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# An asparaginyl endopeptidase processes a microbial antigen for class II MHC presentation

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**Foreign protein antigens must be broken down within endosomes or lysosomes to generate suitable peptides that will form complexes with class II major histocompatibility complex molecules for presentation to T cells. However, it is not known which proteases are required for antigen processing. To investigate this, we exposed a domain of the microbial tetanus toxin antigen (TTCF) to disrupted lysosomes that had been purified from a human B-cell line. Here we show that the dominant processing activity is not one of the known lysosomal cathepsins, which are generally believed to be the principal enzymes involved in antigen processing, but is instead an asparagine-specific cysteine endopeptidase. This enzyme seems similar or identical to a mammalian homologue[1] of the legumain/haemoglobinase asparaginyl endopeptidases found originally in plants[2] and parasites[3]. We designed competitive peptide inhibitors of B-cell asparaginyl endopeptidase (AEP) that specifically block its proteolytic activity and inhibit processing of TTCF *in vitro*. *In vivo*, these inhibitors slow TTCF presentation to T cells, whereas preprocessing of TTCF with AEP accelerates its presentation, indicating that this enzyme performs a key step in TTCF processing. We also show that *N*-glycosylation of asparagine residues blocks AEP action *in vitro*. This indicates that *N*-glycosylation could eliminate sites of processing by AEP in mammalian proteins, allowing preferential processing of microbial antigens.**

To analyse processing of a foreign antigen without making assumptions about which proteases are involved, we incubated a domain of the tetanus toxin antigen (TTCF; relative molecular mass ($M_r$) 47 K) with disrupted lysosomes isolated from the human Epstein–Barr virus (EBV)-transformed B-cell line EDR. The antigen was fragmented to produce a discrete series of products at mildly acidic pH, consistent with an endosomal/lysosomal origin for the protease(s) involved (Fig. 1a). Surprisingly, we could not inhibit lysosomal digestion of TTCF with leupeptin or E64 (trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane), which are both broad-spectrum inhibitors of lysosomal cysteine proteases, or with pepstatin, an inhibitor of the aspartic acid cathepsins E and D (Fig. 1a). In addition, we could not reproduce the lysosomal TTCF-digestion pattern or indeed generate any of these fragments using purified cathepsins L, S, B, D or E (data not shown), all of which have been implicated in the processing of antigens for presentation on class II major histocompatibility complex (MHC) molecules[4–7] (reviewed in refs 8–10). However, further inhibitor studies showed that the activity was sensitive to iodoacetamide, *N*-ethylmaleimide and high concentrations of the diazomethane Z-Phe-Phe-$CHN_2$ (Fig. 1a and data not shown), indicating that one or more cysteine protease(s) was involved.

We sequenced the major TTCF-digestion products (Fig. 1b) to gain more information on this processing activity. The five major fragments arose from three cleavages in the TTCF protein after residues 873, 1,184 and 1,219 (tetanus toxin numbering), all of which are asparagine residues (Fig. 1b). These results indicated that B-cell lysosomes contain one or more unusual cysteine endopeptidase(s) with possible specificity for asparagine. Although not previously described in antigen-presenting cells, an enzyme called legumain or haemoglobinase, which possesses similar properties, has been found in the seeds of leguminous plants[2], in *Schistosoma mansoni*[3] and in mammalian tissues such as kidney and placenta[1]. We partially purified the TTCF-processing activity from a crude B-cell lysosome fraction to establish whether or not it was an asparaginyl endopeptidase. A peak of TTCF-processing activity co-eluted from a cation-exchange resin (~0.4 M NaCl) with an activity capable of cleaving the peptide substrate Z-Val-Ala-Asn-7-(4-methyl)coumaryl-

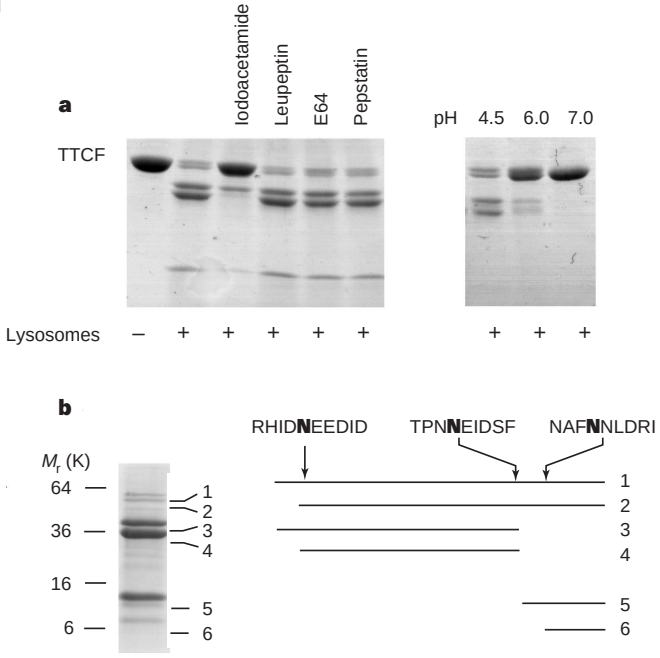


**Figure 1** Processing of TTCF by a leupeptin-insensitive cysteine endopeptidase activity. **a**, TTCF was digested *in vitro* in the presence of disrupted lysosomes (1–2 μg) in the presence or absence of iodoacetamide (1 mM), leupeptin, E64 or pepstatin (each at 0.1 mg $ml^{-1}$). Digestions were in 0.1 M ammonium acetate at pH 4.5, or $Na_2HPO_4$, at pH 6.0 or 7.0. After 4 h at 37 °C, the reactions were separated by Tris-tricine SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie blue. **b**, Cleavage sites. TTCF (labelled 1) and digestion products (numbered 2–6) were separated by Tris-tricine SDS–PAGE, electrophoretically transferred to PVDF membrane and subjected to five cycles of Edman degradation. The N-terminal sequence obtained for each fragment is shown after the asparagine residue printed in bold. Cleavage occurs after asparagines 873 (fragments 2 and 4), 1,184 (fragment 5) and 1,219 (fragment 6) numbered from the whole toxin sequence. Note that the first authentic tetanus toxin residue is Asp 872. There are 24 N-terminal residues that derive from the histidine tagging of TTCF (see Methods). The N-terminal sequence of His-tagged TTCF was obtained for fragments 1 and 3.

amide (Fig. 2a, b). Other protease activities, capable of cleaving the substrates Z-Phe-Arg-NHMec (cathepsin L/B substrate) and Z-Val-Val-Arg-NHMec (cathepsin S substrate), partly overlapped the asparaginyl endopeptidase activity (Fig. 2a). However, these activities were completely inhibited by E64, whereas both the TTCF- and the Z-Val-Ala-Asn-NHMec-processing activities were unaffected (data not shown), confirming that a new cysteine endopeptidase was involved. To compare this activity to the mammalian form of legumain, we generated antisera against peptides based on the human legumain complementary DNA sequence[1]. An antiserum against the peptide KGIGSGKVLKSGPQ crossreacted with purified legumain from pig kidney and with a human B-cell lysosome protein of similar $M_r$ (~35K) that co-eluted with the TTCF- and peptide-substrate-processing activity (Fig. 2c). Reactivity with this protein was blocked specifically by the peptide used as an immunogen (Fig. 2c). Finally, the same processing pattern and cleavage sites were found when TTCF was cleaved either by the partially purified human B-cell asparaginyl endopeptidase or by the purified legumain from pig kidney (Figs 1b and 2d, ref. 1 and data not shown). Thus, EBV-transformed B lymphocytes contain an asparaginyl endopeptidase similar or identical to human legumain and this dominates processing of the native form of the TTCF antigen *in vitro*. We use the acronym AEP to describe the human B-cell enzyme.

As there are currently no specific inhibitors available for asparaginyl endopeptidases, we tested the possibility that asparagine-containing peptides might act as competitive inhibitors. The amino- and carboxy-terminally blocked tetrapeptide Fmoc-Ala-Glu-Asn-Lys-$NH_2$ (AENK) caused substantial inhibition of TTCF processing by pig kidney legumain (Fig. 3a, left panel). Another blocked peptide, Fmoc-Lys-Asn-Asn-Glu-$NH_2$ (KNNE), also inhibited processing but was less effective. Processing of TTCF by B-cell AEP was blocked almost completely by 1 mg $ml^{-1}$ AENK (Fig. 3a, right panel). Importantly, the same concentrations of the glutamine analogues Fmoc-Ala-Glu-Gln-Lys-$NH_2$ (AEQK) and Fmoc-Lys-Gln-Gln-Glu-$NH_2$ (KQQE) had no effect on TTCF processing by asparaginyl endopeptidases (Fig. 3a). To establish that AENK blocked AEP but not other proteases, we measured the hydrolysis, by B-cell lysosomal extracts, of different synthetic peptide substrates in the presence and absence of these tetrapeptides. Increasing concentrations of AENK blocked cleavage of Z-Val-Ala-Asn-NHMec by B-cell lysosomes (Fig. 3b, c), but had no effect on hydrolysis of the cathepsin S substrate Z-Val-Val-Arg-NHMec (Fig. 3d) or the cathepsin B/L substrate Z-Phe-Arg-NHMec (Fig. 3e). In contrast, leupeptin inhibited hydrolysis of the cathepsin S and B/L substrates (Fig. 3d and data not shown), but not Z-Val-Ala-Asn-NHMec (Fig. 3c).

If AEP is important in TTCF processing *in vivo*, then the competing AENK peptide might be expected to interfere with normal loading of TTCF epitopes onto class II MHC molecules. To test this, freshly isolated peripheral blood mononuclear cells (PBMCs) were pre-incubated briefly with AENK, or the control peptide AEQK, and then pulsed with TTCF antigen in the continued presence of one or the other peptide. At different times, the cells were washed, fixed and then co-cultured with different autologous T-cell clones to assess expression of different peptide–MHC complexes. Presentation was detectable within 30 or 60 min of antigen pulsing in the presence of the control peptide (Fig. 4a),

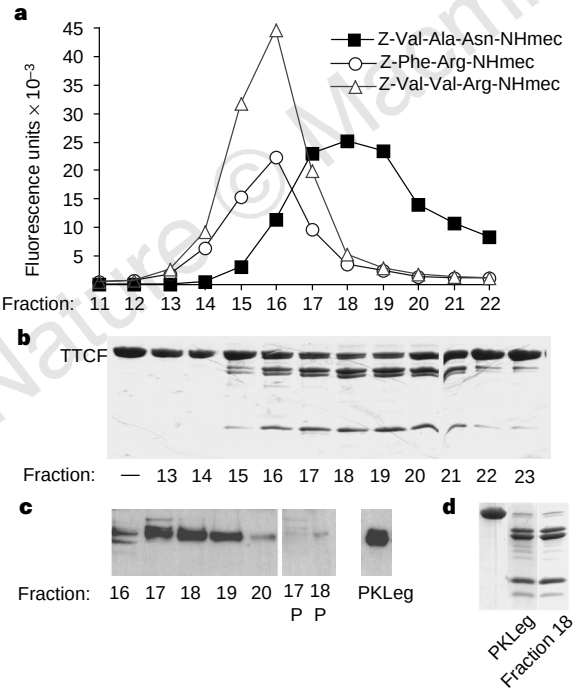


**Figure 2** TTCF is processed by an asparaginyl endopeptidase. **a**, **b**, A B-cell lysosomal extract was applied to a cation-exchange resin (Mono-S) and eluted with a 0.01–1.0 M NaCl gradient. Each fraction was incubated **a**, with the substrates Z-Val-Ala-Asn-NHMec, Z-Phe-Arg-NHMec or Z-Val-Val-Arg-NHMec or **b**, with 10 μg TTCF for 4 h at 37 °C before Tris-tricine SDS–PAGE analysis. **c**, Each fraction was analysed by Tris-tricine SDS–PAGE and western blotting with an affinity-purified sheep antiserum in the presence (17P and 18P) or absence of the immunizing peptide (KGIGSGKVLKSGPQC) from human legumain[1]. **d**, Comparison of TTCF processing with either partially purified B-cell asparaginyl endopeptidase (fraction 18 from **b** above) or purified legumain from pig kidney.

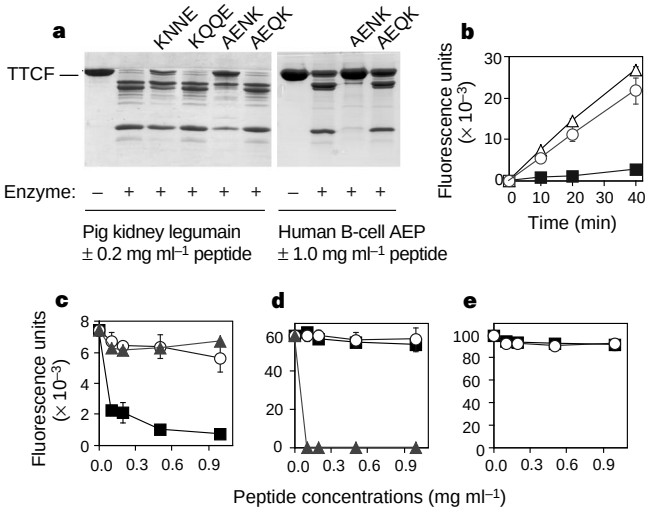


**Figure 3** Specific peptide inhibitors of asparaginyl endopeptidase. **a**, Specific inhibition of TTCF processing by asparagine peptides. TTCF (10 μg) was digested in 20 μl either with purified legumain from pig kidney (0.6 μg $ml^{-1}$) or with human B-cell AEP in the presence or absence of the N- and C-terminally blocked peptides indicated. **b**–**e**, Specific inhibition of AEP activity by Fmoc-Ala-Glu-Asn-Lys-$NH_2$ (AENK). **b**, A crude lysosomal protease mixture was incubated with Z-Val-Ala-Asn-NHMec in the absence (open triangles) or presence of AENK (filled squares) or AEQK (open circles) and release of 7-amino-4-methyl coumarin (NHMec) was measured at 460 nm after 10, 20 or 40 min. **c**–**e**, Hydrolysis of Z-Val-Ala-Asn-NHMec (**c**), Z-Val-Val-Arg-NHMec (**d**) or Z-Phe-Arg-NHMec (**e**) was measured after a 10-min incubation in the presence of increasing concentrations of AENK (filled squares), AEQK (open circles) or leupeptin (filled triangles). Leupeptin was tested in **c, d** only.

but in the presence of AENK presentation to most clones (for example, AK 5, 90 and 6) was undetectable at these early times. We also generated an EBV-transformed B-cell line from donor A.K. and tested the effect of the tetrapeptides on B-cell antigen presentation to the same T-cell clones. The AENK peptide blocked antigen presentation by EBV-B cells very efficiently, whereas the control peptide AEQK had virtually no effect on the kinetics of antigen presentation (Fig. 4b). Thus AENK specifically inhibited antigen presentation by two different populations of antigen-presenting cell.

Antigen presentation to all clones recovered to different extents after longer times of antigen pulsing. We suggest that this recovery is expected given that AENK inhibits by acting as a competitive substrate and is itself hydrolysed by asparaginyl endopeptidases (data not shown). Under these conditions it is not possible to block TTCF processing completely. We also observed that some clones, for example clone 33, were less sensitive to blockade of AEP (Fig. 4a, b). Clonal variations such as these might be explained by a differential requirement for AEP processing, dependent on the location of the T-cell epitope, the antigen dose–response of individual clones, or a combination of these factors. Consistent with the first scenario, we have observed that clones specific for the same peptide region do show similar sensitivities to AEP blockade. For example, clones 5 and 90 and a third clone (AK 20) all recognize the peptide region 1,145–1,156 and show similar sensitivity to AENK (Fig. 4 and data not shown). However, as expected, there was also variation in the antigen dose–response among the clones we tested. Those requiring higher levels of antigen for half-maximal triggering showed, in general, slower kinetics of presentation and greater sensitivity to AEP blockade. We suspect that the apparent differential requirement for AEP processing shown by different clones is due to a combination of epitope location, clone 'affinity' and possibly other factors. Nonetheless, it is clear that presentation of most tetanus toxin T-cell epitopes that have been tested so far is affected by AEP blockade. This is consistent with the hypothesis that initial processing by AEP may be the 'key' that 'unlocks' the native protein for further processing.

As a further test of this hypothesis, we assayed the kinetics of presentation of TTCF that had been predigested *in vitro* with AEP, reasoning that this might accelerate antigen presentation. Cells incubated with TTCF preprocessed with AEP presented T-cell epitopes after only 15–30 min, whereas unprocessed TTCF required 60–120 min before presentation could be detected (Fig. 4c). AENK did not block the rapid presentation of the preprocessed antigen at these times (Fig. 4d), confirming that this inhibitor blocks AEP action specifically on the antigen substrate *in vivo*. However, TTCF preprocessed with AEP still required further cellular processing as it could not be presented by fixed PBMCs (Fig. 4c, d). Taken together, these results support a working model in which AEP initiates TTCF proteolysis but generation of suitable peptides for MHC binding requires further intracellular processing. Native TTCF was a poor substrate for cathepsins E, D, L, S and B (data not shown), but many processing sites, for example for cathepsins E and D, were revealed after antigen unfolding[7].

We did not expect a highly specific enzyme, not previously known to exist in antigen-presenting cells, to dominate processing of the native TTCF antigen *in vitro* and to control the kinetics of its presentation *in vivo*. Other protein 'antigens', such as ovalbumin, RNAse and hen egg lysozyme, are cleaved by AEP and we have detected this activity in different human and murine antigen-presenting cells (data not shown). To our knowledge, this is the first time that a specific protease has been shown to have a central role in processing a defined antigen. The specificity of this enzyme for asparagine indicated that it might have additional significance. Specifically, we wondered whether *N*-glycosylation of asparagine residues might interfere with AEP action on protein and peptide substrates. We tested this hypothesis using both synthetic and natural glycopeptide substrates. The sequence HIDNEEDI, which contains one of the three asparaginyl bonds cleaved by both pig kidney legumain and B-cell AEP (Fig. 1), was synthesized with and without modification of the asparagine residue with *N*-glucosamine. Because the asparagine in this sequence is not part of a consensus glycosylation site (N-X-S/T), we also tested the peptide HIDNESDI, in which Glu in the P2′ position is changed to Ser. Each peptide was incubated with legumain from pig kidney and analysed using high-performance liquid chromatography (HPLC). Whereas

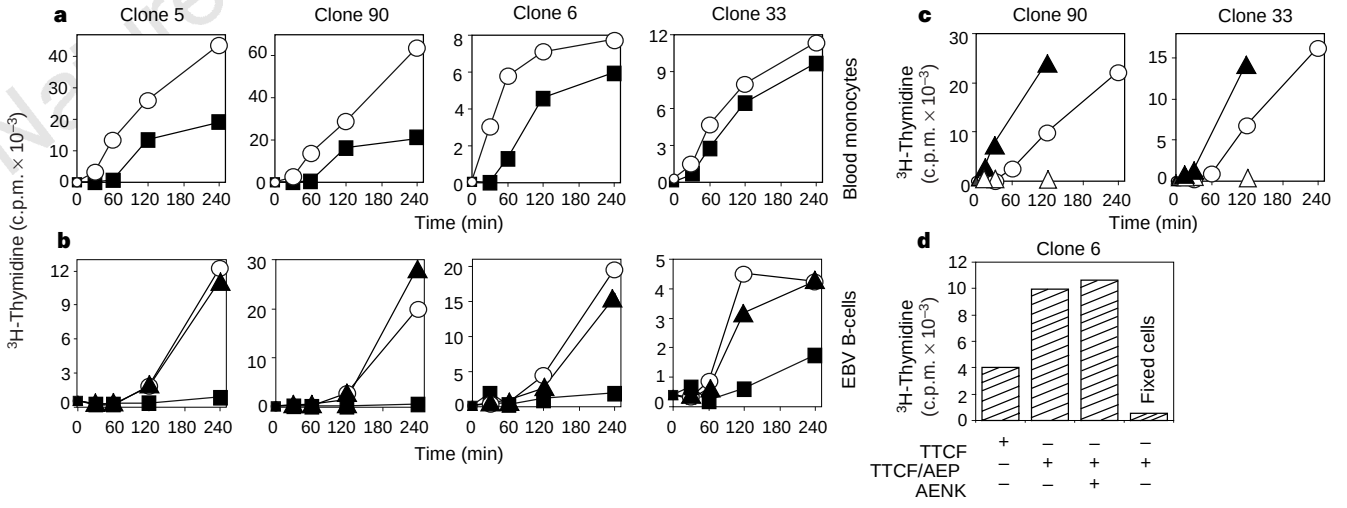


**Figure 4** TTCF processing and presentation requires AEP *in vivo*. **a**, **b**, Peptide inhibitors of asparaginyl endopeptidase slow antigen presentation *in vivo*. **a**, Peripheral blood mononuclear cells or **b**, EBV-transformed B-cells, both from donor A.K., were pre-incubated for 15 min in the presence of AENK (filled squares), AEQK (open circles) or no peptide (filled triangles), before addition of TTCF antigen. When present, peptide was maintained during antigen pulsing at 1 mg ml$^{-1}$ (**a**) or 2 mg ml$^{-1}$ (**b**). At different times the cells were washed and fixed in 0.05% glutaraldehyde and antigen presentation was measured using proliferation of different autologous TTCF-specific T-cell clones. **c**, AEP preprocessing of TTCF speeds antigen presentation. Antigen presentation was measured as in **a**, but using either TTCF (open circles) or TTCF predigested with AEP (filled and open triangles) and either live (open circles and filled triangles) or fixed (open triangles) autologous PBMCs. **d**, Presentation of AEP-preprocessed antigen is no longer inhibited by AENK. Presentation was measured after 60 min of antigen pulsing using EBV-transformed B cells.

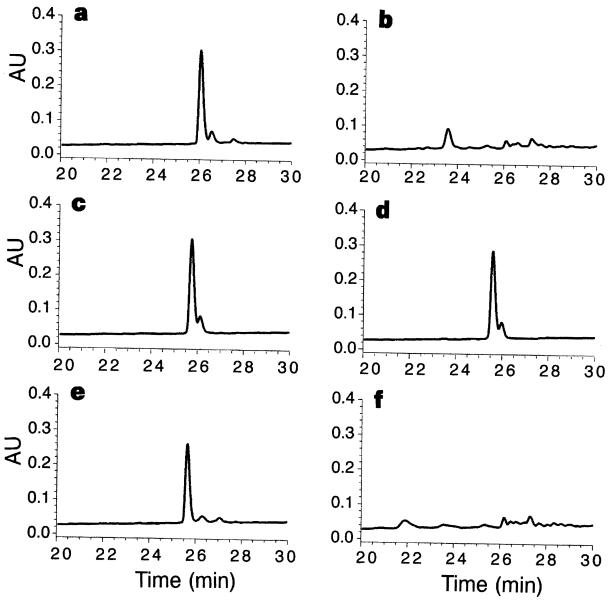


**Figure 5** Asparagine *N*-glycosylation blocks asparaginyl endopeptidase action. The TTCF-based peptides HIDNEEDI (**a**, **b**), HIDN(*N*-glucosamine)EEDI (**c**, **d**) and HIDNESDI (**e**, **f**) were incubated in the presence (**b**, **d**, **f**) or absence (**a**, **c**, **e**) of 50 mU ml$^{-1}$ pig kidney legumain in sodium citrate/phosphate buffer at 30 °C before analysis by reverse-phase HPLC.

the control peptide and the one containing the consensus glycosylation site were completely hydrolysed (Fig. 5a, b, e, f), the HIDN(N-Glc)EEDI peptide was completely resistant (Fig. 5c, d). We also analysed a natural 21-residue tryptic glycopeptide (residues 622–642) purified from human serum transferrin (see Table 1). This peptide contains two asparagine residues, one of which (N630) carries a biantennary oligosaccharide[11]. The same peptide was synthesized without modification of the asparagine and digested in parallel. (Three lysine residues were added to improve solubility.) Both mass spectrometric analysis and N-terminal sequencing of the products confirmed that hydrolysis of both asparagines 630 and 637 occurred in the non-glycosylated peptide (Table 1). However, we detected products from hydrolysis of only N637 in the glycopeptide (Table 1). A second transferrin-derived tryptic glycopeptide (residues 421–452), which contained three asparagine residues, was also digested with AEP. Again, we detected fragments from hydrolysis only at the non-glycosylated asparagine residues 430 and 436 (not shown).

**Table 1 *N*-glycosylation blocks AEP action on a natural glycopeptide**

| | Predicted mass | Observed mass |
|---|---|---|
| Synthetic transferrin peptide (622–642KKK) | | |
| No enzyme | | |
| QQQHLFGS**N**VTDCSG**N**FCLFRKKK | 2,784.36 | 2,784.17 |
| + Asparagine endopeptidase | | |
| QQQHLFGS**N** | 1,058.51 | 1,058.46 |
| VTDCSG**N**FCLFRKKK | 1,745.83 | 1,743.69* |
| FCLFRKKK | 1,069.63 | 1,069.59 |
| Transferrin glycopeptide (622–642) | | |
| No enzyme | | |
| QQQHLFGS**N**(CHO)VTDCSG**N**FCLFR | 4,720.76 | 4,720.2 |
| + Asparagine endopeptidase | | |
| QQQHLFGS**N**(CHO)VTDCSG**N** | 3,996.88 | 3,996.85 |
| FCLFR | 742.91 | 742.23 |
| VTDCSG**N**FCLFR | 1,476.66 | Not observed |
| QQQHLFGS**N**(CHO) | 3,263.13 | Not observed |

Predicted fragment masses were obtained from the human transferrin sequence (Swiss-Prot accession number: P02787 calculated using PeptideMass at the EXPASY server). The transferrin biantennary oligosaccharide (2 sialic acid + 4 *N*-acetylglucosamine + 5 hexose) has a mass of 2,204 coupled to peptide[11]. Variants also observed but not shown are monosialylated forms of the glycopeptides and N-terminal peptide forms that are 17 mass units lower corresponding to N-terminal glutamine pyroglutamization.
* Cyclization of the two cysteine residues in this peptide probably accounts for the observed mass deficit of 2.

Taken together, these data show that *N*-glycosylation blocks attack by asparaginyl endopeptidase at otherwise sensitive sites. Thus it is possible that non-glycosylated microbial proteins such as TTCF will, in general, be more sensitive to processing by AEP. The innate immune system's ability to recognize non-self carbohydrate structures[12,13] might also extend, in the case of prokaryotic microbial proteins, to an 'innate' bias towards processing of non-*N*-glycosylated microbial antigens. Although we do not suggest that glycoproteins are completely resistant to AEP processing, strategic *N*-glycosylation may alter the pathway of proteolysis and unfolding so as to disfavour loading of particular epitopes. Viruses and other pathogens might exploit this to avoid presentation of otherwise immunogenic peptides. Thus, *N*-glycosylation may provide viral glycoproteins with both a physical barrier, for example to neutralizing antibodies[14], and a means of limiting processing by AEP in the class II MHC pathway. This may be one reason why T-cell immune responses can be raised to non-glycosylated recombinant viral glycoproteins that are not recalled by native, fully glycosylated viral glycoprotein[15]. Finally, it is conceivable that disturbances of *N*-glycosylation might result in altered processing of self proteins by AEP, leading to presentation of 'cryptic' epitopes with possible pathological consequences. □

## Methods

**TTCF-processing activity.** Lysosomal fractions from the human B-cell line EDR were prepared on 27% Percoll density gradients as described previously[16] using $^{125}$I-labelled transferrin (internalized for 30 min at 37 °C) and β-hexosaminidase to identify endosomal/plasma membrane and lysosomal fractions, respectively. Lysosomal membranes were collected by centrifugation at 23,000*g* for 1 h, removed from the underlying Percoll pellet, concentrated at 50,000*g*, and stored frozen. For separation of leupeptin-insensitive cysteine protease activity, $7.5 \times 10^9$ cells were homogenized in a ball-bearing homogenizer and nuclei and unbroken cells were removed by centrifugation at 2,000*g* for 10 min. A membrane pellet was collected at 40,000*g* and solubilized in 20 mM sodium acetate, 1 mM EDTA, pH 5.2, containing 0.1% CHAPS. The extract was centrifuged at 2,000*g* for 20 min and then applied to a Mono-S column (Pharmacia) equilibrated in 20 mM sodium acetate, pH 5.2, 10 mM NaCl. Fractions (1 ml) were eluted in 20 mM sodium acetate, pH 5.2, first with a gradient of NaCl (10–300 mM in 5 ml), followed by a 3-ml wash at 300 mM NaCl and then a second NaCl gradient (300–500 mM in 8 ml; 500–1,000 mM in 4 ml). Each fraction was tested for TTCF-processing activity and ability to cleave various fluorogenic peptide substrates. Fluorometric protease assays were done as described[1,17].

**Proteins and peptides.** A histidine-tagged derivative of the C-terminal domain of tetanus toxin was prepared and purified as described[7,18]. This protein includes residues 872–1,315 of the complete toxin (1–1,315 residues) preceded by the sequence MGHGHHHHHHHHHHSSGHIEGRHI. Tetrapeptides were synthesized using Fmoc chemistry leaving the C terminus amidated and the N terminus retaining the Fmoc group. The peptide KGIGSGKVLKSGPQ from the human preprolegumain sequence[1] was coupled by an additional C-terminal cysteine to KLH and BSA and used to immunize a sheep (Scottish Antibody Production Unit, Carluke). Peptide-specific antibodies obtained after three boosts were purified as described[19]. Z-Val-Ala-Asn-NHMec was prepared as described[3]; other substrates were obtained from Sigma or Bachem.

**Antigen presentation.** PBMCs were prepared by Ficoll/Paque centrifugation and used fresh. T-cell clones specific for TTCF were established from donor A.K. as described[20]. Epitope mapping was done using a set of 88 peptides, 17 residues in length and spanning the TTCF sequence (Chiron Mimotopes). Clones AK 6, 90 and 33 recognize regions 1,235–1,246, 1,145–1,156 and 1,125–1,136, respectively. Clone 5 also recognizes the region 1,145–1,156. An EBV-transformed cell line from the same donor was established by standard

methods. Peptides AENK or AEQK were dissolved in water, made isotonic with NaCl and diluted into RPMI growth medium. T-cell-proliferation assays were done essentially as described[20,21]. Briefly, after antigen pulsing (30 μg ml$^{-1}$ TTCF) with tetrapeptides (1–2 mg ml$^{-1}$), PBMCs or EBV-B cells were washed in PBS and fixed for 45 s in 0.05% glutaraldehyde. Glycine was added to a final concentration of 0.1M and the cells were washed five times in RPMI 1640 medium containing 1% FCS before co-culture with T-cell clones in round-bottom 96-well microtitre plates. After 48 h, the cultures were pulsed with 1 μCi of $^3$H-thymidine and harvested for scintillation counting 16 h later. Predigestion of native TTCF was done by incubating 200 μg TTCF with 0.25 μg pig kidney legumain in 500 μl 50 mM citrate buffer, pH 5.5, for 1 h at 37 °C.

**Glycopeptide digestions.** The peptides HIDNEEDI, HIDN(*N*-glucosamine) EEDI and HIDNESDI, which are based on the TTCF sequence, and QQQHLFGSNVTDCSGNFCLFR(KKK), which is based on human transferrin, were obtained by custom synthesis. The three C-terminal lysine residues were added to the natural sequence to aid solubility. The transferrin glycopeptide QQQHLFGSNVTDCSGNFCLFR was prepared by tryptic (Promega) digestion of 5 mg reduced, carboxy-methylated human transferrin followed by concanavalin A chromatography[11]. Glycopeptides corresponding to residues 622–642 and 421–452 were isolated by reverse-phase HPLC and identified by mass spectrometry and N-terminal sequencing. The lyophilized transferrin-derived peptides were redissolved in 50 mM sodium acetate, pH 5.5, 10 mM dithiothreitol, 20% methanol. Digestions were performed for 3 h at 30 °C with 5–50 mU ml$^{-1}$ pig kidney legumain or B-cell AEP. Products were analysed by HPLC or MALDI-TOF mass spectrometry using a matrix of 10 mg ml$^{-1}$ α-cyanocinnamic acid in 50% acetonitrile/0.1% TFA and a PerSeptive Biosystems Elite STR mass spectrometer set to linear or reflector mode. Internal standardization was obtained with a matrix ion of 568.13 mass units.

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# Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer

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**Fas ligand (FasL) is produced by activated T cells and natural killer cells and it induces apoptosis (programmed cell death) in target cells through the death receptor Fas/Apo1/CD95 (ref. 1). One important role of FasL and Fas is to mediate immune-cytotoxic killing of cells that are potentially harmful to the organism, such as virus-infected or tumour cells[1]. Here we report the discovery of a soluble decoy receptor, termed decoy receptor 3 (DcR3), that binds to FasL and inhibits FasL-induced apoptosis. The DcR3 gene was amplified in about half of 35 primary lung and colon tumours studied, and DcR3 messenger RNA was expressed in malignant tissue. Thus, certain tumours may escape FasL-dependent immune-cytotoxic attack by expressing a decoy receptor that blocks FasL.**

By searching expressed sequence tag (EST) databases, we identified a set of related ESTs that showed homology to the tumour necrosis factor (TNF) receptor (TNFR) gene superfamily[2]. Using the overlapping sequence, we isolated a previously unknown full-length complementary DNA from human fetal lung. We named the protein encoded by this cDNA decoy receptor 3 (DcR3). The cDNA encodes a 300-amino-acid polypeptide that resembles members of the TNFR family (Fig. 1a): the amino terminus contains a leader sequence, which is followed by four tandem cysteine-rich domains (CRDs). Like one other TNFR homologue, osteoprotegerin (OPG)[3], DcR3 lacks an apparent transmembrane sequence, which indicates that it may be a secreted, rather than a membrane-asscociated, molecule. We expressed a recombinant, histidine-tagged form of DcR3 in mammalian cells; DcR3 was secreted into the cell culture medium, and migrated on polyacrylamide gels as a protein of relative molecular mass 35,000 (data not shown). DcR3 shares sequence identity in particular with OPG (31%) and TNFR2 (29%), and has relatively less homology with Fas (17%). All of the cysteines in the four CRDs of DcR3 and OPG are conserved; however, the carboxy-terminal portion of DcR3 is 101 residues shorter.

We analysed expression of DcR3 mRNA in human tissues by northern blotting (Fig. 1b). We detected a predominant 1.2-kilobase transcript in fetal lung, brain, and liver, and in adult spleen, colon and lung. In addition, we observed relatively high DcR3 mRNA expression in the human colon carcinoma cell line SW480.

To investigate potential ligand interactions of DcR3, we generated a recombinant, Fc-tagged DcR3 protein. We tested binding of DcR3–Fc to human 293 cells transfected with individual TNF-family ligands, which are expressed as type 2 transmembrane proteins (these transmembrane proteins have their N termini in the cytosol). DcR3–Fc showed a significant increase in binding to cells transfected with FasL[4] (Fig. 2a), but not to cells transfected with TNF[5], Apo2L/TRAIL[6,7], Apo3L/TWEAK[8,9], or OPGL/TRANCE/