A proteolytic fragment from human link protein is taken up and processed by monocytes and B cells

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Mild digestion of ^{125}I -labelled human proteoglycan aggregates with trypsin or stromelysin produced specific peptides that were taken up rapidly by THP-1 monocytes. SDS/PAGE of undigested aggregate showed that the three components of molecular mass 48, 44 and 41 kDa, corresponding to isoforms of link protein originally present, had been converted into a single component of 41 kDa by trypsin treatment, and that fragments of 6–12 kDa were present in fractions containing the high-uptake peptide. Separate proteolysis of isolated proteoglycan monomer and link protein confirmed that the specific high-uptake fragment was derived from link protein. Uptake of the link fragment was rapid, reaching a maximum after 5 min, and specific, since it was blocked by metabolic or serine proteinase inhibitors and at 4 °C. After uptake the cleaved fragment was processed further, with 50 % of the radiolabel being released as degraded peptides within 5 min. In contrast, accumulation of whole aggregate reached a maximum after 45 min and only 50 % had been released after 2 h. Uptake of aggregate was less affected by inhibitors or at low temperature, suggesting that a separate mechanism existed for its turnover. The aggregate was transported to lysosomes after uptake, although the link fragment did not sediment with either lysosomes or plasma membranes, suggesting that it was present in the cytoplasm or in very labile vesicles. However, the mode of handling of the peptide by the cells remains unclear. The link fragment was taken up by several different monocytic and B cell lines, but not by mouse fibroblasts or peritoneal macrophages. These data suggest that a surface serine proteinase on monocytes and B cells enables them to process and take up a fragment of link protein derived by extracellular proteolysis.

INTRODUCTION

Proteoglycans (PGs) are one of the major constituents of cartilage matrix. Under appropriate conditions they are able to induce inflammation, as well as themselves being a target for degradation in autoimmune diseases such as rheumatoid arthritis, ankylosing spondylitis and relapsing polychondritis [1–7]. For example, in a mouse model of inflammatory disease the use of chondroitinase-treated PG as an antigen can induce polychondritis, showing that, in this model, the target epitopes for the autoimmune response reside in the PG protein core rather than in its carbohydrate side-chains [8,9].

Synovial fluids taken from patients with rheumatoid arthritis often contain large numbers of activated monocytes and macrophages [10,11] which have the capacity to act as antigen-presenting cells in immune reactions. Increased infiltration by these cells is frequently accompanied by large increases in lysosomal hydrolases and proteases in synovial fluids in both naturally occurring and experimental models of arthritis [12]. Macrophages also secrete a number of cytokines, including interleukin-1 and tumour necrosis factor α , which can promote destruction of cartilage matrix and bone [13–15]. The presence of these cytokines in synovial fluid may stimulate macrophages or chondrocytes to secrete enzymes which can degrade cartilage matrix, exposing epitopes which may initiate or prolong an antigen-driven arthritis. This probability is rendered likely by the fact that the neutral metalloproteinase stromelysin, an enzyme that is able to degrade PG, has been found in explant cultures from rheumatoid joints [16] and in animal models of rheumatoid arthritis [17]. The metalloproteinase collagenase and a so-far-unidentified Ca^{2+} - and Mg^{2+} -independent neutral proteinase, with trypsin-like activity, have also both been identified in rheumatoid synovial fluids [18]. In addition, α_1 -proteinase in-

hibitor, an inhibitor of elastase and other proteinases, is often inactivated by oxidation in rheumatoid joints [19].

Some types of cell express receptors via which PG can be rapidly internalized. For example, dermatan sulphate PGs are taken up into fibroblasts by a receptor that recognizes appropriate sequences on the protein core [20,21]. The PGs are then transported to lysosomes where the protein core is degraded first, followed by the dermatan sulphate side-chains. Liver endothelial cells, on the other hand, take up chondroitin sulphate PGs (CSPGs) by a different receptor that can also recognize carbohydrate structures present on hyaluronic acid [22–24]. Similarly, after internalization, CS is transported to lysosomes where it is degraded.

In addition to the proteolytic breakdown of PG which occurs during joint disease, non-pathogenic proteolysis of cartilage PG occurs as part of the normal process of turnover and aging. Thus in normal cartilage cleavage of core protein results in smaller PG subunits [25], and link protein is decreased in size from the 48 and 44 kDa forms to a 41 kDa form [16]. Little is yet known, however, about the uptake and breakdown of PG by cells such as macrophages that are able to present antigens, are found in large numbers at sites of inflammation and may play a role in the pathogenesis of inflammatory joint diseases. We therefore set up an experimental system to compare the uptake and breakdown of whole human PG aggregates with that of proteolytic fragments, such as those which might result from extracellular proteolysis of intact aggregates present in cartilage. For this we used PG aggregates, monomer and link protein prepared from human articular cartilage and THP-1 cells, a human monocytoid cell line that displays many of the characteristics of normal macrophages, including the ability to express Fc and C3 receptors and class II antigens [26]. The results of this investigation showed that there were clear differences in rates of uptake, turnover and

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; CS, chondroitin sulphate; FITC, fluorescein isothiocyanate; GAG, glycosaminoglycan; PG, proteoglycan; PBS, phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride.

subcellular location of whole PG aggregates in comparison with certain specific peptides derived from them by extracellular proteolysis.

MATERIALS AND METHODS

Materials

Aggregated PGs, link protein and PG monomer from human articular cartilage were a gift from M. Bayliss, Kennedy Institute of Rheumatology [27]. Tissue culture media, supplements and Dulbecco's phosphate-buffered saline (PBS) were supplied by Flow Laboratories, Rickmansworth, Herts., U.K., and sera were from Imperial Laboratories, Salisbury, Wilts., U.K. Percoll and density marker beads were obtained from Pharmacia (U.K.). 4-Methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside substrate was purchased from Koch-Light, Watford, Herts., U.K. ^{125}I and $[2\text{-}^3\text{H}]\text{AMP}$ were supplied by Amersham International. Trypsin (EC 3.4.21.4), free from chymotrypsin activity, was supplied by Sigma. Stromelysin (EC 3.4.23.-) was a gift from Dr. G. Murphy, Strangeways Laboratories, Cambridge, U.K.

Assays

The activity of β -hexosaminidase was determined fluorimetrically using 4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside as substrate [28]. Plasma membranes were detected by their 5'-nucleotidase activity [29] using $[2\text{-}^3\text{H}]\text{AMP}$ as a substrate. The concentration of sulphated glycosaminoglycans (GAGs) in Sepharose CL-6B fractions was determined by a modification of the dye-binding assay [30]. Samples of 40 μl were added to 250 μl of Coomassie Blue in a 96-well microtitre plate and read at 600 nm on a Bio-Rad model 2550 EIA reader.

Iodination and fluorescein isothiocyanate (FITC) labelling of PGs

PG was iodinated using 1 mCi of ^{125}I and four Iodo-Beads added to 1 mg of PG in 300 μl of PBS. After 20 min at 4 °C the solution was removed from the beads and free iodine was separated from labelled PG on Sephadex G-25 equilibrated with PBS [31]. Some of this material was then given an additional FITC label [32] by dissolving 1 mg of PG in 0.5 ml of 0.1 M- NaHCO_3 buffer, pH 9.3, after which 20 μg of FITC was added. The solution was left overnight at 4 °C, and unbound FITC was removed on Sephadex G-25.

Proteolytic digestion of PGs

Samples of iodinated PG were subjected to proteolysis using either trypsin (1.5 mg of PG/3 mg of Tos-Phe- CH_2Cl -treated trypsin) for 6 h at 37 °C in 0.05 M-Tris/0.1 M-NaCl buffer, pH 7.5, or stromelysin (1.5 mg of PG/1 unit of stromelysin) for 16 h at 37 °C in 0.01 M- CaCl_2 /0.05 M-Tris/0.1 M-NaCl, pH 7.5. Trypsin activity was blocked with soya bean trypsin inhibitor, and stromelysin activity was stopped by freezing samples at -20 °C until digested fragments were separated from the proteinase by gel filtration.

Gel chromatography

Gel chromatography was carried out using 80 ml bed volume columns of Sepharose CL-2B and CL-6B, a 60 ml Sephadex G-50 column and a 30 ml Sephadex G-25 column, all equilibrated with PBS and eluted at a flow rate of 1 ml/min. Sample volumes of 2 ml were loaded on to Sepharose columns, 1.5 ml on to G-50 and 1 ml on to G-25. Fraction volumes of 0.5 ml were collected from Sephadex G-25 and 1 ml from each of the other columns. For chromatography of the PG fragment stripped from the cells

in acid buffer or secreted into the culture medium, columns were equilibrated and eluted in 1 % (w/v) SDS in water to minimize binding to the Sephadex.

SDS/polyacrylamide gel electrophoresis

SDS/PAGE was carried out on 10–20 % and 2.5–20 % gradient slab gels [33]. Molecular mass markers in the range 200–14.3 kDa were purchased from Amersham (CFA-626). Gels were dried and kept in contact with Kodak XAR-5 X-ray film for 2–4 days at -70 °C.

Uptake of labelled PGs

Cells were harvested, washed twice in Dulbecco's modified Eagle's medium (DMEM) and pelleted (2×10^6 cells/tube) in 1.5 ml Eppendorf tubes. Pellets were resuspended in 100 μl of DMEM, and 1×10^5 c.p.m. of PGs or peptides in 100 μl of PBS was added for an appropriate period of time. The cells were then washed three times in PBS at 4 °C and counted for radioactivity in a Wallac LKB Compugamma γ -radiation counter. Inhibition experiments were carried out after preincubation with the metabolic inhibitors 2-deoxyglucose and NaN_3 and the proteinase inhibitors phenylmethanesulphonyl fluoride (PMSF), phenanthroline, pepstatin and iodoacetamide to inhibit serine, metallo-, aspartate and thiol proteinases respectively. The concentrations and preincubation times used for each inhibitor were: PMSF, 2 mM, 15 min; 2-deoxyglucose, 25 mM, 120 min; NaN_3 , 10 mM, 120 min; iodoacetamide, 1 mM, 30 min; *o*-phenanthroline, 1 mM, 30 min; pepstatin, 2 mg/ml, 30 min. In additional experiments cells were precooled to 4 °C and incubated for a further 1 h at this temperature with labelled peptide.

Cell fractionation

Cells of the human monocytoid line THP-1 [34] were cultured in DMEM supplemented with 10 % heat-inactivated foetal calf serum, glutamine and penicillin/streptomycin at 37 °C in CO_2 /humidified air (1:19). They were collected by centrifugation, suspended in 2 ml of 0.25 M-sucrose/3 mM-imidazole, pH 7.5, at 4 °C and disrupted by nitrogen cavitation after pressurization for 15 min at 450 kPa in a nitrogen bomb [35]. The homogenate was centrifuged at 800 *g* (r_{av} 8 cm) for 5 min to remove nuclei and remaining whole cells. BSA (10 mg) was added to the post-nuclear supernatant, which was layered over 35 ml of Percoll/0.25 M-sucrose (starting density 1.05 g/ml) and centrifuged at 45000 *g* (r_{av} 10.5 cm) for 60 min. In experiments using the tryptic PG fragment, the cells were disrupted in a sample volume of 8 ml and layered over 29 ml of Percoll to allow a more complete separation of plasma membranes from soluble cell constituents.

RESULTS

Degradation and uptake of whole PG aggregate

Whole PG aggregates were radiolabelled with ^{125}I , re-chromatographed on Sepharose CL-2B, and the material that was still large enough to be excluded from the column (57 % of the total label) was subjected to mild digestion with trypsin or stromelysin before a final separation on Sepharose CL-6B (Fig. 1a). Samples from this profile were tested by the dye-binding assay, which showed that sulphated GAG was present in those fractions with a K_{av} of less than 0.55, equivalent to a size of 20 kDa or greater. No GAG was detectable in fractions eluted after this point (Fig. 1b). Samples of trypsin-treated PG that had also been labelled with FITC showed almost identical radiolabel and fluorescence profiles (Figs. 1a and 1b), indicating that most or all of the fragments produced by proteolysis were also labelled

Fig. 1. Sepharose CL-6B chromatography of PG aggregates

(a) Iodinated PG (1 mg) was digested with either trypsin ($\square$) or stromelysin ($\bullet$), as described in the Materials and methods section, or left untreated ($\triangle$). Each sample was loaded on to Sepharose CL-6B and the fractions eluted were assayed for radiolabel. Fractions of 1 ml were collected. (b) A sample of iodinated PG that had been labelled with FITC and then treated with trypsin was chromatographed on Sepharose CL-6B. Fractions were tested for sulphated GAG by the dye-binding assay ($\blacksquare$), and FITC-labelled protein was measured by fluorescence ($\circ$). (c) Selected fractions from the CL-6B profiles of undegraded PG aggregate ($\blacksquare$) and from samples that had been digested with trypsin ($\hatchedbox$) and stromelysin ($\square$) were incubated for 5 min at 37 °C with 2×10^6 THP-1 cells. After incubation cell-associated radioactivity was expressed as a percentage of total c.p.m. offered to the cells. Assays were carried out in duplicate using PG preparations from two different individuals. In a series of five experiments the percentage of cell-associated radioactivity in each fraction varied by less than $\pm 5\%$ of the mean percentage uptake.

Fig. 2. SDS/PAGE of trypsin-treated PG

Samples from selected fractions from the CL-6B profile shown in Fig. 1 (10000 c.p.m.) were analysed by SDS/PAGE on a 2.5–20 % gradient gel (a) and on a 10–20 % gradient gel (b) as described in the Materials and methods section. The gels were then fixed and dried, and radiolabelled PG proteins were visualized by autoradiography.

with ^{125}I . Selected fractions from each profile were incubated at 37 °C for 5 min with THP-1 cells which were then washed, and the amount of radiolabelled PG taken up was determined. This test showed that some fractions which were eluted shortly before V_t on the CL-6B column ($K_{av} = 0.86$) contained peptides that were taken up at least three times more rapidly by the cells (Fig. 1c) than material from other parts of the profile.

Undigested iodinated PG aggregates were also analysed by SDS/PAGE and autoradiography, which showed that in addition to the high-molecular-mass material, three relatively small components with apparent molecular masses of 48, 44 and 41 kDa were present, the largest of these being the most abundant. After mild trypsin treatment, the component with the lowest molecular mass predominated, with a small quantity of the higher-molecular-mass form still remaining (Fig. 2a). These components corresponded in size to the three different forms of link protein described previously [16] which can be separated from aggregates by SDS/PAGE. Other, more polydisperse, fragments generated by trypsin treatment were present in the high-uptake fractions eluted towards the end of the CL-6B profile, with apparent molecular masses ranging from 6 to 12 kDa on SDS/PAGE (Fig. 2b). In a further purification, those fractions containing the high-uptake fragments were pooled, concentrated and re-

Fig. 3. Chromatography of labelled peptides on Sephadex G-50

Fractions 60–90 from the Sepharose CL-6B elution profiles of radiolabelled PG that had been digested with (a) trypsin or (b) stromelysin were pooled, rechromatographed on Sephadex G-50 (—) and their radioactivity counted. Fractions from each peak separated were then tested for association with THP-1 cells (■). Radiolabel recovered was expressed as a percentage of total c.p.m. offered; 1 ml fractions were collected.

chromatographed on Sephadex G-50. Several components were separated by this final step, one of which, with a K_{av} on G-50 of 0.69, was taken up at a very high rate, amounting to 10–12% of the total present in 5 min (Fig. 3).

Degradation and uptake of PG monomer and link protein

In a further series of experiments, PG monomer and link protein were labelled separately and trypsin-treated, and fractions were separated from them by chromatography on Sepharose CL-6B tested against THP-1 cells, to determine from which component the high-uptake fragment was derived (Fig. 4). The elution profile of trypsin-treated monomer did not contain the low-molecular-mass component produced by trypsin treatment of aggregate, and none of the fractions from the profile contained a high-uptake peptide. In contrast, when link protein was treated with trypsin and chromatographed, some of the degraded material aggregated and was eluted with the void volume, and a high-uptake component was produced which was eluted from the column in a position corresponding to that of the high-uptake peptide produced from whole aggregate (Fig. 4). Intact non-trypsin-treated link protein was not, however, recognized as a high-uptake protein.

Fig. 4. Chromatography of tryptic fragments from PG monomer and link protein

(a) Samples of ^{125}I -labelled PG monomer (▲), link protein (○) or whole PG aggregate (□) were digested with trypsin and chromatographed on Sepharose CL-6B, and the fractions eluted were assayed for radioactivity. Fractions of 1 ml were collected. The position of undegraded link protein, which moved on gel filtration as a single well-defined peak, is indicated by the arrow. (b) Selected fractions from each elution profile were incubated for 5 min at 37 °C with THP-1 cells. After washing, cell-associated radiolabel was measured: ■, monomer; ▨, link protein; ■, whole PG aggregate.

Rates of uptake and turnover

The rate of uptake of this specific fragment was compared with that of the whole PG aggregate from which it was derived. Whereas uptake of the fragment was rapid, reaching a maximum after only 5 min incubation, the aggregate was taken up relatively slowly, with accumulation peaking at 45–60 min (Fig. 5). The initial rapid uptake of the fragment was followed by a transient drop in cell-associated radiolabel, suggesting that turnover and release was occurring. When cells were incubated with the labelled fragment for 5 min and then transferred to fresh medium, radiolabel was released rapidly, with 50% being shed into the medium within 5 min. In contrast, turnover of whole PG aggregate was considerably slower, with 50% release being reached only after a 2 h incubation. These data suggested that different mechanisms for the uptake and turnover of whole PG aggregate and of the high-uptake fragment generated from it by proteolysis were utilized by the THP-1 cells.

To determine whether the high-uptake peptide was degraded during such rapid turnover, its size profile was compared at different times after uptake. Before incubation the peptide was excluded from Sephadex G-25, but the radiolabel extracted from the cells in acetate buffer (pH 3.5) after a 5 min incubation had been greatly decreased in size and ran as a homogeneous peak shortly before V_t (Fig. 6). Control samples of peptide remained undegraded when they were incubated in this buffer for 20 min.

Fig. 5. Uptake and release of whole PGs and proteolytic fragments

High-uptake fractions of trypsin-treated PGs (fraction 30 from a Sephadex G-50 column; ○) and undigested PG (fraction 20 from a CL-6B column; ●) were incubated for increasing periods of time with THP-1 cells. After washing, cell-associated radiolabel was counted. After cells had been incubated with the tryptic fragment (□) for 5 min and the aggregate (■) for 45 min to allow maximal accumulation, cells were transferred to fresh medium and aliquots were assayed at various intervals of time to determine the amount of radioactivity that remained cell-associated.

Fig. 6. Processing of tryptic fragments by THP-1 cells

Cells were incubated for 5 min with fraction 60 from the trypsin-treated PG profile shown in Fig. 1. They were then either placed in acetate buffer, pH 3.5, for 20 min, or chased in fresh culture medium for 30 min. The acetate extract and the chase medium were then subjected to chromatography on Sephadex G-25: □, starting material; ●, radiolabel extracted in acetate buffer; △, radiolabel secreted by the cells. Fractions of 0.5 ml were collected.

Other experiments in which tryptic fragments were incubated with cells for only 30 s, washed in ice-cold PBS and then extracted with acetate buffer showed that they had already become decreased in size (results not shown). When cells were transferred to fresh medium after uptake, it was found that those fragments released into the culture supernatant had been decreased in size even further, being retarded on Sephadex G-25 and eluted after V_0 , despite the presence of 1% (w/v) SDS in the buffer. Without added SDS, secreted radiolabelled fragments were retarded by as much as 2 column volumes (results not shown).

Subcellular location of radiolabel after uptake

To determine the intracellular location of the PG aggregate and the tryptic fragment after uptake, cells were disrupted by N_2

Fig. 7. Subcellular location of tryptic fragments and PG aggregate

(a) THP-1 cells were incubated for 10 min with whole PG aggregate, washed in PBS at 4 °C and then either disrupted immediately or incubated in fresh culture medium for 30 min before disruption by N_2 cavitation. The post-nuclear supernatant from the cell lysates was centrifuged on a 1.05 g/ml starting density Percoll gradient, fractionated and assayed for distribution of radiolabel (■, 10 min pulse; ○, 30 min chase), the lysosomal enzyme marker β -hexosaminidase (▲) and density (—). (b) THP-1 cells were incubated for 10 min with the high-uptake tryptic fragment of PG (CL-6B fraction 60), washed in PBS and chased for 20 min in fresh medium before disruption by N_2 cavitation. The post-nuclear supernatant was centrifuged on a 1.05 g/ml starting density Percoll gradient which was then fractionated and assayed for the lysosomal enzyme marker β -hexosaminidase (○), the plasma membrane enzyme marker 5'-nucleotidase (△), radiolabel (■) and density (—).

cavitation and subcellular fractionation carried out using Percoll density gradients. Pulse-chase experiments showed that the whole aggregate was transported by lysosomes via endosomes within 30 min of uptake (Fig. 7a). When cells were allowed to take up the tryptic fragment, however, for periods of time ranging from 5 to 45 min, radiolabel was always released into the supernatant solution immediately on disruption of the cells. Label was never found associated with lysosomes, nor did it appear to be attached to the plasma membrane, from which it could be readily separated on the gradient (Fig. 7b). When cells were disrupted by alternative methods, including freeze-thawing or by several passages through a syringe needle, this also resulted in the release of the labelled fragment, which remained in the supernatant solution when the plasma membrane was pelleted by high-speed centrifugation (results not shown). These results indicated that once the peptide had been taken up and cleaved by the cells, the remaining radiolabelled portion of the fragment had no affinity for the plasma membrane. The rapidity with which this fragment was released on disruption by mild methods contrasted with the relatively slow removal observed when cells were extracted for 20 min in acetate buffer, pH 3.5.

Table 1. Inhibition of uptake of PG peptides

THP-1 cells were incubated for 5 min at 37 °C with the high-uptake tryptic PG fragment with or without the addition of proteinase and metabolic inhibitors, or incubated for 1 h at 4 °C. To measure inhibition of uptake of whole PGs, cells were incubated with radiolabelled material for 1 h. The cells were then washed and cell-associated radioactivity was counted.

Inhibitor	Inhibition (%)
PMSF	84
4 °C	91
Iodoacetamide	19
2-Deoxyglucose	61
Pepstatin	0.5
NaN ₃	66
Phenanthroline	0.5

Fig. 8. Uptake of peptides by mouse and human cells

The high-uptake tryptic fragment from PG aggregate was incubated with a number of cell lines and with unstimulated CBA mouse peritoneal macrophages (mouse mφ) for 5 min at 37 °C, after which they were washed and cell-associated radioactivity was measured as described in the Materials and methods section.

Effect of inhibitors on uptake

When the number of cells used in an assay was increased from 2×10^6 to 3.2×10^7 in a series of experiments without an increase in peptide concentration, the percentage uptake did not increase but remained constant, amounting to about 10% of the total radiolabel. Thus the high-uptake fragment accounted for at least 10% of this fraction, but may have been even higher than this because of the high rate of release of label after uptake. The decrease in size of the tryptic peptide noted after it had been extracted from the cells in acetate buffer at pH 3.5 suggested that a proteinase may be involved in its uptake. To examine this further, the uptake assay was carried out at 4 °C and the cells were also treated with a panel of proteinase inhibitors. In addition, since the fragment was cell-associated after uptake but appeared, from the subcellular fractionation data, not to be bound to the plasma membrane, we determined whether uptake of the fragment was an active process and might therefore be sensitive to metabolic inhibitors. The results of these experiments (Table 1) showed that uptake of the PG fragment was almost completely abolished at 4 °C (91% inhibition) and remained very low even after 60 min incubation. Uptake was also strongly inhibited when the cells were treated with the serine proteinase inhibitor PMSF (84% inhibition), but only weakly inhibited by the thiol proteinase inhibitor iodoacetamide and not at all by

the metalloproteinase inhibitor phenanthroline or the aspartic proteinase inhibitor pepstatin. Uptake was, however, inhibited by over 60% when cells were preincubated with the metabolic inhibitors 2-deoxyglucose and NaN₃. In comparison, uptake of whole PG aggregate was decreased only 38% and 32% at 4 °C and by PMSF respectively, suggesting that it was taken up by a different mechanism.

Uptake by different cell types

In addition to THP-1 cells, the human monocytic cell lines HL-60 and U937 and a range of other cell types were tested for their ability to take up proteolytic fragments of human PG. As shown in Fig. 8, all of the monocytic and B cell lines took up the fragment derived from link protein rapidly at rates varying from 3 to 10% in 5 min, whereas resident mouse peritoneal macrophages and 3T3 fibroblasts had no affinity for the peptide. These data showed that uptake of the fragment was highly specific and not due simply to non-specific pinocytosis or generalized adsorption to cell membranes.

DISCUSSION

When human PG aggregates were partially degraded with either trypsin or stromelysin, specific fragments with an apparent molecular mass of 6–12 kDa on SDS/PAGE and a K_{av} of 0.69 on Sephadex G-50 were taken up rapidly and processed by THP-1 monocytes (Figs. 1–3, 5 and 6). No equivalent fragments were produced from separate digests of PG monomer, but high-uptake peptides were derived from link protein by proteolysis (Fig. 4). Undegraded link itself was not, however, recognized as a high-uptake protein.

Experiments in other laboratories have shown that the link protein extracted from human cartilage can be separated into three components of 48, 44 and 41 kDa by SDS/PAGE [16]. Link protein is susceptible to cleavage by trypsin and stromelysin near its *N*-terminus to yield peptides 13 and 16 amino acids long respectively, which may be glycosylated [16]. In our experiments three components of 48, 44 and 41 kDa were separated from whole PG aggregate, but only the 41 kDa component was detected after trypsin treatment, suggesting that these components were also derived from link protein and had been degraded by proteolysis to release a component of about 7 kDa. These findings were similar to those described by Nguyen *et al.* [16], who showed that fragmentation of link protein by stromelysin *in vitro* was due to the removal of a small *N*-terminal peptide and produced similar link components to these extracted from cartilage. Furthermore, since stromelysin is present in an active form in rheumatoid joints, degradation of link protein and production of this *N*-terminal peptide might well be accelerated during inflammation. Trypsin treatment of our preparations of purified link protein, in which the 48 kDa form was predominant, produced a cleavage product of apparent molecular mass 41 kDa and an additional diffuse band in the 6–12 kDa region, identical to the fragments produced by digestion of whole aggregate (Fig. 2b). Some species of link protein are glycosylated near their *N*-terminus [16]. The diffuse resolution of the small cleavage product may be due, therefore, to differences in its degree of glycosylation, since it was produced by limited digestion of pure protein substrate by a single proteinase. If the high-uptake fragment were derived from the *N*-terminal region of link protein, then its glycosylation would also account for the fact that its apparent molecular mass was slightly greater than expected.

Because only monocytic and B cell lines took up the fragment, whereas resident mouse peritoneal macrophages and fibroblasts did not, uptake was clearly specific and not due simply to generalized, non-specific pinocytosis or binding. The fact that

uptake of this cleaved fragment was almost completely inhibited by the serine proteinase inhibitor PMSF, as well as at low temperature and by metabolic inhibitors (Table 1), coupled with the decrease in its size observed after uptake (Fig. 6), suggested that it was first cleaved by a serine proteinase and then actively internalized. Macrophages stimulated with thioglycollate are known to synthesize the serine proteinase plasminogen activator and to express increased numbers of surface receptors to which the enzyme can bind [36]. Another membrane-bound serine proteinase has been described in human B cells transformed with Epstein-Barr virus [37], and the serine proteinase myeloblastin is expressed by actively dividing cells of the myeloid lineage, including HL-60 and U937 monocytes [38]. One or more of these membrane-associated proteinases may therefore be responsible for the initial cleavage of the link peptide and may be an essential requirement for uptake of the fragment by monocytes and B cells. The well-defined single radioactive component extracted from the cells at pH 3.5 shortly after uptake indicated that this initial essential cleavage was highly specific. Subsequent further rapid degradation, presumably by other proteinases, produced still smaller peptides that were released back into the medium. Although the suppression of uptake by metabolic inhibitors and at 4 °C and the non-association of the label with the plasma membrane observed after subcellular fractionation suggested that the peptide had been internalized, the possibility that the small ligand created by the proteinase was released from the plasma membrane during processing cannot be excluded.

The biological consequences of uptake and processing of this fragment from link protein remain to be determined. If the peptide were indeed derived from the *N*-terminal region of link protein, some of it would be produced under normal circumstances during maturation and aging of cartilage as link protein is degraded from a 48 kDa to a 41 kDa species. The increase in activity in stromelysin observed in rheumatoid joints may accelerate production of the peptide, and its uptake by monocytes and B cells could be the initial stage in induction of an autoimmune response to this peptide. Activated class II antigen-bearing macrophages and B cells are normally present in rheumatoid joints [10,11,39] and may express the specific surface serine proteinase that appears to be essential before uptake of the link fragment can take place. Myeloblastin, for example, is expressed only by actively dividing cells and not by cells induced to differentiate and to stop dividing by agents such as dimethyl sulphoxide and phorbol esters [38]. Recent data have shown that antigen-presenting cells are able to process small proteins after uptake by limited proteolysis within endosomes [40,41]. These processed peptides can then bind to class II antigens, which are recycled back to the plasma membrane. Although monovalent ligands usually follow this pathway after internalization [42], large multivalent ligands appear to follow an alternative route, and are not recycled but instead are transported to the lysosomes for degradation [43,44]. Similar differences were observed in our experiments between the lysosomal location of large multivalent PG aggregates and the rapid recycling of the smaller peptide fragments derived from them.

The apparent cytoplasmic location of the radiolabelled fragment seen after uptake was an unexpected finding. However, ATP-dependent transport of peptides and proteins across membranes has been described previously in several systems. For example, cytoplasmic peptides are thought to be transported across the membranes of the endoplasmic reticulum into the lumen, where they are able to bind to class I molecules [45]. Other proteins such as thioredoxin and interleukin 1 that lack hydrophobic signal sequences appear to be secreted by a mechanism that involves direct transmembrane transport [46]. Uptake of the link fragment may take place by a similar mechanism, enabling

the peptide to be transported directly into the cytoplasm. The lack of binding of the peptide to the cells at 4 °C, the blocking of uptake with metabolic inhibitors and the non-association of the peptide with the plasma membrane following subcellular fractionation all suggest that any initial binding of the peptide is followed rapidly by internalization. However, distinct stages of binding and internalization have not yet been delineated and the mode of handling of the peptide remains unclear. An alternative explanation is that the peptide was taken up into labile vesicles, which were lysed during cell disruption procedures. Although the ¹²⁵I-labelled portion of the tryptic fragment of link protein did not appear to undergo processing in endosomes or lysosomes, the location of any unlabelled peptides that might be derived from it remains unknown. Thus the rapid uptake and cleavage of the specific stromelysin and tryptic fragments derived from PG aggregates seen in these experiments may be one mechanism by which macrophages generate potentially auto-antigenic fragments from PGs that have already been partially degraded extracellularly. Alternatively, this processing of peptide fragments by cells such as THP-1, which normally express only low levels of class II antigen unless stimulated to do so [26], may render potentially auto-antigenic fragments 'safe' before they can be presented as antigen by class II-bearing cells.

Knowledge of the amounts and location of this link-protein-derived peptide in tissues from normal and inflamed joints would provide important information about the turnover of PG during normal and pathological catabolism of cartilage matrix. Its presence in normal cartilage would indicate that no mechanism exists for its clearance, presumably due to the absence of cells able to bind and degrade the fragment. The absence of the link peptide fragment in rheumatoid joints, despite the presence of enzymes such as stromelysin that are able to degrade link protein and release the peptide, would indicate either that cells able to take up the fragment rapidly were present or that it was degraded completely soon after formation. Alternatively, because of its small size the peptide would be able to diffuse rapidly out of joints into the circulation where, depending on its half-life, it might be detectable as a marker of cartilage breakdown.

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