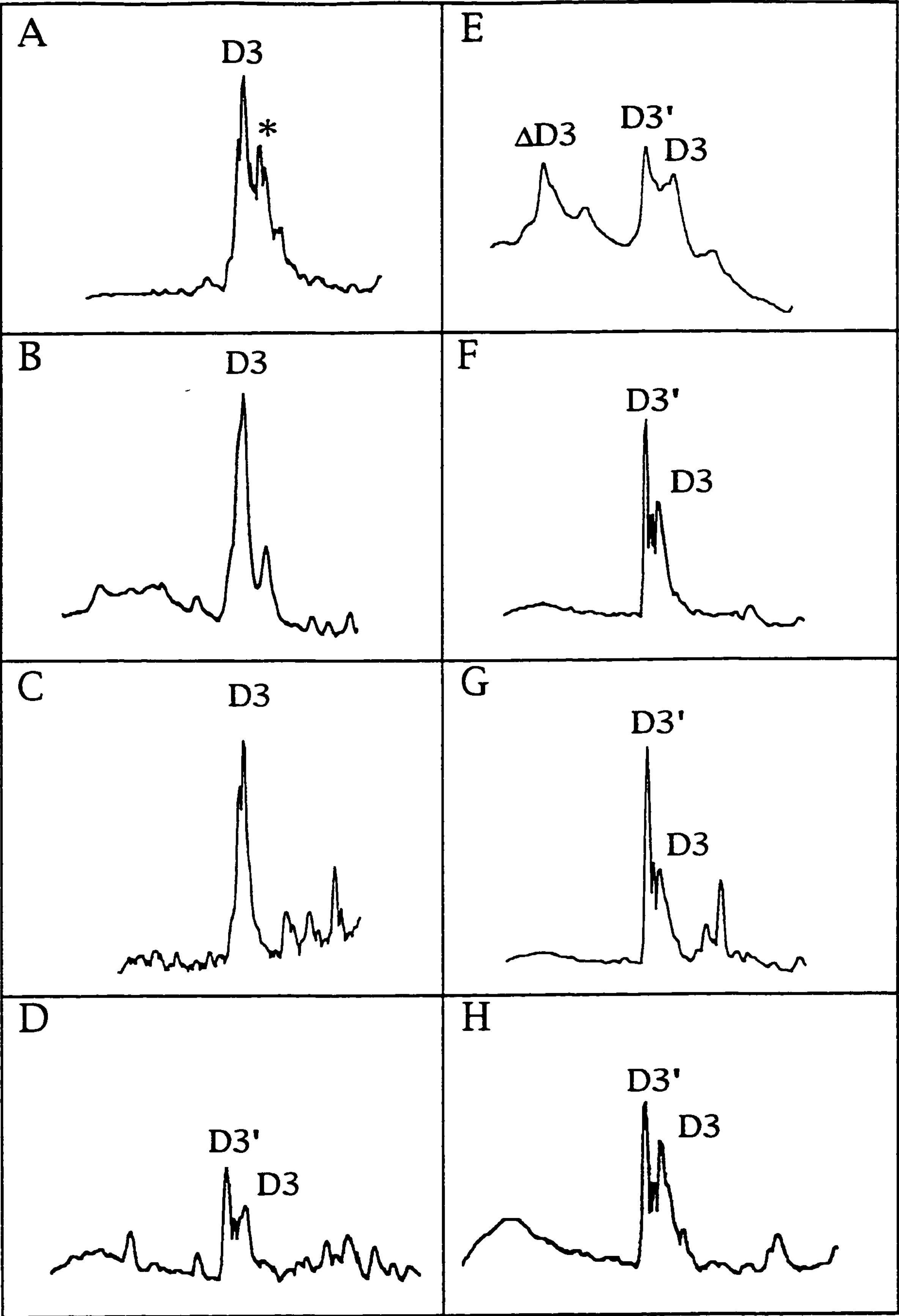

A
D3
*
B
D3
C
D3
D
D3'
D3
E
ΔD3
D3'
D3
F
D3'
D3
G
D3'
D3
H
D3'
D3

Table 1. Post-translational modification of lysine residues in type II collagen from cartilage. Location of modified lysine residues in CB peptides 12 and 10 along with their triple helical location and surrounding sequence. K=lysine, K*=hydroxylysine, K^{G}=galactosylhydroxylysine, K^{GG}=glucosylgalactosylhydroxylysine, X= undetermined.

CB PEPTIDE	CB12		CB10	
LOCAL SEQUENCE	DGAKGEA	PGVKGES	VGEKGPE	DGPKGAR
RESIDUE NUMBER	99	108	573	729
2 Y.O. CONTROL	K^G	$K^G K^* K$	K	K^G K
24 Y.O. CONTROL	K^G	$K^G K^*$	K	K^G K
45 Y.O. CONTROL	K^G	$K^G K^*$	K	K^G K
OSTEOARTHROSIS S493	K^G	$K^G K^*$	K	K^G $K^* K$
KNIEST DYSPLASIA Δ102-108	K^{GG} K^G	$K^{GG} K^G$	K	K^{GG} K^G K
SEMD S841	K^G $K^{GG} K^*$	$K^* K^G$	K	K^{GG} K^G K
HYPOCHONDROGENESIS E853	$K^{GG} K^G$	$K^G K^*$	K	K^{GG} K K^G
ACHONDROGENESIS R691	$K^{GG} K^G$	X	K	X

at cycle 13 (position 108; table1). The peptide map from the two year old control also contained a later resolving peak indicated by the asterisk in panel A. The peptide was identified as an undermodified version of peptide D3. Microsequence analysis of this peptide yielded galactosylhydroxylysine at cycle 4 and an equal ratio of lysine, hydroxylysine, and galactosylhydroxylysine. There was a minor amount of this undermodified version of peptide D3 also present in the 24 year old control (panel B).Peptide D3 from the diseased cartilages (panels D-H) partially resolved into two main peaks. A peak in the normal elution position for peptide D3 was present and an earlier eluting version labeled D3'. Microsequence analysis of peptide D3' revealed it to be an overmodified version of peptide D3.

Peptide D3 from the osteoarthrosis cartilage (panel D; table1) contained the least amount of D3' and was the least overmodified of the diseases studied. Microsequence analysis yielded the same result as the controls with predominantly galactosylhydroxylysine at cycle 4 and a mixture of predominantly hydroxylysine and galactosylhydroxylysine at cycle 13.

Peptide D3 resolved into three peaks from the Kniest Dysplasia cartilage. The protein defect was a seven amino acid deletion in this peptide and is labeled ΔD3 (Bogaert et. al. 1994.; Panel E; table1). Peptide D3' was the most overmodified of the probands studied. Cycle 4 yielded approximately a 2 to 1 ratio of glucosylgalactosylhydroxylysine to galactosylhydroxylysine. Cycle 13 yielded approximately a 1 to 1 ratio of glucosylgalactosylhydroxylysine to galactosylhydroxylysine.

Sequence analysis of peptide D3' from the hypochondrogenesis and achondrogenesis probands (panels G and H; table1) were the same. Cycle 4 yielded a 1 to 1 ratio of glucosylgalactosylhydroxylysine to galactosylhydroxylysine and cycle 13 from the hypochondrogensis cartilage yielded a 1 to 1 ratio of galactosylhydroxylysine to hydroxylysine. The sequence yield was too low to analyze for the achondrogenesis proband at cycle 13.

Peptide D3 from the SEMD proband (panel F; table1) was overmodified compared to the controls but less so than the Kniest, hypochondrogenesis, and achondrogenesis probands. Cycle 4 yielded roughly a 2 to 1 ratio of galactosylhydroxylysine to glucosylgalactosylhydroxylysine with a small portion of

hydroxylysine also present. Cycle 13 yielded a 3 to 2 ratio of hydroxylysine to galactosylhydroxylysine.

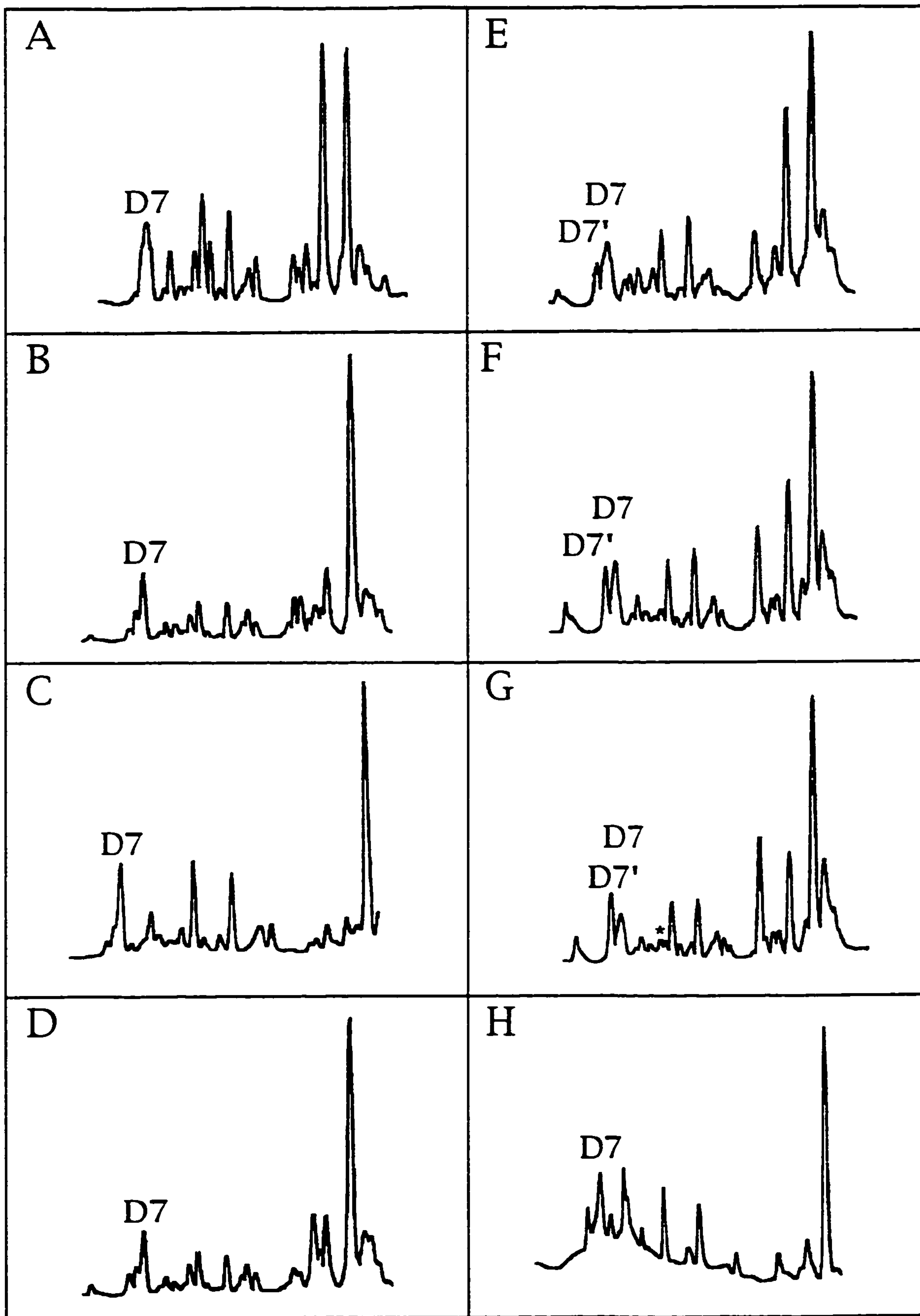
Figure 21 shows the early portion of reverse phase HPLC elution profiles of endoproteinase asp-n digested $\alpha 1$(II) CB10. Two Y-position lysines at position 573 and 729 of the triple helical domain were analyzed by microsequencing. Sequence analysis of the lysine at 573 yielded only lysine for both controls and experimentals (table 1).

Sequence analysis of peptide D7 from the controls (panels A,B, and C; table1) yielded predominantly galactosylhydroxylysine and lysine at cycle 4 (position 729). A minor amount of hydroxylysine and glucosylgalactosylhydroxylysine were also detected at cycle 4 from the 2 and 24 year old controls.

Peptide D7 from the osteoarthrosis proband (panel D; table1) was similar to the controls. Cycle 4 yielded predominantly galactosylhydroxylysine, hydroxylysine, and lysine. A very minor amount of glucosygalactosylhydroxylysine was also present.

Peptide D7 and an overmodified version D7' from the Kniest, SEMD, and hypochondrogenesis probands (panels E,F, and G; table1) were all similarly overmodified compared to the controls. Cycle 4

Figure 21. Early portion of the elution profile on reverse phase HPLC of endoproteinase digested CB10 from: 2 year old control (A), 24 year old control(B), 45 year old control(C), osteoarthrosis(D), Kniest dysplasia(E), SEMD(F), hypochondrogenesis(G), achondrogenesis(H). Peptide D7 is identified as is the overmodified version of it, peptide D7'. The asterisk in panel G identifies the glutamate for glycine substitution in the hypochondrogenesis proband.


A
D7
B
D7
C
D7
D
D7
E
D7
D7'
F
D7
D7'
G
D7
D7'
*
H
D7

yielded a 2 to1 ratio of glucosylgalactosylhydroxylysine to galactosylhydroxylysine and lysine. Peptide D7/D7' from the hypochondrogenesis proband was quantitatively the most overmodified as it yielded greater than 70% glucosylgalactosylhydroxylysine. The sequence yield for peptide D7 from the achondrogenesis proband (Panel H) was too low for analysis, although the peptide map suggests little to no overmodification or presence of D7'.

Discussion

The abnormal type II collagen was post-translationally overmodified primarily in the domain amino terminal to the mutation site. This is believed to occur in fibrillar collagens because either there is a delay in folding from the defective site towards the amino terminus and/or the structure of the triple helix is altered from the site. In either case the net result is an increase in time for the ER resident hydroxylases and glycosyl transferases to act upon a substrate of nontriple helical collagen molecules. Once the molecules are folded into a stable triple helix the enzymes are no longer able to

modify proline and lysine, and the molecules are transported to the golgi apparatus for secretion.

In the delayed-folding model it is predicted that lysines near the amino terminal would be overmodified to a greater extent than towards the carboxy end. Whereas in the altered structure model the extent of overmodification of lysines would not be dependent on their position but rather be dependent upon the extent to which the triple helix is altered. Sequence analysis of lysyl residues at positions 99, 108, and 729 of the triple helical domain in this study supports the altered structure model.

The osteoarthrosis and SEMD phenotypes were the result of serine substitutions for glycine at position 493 and 841 of the triple helical domain. If a delay in folding occurred at each mutation site then lysine 99 should be overmodified in both cases. The lysine at position 99 was not overmodified in the osteoarthrosis tissue and only slightly overmodified in the SEMD tissue. Position 493 may be more tolerant of glycine substitutions and may be in a region less critical to the stability of the triple helix. Whereas position 841 may be in a region more critical to helix stability and a glycine substitution here may alter the structure of the triple helix greater

than a substitution at position 493. The hypochondrogenesis and achondrogenesis phenotypes were the result of glutamate and arginine substitutions for glycine. Since the side chains of glutamate and arginine are large, these substitutions would be expected to alter the structure of the triple helix greater than a less bulky serine for glycine substitution. Indeed the lysine at 99 from these two cases was highly overmodified.

The Kniest phenotype was the result of a deletion of seven amino acids. This mutation in COL2A1 is unique in that it disrupts the Gly-X-Y repeat of the collagen triple helical domain. The consequence of this mutation would produce hetertrimeric molecules assembled form shortened and normal chains. Two possible molecules may be formed (figure 18). In model A the triple helix is out-of-register amino terminal from a hiatus at the deletion site. In model B the triple helix is in-register from the deletion site accomodated by a looping out of the normal chain sequence.

Like the hypochondrogenesis and achondrogenesis cases, lysine 99 from the Kniest tissue was also highly overmodified. This occurs due to the altered structure amino terminal to and at the deletion site.

Sequence analysis of the lysyl residue at position 108 revealed galactosylhydroxylysine and hydroxylysine for the healthy and diseased tissues. This site appears to resist any further modification except for the Kniest tissue which was highly overmodified and yeilded glucosylgalactosylhydroxylysine. The overmodification occurred in the normal α chains of heterotrimers containing normal and shortened α chains. In these molecules the normal chains at the deletion site remain nonhelical and are therefore accessible to enzymatic overmodification.

The lysine 573 resists any modification in healthy and diseased tissues since only lysine is found on sequence analysis. Perhaps the presence of glutamate 572 sterically inhibits the hydroxylase. Yet another possibility is that we were unable to locate the modified lysine from these peptide maps. Since in an earlier study (chapter 3) the hypochondrogenesis cartilage yielded on sequence analysis a 1:1 ratio of hydroxylysine and lysine.

The lysyl residue at position 729 is overmodified in collagen molecules containing sequence abnormalities that are carboxy terminal to this site and also in the type II collagen from Kniest cartilage.

In summary, mutations in the gene COL2A1 that result in structural defects in type II collagen appear to alter the molecule amino terminal to the defect and in one unique mutation carboxy terminal to the defect as well. The degree to which the structure is altered is reflected in the amount of post-translational overmodification of the molecule.

CHAPTER VI

SUMMARY

Collagen is one of the most abundant proteins in the human body and is an essential element in the structural framework of our connective tissues. The main molecular feature common to all collagens is the collagen triple helical domain. The collagen triple helix consists of three coiled alpha chains coiled around each other into a triple helix. In order to form the triple helix it is important that glycine be present every third amino acid in the primary structure of each alpha chain.

Mutations in the genes for collagen type I that disrupt the Gly-x-y primary structure produces osteogenesis imperfecta(Byers1993). Mutations in COL2A1 that disrupt the primary structure of collagen type II produce a spectrum of disease phenotypes within the chondrodysplasias (Chapters 3 and 4). Analogous to the mutations in osteogenesis imperfecta, those mutations which alter the structure of type II collagen and are secreted into the extracellular matrix produce a more severe disease phenotype than for those which only reduce the amount of collagen secreted into the matrix. A reduction

in type II collagen production produces the Stickler phenotype (Ahmad et al., 1991), the mildest of the type II collagenopathies.

In this study defective collagen molecules were found to be post-translationally overmodified amino terminal to the defect (Chapter 5). The amount of overmodification was not greater further away from the defect towards the amino terminus. Therefore, a delay in folding of the molecule from the carboxyl end towards the amino end could not account solely for all of the overmodification observed. Alternatively the overmodification may be due to an altered structure that exists amino terminal to the defect or a folding, unfolding, and refolding mechanism may exist.

This study of inherited diseases not only gives us insight into the pathogenesis of disease but also a better understanding of normal human biochemistry.

UPDATE

This dissertation was written in 1994 and a number of mutations have been identified since. The following is an overview of type II collagen mutations to date.

The spectrum of type II collagen mutations ranges from Wagner/Stickler syndrome at the mild end to lethal achondrogenesis/hypochondrogenesis at the severe end. There has been one report of Wagner syndrome which appears to be a mild variant of Stickler syndrome. In this report an aspartate substitution for glycine67 was identified (Korkko et al., 1993). This is the most amino terminal glycine substitution to date and one of the mildest phenotypes.

In the Stickler syndrome all the mutations result in a premature termination codon within the triple helical domain (Ahmad et al., 1991, Brown et al., 1992, Ahmad et al., 1993, Ritvaniemi et al., 1993, Williams et al., 1994, Williams et al., 1996). The phenotype is the result of a genotype that produces 50% of the normal deposition of normal collagen type II. There is one reported case of a premature stop codon in the C-propeptide of type II collagen (Zabel et al., 1996). This mutation produced the spondyloperipheral dysplasia phenotype.

There is one report of a nontermination mutation that produces a Stickler-like syndrome (Ballo et al., 1998). In this report a cysteine substitution for arginine704 was identified. The jump from genotype

to phenotype is not apparent for the arginine to cysteine mutations, although if you were to assume that this phenotype was less severe than the R789C mutation (Chan et al., 1993 & 1995) that produces SEDc and more severe than the R75C (Williams et al., 1993, Bleasel et al., 1995 & 1996) and R519C (Ala-Kokko et al., 1990, Williams et al., 1995, Bleasel et al., 1998) that produce a mild SED phenotype with precocious osteoarthritis, than there may be a gradient of arginine to cysteine mutations with milder phenotypes from the amino end and more severe phenotypes from the carboxyl end.

The arginine to cysteine mutations at 75 and 519 that produce precocious osteoarthritis have been found in multiple unrelated families. These sites may be "hot spots" on COL2A1 related to the hypermutability of the CG dinucleotide (Bleasel et al., 1996). Not all of the cases of mild SED with osteoarthritis are due to argenine to cysteine mutations. A G493S (unpublished data), G691R, and G976S (Williams et al., 1995) have been identified. How these glycine substitutions correlate to phenotype is unclear at this time.

In Kniest dysplasia it is becoming clear that most of the phenotypes are due to deletion mutations in the amino half of the type II collagen molecule. Deletions of exons 12,14, 15, 18, 21, and

24 (Winterpacht et al., 1993 & 1994, Mortier et al., 1995, Frenandes et al., 1998, Weis et al., 1998, Spranger et al., 1997), and deletions of the last seven amino acids in exon 12 (Bogaert et al., 1994, Chen and Cole 1996, Chen et al., 1996, Cole 1997). The seven amino acid deletions in exon 12 have been identified in 5 presumably unrelated individuals and occurs in another "hot spot" at a CG dinucleotide. The molecules produced in the Kniest phenotype are shorter than normal type II collagen molecules with normal alpha chains looping out of the helix at the deletion site (Fernandes et al., 1998, Weis et al., 1998). The extracellular matrix consists of a decrease in the deposition of collagen with about 50% mutant chains. Normal type II collagen homotrimers would only account for 12.5% of the molecules with about 75% shortened heterotrimers of mutant and normal alpha chains.

Not all of the Kniest phenotypes are due to deletions in the amino half of the molecule. A deletion of six amino acids (1007-1012) near the carboxyl end of the triple helical domain has been identified (Winterpacht et al., 1996). This and an aspartate for glycine103 substitution (Wilkin et al., 1994) are the exceptions.

Not all exon deletions result in the Kniest phenotype. Deletions of exons 20, 36, 40 and 48 (Tiller et al., 1995, Spranger 1994, Mortier et al., 1995, Williams et al., 1995, Lee et al., 1989) result in the SED phenotype and for the exon 40 deletion the osteochondrodysplasia phenotype.

In the SED family (achondrogenesis, hypochondrogenesis, and SEDc) and SEMD those mutations identified so far include the above deletions, an insertion (Tiller et al., 1990) and about 21 glycine substitutions (Kaitila et al., 1996, Bonaventure et al., 1995, Chan et al., 1995, Mundlos et al 1996, Kuivaniemi et al., 1997). The general trend for the glycine substitutions appear to be located nearer to the carboxyl end of the molecule. Exceptions to this trend do occur and as yet are not wholly clear at this time.

The matrix from the SED and SEMD cartilages contain a decreased deposition of type II collagen with about 50% mutant chains, while the more severe SEDs and achondrogenesis/hypochondrogenesis cartilages can range from 0 to 50% deposition of type II collagen with 0 to 50% mutant chains. In some of the severe lethal achondrogenesis and hypochondrogenesis

cases there is no type II collagen produced and type I and III collagens are produced instead.

The relationship of genotype to phenotype in the chondrodysplasias is becoming less muddy with the increasing number of mutations reported. Part of the difficulty in assigning these relationships lies in the diagnosis. What one clinician may call a severe Kniest dysplasia another may call SED.

Another part of the difficulty may also depend on the genetic composition of the patient. A report of Kniest dysplasia was the result of an exon 12 deletion in one patient, while the mother of the patient had a somatic mosaicism for the same mutation and was diagnosed with Stickler syndrome (Winterpacht et al., 1993).

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Major Research Interests

Endodontics
Mineralized tissue
Biochemistry

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Presentations

Bogaert. R., Weis. M.A., and Eyre, D.R. Detection and Characterization of a Mutation in Type II Collagen. AADR Annual Meeting, March 1991, Boston, Mass., Poster Discussion

Bogaert, R. and W.K. Ramp. Collagen dressings as delivery systems for calcium hydroxide. IADR/AADR Annual Meeting, March 1988, Montreal, Canada. Poster presentation.

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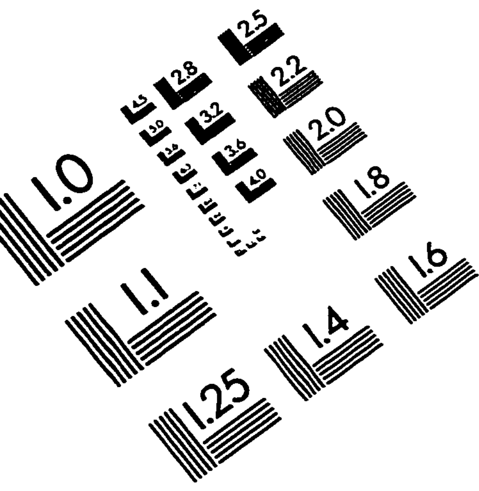
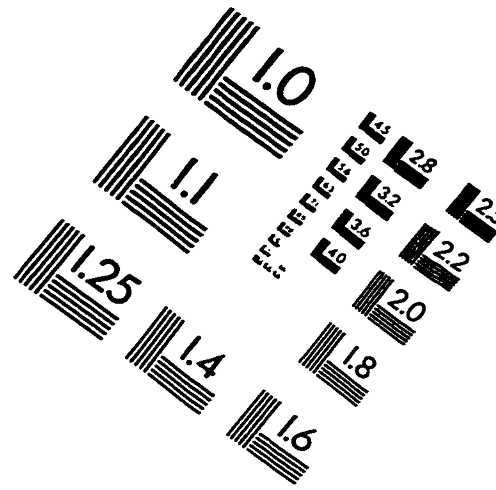
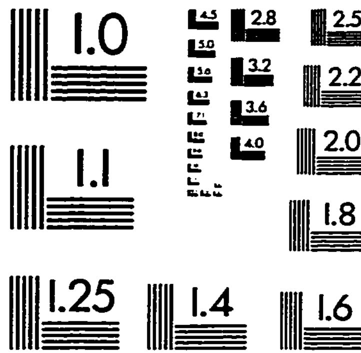
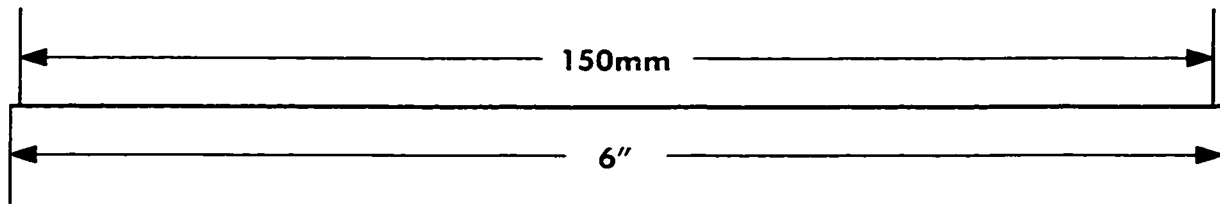
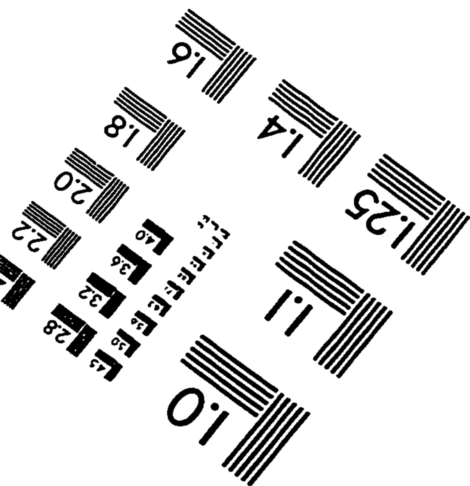
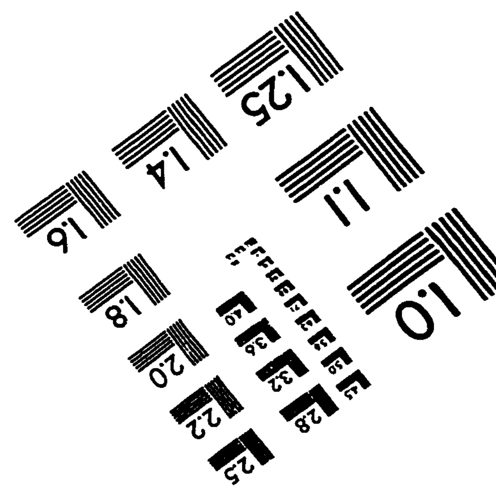
Eyre DR, Wu JJ, Diab M, **Bogaert R**, Weis MA, Knigge P. Cartilage collagen heteropolymers: Implications for assembly, degradation and heritable disease. 2nd Pan-Pacific Connective Tissue Societies Symposium, September 12-17, 1993, Bali, Indonesia.

IMAGE EVALUATION TEST TARGET (QA-3)

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