Link peptide cartilage growth factor is degraded by membrane proteinases

Michael F. DEAN¹ and Paul SANSOM

Department of Veterinary Basic Sciences, The Royal Veterinary College, University of London, Royal College Street, London NW1 0TU, U.K.

The peptide DHLSDNYTL DHDRAIH (Link N), cleaved from the N-terminus of the link protein component of cartilage proteoglycan aggregates by the action of stromelysin, can act as a growth factor and stimulate synthesis of proteoglycans and collagen in articular cartilage [McKenna, Liu, Sansom and Dean (1998) *Arthritis Rheum.* **41**, 157–161]. The mechanism by which this biologically active peptide is degraded and inactivated was investigated using U937 monocytes as a model cell. Time-course experiments showed that two major proteases, an initial serine proteinase followed by a metalloproteinase, acted in sequence. Analysis of the resulting fragments showed that the serine endopeptidase cleavage was at the Leu³–Ser⁴ bond to produce the peptide SDNYTL DHDRAIH. The terminal serine could

then be removed from the resulting peptide by an aminopeptidase. A second metalloproteinase liberated the peptides SDNYTL or DNYTL from DHDRAIH by cleavage at the Leu⁸–Asp¹⁰ bond. The DNYTL peptide intermediate was degraded too rapidly to allow sequencing and sequential aminopeptidase cleavages removed further amino acids from the N-terminus of the remaining DHDRAIH peptide. The identical patterns of breakdown that occurred when either whole cells or purified plasma membranes were used indicated that proteolysis and inactivation of Link N was carried out entirely by membrane-associated enzymes.

Key words: cartilage, cell membranes, link peptide, proteinase.

INTRODUCTION

Normal articular cartilage is in a state of dynamic equilibrium, with turnover and repair regulated by the interaction of chondrocytes with a number of factors that may act during development, growth and maintenance. Among these are transforming growth factor β and insulin-like growth factor-1, which can up-regulate synthesis of aggrecan, collagen, link protein and hyaluronan [1–3]. Another growth factor, human osteogenic protein-1, can stimulate synthesis of proteoglycans and collagen type II in chondrocytes cultured in alginate beads [4]. In contrast, cytokines such as interleukin-1 and tumour necrosis factor α have opposite effects and can induce chondrocytes to down-regulate proteoglycan and type-II collagen synthesis [5,6] and increase synthesis of stromelysin and collagenase [7,8]. An imbalance in any of these control mechanisms can lead eventually to a loss of cartilage.

Synthesis and degradation of cartilage matrix may also be regulated by interaction of chondrocytes with matrix molecules themselves, or fragments derived from them by proteolysis. One of these is link protein, which comprises approx. 0.05% of the wet weight of cartilage [9], is synthesized as a single gene product [10] and is glycosylated to generate two mature forms of 48 kDa and 44 kDa. Its primary role in articular cartilage is to stabilize the interaction of the proteoglycan aggrecan with hyaluronan [11]. As cartilage ages, a 41-kDa form of link protein resulting from cleavage of the His¹⁶–Ile¹⁷ bond by the enzyme stromelysin accumulates [12,13]. We postulated that this cleaved N-terminal peptide, called the N-terminal link-protein peptide or Link N, may have an additional function as a cartilage growth factor [14]. Nanomolar concentrations of this peptide were shown subsequently to stimulate synthesis of proteoglycans and collagen in normal articular cartilage from a wide age range of subjects [15,16]. The newly synthesized proteoglycans had a normal composition and were therefore capable of being integrated into a viable cartilage matrix [17].

A number of other biologically active proteins involved in joint metabolism are synthesized as propeptides that require specific proteolysis to become active [18]. These peptides have relatively short half-lives before they themselves are degraded and inactivated [19]. For example, substance P is found in increased levels in rheumatoid synovial fluid [20] and may contribute to early arthritis by activating inflammatory cells [21,22]. Its hydrolysis by the membrane proteinase CD10 produces a fragment (SP7-11) that can induce synthesis of collagenase by chondrocytes [23]. Another active peptide, neuropeptide Y, can increase the inflammatory response within joints [24] and may be degraded by cell-surface peptidases such as CD10 and CD26, which regulate its interconversion between receptor-selective forms and degraded inactive metabolites [25]. Many of these membrane peptidases are distributed widely [26], suggesting they have an important function in the regulation of peptide-mediated control of cell metabolism by either inactivating biologically active peptides or cleaving inactive peptides to form new active intermediates [27–30].

To determine whether link peptide is degraded by a similar mechanism, the sites of cleavage of link peptide were investigated using U937 monocytes because of the similarity of their proteolytic profile to those of the main cell types found in synovial fluids during inflammatory episodes [31,32]. During these periods of inflammation degradation of Link N would be expected to be maximal. We now present data that show that link peptide itself is also rapidly degraded in a specific manner by plasma-membrane-associated peptidases. This may be the primary mechanism by which the effects of this peptide growth factor are controlled.

MATERIALS AND METHODS

Materials

Enzyme inhibitors were supplied by Sigma (Poole, Dorset, U.K.) and HPLC-grade solvents by Merck (Dagenham, Essex,

Abbreviations used: DFP, di-isopropylfluorophosphate; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane; Link N, N-terminal link-protein peptide.

¹To whom correspondence should be addressed (e-mail mdean@rvc.ac.uk).

U.K.). Tissue-culture medium, serum and Dulbecco's PBS were from Imperial Laboratories (Andover, Hants., U.K.). Protein assay reagents were obtained from Bio-Rad Laboratories (Hemel Hempstead, Herts., U.K.) and Na¹²⁵I was from Amersham (Little Chalfont, Bucks., U.K.). All other reagents were from Sigma and were of analytical grade.

Synthetic peptides

The N-terminal peptide DHLSDNYTLDDHRAIH (Link N) was synthesized at the National Institute for Medical Research, Mill Hill, London, U.K., using stepwise solid-phase synthesis with Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry on an Applied Biosystems 431a Automatic Peptide Synthesiser. Its homogeneity was determined by C₄ reversed-phase HPLC and fast-atom bombardment MS. It was purified on a Gilson HPLC fitted with a 250 mm × 4.6 mm Dynamax 300 Å C₁₈ reversed-phase column (Rainin Instrument Co.) using a linear gradient of 0.1 % trifluoroacetic acid (solvent A) and acetonitrile/0.086 % trifluoroacetic acid (solvent B) at a flow rate of 1.0 ml/min. Unlabelled peptides were detected by peptide-bond absorption at 206 nm using a Gilson System Controller and the radioactivity of iodinated peptides measured with a Berthold LB-206 on-line monitor and flow cell. Column performance was monitored using standard samples of trypsinized horse cytochrome *c* eluted with a 5–45 % gradient of solvent B.

Radio-iodination of peptides

Iodo-beads were added to 500-μl aliquots of Link N dissolved at a concentration of 1 mg/ml in PBS. After 5 min, 500 μCi of Na¹²⁵I was added, the mixture agitated for 30 min and the iodinated peptide solution removed and stored at 4 °C. Incorporation was routinely > 99.9 %, to produce a specific radioactivity of 1.0 mCi/mg of Link N. Working solutions of iodo-Link N were prepared by dilution of this stock peptide with RPMI 1640 culture medium or PBS.

Preparation of membranes

Samples of cells were washed with PBS and suspended at a concentration of 1 × 10⁸ in 5 ml of buffer C (5 mM Tris/HCl, 1 mM sodium bicarbonate, 5 mM magnesium chloride, 1 mM calcium chloride, pH 7.4) for 20 min to allow swelling. After 20 min at a pressure of 65 psi (≡ 448.5 kPa) in a Nitrogen Bomb the cells were disrupted by cavitation and nuclei and cell debris removed by centrifugation at 1000 *g*. The lysate was pelleted at 100 000 *g* for 1 h (in a Beckman TL-100 ultracentrifuge), washed with 10 mM Tris/HCl, pH 7.4, and resuspended in 200 μl of 10 mM CHAPS/10 mM Tris/HCl, pH 7.4. Aliquots of this solution were used to measure the effects of whole-cell lysates on cleavage of Link N. Subcellular fractions were prepared on discontinuous sucrose gradients [33]. The lysate (A layer) was layered over a gradient of 2 ml of 20 % (w/v) sucrose (B layer), 42 % sucrose (C layer) and 70 % sucrose (D layer) in lysis buffer and centrifuged at 52 000 *g* for 60 min. The microsomes (A/B interface), plasma membranes (B/C interface) and lysosomal fraction (C/D interface) were then diluted with buffer and pelleted at 100 000 *g* for 60 min. The plasma-membrane fraction pellet was resuspended in 200 μl of Tris buffer, pH 7.4, and this stock was diluted when required for peptide-cleavage experiments to produce a membrane solution equivalent to 5 × 10⁶ cells per 50 μl. The protein content of each fraction was determined with the Bio-Rad protein reagent using bovine serum globulin as a standard. This procedure successfully purified plasma membranes, resulting in a 5-fold enrichment of leucine aminopeptidase

from 1.0 to 5.3 units/mg in a fraction that contained less than 10 % of the total protein. There was an accompanying 3-fold enrichment of β-D-glucuronidase from 1.0 to 2.7 units/mg in the lysosomal fraction.

Enzyme assays

To determine the leucine aminopeptidase activity of membranes, 50-μl aliquots were mixed with 150 μl of leucine *p*-nitroanilide (1 mg per ml in 10 mM Tris/HCl, pH 7.4) in a 96-well microtitre plate. Absorbance was read at 405 nm after 1 h at 37 °C and a unit of activity calculated as being equivalent to the increase in absorbance produced by 1 mg of membrane in 1 h. β-D-Glucuronidase activity was used as a marker of lysosomal membranes. For this, 10 μl of membrane were mixed with 2 ml of 1 mM 4-methylumbelliferyl β-D-glucuronide in 0.1 M sodium acetate, pH 4.5, at 37 °C for 30 min. The reaction was stopped with 3 ml of 0.4 M glycine buffer, pH 10.4, and the rate of release of methylumbelliferone measured on a Locarte fluorimeter. A unit of activity was equivalent to the change in fluorescence produced by 1 mg of membrane in 30 min.

Proteolysis of Link N by U937 monocytes

Human U937 monocytes were maintained as suspension cultures in RPMI 1640 medium containing 5 % heat-inactivated foetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine in a humidified atmosphere of 5 % CO₂ at 37 °C. Cell viability routinely exceeded 95 % by Trypan-Blue exclusion. Aliquots of cells (50 μl, containing 10⁶ cells in serum-free RPMI) were mixed with aliquots of iodinated Link N (0.1 μCi in 50 μl of RPMI) and incubated at 37 °C. For membrane experiments, aliquots of plasma membranes (50 μl, equivalent to 5 × 10⁶ cells) were added to 50 μl of iodo-Link N and incubated at 37 °C. Proteolysis was terminated by the addition of 200 μl of 0.1 % trifluoroacetic acid followed by filtration of the samples (pore size 0.22 μm, Millipore) and proteolysis measured by HPLC. When proteinase inhibitors were used these were dissolved in ethanol or DMSO to produce 100-times-concentrated stock solutions. These were added to cell or membrane samples to give the required final concentrations 10 min before the addition of Link N. Control experiments used equivalent volumes of inhibitor solvents alone.

Sequence analysis of peptides

Degradation of Link N was measured by incubation of 100-μl samples of unlabelled peptide (1 mg/ml in PBS) at 37 °C with 10⁷ U937 monocytes or with 50 μl of a 2 μg/ml solution of plasma membranes (equivalent to 2.5 × 10⁷ cells). Incubation was terminated by adding 200 μl of 0.1 % trifluoroacetic acid and filtration through a 0.22-mm filter. The resulting peptides were separated by reversed-phase HPLC and analysed by solid-phase sequencing (Protein Sequencing Service at the National Institute for Medical Research). Some peptides were also analysed by fast-atom bombardment MS (Department of Chemistry, University of Manchester Institute of Science and Technology, Manchester, U.K.).

RESULTS

Proteolysis of Link N

Initial time-course experiments were carried out using U937 cells in RPMI medium, which contained components that interfered with the detection of peptides at both 206 nm and 280 nm. To

Figure 1 Processing of iodinated Link N by U937 cells

Iodinated Link N ($0.2 \mu\text{Ci}$, equivalent to 200 ng of peptide) was incubated with 1×10^6 U937 cells in $100 \mu\text{l}$ of RPMI 1640 and aliquots taken for analysis by C_{18} reversed-phase HPLC. Radiolabel was detected by on-line gamma counting and expressed as c.p.m./ml.

Table 1 Effect of proteinase inhibitors on cleavage of iodinated Link N by U937 cells

U937 cells (1×10^6) were pre-incubated for 10 min with inhibitors prior to the addition of $0.2 \mu\text{Ci}$ of iodinated Link N. Incubation was terminated after 60 min by addition of 0.2% trifluoroacetic acid, and aliquots analysed by C_{18} reversed-phase HPLC. Values are the percentage of total counts present in each peak. Peak numbers are those indicated in Figure 1.

Treatment	Peak 1 (% Total counts)	Peak 2 (% Total counts)	Peak 3 (% Total counts)
Control	45	27	28
0.2 mM PMSF	86	8	6
1.0 mM DFP	46	25	29
1.0 mM Phenanthroline	69	28	3
1.0 mM EDTA	45	35	20
1.0 mM E64	45	26	28
2.0 mM Iodoacetamide	78	18	4
2.0 mM Pepstatin A	45	27	28

avoid complications in interpretation of chromatographs due to these interfering components, an alternative method of peptide detection was used. It had been shown previously that ^{125}I could

Figure 2 Processing of iodinated Link N by U937 plasma membranes

Iodinated Link N ($0.2 \mu\text{Ci}$, equivalent to 200 ng of peptide) was incubated with a plasma-membrane fraction equivalent to 5×10^6 cells in a volume of $100 \mu\text{l}$ and aliquots taken for analysis by C_{18} reversed-phase HPLC. Radiolabel was detected by on-line gamma counting and expressed as c.p.m./ml.

be incorporated into the Tyr⁷ residue of Link N, and that this radiolabel could be used to measure cleavage [14]. Iodinated Link N peptides could be detected by gamma counting without interference from exogenous factors. Cleavage of this labelled peptide was rapid, resulting in conversion of Link N peptide (Figure 1, peak 1, retention time 16.0 min) into a single labelled product (Figure 1, peak 3, retention time 11.6 min) in less than 4 h under the conditions used (Figure 1). An additional peak with a retention time of 15.1 min accumulated and then disappeared, suggesting that there were two sequential cleavages; conversion of peak 1 into the intermediate 2, which was in turn converted into 3. Incubation of iodinated Link N in medium alone induced no breakdown of the peptide and medium that had been conditioned by prior incubation with monocytes also had no effect, indicating that the enzymes responsible for these cleavages were cell-associated and had not been secreted. Breakdown of

Table 2 Effect of proteinase inhibitors on cleavage of iodinated Link N by U937 plasma membranes

U937 plasma membranes, equivalent to 5×10^6 cells, were pre-incubated for 10 min with inhibitors before the addition of 0.2 μ Ci of iodinated Link N. Incubation was terminated after 60 min by addition of 0.2% trifluoroacetic acid, and aliquots analysed by C_{18} reversed-phase HPLC. Values are the percentage of total counts present in each peak. Peak numbers relate to those indicated in Figure 2.

Treatment	Peak 1 (% Total counts)	Peak 2 (% Total counts)	Peak 3 (% Total counts)
Control	41	10	49
0.2 mM PMSF	80	5	15
1.0 mM DFP	42	9	49
1.0 mM Phenanthroline	83	16	1
1.0 mM EDTA	42	14	44
1.0 mM E64	41	10	49
2.0 mM Iodoacetamide	88	11	1
2.0 mM Pepstatin A	41	10	49

Link N was inhibited almost completely by the serine proteinase inhibitor PMSF, by the metalloproteinase inhibitor 1,10-phenanthroline and also by iodoacetamide (Table 1), although none of these prevented cleavage completely. In contrast, di-isopropyl-fluorophosphate (DFP), *trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane (E64) and pepstatin A had no effect. EDTA did not prevent breakdown of the starting peptide but did inhibit conversion of the intermediate peak 2 into peak 3, suggesting that different enzymes were responsible for each cleavage. The inhibitor solvents alone (DMSO or ethanol) did not inhibit proteolysis.

When whole-cell homogenates were tested on Link N before membrane fractionation, the peptide was degraded completely after 15 min of incubation. No clear sequence of cleavages could be deduced, unlike the distinct pattern of cleavages observed when whole cells were used. When purified plasma membranes in

PBS were used in place of whole cells, however, an almost identical pattern of cleavage was seen. Link N (Figure 2, peak 1, retention time 16.0 min) was again converted into a single labelled final product (Figure 2, peak 3, retention time 11.7 min) in 6 h. An intermediate peak (Figure 2, peak 2, retention time 15.0 min) again accumulated but less rapidly, reaching a maximum of 15% of the total label after 2 h of incubation, compared with a maximum of 40% when the peptide was degraded by whole cells.

As with whole cells, breakdown of the peptide by membranes was inhibited by PMSF, 1,10-phenanthroline and iodoacetamide (Table 2). EDTA did not affect breakdown of the starting peptide but did inhibit further processing of the intermediate peak 2. Again, DFP, E64 and pepstatin A had no significant effect on either cleavage. These data showed that the pattern of proteolysis observed with intact cells was duplicated by plasma membranes and involved at least two sequentially acting enzymes. The relative activities of each of these had altered slightly during membrane preparation, giving rise to the small differences observed in the relative rates of cleavage. The specific inhibitors of membrane proteinases captopril and phosphoramidon (10 μ M) had no measurable effect on cleavage of Link N but bestatin, an aminopeptidase inhibitor, reduced breakdown to less than 5% of uninhibited controls.

Determination of cleavage sites

To produce sufficient quantities of peptides for direct amino acid analysis and mass analysis, microgram quantities of unlabelled Link N in PBS, which contained no interfering peptides, were used as a substrate to determine the sites of cleavage. Four major peaks were resolved when the peptide was degraded by intact cells (Figure 3). Peak c1, the parent peptide, was not sequenced. Peak c2 (retention time 14.0 min) and peak c3 (9.8 min) were both resolved as double peaks, which is often characteristic of a C-terminal histidine residue, which may be present as both D and L isomers that can be distinguished when peptides are detected by absorption at 206 nm. Peak c4 (6.8 min) was resolved as a single

Figure 3 Cleavage of Link N by U937 cells

Link N (100 μ g) was incubated for 4 h with 5×10^6 cells in 200 μ l of PBS and the peptides separated for sequence analysis by C_{18} reversed-phase HPLC. Peptides were detected by absorbance of peptide bonds at 206 nm and expressed as a percentage of maximal absorption on an adjustable scale.

Figure 4 Cleavage of Link N by U937 plasma membranes

Link N (100 μ g) was incubated for 24 h with plasma membranes equivalent to 1×10^7 cells in 200 μ l of PBS and the peptides separated by C_{18} reversed-phase HPLC. Peptides were detected by absorbance of peptide bonds at 206 nm and expressed as a percentage of maximal absorption on an adjustable scale.

component and therefore did not contain sequences from the C-terminal end of the peptide. The supernatant solution from cells that had been incubated in PBS for 24 h contained no detectable peptides and there was no breakdown of Link N when it was incubated in PBS alone, showing that each of these components had been derived by cell-mediated proteolysis.

The similarity in retention times of peaks 2 and 3 (Figure 1) resulting from cleavage of either iodinated or unlabelled Link N suggested that they had identical sequences. Analysis of these products showed that peaks **c2** and **c3** were N-terminally truncated forms of Link N with sequences DNYTLDDHRAIH and DHDRAIH respectively. Although peak **c3** did not contain any measurable tyrosine, its retention time corresponded to that of peak 3 derived from iodinated Link N, suggesting that it may have co-eluted with a minor tyrosine-containing peptide having an identical retention time. No clearly defined sequence could be assigned to peak **c4**. The identities of peaks **c2** and **c3** were confirmed by mass spectral analysis, which gave values of 1472 Da and 862 Da, respectively, identical to those predicted from their amino acid compositions.

Analysis of the peptides produced from Link N by isolated plasma membranes showed that the same three peaks (peaks **m2**, **m3** and **m4**, Figure 4), corresponding to peaks **c2**, **c3** and **c4** (Figure 3) were present. Peak **m2** (retention time 14.0 min) and peak **m3** (retention time 9.8 min) again had the double peak characteristic of a C-terminal histidine and corresponded to peaks 2 and 3 derived from iodinated Link N (Figure 2). Peak **m4** (retention time 6.8 min) was again present as a single peak and two further peptides, present in only trace amounts when whole cells were used, were resolved. Peptide **m5** had a retention time of 16.4 min, greater than that of the starting peptide and peak **m6** (retention time 9.0 min) had the double peak characteristic of a C-terminal histidine. These additional peaks may have been present in larger proportions than when whole cells were used because of the increased time of incubation used for membrane experiments.

Sequence analysis of the peptides produced by plasma membranes showed that there was a somewhat more complex mechanism of breakdown than had been observed with intact

Table 3 Sequences of Link N cleavage fragments produced by plasma membranes

The sequences of the cleavage fragments are those present in each of the components shown in Figure 4.

Peak	Retention time (min)	Sequence
m2	14.0	SDNYTLDDHRAIH DNYTLDDHRAIH NYTLDDHRAIH
m3	9.8	DHDRAIH
m4	6.8	Y, L
m5	16.4	Not sequenced
m6	9.0	DRAIH

cells (Table 3). Peak **m2** was a mixture of three unresolved peptides with the same retention times but each with a different N-terminus. These consisted of the sequence DNYTLDDHRAIH together with two related peptides containing one more or one less amino acid. Peak **m3** was again identified as DHDRAIH and did not therefore contain tyrosine, although its retention time suggested that it may have corresponded to peak 3 derived from iodinated Link N. The new peak **m6** was a peptide from which two further N-terminal amino acids had been removed to produce the product DRAIH. Peak **m4** contained free tyrosine and leucine but peak **m5** could not be identified by direct sequence analysis.

DISCUSSION

Earlier experiments have shown that the cartilage-derived growth factor Link N is rapidly degraded by a variety of different cell types, including chondrocytes and macrophages [14]. A similar method of inactivation has been described for a number of other small, biologically active peptides [26]. Proteinases not normally synthesized by chondrocytes have been identified in articular cartilage and are thought to originate from neutrophils, macro-

Figure 5 Proposed scheme for cleavage of Link N

(a) The initial scheme proposed for cleavage of Link N by intact cells involved the first cleavage by a serine protease E1 at Ser⁴-Asp⁵ followed by a cleavage by the metalloprotease E2 at Leu⁹-Asp¹⁰. **c2** and **c3** are peptides with known sequences, **c3** and **c4** are deduced assignments. (b) The scheme proposed for cleavage of Link N by purified plasma membranes. An obligatory initial cleavage by a serine protease E1 at Leu³-Ser⁴ is followed by sequential aminopeptidase cleavages. Ea represents known sites of aminopeptidase cleavage and Eb sites of cleavage that must occur to liberate free tyrosine. The endopeptidase represented by Ec corresponds to the EDTA-inhibitable enzyme E2. The peptides designated **m2**–**m6** correspond to peaks **m2**–**m6** depicted in Figure 4.

phages, synoviocytes and lymphocytes [34]. One or more of these proteinases may be responsible for cleavage and inactivation of Link N. U937 monocytes were therefore chosen to investigate the mechanism and sites of cleavage because of the similarity of their proteinase profile to those of the main cell types found in synovial fluids during episodes of inflammation [31,32] when degradation of Link N would be expected to be maximal.

Time-course experiments using radio-iodinated Link N showed that at least two cell-associated proteinases were involved in its cleavage to produce a final labelled product. Since PMSF abolished degradation of Link N almost completely, this first cleavage appeared to be essential before cleavage of the resulting intermediate radiolabelled peptide by the second, EDTA-sensitive, peptidase could take place. Phenanthroline inhibited both enzymes, suggesting that the enzyme responsible for the first cleavage may have been a metal-dependent serine protease. Although iodoacetamide also inhibited breakdown, the lack of action of E64, a specific thiol-protease inhibitor, indicated that this may have been a non-specific effect.

When unlabelled Link N was used as a substrate, several additional peptides that did not contain a labelled tyrosine were resolved. Sequencing of these suggested that the initial cleavage of the peptide at Ser¹-Asp⁵ by an unknown serine proteinase E1 had released the peptide DHLS (corresponding to component **c4**) from Link N. The subsequent cleavage of the remaining peptide DNYTL-DHDRAIH at the Leu⁹-Asp¹⁰ bond by the metalloproteinase E2, as depicted in Figure 5(a), released the remaining peptide DHDRAIH from DNYTL, which may

have co-eluted with peak **c3** but have been degraded too rapidly to be detected during sequencing. This would account for the presence of tyrosine in this fraction when iodinated peptide was used as a substrate.

The profile of Link N breakdown produced by U937 plasma membranes (Figure 4) was almost identical to but more complex than that seen with whole cells (Figure 3), indicating that the mechanism of degradation was essentially the same and involved membrane-associated proteinases. However, two additional minor peaks were detected that were present as trace amounts only when whole cells were used. Peak **m2** contained two additional peptides with either one more or one less amino acid in addition to the DNYTL-DHDRAIH sequence. These data suggested that the initial serine proteinase E1 had removed a tripeptide DHL, not a tetrapeptide, and that the resulting sequence SDNYTL-DHDRAIH had undergone further processing by the action of aminopeptidases. The sequence data for the breakdown of Link N by whole cells showed that trace quantities of serine were present as an alternative first residue to aspartate, indicating that when intact cells were used, cleavage of serine from the newly generated N-terminal of SDNYTL-DHDRAIH was extremely rapid but was reduced when purified membranes were used. When the sequences of the peaks were determined, **m2** was shown to contain a Tyr⁷ residue as expected and probably corresponded to the radiolabelled intermediate peak 2. However, **m3** was too truncated to contain tyrosine and it appeared that following cleavage of DHLS from Link N at Ser⁴-Asp⁵, the E2 cleavage liberated the peptide DNYTL from DNYTL-DHDRAIH by cleavage at Leu⁹-Asp¹⁰. Although DNYTL was not detected by sequence analysis, it may have co-eluted with DHDRAIH, explaining the localization of the labelled peak **m3** derived from iodinated Link N to this region of the chromatograph. Peptide DNYTL was then degraded rapidly by the subsequent action of aminopeptidases and endopeptidases, as depicted in Figure 5(b). The initial cleavage by a serine-dependent protease was followed by a metallo-endopeptidase that cleaved the remaining peptide into two and sequential trimming by one or more aminopeptidases then released a series of sub-peptides.

The U937 cells used in these experiments express both elastase and cathepsin G, two proteinases that are important in the degradation of articular cartilage, either directly or through modulation of metalloproteinase activity [35–37]. Although these enzymes are normally lysosomal in origin, they have been purified from U937 plasma membranes [38] and either of these may have been responsible for this first cleavage. However, Link N was not cleaved by either a commercial preparation of human leucocyte elastase or by stromelysin itself (results not shown).

U937 plasma membranes also express a number of metalloproteinases. These include dipeptidyl peptidase IV (CD26), aminopeptidase N (CD 13), and angiotensin-converting enzyme [39,40]. A membrane-bound form of the metalloproteinase aggrecanase, which is inhibited by EDTA and the endopeptidase 24.11 (CD10) have also been described on chondrocytes [41,42]. Breakdown of Link N was not, however, inhibited by phosphoramidon, a specific inhibitor of endopeptidase 24.11, or by captopril, an inhibitor of angiotensin-converting enzyme. A membrane form of the thiol-dependent metalloendopeptidase thimet oligopeptidase has been described [43,44], although it is not known whether this is expressed on U937 cells. This enzyme is able to cleave peptides ranging from 6 to 17 residues in length and like the Link N peptidases is inhibited by EDTA and phenanthroline but is unaffected by DFP or phosphoramidon [43]. Thimet oligopeptidase is known to inactivate a number of biologically active peptides including bradykinin, neurotensin, angiotensin,

substance P and α endorphin and may therefore have been the enzyme responsible for one or more of the endopeptidase cleavages of Link N. Bestatin, a specific inhibitor of aminopeptidases, also inhibited cleavage of Link N, indicating that one or more of this group of enzymes may have been responsible for some of the additional N-terminal cleavages observed.

Membrane-peptidase-mediated inactivation of a large number of other biologically active peptides has been well documented [39,40]. Since data from previous experiments have shown that N- and C-terminally truncated link peptides with the sequences SDNYTLDDHDR and DNYTLDDHDR lost their ability to stimulate proteoglycan synthesis [15], it is probable that Link N was also inactivated by rapid specific cleavage mediated by membrane-associated proteinases.

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