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Asparagine Deamidation Perturbs Antigen Presentation on Class II Major Histocompatibility Complex Molecules*

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Post-translational protein modifications can be recognized by B and T lymphocytes and can potentially make "self"-proteins appear foreign to the immune system. Such modifications may directly affect major histocompatibility complex-restricted T cell recognition of processed peptides or may perturb the processing events that generate such peptides. Using the tetanus toxin C fragment protein as a test case, we show that spontaneous deamidation of asparagine residues interferes with processing by the enzyme asparagine endopeptidase (AEP) and contributes to diminished antigen presentation. Deamidation inhibits AEP action either directly, when asparagine residues targeted by AEP are modified, or indirectly, when adjacent Asn residues are deamidated. Thus, deamidation of long-lived self-proteins may qualitatively or quantitatively affect the spectrum of self-peptides displayed to T cells and may thereby contribute to the onset or exacerbation of autoimmune disease.

Asparagine deamidation is the most common spontaneous post-translational protein modification and is increasingly recognized as having an important impact on protein function. For example, a recent study demonstrated that the DNA-damaging antineoplastic drug cisplatin induced the deamidation of two asparagine residues in a flexible loop of the antiapoptotic protein Bcl-xL, preventing its interaction with other proapoptotic Bcl family members and thereby inducing apoptosis (1). Other studies document the deleterious effects of asparagine deamidation on the CD4 binding activity of human immunodeficiency virus gp120 (2), the co-mitogenic and receptor binding activity of murine IL1-β [1] (3), and the angiogenic activity of angiogenin (4).

Immune recognition of proteins and peptides can also be affected by deamidation/isomerization of asparagine and glutamine residues. For example, T cells have been described that specifically recognize peptides containing asparagine residues that have either cyclized (5) or become deamidated to aspartic acid (6, 7). As has been pointed out (8), the appearance of such modifications in "self"-proteins may disturb the delicate balance that the immune system establishes between tolerance to self-proteins on the one hand but an ability to respond to foreign entities on the other. For example, mice showed no response to unmodified forms of a self-cytochrome *c* peptide (90–104) or to a small nuclear ribonucleoprotein D peptide (65–79) but developed strong T and B cell responses to forms of these peptides in which a single Asp residue had been modified to iso-Asp (9). Importantly, the B cell response to the iso-Asp containing peptide cross-reacted with the native protein, suggesting a plausible chain of events initiated by post-translational protein modification that may lead to autoreactivity. In the case of α-gliadin, a single glutamine residue, likely deamidated as a result of tissue transglutaminase action, is found in two overlapping T cell epitopes recognized by T cells from human leukocyte antigen-DQ2-positive celiac disease patients (10, 11).

Proteolytic processing reactions are required before T cell epitopes are captured and displayed on major histocompatibility complex molecules. In the class II major histocompatibility complex pathway, a number of endosomal/lysosomal processing enzymes participate in these reactions (12), including a cysteine protease known as asparagine endopeptidase (AEP) or legumain that is highly specific for cleavage at Asn residues (13, 14). The identification of this enzyme raised the possibility that asparagine deamidation may additionally affect T cell recognition of proteins indirectly, by influencing the processing reactions that generate T cell epitopes. Using the tetanus toxin C fragment (TTCF) antigen as a test case, we explore this possibility here and demonstrate that spontaneous Asn deamidation readily occurs and that this can influence antigen processing and presentation.

EXPERIMENTAL PROCEDURES

TTCF and TTCF Mutants—The TTCF, incorporating a hexa-histidine tag, was produced in BL21 *Escherichia coli* and purified using nickel-agarose as described (15). Single point mutations were introduced using the QuikChange mutagenesis kit (Stratagene) according to manufacturer's instructions. Asn-1183 was mutated to Asp using the following mutagenic PCR primers: forward, 5′-CAA ACG CTA CAC TCC GGA CAA CGA AAT CGA TTC-3′; reverse, 5′-GAA TCG ATT TCG TTG TCC GGA GTG TAG CGT TTG-3′.

Peptides—TTCF peptides comprising residues 1178–1191 of the tetanus toxin sequence (KRYTP**N**NEIDSFVK, KRYTP**D**NEIDSFVK, KRYTP**iso-D**NEIDSFVK) were synthesized by *N*-(9-fluorenyl)methoxycarbonyl) biochemistry by Dr. Graham Bloomberg at the University of Bristol. Residue 1183 is highlighted in bold.

Deamidation of TTCF—To permit spontaneous deamidation of TTCF, the protein was filter-sterilized and incubated in phosphate-buffered saline for 4–6 weeks at 37 °C (TTCF37). A control sample was incubated in parallel at 4 °C (TTCF4).

Proteolytic Cleavage of TTCF by AEP—TTCF (0.5 mg/ml) was incubated at 37 °C with 10 mU/ml recombinant human AEP (prepared as described (17)) in 50 mM acetate, pH 4.5, 5 mM dithiothreitol. At timed intervals, the reaction was stopped by the addition of 1 mM iodoacetamide. For pepsin digestions, TTCF (0.5 mg/ml) was incubated with 5 mU/ml pepsin (Sigma) in 50 mM sodium citrate, pH 4.0, 4 M urea.

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[1] The abbreviations used are: IL, interleukin; TTCF, tetanus toxin C fragment; AEP, asparagine endopeptidase; MBP, myelin basic protein; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MALDI-TOF/TOF, MALDI-time of flight/time of flight; MS, mass spectrometry; Bi, biotinylated.

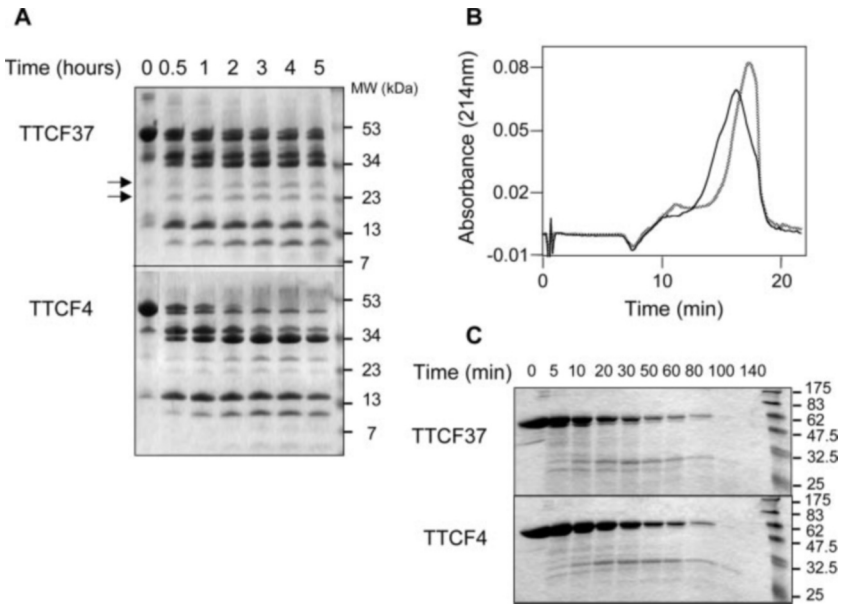

FIG. 1. **Evidence for deamidation of TTCF.** *A*, TTCF37 and TTCF4 were digested with AEP as described under "Experimental Procedures." 5-μg aliquots were removed at the indicated times and analyzed by SDS-PAGE. *Arrows* indicate two minor AEP cleavage products that are more prevalent in the digestion of TTCF37. *MW*, molecular mass. *B*, cation exchange chromatography of TTCF4 (*dashed line*) and TTCF37 (*solid line*) was used to analyze charge differences between the two proteins. *C*, TTCF37 and TTCF4 were incubated with pepsin, and 5-μg samples were removed at the indicated times and analyzed by SDS-PAGE.

Proteolytic peptides were visualized on 12% Tris-Tricine SDS gels.

Measurement of Iso-Aspartic Acid in TTCF—Iso-aspartyl residues were detected using the ISOQUANT™ protein deamidation detection kit (Promega Corp. Madison, WI) according to the manufacturer's instructions. Briefly, TTCF was incubated for 30 min with protein iso-aspartyl methyl transferase and *S*-adenosyl-L-methionine. The formation of the methylation byproduct, *S*-adenosyl homocysteine, was detected by reversed phase HPLC. An *S*-adenosyl homocysteine standard curve was generated by calculating the area of the *S*-adenosyl homocysteine peak and used to estimate levels of iso-aspartic acid in unknown samples.

Cell Lines and Media—T cell hybridomas specific for TTCF were generated as described (16, 18) and were maintained in RPMI supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 μg/ml kanamycin, and 50 μM β-mercaptoethanol. The hybridoma specificities have been defined as follows: 1H3 recognizes amino acids 1145–1161, 1H3 and 4E4 recognize amino acids 950–966, and 5B12 recognizes amino acids 900–915 (18). The murine B cell line LB27.4 was maintained in RPMI supplemented with 10% fetal calf serum, 2 mM glutamine, 100 μg/ml kanamycin, and 50 μM β-mercaptoethanol.

T Cell Assays—In antigen titration assays, TTCF was added at the indicated concentrations to wells containing 1×10^5 antigen-presenting cells, together with 1×10^5 hybridomas. Assays were done in duplicate or triplicate in 96-well U-bottomed plates. After 24 h, the supernatants were removed, and the amount of IL-2 was determined by enzyme-linked immunosorbent assay using a mouse IL-2 OptEIA set (Pharmingen). In kinetic assays, antigen-presenting cells were incubated at $0.5–1 \times 10^7$/ml with 50 μg/ml TTCF. Cells were harvested at the indicated time points, fixed with 0.05% glutaraldehyde for 45 s, and quenched with 0.1 M glycine. Fixed cells (1×10^5/well) were added to an equivalent number of hybridomas, and the released IL-2 was measured after 24 h.

Chromatography—Preparative and analytical chromatography was done on a SMART HPLC system (Amersham Biosciences). Cation exchange analysis of TTCF4 and TTCF37 was done using a Mini S 3.2/3 column (Amersham Biosciences) in 50 mM sodium acetate, pH 5.0, over a 0–1 M NaCl gradient. Reversed phase chromatography was done on a C2/C18 SC 2.1/10 column (Amersham Biosciences) over a gradient ranging from 0.05% trifluoroacetic acid in water to 0.045% trifluoroacetic acid in 80% acetonitrile.

Mass Spectrometry—TTCF was digested with sequencing grade trypsin (Promega) in 20 mM ammonium bicarbonate at an enzyme:protein ratio of 1:50 (w/w). Tryptic peptides were separated by reversed phase chromatography and concentrated in a vacuum centrifuge. HPLC fractions were analyzed individually on a Applied Biosystems Voyager-DE STR mass spectrometer. 1 μl of each crude peptide fraction was mixed with 1 μl of matrix (5 mg/ml α-cyano-4-hydroxycinnaminic acid in 50% acetonitrile, 48% water, 2% ammonium di-citrate) on a stainless steel MALDI plate and allowed to crystallize at room temperature. Fractions were analyzed individually on a Applied Biosystems Voyager-DE STR mass spectrometer. Positive ion MS spectra were acquired in reflectron mode. The extraction delay time was 180 ns, and the acceleration voltage set at 20 kV. Spectra were calibrated using an external standard.

Whole tryptic TTCF digests derived from native and deamidated samples were analyzed by MALDI-TOF/TOF tandem MS analysis. The Applied Biosystems 4700 proteomics analyzer was used to determine the sites of deamidation. 1 μl from each digest was spotted onto a stainless steel MALDI target plate and allowed to dry. 1 μl of matrix was added to each digest sample and allowed to co-crystallize. Positive ion MS spectra were acquired in reflectron mode for each digest. Peptides 1214–1223 (DGNAFNNLDR) and 1179–1191 (YTPNNEIDSFVK) were sequenced by tandem MS analysis to determine the site of deamidation.

RESULTS

"Aged" Tetanus Toxin C Fragment Resists AEP Digestion—We have shown previously that processing of TTCF is initiated by the asparagine-specific endopeptidase AEP and that glycosylation of target Asn residues or mutagenesis to Gln drastically inhibits AEP action (14, 16). Asparagine deamidation, which leads to the generation of aspartic or iso-aspartic acid (19), might also be expected to reduce the efficiency of AEP action. Using TTCF as a model antigen, we first tested whether spontaneous asparagine deamidation occurred in this protein and whether processing by AEP was affected. Aliquots of recombinant TTCF, dissolved in sterile phosphate-buffered saline, were stored either at 4 °C or at 37° for 4–6 weeks. No precipitation or autodigestion occurred, and the concentration of protein was the same at the end of the incubation period. The protein was then subjected to digestion with purified AEP, and the products were analyzed on 12% SDS gels. As shown previously (14), a discrete set of digestion products is observed following cleavage at Asn residues 873, 1184, and 1219. Intact TTCF37 still persisted after 5 h of digestion, whereas TTCF4 was completely digested after 2–3 h of processing (Fig. 1*A*). At higher levels of AEP, additional Asn residues in TTCF are targeted.[2] Peptides generated from cleavage by AEP at these minor sites were more prevalent in a digest of TTCF37, providing further evidence that aging TTCF perturbed the normal pattern of proteolytic degradation.

Since asparagine deamidation leads to a net increase in negative charge and a gain of 1 mass unit, protein and peptide deamidation can be readily detected. Deamidation of Asn generates a mixture of Asp and iso-Asp, with L-iso-Asp comprising 60–80% (19). Thus, an additional diagnostic for deamidation is

[2] C. X. Moss, unpublished data.

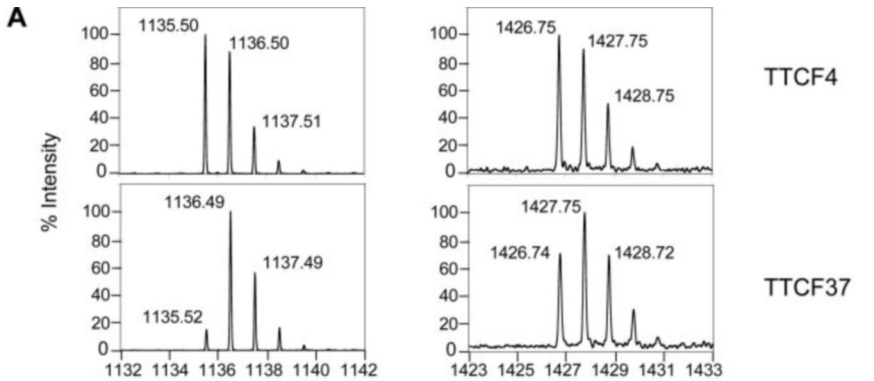

FIG. 2. **Identification of deamidated Asn residues.** *A*, MALDI-TOF mass spectra of tryptic peptides derived from TTCF4 (*upper panels*) and TTCF37 (*lower panels*). The regions of the spectrum containing peptide 1214–1223 (theoretical mass 1135.51, *left panels*) and peptide 1180–1191 (theoretical mass 1426.68, *right panels*) are shown. *B*, the peptides shown in *A* were sequenced by tandem mass spectrometry. The fragment peptides derived from TTCF37 are listed. The Asp residue generated by deamidation is highlighted in *bold*.

B

Expected mass	Observed mass	Peptide(s)
175.12	175.09	R
290.15	290.13	DR
403.23	403.20	LDR
517.27	517.23	NLDR
631.32	631.30 632.26	NNLDR **D**NLDR
778.38	779.34	F**D**NLDR
849.42	849.39 850.38	AFNNLDR AF**D**NLDR
963.46	963.46 964.45	NAFNNLDR NAF**D**NLDR
1020.49	1020.48 1021.47	GNAFNNLDR GNAF**D**NLDR

Expected mass	Observed mass	Peptide(s)
246.18	246.19	VK
393.25	393.25	FVK
480.28	480.27	SFVK
595.31	595.31	DSFVK
708.39	708.38	IDSFVK
837.44	837.42	EIDSFVK
951.48	951.49	NEIDSFVK
1065.52	1065.47 1066.55	NNEIDSFVK **D**NEIDSFVK
1162.57	1162.62 1163.61	PNNEIDSFVK P**D**NEIDSFVK

quantitation of iso-aspartic acid using the enzyme protein-L-iso-aspartate *O*-methyltransferase, which methylates the unusual α-carboxylic acid group on iso-aspartic acid. As shown in Fig. 1*B*, TTCF37 eluted from a cation exchange column before TTCF4, consistent with the former having gained net negative charge as a result of deamidation. In addition, direct analysis of iso-aspartic acid content showed that TTCF4 contained 1.08 mol of iso-Asp/mol of TTCF, whereas TTCF37 contained 3.56 mol/mol, a 3.3-fold increase. Thus, TTCF stored at 37 °C undergoes spontaneous deamidation and becomes more resistant to digestion by AEP.

To address the possibility that other post-translational modifications were introduced into TTCF during the aging process, we examined the degradation of TTCF by other proteolytic or chemical agents. As pepsin prefers to cleave between pairs of hydrophobic residues, we predicted that deamidation of Asn would have no effect on substrate cleavage. As shown in Fig. 1*C*, pepsin degraded both TTCF37 and TTCF4. There was no significant difference in either the kinetics of degradation or in the pattern of fragments generated. In addition, both TTCF37 and TTCF4 were equally good substrates for CNBr digestion (data not shown), suggesting that methionine oxidation had not occurred to a significant extent. Taken together, these data indicate that the *in vitro* aging process did not grossly affect the susceptibility of TTCF to proteolytic or chemical processing.

Asparagine Residues at and Adjacent to AEP Cleavage Sites Are Deamidated in TTCF37—To establish whether AEP processing sites had become deamidated in TTCF37, we subjected TTCF4 and TTCF37 samples to tryptic digestion and resolved the peptide products by reversed phase-HPLC (see "Experimental Procedures"). Individual fractions were analyzed by MALDI-TOF mass spectrometry. We identified two tryptic peptides with an isotopic distribution consistent with a proportion of the peptide having gained 1 mass unit. Interestingly, these were peptides containing AEP cleavage sites 1184 and 1219. As shown in Fig. 2*A*, the most abundant isotope for peptide 1214–1223 (DGNAFNNLDR) was the monoisotopic mass 1135.51 Da for TTCF4, but for TTCF37, the second isotopic mass of 1136.51 Da was the most abundant. Similarly, the isotopic distribution for peptide 1179–1191 (YTPPNNEIDSFVK) shifted upwards by one unit to 1427.68 Da. Although each peptide contains two adjacent Asn residues, AEP only cleaves at one site in each peptide (14), illustrating that Asn sequence context plays an important, but as yet poorly understood, role in determining AEP target sites (16, 20). To determine which Asn residue(s) had become deamidated, we sequenced each peptide using a MALDI-TOF/TOF tandem MS instrument. Inspection of the mass profile for each fragment ion generated from TTCF37 allowed us to identify the Asn residue that had become deamidated. As shown in Fig. 2*B*, the analysis for the 1214–1223 peptide demonstrated that residue 1219, which is the first of the paired Asn residues, had become converted to Asp/iso-Asp. When the analysis was performed for the 1179–1191 peptide, it was once again the first Asn residue of the pair (Asn-1183) that had become deamidated. No evidence was found for deamidation of Asn-1184, which is the AEP target in this region of TTCF (14). Thus, we find evidence for deamidation in two regions of the TTCF molecule targeted by AEP. In one, the Asn cleaved by AEP is deamidated. In the other, an Asn residue immediately adjacent to an AEP cleavage site is similarly modified.

Altered Presentation of Deamidated TTCF—Processing by AEP, particularly at Asn-1219, is required for optimal presentation of most T cell epitopes in TTCF (16). We therefore tested TTCF4 and TTCF37 in antigen presentation assays. For this, we used a panel of murine T cell hybridomas that recognize different epitopes in the TTCF antigen. We found no evidence for deamidation of Asn residues lying within these T cell epitopes; therefore, any effects are likely to be due to antigen uptake and/or processing rather than to the presentation of altered peptide ligands. We found that TTCF37 was much less

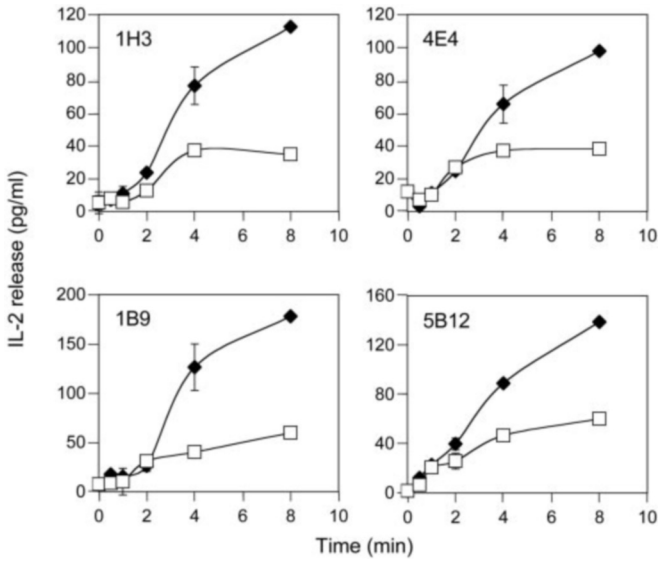

FIG. 3. **Effect of deamidation on T cell epitope presentation.** TTCF4 (*solid diamonds*) or TTCF37 (*open squares*) were incubated with splenocytes for the indicated time periods. Fixed splenocytes were incubated with the T cell hybridomas 1H3, 1B9, 4E4, or 5B12 and the secreted IL-2 detected by enzyme-linked immunosorbent assay.

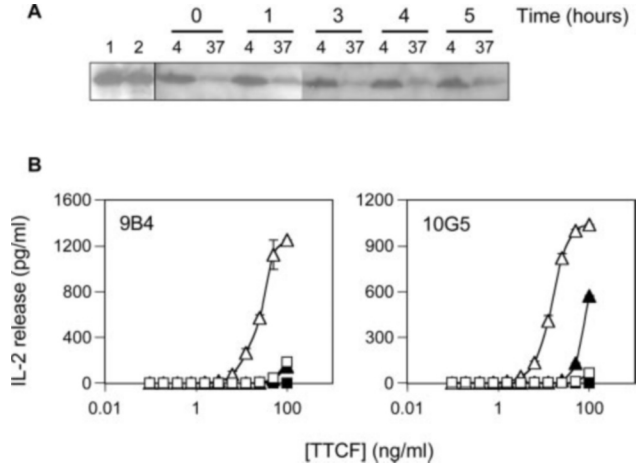

FIG. 4. **Differences in antigen presentation are partially due to differences in endocytosis of TTCF37.** *A*, splenocytes were incubated with 25 μg/ml biotinylated TTCF4 (*odd lanes*) or biotinylated TTCF37 (*even lanes*). At the indicated times, 2.5×10^5 cells were removed and washed, and lysates were analyzed by Western blotting with streptavidin-horseradish peroxidase. The amount of biotin conjugated to each protein was compared by loading 20 ng each of biotinylated TTCF4 (*lane 1*) and biotinylated TTCF37 (*lane 2*). B, TTCF4 (*triangles*) or TTCF37 (*squares*) was added to LB27.4 cells together with 10 μg/ml TTCF-specific monoclonal antibodies 9B4 or 10G5 (*open symbols*) or without antibody (*filled symbols*). Both panels show presentation to the T cell hybridoma 5B12 as measured by IL-2 release.

efficient than TTCF4 in generating a T cell response in all hybridomas tested (Fig. 3), consistent with diminished processing of TTCF37. However, we were surprised by the magnitude of the inhibitory effect, which was greater than that obtained by mutagenesis of key AEP cleavage sites (16). This raised the possibility that deamidation and/or some other event occurring during aging was affecting not only processing of TTCF but some other parameter, for example, the efficiency of its uptake into antigen-presenting cells.

TTCF37 Is Taken Up Less Efficiently by Antigen-presenting Cells—To investigate the possibility that TTCF37 was taken up less efficiently by antigen-presenting cells, we labeled TTCF and directly monitored the level of cell association. TTCF4 and TTCF37 were biotinylated, and the amount of biotin incorporated was shown to be equivalent in each case (Fig. 4*A*, *lanes 1* and *2*). When both forms of antigen were incubated with splenocytes (Fig. 4*A*), less Bi-TTCF37 was found associated with splenocytes at all time points when compared with Bi-TTCF4. This difference was evident even on cells incubated with Bi-TTCF4 or Bi-TTCF37 at 4 °C, indicating differential binding of the two TTCF forms to the cell surface. Although this differential association does not necessarily reflect the relative levels of TTCF4 and TTCF37 that are actually taken up and processed, it clearly indicates that diminished presentation of TTCF37 to T cells is likely to be due in part to weaker association with splenocytes when compared with TTCF4.

Diminished AEP Processing Also Inhibits Presentation—We next asked whether the deficit in TTCF37 presentation was due to reduced processing by AEP in addition to diminished uptake. To address this, we needed to ensure that equal amounts of TTCF4 and TTC37 were taken up by cells, and we achieved this by using FcR-mediated uptake. Cells were provided with low concentrations of antigen, together with the TTCF-specific monoclonal antibodies 9B4 or 10G5. These antibodies bind equally well to both TTCF4 and TTCF37 as shown by enzyme-linked immunosorbent assay, and cell association of the two TTCF forms was now virtually equivalent (data not shown). As expected, inclusion of either antibody boosted presentation, indicating that the endocytic route was FcR-mediated (Fig. 4*B*). However, even under these conditions of comparable antigen association with cells, presentation of TTCF37 was markedly worse than TTCF4 (Fig. 4*B*). Thus, both reduced uptake and altered processing seem to contribute to the deficiency in presentation of peptides from TTCF37.

Asp but Not Iso-Asp Is Tolerated Adjacent to the Asn-1184 AEP Cleavage Site—Although our earlier studies demonstrated that Asn-1219 was the most important AEP cleavage site for maximal presentation efficiency, mutagenesis of Asn-1184 also had a measurable deleterious effect on presentation to different T cell clones and hybridomas (16)[2]. We therefore asked whether deamidation of the adjacent residue 1183 might indirectly affect AEP processing at Asn-1184 and therefore contribute to the overall deficit in presentation of TTCF37. Mutation of Asn-1183 to Asp had little effect on *in vitro* digestion by AEP (Fig. 5*A*) nor on presentation of TTCF (Fig. 5*B*). However, natural Asn deamidation generates more iso-Asp relative to Asp. Because this induces a change in the conformation of the polypeptide chain, we reasoned that this might have a more profound effect on AEP cleavage at the adjacent (1184) Asn residue. To test this, we performed AEP digestions on synthetic peptides encompassing residues 1178–1191 of TTCF, which includes the 1184 cleavage site. We included Asn, Asp, or iso-Asp at position 1183. As shown in Fig. 5*C*, the time course of digestion of the Asn-1183 and Asp-1183 peptides was comparable, consistent with the modest effect when this mutation was expressed in TTCF protein. However, the presence of iso-Asp at 1183 markedly reduced the efficiency of digestion of the peptide. After 4 h of digestion, almost 60% of this peptide remained when compared with 10% of the wild type or 1183Asp peptides (Fig. 5*B*). Thus, it seems likely that at least three factors contribute to diminished presentation of TTCF37: reduced uptake, direct modification of the AEP cleavage site at Asn-1219, and indirect modification of the site at 1184 as a result of iso-Asp generation at 1183 (Fig. 6).

DISCUSSION

Here we demonstrate a distinct and novel effect of deamidation on T cell recognition of a protein antigen due, at least in part, to altered antigen processing. Our experiments were prompted by our recent studies on the asparagine-specific en-

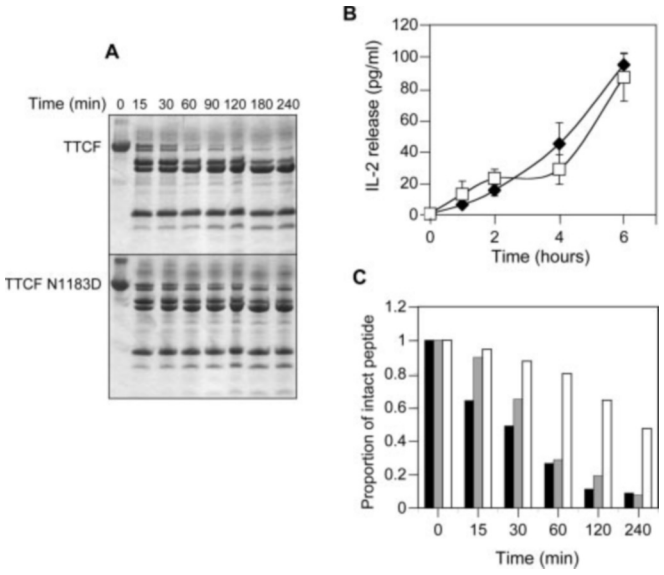

FIG. 5. **The effect of Asp and iso-Asp adjacent to an AEP cleavage site.** *A*, TTCF, and the mutant TTCF N1183D were digested with AEP and analyzed by SDS-PAGE. *B*, presentation of TTCF (*solid diamonds*), TTCF N1183D (*open squares*) to T cell hybridoma 4E4. *C*, synthetic peptides spanning residues 1178–1191 and incorporating Asn (*black bars*), Asp (*gray bars*), or iso-Asp (*white bars*) were digested with AEP. The degree of peptide cleavage was determined by reversed phase chromatography. The amount of intact peptide at each time point is expressed as a proportion of the value at time 0.

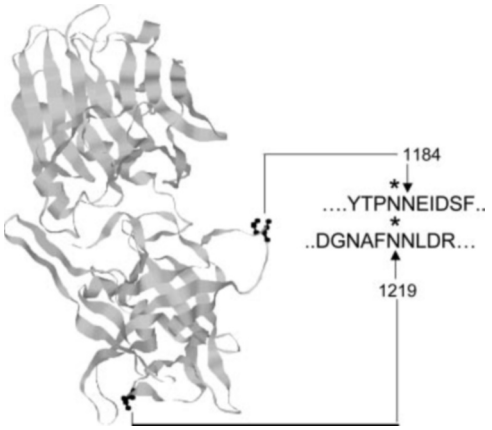

FIG. 6. **TTCF structure and the location of deamidated AEP processing sites.** The locations of AEP sites Asn-1184 and Asn-1219 are shown as *ball-and-stick* features on a *ribbon* model of TTCF. Deamidation occurs at Asn residues 1219 and 1183 (*asterisked*). Sequences surrounding the AEP cleavage sites and deamidated Asn residues are shown.

dopeptidase AEP (or legumain) found in human and murine APCs (21). Mammalian AEP or legumain is found in diverse cells and tissues including human and murine antigen-presenting cells (13). It contributes to productive processing of antigens such as tetanus toxin and is one of the proteases that initiates removal of the invariant chain chaperone, which guides early class II major histocompatibility complex biosynthetic events (14, 22). We found evidence for deamidation of a key AEP processing site (Asn-1219) and evidence that a second site (Asn-1184) was indirectly affected by deamidation and β-isomerization of a neighboring Asn residue (Fig. 6). Deamidation altered the pattern of TTCF processing by AEP and suppressed presentation of different T cell epitopes in TTCF, consistent with our earlier studies on mutagenesis of key AEP processing sites (16). However, reduced uptake of TTCF37 also contributed to its poor presentation when compared with TTCF4. The reasons for this are not yet fully understood. The overall net increase in negative charge that accompanies conversion of Asn to Asp/iso-Asp (Fig. 1) may diminish binding given that the cell surface is also negatively charged. However, a mutant form of TTCF in which Asn-1183 and -1219 were changed to Asp was taken up equally well by cells (data not shown). However, it is also possible that deamidation of additional Asn residues in TTCF inhibits more specific interactions than TTCF makes with cells. For example, TTCF is known to bind gangliosides as well as other receptor structures on neuronal and possibly other cells (23, 24). Although we were not able to identify modifications other than Asn deamidation, it remains a possibility that other modifications could have been introduced during *in vitro* aging and may contribute to the altered cell association of TTCF37. By complexing TTCF4 and TTCF37 with anti-TTCF antibodies that then drive uptake via Fc receptors, we could largely eliminate differential uptake of TTCF4 and TTCF37 (see also Ref. 16). Even under these conditions, presentation of TTCF37 was significantly worse than TTCF4 due, we suggest, to deamidated AEP processing sites.

In the case of the TTCF antigen, we did not observe instances of enhanced T cell epitope presentation as a result of Asn deamidation because of diminished uptake and presumably because AEP action on TTCF is required to initiate processing and is the first step for optimal presentation of most T cell epitopes (16). However, it is possible that loss of processing sites (for AEP or other enzymes) as a result of post-translational protein modifications might enhance the presentation of T cell epitopes in other antigens/autoantigens. This idea is supported by recent evidence that proteolytic processing reactions, for both class I and class II major histocompatibility complex epitopes, can be destructive as well as productive (25–27). An example of this, which also illustrates how direct and indirect effects of Asn deamidation on immune recognition can be linked, is seen in the case of another AEP substrate, myelin basic protein (MBP). A T cell epitope lying between residues 85–99 in MBP is destroyed by AEP cleavage at Asn-94 (25, 26). We have shown that presentation of this peptide to MBP-specific T cells is inversely proportional to AEP activity and have suggested that T cells specific for this region of MBP escape tolerization in the thymus because of this destructive processing reaction (26). Deamidation of Asn-94 would eliminate a dominant AEP processing site in MBP, allowing survival of the 85–99 peptide with a change to Asp or iso-Asp in position 94, which might appear foreign to the immune system. T cell reactivity to MBP85–99 is thought to be important in the inflammatory reactions that occur in multiple sclerosis, and some (although not all) T cells isolated from multiple sclerosis patients that are specific for MBP 85–99 are able to recognize Asp in position 94 (28). However, at this point, this scenario is speculative, and our initial attempts to demonstrate deamidation of Asn-94 in the MBP 85–99 peptide over the short time scales used in our TTCF studies have not been successful. Rates of deamidation of asparagine residues are dependent on a number of variables, among them the amino acid residue C-terminal to the Asn and the conformational flexibility of the polypeptide chain in that region. Although the slow turnover of MBP would in principle allow deamidated Asn residues to accumulate over time, the residue that follows Asn-94 in MBP, Ile-95, is not optimal for deamidation of Asn-94 (29, 30). Nonetheless, it remains an intriguing possibility that deamidation of Asn-94 occurs in MBP over longer time scales and that this contributes to the autoreactivity to this protein seen in multiple sclerosis. In TTCF, we found that two different surface loops, each containing a pair of Asn residues, were deamidated on the first Asn of the pair (Fig. 6). The most comprehensive studies on rates of Asn deamidation did not include a second

Asn as the C-terminal residue (presumably because of its own potential instability), but Asp, although not optimal, was permissible (29, 30). The structure of the TTCF antigen shows that the loop containing Asn-1183/-1844 is exceptionally exposed and might be expected to be flexible in solution (31, 32) (Fig. 6).

It is interesting to speculate that loss of AEP sites as a result of deamidation might concomitantly generate new sites for proteases specific for Asp in the P1 position. The caspases are Asp-specific proteases, although the distinct compartmental localizations of AEP (endosomes/lysosomes) and caspases (cytosol) might rule out their involvement. Another Asp-specific protease is granzyme B found in the cytolytic granules of T and NK cells. Interestingly, Casciola-Rosen *et al.* (33) have demonstrated that granzyme B generates unique fragments from many known and putative human autoantigens. They propose that by so doing granzyme B action, for example, on apoptotic cell debris, might trigger an autoreactive response. They propose that sensitivity to granzyme B (but not to caspase 8, which has similar specificity) is a hallmark of candidate autoantigens. If this hypothesis is correct, it is interesting to speculate that Asn deamidation might produce new granzyme B substrates and that this might be a further connection between Asn deamidation and autoreactivity.

In summary, extrapolating from the example of tetanus toxin, we speculate that long-lived self-proteins, the Asn residues of which become spontaneously deamidated, may be differently processed by AEP when compared with their younger, and in particular their thymically expressed, counterparts. Post-translational modifications to proteins constitute a potential hazard to immune tolerance because there is no certainty that a particular modification will be represented in the protein substrate "mix" available for thymic antigen presentation. Differential processing of self-proteins, between the thymus, where central tolerance is established, and peripheral tissues, is thought to be one of the factors that create permissive conditions for induction and/or maintenance of autoimmune disease (8, 34). Finally, our results, and earlier results reporting that storage of peptides led to distinct immune recognition patterns as a result of deamidation (5–7), raise the possibility that long term storage of vaccines in solution might lead to deamidation of Asn residues that are either important for T cell recognition or key AEP target residues and consequently influence processing, and thus indirectly, the display of important T cell epitopes.

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