N-glycosylation of human interferon- γ : glycans at Asn-25 are critical for protease resistance

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Human interferon- γ (IFN- γ) is a secretory, dimeric glycoprotein that forms a compact globular structure with potential N-linked glycosylation sites at Asn-25 and Asn-97 on the surface of the dimer. In natural leucocyte IFN- γ (nIFN- γ), 52%, 39% and 9% of the monomers are core-glycosylated in two, one or none of the potential N-glycosylation sites respectively. Chemical cross-linking of nIFN- γ with glutaraldehyde revealed that 4, 3, 2 or 1 glycosylation sites occupied 28%, 40%, 26% and 6% of the dimers respectively. In baculovirus-produced wild-type (Wt) and N-linked glycosylation site-defective mutant (N25Q or N97Q, Asn-25 or Asn-97 substituted by Gln) IFN- γ proteins, the extent of core glycosylation of monomers reflected the glycan composition of dimers. This suggests that dimers are formed

randomly and independently of glycosylation. The glycan residues of IFN- γ , especially at Asn-25, play an important role in protease resistance. Unglycosylated recombinant IFN- γ proteins (from *Escherichia coli* and baculovirus) and N25Q IFN- γ were sensitive to crude granulocyte protease, purified elastase, cathepsin G and plasmin degradation. Fully glycosylated nIFN- γ and baculovirus Wt and N97Q IFN- γ showed full or partial resistance to these proteases. These results emphasize the importance of glycan residues, especially at Asn-25, in the proteolytic stability of human IFN- γ . Whether the differential glycosylation of n- and recombinant IFN- γ (rIFN- γ) is reflected in their biological activities in tissues or their clinical applicability is not known.

INTRODUCTION

Human interferon- γ (IFN- γ) is a secretory glycoprotein produced by activated T lymphocytes and natural killer cells. It is a potent regulatory molecule with antiviral, antiproliferative and immunomodulatory activities [1–3] and is an important member of the cytokine family. The biologically active form of IFN- γ is a homodimer. The monomeric subunits are non-covalently bound to each other in an antiparallel fashion, forming a compact globular structure [4,5]. Human IFN- γ contains two potential N-linked glycosylation sites at positions Asn-25 and Asn-97 of its 143-amino-acid-long mature polypeptide chain. Based on the three-dimensional structure of the IFN- γ dimer, the N-glycosylation sites are situated on the surface [4]. Relatively little research effort has been devoted to the functions of glycan residues of IFN- γ . Unglycosylated recombinant interferon- γ (rIFN- γ) molecules (*Escherichia coli*-produced) have a specific activity comparable with that of native IFN- γ (nIFN- γ) [6]. Recent evidence suggests that glycosylated IFN- γ molecules have a 2-fold higher specific activity compared with their unglycosylated counterparts [7]. Glycosylation at Asn-25 is essential in the folding and dimerization of newly synthesized IFN- γ molecules [7]. Analysis of nIFN- γ has, however, revealed that not all glycosylation sites of IFN- γ are used. In gel electrophoresis three different molecular mass monomers of IFN- γ are typically seen, 16 kDa, 20 kDa and 24 kDa forms, corresponding to molecules with none, one or both of the glycosylation sites occupied respectively [7–9]. It is not yet known how these different forms are represented in IFN- γ dimers. Proper glycosylation of IFN- γ has been shown to increase the survival time of biologically active IFN- γ in the circulation [10,11]. The nIFN- γ has also been shown to be resistant to crude protease preparations obtained from human granulocytes [12]. Serine proteases, elastase and cathepsin G are important enzymes occurring in abundance in human leucocytes. They are involved

in a number of cellular processes, being able, among other things, to degrade extracellular matrix proteins, complement component C3, tumour necrosis factor- α (TNF- α) and interleukin (IL)-6 [13–16]. Elastase and cathepsin G can also process the inactive IL-1 β precursor into its active form [17]. Plasminogen, the precursor form of plasmin, is present in abundance in the blood and tissues and is converted into plasmin by plasminogen activators. Plasmin is a serine protease involved in fibrinolysis and extracellular matrix degradation [18]. As shown recently, it is also able to degrade other substrates, e.g. unglycosylated rIFN- γ [19].

In this work we analysed the extent of glycosylation on IFN- γ dimers using purified nIFN- γ and differentially glycosylated rIFN- γ molecules. Our findings provide evidence that dimerization of IFN- γ molecules is random and unrelated to the extent of glycosylation. In addition, baculovirus-produced rIFN- γ molecules lacking either one or both of the N-linked glycosylation sites [7] were used to demonstrate the importance of glycosylation, especially the glycan residue at Asn-25, in resistance to the common cellular proteases elastase, cathepsin G and plasmin.

EXPERIMENTAL

Interferons

The nIFN- γ was produced in leucocyte cultures and partially purified by ion exchange on a CM-Sephadex C-50 (Pharmacia) column [20] followed by further purification in an immunoaffinity column using monoclonal anti-IFN- γ antibodies [21]. The specific antiviral activity was $(1\text{--}2) \times 10^7$ international units (i.u.)/mg of IFN- γ protein. Differentially glycosylated rIFN- γ proteins were produced in *Spodoptera frugiperda* (Sf9) insect cell cultures [22] as described by Sareneva et al. [7]. The rIFN- γ proteins obtained were named as follows: (i) Wt, fully glycosylated rIFN- γ (wild-type); (ii) N25Q, Asn at position 25 substituted by Gln;

Abbreviations used: IFN- γ , interferon- γ ; nIFN- γ , natural leucocyte IFN- γ ; rIFN- γ , recombinant IFN- γ ; IL, interleukin; Sf9, *Spodoptera frugiperda* cells; TNF- α , tumour necrosis factor α ; Wt, wild type.

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(iii) N97Q, Asn-97 substituted by Gln; and (iv) N25Q, N97Q, asparagines both at 25 and 97 substituted by Gln. The specific antiviral activities were 1.0×10^7 i.u./mg of protein for the glycosylated forms and 0.5×10^7 i.u./mg of protein for the unglycosylated (N25Q, N97Q) form of IFN- γ [7]. Human rIFN- γ produced in *E. coli* was kindly provided by Dr. Julian Davies (Biogen).

Chemical cross-linking of IFN- γ

Immunoaffinity-purified nIFN- γ was cross-linked with glutaraldehyde [0.06% (v/v) final concentration; Electron Microscopy Sciences] for 15 min at 23 °C and analysed by SDS/PAGE followed by Coomassie Blue staining. The rIFN- γ proteins were cross-linked in cell extracts after metabolic labelling. Recombinant baculovirus-infected Sf9 cells were grown as monolayers in 60 mm $\times$ 15 mm culture plates at a density of 3×10^6 cells per plate. At 36 h post-infection, the plates were washed with methionine-free Grace's insect cell medium, preincubated for 15 min in the same medium and labelled with [35 S]Met (50 μ Ci/ml, 1000 Ci/mmol, Amersham) for 2 h at 27 °C. The cells were washed with ice-cold PBS, collected and suspended in a small volume of solution containing 0.2% (v/v) Triton X-100 and glutaraldehyde at a final concentration of 0.02–0.08% (v/v). After 30 min at 23 °C, the cells were lysed with immunoprecipitation buffer (150 mM NaCl/5 mM EDTA/0.5% Triton X-100/50 mM Tris, pH 7.4). The cell debris was removed by centrifugation (12000 rev./min, 2 min) and the supernatant was used for immunoprecipitation. The supernatants were incubated with rabbit anti-IFN- γ antibodies [7] bound to Protein A–Sepharose CL-4B (Pharmacia) in a total volume of 500 μ l. The precipitated proteins were analysed by SDS/PAGE followed by autoradiography.

Gel-filtration chromatography

Partially purified nIFN- γ and samples of Wt or mutant rIFN- γ baculovirus-infected Sf9 insect cell culture media were applied on to a HiLoad 26/60 Superdex 75 (Pharmacia) gel-filtration column. The column was eluted with buffer containing 500 mM NaCl, 5 mM EDTA, 10 mM 2-mercaptoethanol and 25 mM Tris/HCl (pH 6.8). Fractions (2.5 ml) were collected (stabilized with 0.1% BSA) and the presence of IFN- γ determined by biological assay and immunoblotting. Molecular-mass markers (Sigma), albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa) were used as standards in gel filtration. The void volume of the column was determined using Blue Dextran.

Biological assay for human IFN- γ

The specific antiviral activity of IFN- γ was determined by vesicular stomatitis virus (VSV) plaque reduction in HEP2 cells [23]. The results are presented as i.u./ml using an international IFN- γ standard preparation.

Protease sensitivity of different IFN- γ molecules

Crude cell extract (granulocyte protease) from cultured human leucocyte suspensions was obtained as described [12]. Cathepsin G (C-0288), elastase (E-1508) and plasmin (P-4895), all of which were obtained from Sigma, were diluted in PBS at a final concentration of 225 m-units/ml. The protease (90 μ l) and 10 μ l of partially (CM-Sephadex stage) purified IFN- γ (4×10^3 – 1×10^4 i.u./ml containing 0.1% BSA) were mixed and incubated at 37 °C. Samples were taken at different times, diluted 1:10 in Minimal Essential Medium supplemented with 10% (v/v) fetal

bovine serum and stored at –70 °C, until assayed for IFN- γ activity.

SDS/PAGE, autoradiography and quantification

The proteins were analysed by separating in 10% (IFN- γ dimers) or 14% (IFN- γ monomers) SDS/PAGE [24]. The gels were either stained with Coomassie Brilliant Blue or were fixed, amplified (Amplify, Amersham) and exposed to Amersham Hypermax films. The absorbances of the bands in Coomassie Blue-stained gels or in autoradiograms were quantified by a Millipore BioImage scanner.

RESULTS

Extent of core glycosylation of IFN- γ dimers

We evaluated the extent of glycosylation by chemical cross-linking with glutaraldehyde to create covalently cross-linked IFN- γ dimers. The apparent molecular masses of differentially core-glycosylated IFN- γ dimers were determined by SDS/PAGE and gel filtration (see below). The highly purified human leucocyte nIFN- γ was cross-linked with various concentrations of glutaraldehyde (0–0.12%, v/v). A good resolution of cross-linked IFN- γ dimers in gels was obtained by using relatively low concentrations of glutaraldehyde. Four different molecular-mass variants are seen in the dimeric area (32–46 kDa) of cross-linked nIFN- γ after analysis in SDS/PAGE (Figure 1a). The molecular masses of the nIFN- γ dimers were 34, 38, 42 and 46 kDa, which

Figure 1 Extent of glycosylation of IFN- γ dimers

(a) Immunoaffinity-purified nIFN- γ was cross-linked with glutaraldehyde for 15 min, proteins were separated in SDS/10%-PAGE (left-hand panel) and stained with Coomassie Brilliant Blue. nIFN- γ without cross-linking is shown on the right-hand panel (SDS/14%-PAGE). (b) Wt IFN- γ baculovirus-infected Sf9 cells were metabolically labelled and permeabilized cells were cross-linked with glutaraldehyde as described in the Experimental section. The cells were lysed and the cell debris was removed by centrifugation. Supernatants were immunoprecipitated with anti-IFN- γ antibodies followed by analysis in SDS/14%-PAGE and autoradiography. IFN- γ dimer and monomer protein bands are shown by arrows. The number beside the arrow refers to the number of N-glycosylation sites (0–4) occupied. Migration of molecular-mass markers is indicated.

Table 1 Relative amounts of core glycosylation in natural and recombinant IFN- γ molecules

Monomers were quantified (from Figures 1 and 2) by densitometry from Coomassie Blue-stained SDS/PAGE gels (nIFN- γ) or from autoradiograms after metabolic labelling, immunoprecipitation and SDS/PAGE (IFN- γ Wt). Dimers were quantified after cross-linking with glutaraldehyde.

Interferon	Number of N-glycosylation sites	Monomers (%)	Dimers (%)	
			Calculated*	Observed
nIFN- γ (leucocyte)	4	—	27	28
	3	—	40	40
	2	52	25	26
	1	39	7	6
	0	9	1	0
IFN- γ Wt (baculovirus)	4	—	7	6
	3	—	26	25
	2	26	38	36
	1	51	24	26
	0	23	5	7

* Assuming random combination of dimers.

correspond, respectively, to differentially glycosylated IFN- γ dimers with one to four of the potential glycosylation sites used. The amounts of differentially glycosylated nIFN- γ monomers and cross-linked dimers (from Figure 1a) were quantified by densitometry; the data are shown in Table 1. The observed distribution of different molecular-mass dimers correlated well with the calculated values, assuming random association (based on core glycosylation of monomers) of monomers into dimers. The results show that 28 % of the nIFN- γ dimers were completely glycosylated (all four sites occupied) and 40 % were triply, 26 % doubly and 6 % singly glycosylated. The amount of unglycosylated dimers remained under the detection limit of the assay. For comparison, insect cells infected with Wt rIFN- γ baculovirus were metabolically labelled with [35 S]methionine and cross-linked with glutaraldehyde (Figure 1b). Densitometric quantification of the IFN- γ dimer bands revealed that the extent of dimer glycosylation correlated with the expected pattern derived from the core glycosylation of monomers (Table 1).

A similar analysis was carried out for IFN- γ molecules with Asn-25 and/or Asn-97 mutated to Gln. The results of metabolic labelling followed by cross-linking, immunoprecipitation and SDS/PAGE analysis suggest that the dimer composition of these constructs is dependent on the extent of core glycosylation and apparently the dimerization occurs randomly (Figure 2).

Partially purified samples of nIFN- γ from leucocytes and different rIFN- γ molecules produced by baculovirus were also analysed by gel-filtration chromatography (Figure 3). Gel filtration was used to estimate the molecular masses and homogeneity of different IFN- γ preparations. The gel-filtration patterns of various forms of IFN- γ molecules were homogeneous and symmetrical and correlated well with the observed composition of dimers (compare with Figures 1 and 2). Based on the antiviral activity of the proteins, we determined the relative molecular masses of different IFN- γ preparations by gel filtration: nIFN- γ , 52 kDa; Wt, 44 kDa; N25Q and N97Q, both 37 kDa; and N25Q, N97Q, 32 kDa. Antiviral activity was found only in fractions corresponding to dimers; there was none in the monomeric fractions. This finding was confirmed by Western blotting: IFN- γ -specific staining was noted only in fractions that showed antiviral activity (results not given).

Figure 2 Dimer patterns of differentially core-glycosylated IFN- γ molecules

Monolayers of Sf9 cells were infected with Wt IFN- γ and different glycosylation-site mutant baculoviruses and were metabolically labelled as described in the Experimental section. Cells were treated with 0.04 % (v/v) glutaraldehyde (a) or left untreated (b). Immunoprecipitates were subjected to SDS/PAGE under reducing conditions. IFN- γ dimers and monomers are shown by arrows and the number beside the arrow refers to the extent of glycosylation of the IFN- γ molecules. Migration of molecular-mass markers is shown on the right.

Glycosylation at Asn-25 of IFN- γ is essential for protease resistance

We have previously shown that glycan residues of IFN- γ play an important role in the dimerization of human IFN- γ [7]. As well as being important in the biogenesis of the molecule, they also appear to confer relative resistance to cellular proteases [12]. To specifically reveal the role of each (Asn-25 or Asn-97) of the glycosylation sites in proteolytic resistance, we incubated nIFN- γ and differentially glycosylated baculovirus-produced IFN- γ molecules with crude granulocyte (serine) protease [12] and determined the remaining IFN- γ activity at various times after protease treatment. IFN- γ molecules with intact N-glycosylation sites (nIFN- γ and Wt) were relatively resistant to the enzyme, whereas the unglycosylated N25Q, N97Q was rapidly inactivated by the protease (Figure 4). After 5 h incubation at 37 °C, only 1 % of the initial antiviral activity of the unglycosylated N25Q, N97Q remained. In partially glycosylated IFN- γ , protease re-

Figure 3 Gel-filtration profiles of nIFN- γ , Wt and glycosylation-site mutant IFN- γ proteins

The nIFN- γ and baculovirus-produced Wt and different glycosylation mutant IFN- γ proteins were separately applied to a Superdex 75 gel-filtration column [input $(3-8) \times 10^5$ i.u.]. The IFN- γ activity of the fractions was determined and is shown as i.u./ml. Relative mobility of molecular-mass-marker proteins and void volume (V_0) are shown by arrows.

sistance was dependent on the site of IFN- γ N-glycosylation. Glycans at Asn-25 (IFN- γ N97Q) were critical for maintaining the resistance against crude granulocyte protease. The loss of antiviral activity in IFN- γ N25Q (Asn-97-glycosylated) was comparable with that of the completely unglycosylated IFN- γ N25Q, N97Q (Figure 4).

The nIFN- γ , rIFN- γ (produced in *E. coli*) and baculovirus-produced rIFN- γ molecules were treated with elastase, cathepsin G and plasmin. The residual antiviral activities of IFN were determined for samples taken at different times after incubation at 37 °C (Figure 5). The left-hand panels show the effects of proteases on nIFN- γ and rIFN- γ (*E. coli*). The nIFN- γ from human leucocytes was completely resistant to elastase, whereas some loss in antiviral activity (50–60 %) was seen after incubation with cathepsin G or plasmin. In 1 h the antiviral activity of rIFN- γ was reduced to 30 % with elastase and cathepsin G treatment, and to 5 % with plasmin treatment (Figure 5). Longer treatment of IFN- γ with these enzymes resulted in a clear loss of biological activity and at the end of the incubation period only 0.5–2 % of the initial antiviral activity remained. The effect of the proteases on antiviral activities of baculovirus-produced IFN- γ Wt and glycosylation mutants is shown in the right-hand panels of Figure 5. The effects of elastase, cathepsin G and plasmin on differentially glycosylated IFN- γ were similar to the effect of the crude granulocyte protease (cf. Figures 4 and 5). Wt and N97Q forms of IFN- γ were more resistant to all of these enzymes than were N25Q and unglycosylated N25Q, N97Q IFN- γ molecules.

To establish whether there were any differences in the heat stability of Wt and N25Q, N97Q IFN- γ molecules, we incubated

Figure 4 Effect of crude granulocyte protease on differentially glycosylated IFN- γ proteins

The nIFN- γ from human leucocytes, baculovirus-produced Wt and glycosylation-deficient IFN- γ proteins (N25Q; N97Q and N25Q, N97Q) were incubated with crude extract of granulocyte protease [12] for different times at 37 °C. Samples were taken at times indicated and IFN- γ activity was determined by biological assay and compared with the initial IFN- γ activity.

these proteins at 37 °C for several days in the absence of proteases. Only minor differences between the two IFN molecules were seen. The biological activity of IFN- γ Wt remained constant for 2 days, and after 4 days 70 % of the activity still remained (Figure 6). The biological activity of unglycosylated IFN- γ (N25Q, N97Q) decreased to 65 % at day 1 and was 30 % of the initial activity at the end of the incubation period.

DISCUSSION

Biologically active IFN- γ exists as a homodimer. Based on its three-dimensional structure, IFN- γ forms a compact, globular structure [4,5]. The three-dimensional structures of human and rabbit IFN- γ and the sequence data of different mammalian IFN- γ molecules suggest that the structure is essentially the same for all IFN- γ molecules [4,5,25]. The dimer is stabilized by unique intertwining of six α -helices derived from each of the subunits. Such a unique structure is found in only one other molecule, namely, the HIV p17 protein dimer [26]. The two N-glycosylation sites of IFN- γ are on the surface of the dimer [4] and are schematically shown in Figure 7. The first glycosylation site at Asn-25 is at the end of a long flexible loop between the A and B helices. All mammalian IFN- γ molecules have the first N-linked glycosylation site in the same expected (between A and B helices) position, although in non-primates it is at position Asn-16. In human IFN- γ , the second N-linked glycosylation site at Asn-97 is situated in α -helix E, which appears to be more hidden than site Asn-25 (Figure 7). In other animal species, the location of the second glycosylation site is more varied, being located either in the loop between the D and E helices (bovine and pig) or in helix D (mouse and rat) further away from the first glycosylation site [4,25]. The proximity of Asn-25 and Asn-97 N-

Figure 5 Effect of elastase, cathepsin G and plasmin on IFN- γ activity

The nIFN- γ , *E. coli*-produced (rIFN- γ), and baculovirus-produced Wt and glycosylation-site mutant IFN- γ proteins were treated with elastase, cathepsin G and plasmin at 37 °C. Antiviral activity (%) of IFN- γ at different incubation times is given. Enzymes and IFN- γ proteins as shown in the Figure.

linked glycosylation sites in the dimer structure of human IFN- γ may give rise to the observed glycosylation heterogeneity of the monomers [7–9]. The monomers of nIFN- γ were fully glycosylated in about 50% of the cases, and about 40% of the molecules had only one glycosylation site occupied. This led to considerable heterogeneity of the IFN- γ dimers. However, most

Figure 6 Heat stability of Wt and N25Q, N97Q IFN- γ proteins

Baculovirus-produced glycosylated (Wt) and unglycosylated (N25Q, N97Q) IFN- γ were incubated at 37 °C for 1–4 days. Samples were taken at different times and antiviral activity was determined. IFN- γ activity is given in relation to initial activity.

Figure 7 Schematic representation of N-glycosylation sites in the IFN- γ dimer structure

The three-dimensional model structure of human IFN- γ homodimer. The α -helices (A–F) are represented as cylinders, and the non-helical regions are shown as tubes. The putative N-glycosylation sites are indicated by arrows. Each of the monomers is shown as white (A–F) or stippled (A'–F') shading. Redrawn and modified with permission from the authors [4].

dimers (> 90%) have either four, three, or two glycan residues (Figure 1, Table 1) as expected from the proportions of differentially glycosylated monomers. We have previously demonstrated

that the first N-linked glycosylation site is glycosylated co-translationally, whereas the second site is less efficiently used and may also be glycosylated post-translationally [7]. Dimerization of IFN- γ is also rapid ($t_{1/2}$ = 5 min) and may already have started during or immediately after translation. Rapid folding and dimerization of IFN- γ , and the proximity of the two glycosylation sites in the three-dimensional structure (Figure 7), may explain the glycosylation heterogeneity of IFN- γ dimers. Biochemical and cell biological evidence [7] would suggest that, in the dimers, Asn-25 is more often occupied than Asn-97.

Both glycosylated and unglycosylated IFN- γ dimers are biologically active. Amino acid residues at both the N- and C-terminal ends of the molecule are important for its biological activity [27–29]. The symmetrical structure of the molecule would also suggest that IFN- γ binds to two separate receptor molecules and induces receptor dimerization [30]. The sites of IFN- γ apparently interacting with its receptor are composed of segments from the N- and C-termini of different IFN- γ subunits [3,25] that are close to one another (helices A and F' or A' and F; Figure 7).

Protease treatment of glycosylated and unglycosylated IFN- γ molecules revealed striking differences. Unglycosylated baculovirus- and *E. coli*-produced rIFN- γ were susceptible to crude granulocyte protease and purified elastase, cathepsin G and plasmin (Figures 4 and 5). The nIFN- γ and baculovirus-produced Wt IFN- γ molecules were relatively resistant to these enzymes. Some loss of biological activity, more clearly seen for Wt IFN- γ , was, however, evident. Of interest was the difference between IFN- γ molecules N25Q and N97Q. Even though the extent of glycosylation was similar (Figures 2 and 3), they exhibited clear differences in their sensitivity to proteases, to the crude granulocyte protease in particular. The antiviral activity of N25Q IFN- γ (the first glycosylation site mutated) was lost rapidly within a few hours, whereas the N97Q IFN- γ retained its biological activity in a fashion similar to Wt IFN- γ and nIFN- γ , indicating that the glycan residues at Asn-25 may sterically hinder the access of proteases into the vicinity of helices A and B of one monomer and helix F' of the other (Figure 7). This fits well with the concept that this region is important in the biological activity of IFN- γ and that it is also involved in the interaction of IFN- γ with its receptor [3,25,31]. Each of the complex glycan residues increases the apparent molecular mass of the IFN- γ protein by approximately 3–4 kDa. The sugar chains possibly cover a relatively large surface area of the IFN- γ dimer, thus being able to cover those parts of the molecule that would be prone to proteolysis. Unfortunately, we do not know how the glycan residues are positioned in the three-dimensional structure of IFN- γ , since the structure has only been revealed for unglycosylated recombinant molecules [4,5].

Other cytokines are also susceptible to proteolytic inactivation. TNF- α and unglycosylated rIL-6 have been shown to be inactivated by cellular serine proteases elastase and cathepsin G [15,16]. In some cases, proteolytic degradation leads to activation of the molecule. The IL-1 β precursor can be activated by elastase and cathepsin G cleavage, which occurs close to the natural IL-1-converting enzyme cleavage site [17]. Plasmin and the plasminogen activator/plasmin system may also be involved in cytokine regulation, since monocytic U937 cell-surface plasmin was seen to be able to control the biological activity of *E. coli*-produced rIFN- γ [19]. These observations are in good agreement

with our results. It will be of interest to see whether proteolytic activity is a common mechanism in regulating cytokine activity in tissues.

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