A Common Autoimmunity Predisposing Signal Peptide Variant of the Cytotoxic T-lymphocyte Antigen 4 Results in Inefficient Glycosylation of the Susceptibility Allele

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A common T17A polymorphism in the signal peptide of the cytotoxic T-lymphocyte antigen 4 (CTLA-4), a T-cell receptor that negatively regulates immune responses, is associated with risk for autoimmune disease. Because the polymorphism is absent from the mature protein, we hypothesized that its biological effect must involve early stages of protein processing, prior to signal peptide cleavage. Constructs representing the two alleles were compared by in vitro translation, in the presence of endoplasmic reticulum membranes. We studied glycosylation by endoglycosidase H digestion and glycosylation mutant constructs, cleavage of peptide with inhibitors, and membrane integration by ultracentrifugation and proteinase K sensitivity. A major cleaved and glycosylated product was seen for both alleles of the protein but a band representing incomplete glycosylation was markedly more abundant in the predisposing Ala allele (32.7 ± 1.0 versus 10.6% ± 1.2 for Thr, p < 10−8). In addition, differential intracellular/surface partitioning was studied with co-transfection of the alleles fused to distinct fluorescent proteins in COS-1 cells. By quantifying fluorescence with confocal microscopy we found a higher ratio of cyan fluorescent protein; YFP, yellow fluorescent protein. 

The cytotoxic T-lymphocyte antigen 4 (CTLA-4), a disulfide-linked homodimer expressed on the cell surface of activated T-cells is responsible for the attenuation of immune response by binding to ligands (B7.1 and B7.2) expressed on the surface of antigen presenting cells (1–4). Recent reports have implicated CTLA-4 in the modulation of autoimmune responses (5–11) and in the maintenance of peripheral tolerance (1, 12, 13). The CTLA-4 knockout mouse exhibits a severe lymphoproliferative disorder, autoimmune disease, and early lethality, demonstrating the importance of CTLA-4 in the modulation of T-cell response (14, 15). Patients with the Chediak-Higashi syndrome present symptoms not unlike those of the CTLA-4−/− mouse, because of a defect in the CTLA-4 cycling pathway caused by mutations in the lysosomal trafficking regulator gene (LYST) (16). More subtle reductions in expression or function of CTLA-4 may determine susceptibility to common autoimmune diseases.

Indeed, common CTLA-4 polymorphisms have been found to confer susceptibility to type 1 diabetes (17–23), thyroid disease (22, 24–33), and several other autoimmune disorders (34–38). By the transmission disequilibrium test, association with diabetes has been narrowed down to a haplotype encompassing CTLA-4 but not adjacent genes (21, 27). The haplotype consists of three CTLA-4 polymorphisms in tight linkage disequilibrium (LD) with each other that includes a C→T transition in the promoter, a signal peptide amino acid substitution (T17A) (49) and a microsatellite (AT)n repeat in the 3′-untranslated region (21, 27, 39). Because of the tight LD, contribution to diabetes susceptibility cannot be genetically dissected and functional studies are required to define the etiological variant(s). We decided to focus on the nonsynonymous signal peptide polymorphism as the most likely candidate. Two recent reports present evidence that T-lymphocytes from subjects homozygous for the diabetes predisposing G (Ala) allele of the CTLA-4 signal peptide showed enhanced proliferation and cytokine production after in vitro stimulation compared with cells from homozygotes for the protective A (Thr) allele (40, 41). Although Muuer et al. (41) presented some nonquantitative evidence of defective CTLA-4 targeting to the cell surface by confocal microscopy, neither study addressed the molecular mechanism for the differential behavior of T-lymphocytes from homozygotes for each genotype.

Because the signal peptide is co-translationally cleaved in the endoplasmic reticulum (ER) and is not a part of the mature protein, we hypothesized that the T17A polymorphism determines differential targeting to the cell surface by altering early intracellular trafficking of CTLA-4. Signal peptides function in directing ribosome-bound nascent polypeptides to the (ER) membrane where they assure the translocation of growing polypeptide chains into the ER lumen. In conformity with most signal sequences, the CTLA-4 signal peptide has three distinct regions (Fig. 1): a predicted hydrophobic sequence of 12 amino acids flanked by two helix-breaking prolines, a small polar...
COOH terminus (C-) region encompassing the recognition site for signal peptide cleavage, and a long NH₂ terminus (N-) region of 20 amino acids that includes the Thr to Ala substitution at position 17. As shown in Fig. 1A, the Ala allele introduces a hydrophobic amino acid in a highly conserved position, occupied by a serine or threonine in 24 of 25 other species found in a BLAST search we performed (four species are shown for illustration purposes in Fig. 1A). This change somewhat alters hydrophobicity and α-helix propensity, two properties known to be important in signal peptide function (Fig. 1B). We found that the threonine to alanine change resulted in increased hydrophobicity and in a higher propensity to form α-helices in the area directly adjacent to the change.

The work reported here was aimed at testing the hypothesis that the T17A substitution in the signal peptide of CTLA-4 alters the early ER trafficking and/or processing of CTLA-4 and leads to its differential expression on the cell surface. To test our hypothesis we used a cell-free in vitro translation system suited for examining early ER transport events. To also look for decreased expression at the cell surface, the hypothesized ultimate consequence of defective early processing, we examined allelic differences in intracellular versus cell surface CTLA-4 levels in a dual-transfection system with fusion of each allele to a different fluorescent protein and simultaneous quantification by confocal microscopy. The results suggest defective ER processing of a significant portion of the CTLAAla27 molecules resulting in an aberrantly glycosylated product and decreased cell surface expression.
Experimental Procedures

Construction of DNA Plasmids—Full-length CTLA-4 was amplified by reverse transcriptase-PCR from cDNA of a heterozygous individual for a putative CTLA4 signal peptide polymorphism (codon 17). For the truncation fusion proteins, a forward primer containing the linker NheI (underlined), TATGCTAGCCGTGGGCGCTTGACGAGG, and an anti-

ence primer containing the linker AgeI (underlined), with two additional bases (lowercase) ACAACCCGTTCTATGGGAAATAATAGGG, ensured an in-frame, seamless fusion protein. PCR was performed with high fidelity polymerase/Taq mix. Adenine 3' overhangs were added and the PCR product was cloned into pCR2.1 (Invitrogen, Carlsbad, CA). Clone genotyping was done by PCR and digestion with Fnu4HI (New England Biolabs, Beverly, MA). Full-length CTLAThr17 and CTLAAla17 digested with NheI + AgeI purified from plasmid were fused to restricted NheI + AgeI ECFP and EFYP (Clontech, Palo Alto, CA).

For in vitro transcription, full-length CTLA-4 with the termination codon was amplified from plasmid with the mutagenic antisense primer CACCCCGTTGATGGGGAAATAATAGGG (the stop codon is in lowercase and the AgeI linker is underlined). Adenine 3’ overhangs were added to the PCR product and ligated to pCR2.1. T-vector and clones were selected for orientation downstream of the T7 promoter.

Truncation of amino acids Tyr201–Asn223 from the CTLA-4 COOH-terminal results in a mutant constructively directed to the cell surface with no requirement for the machinery that normally directs this translocation in activated T-cells (43). The corresponding construct was prepared by PCR amplification from cDNA of a heterozygous individual using the antisense primer TATGCTAGCCGTGGGCGCTTGACGAGG (underlined) and the PCR product was cloned into pCR2.1. COOH-truncated CTLAThr17 and CTLAAla17 will be denoted herein as CTLAThr-17 and CTLAAla-17 (in reference to Gly200, the last amino acid in the truncated constructs (numbering of all constructs assumes an uncleaved signal peptide of 37 amino acids). The truncated DNA plasmids were then subcloned in-frame into ECFP and EFYP plasmids, using the NheI + AgeI sites. All resulting DNA constructs were sequenced.

N-Glycosylation Mutants—The two predicted N-linked glycosylation sites in the CTLA-4 sequence were deleted by site-directed mutagenesis by the mismatched primer method (44) using the QuikChange multi-

site-directed mutagenesis kit (Stratagene, La Jolla, CA). Asparagine 113 and asparagine 145 were converted to aspartate residues in CTLAThr17 and CTLAAla17. Both sites were mutated in each allele yielding CTLAThr-17(N113D/N145D) and CTLAAla17(N113D/N145D) or one at a time, yielding CTLAThr-17(N113D), CTLAAla17(N113D), and CTLAThr-17(N145D), and CTLAAla-17(N145D). All mutant constructs were verified by sequencing.

RESULTS

N-Glycination is important for proper intracellular transport, and CTLAAla17 Is Inefficiently Processed in the ER—When otherwise identical full-length CTLAAla17 and CTLAThr17 cDNA constructs were in vitro transcribed and translated in the absence of microsomal membranes, [35S]methionine-labeled protein products from both allelic forms migrated with an apparent molecular weight of 26,000. This is consistent with the calculated molecular mass of monomeric, uncleaved, unglyco-

sylated CTLA-4 (Fig. 2A, lanes 1 and 4). Upon addition of microsomal membranes translation products of 29 kDa were apparent for both alleles, corresponding to the size change expected from high mannose glycosylation in two positions and signal peptide cleavage. In addition, the CTLAThr17 reaction (Fig. 2A, lanes 2 and 3) contained an intermediate band migrating with an apparent molecular weight of 25,000. In multiple experiments, this band was absent or only faintly visible in the CTLAThr17 lane. Quantitatively, the CTLAThr17 25-kDa product represented 32.7% ± 1.0 of total processed CTLAAla17 versus 10.6% ± 1.2 for the CTLAThr17 allele (Fig. 2B) (p < 1 × 10−8, n = 11 independent experiments). This ratio was inde-

pendent of the amount of microsomal membranes in the reaction, within a range from 0.8 to 2.8 eq membranes added to the reticulocyte lysate. One observation worth noting was that translation efficiency was substantially increased in the presence of microsomal membranes suggesting that co-transla-

tional processing increases translation efficiency. Having established that a portion of CTLAAla17 molecules is tran-

siently localized at the ER membrane, we used proteinase K digestion. Translation products were treated with 100 mM CaCl2, and incubated for 1 h with 20 μg/ml proteinase K (Sigma) with or without Triton X-100 (1%) at 0 °C. The reaction was terminated with phenylmethylsulfonyl fluoride (2 mM final concentration). As an addi-

tional measure of cytosolic/ER partition the translation products were separated by ultracentrifugation. Briefly, reactions were diluted 100-fold with Na2CO3 (pH 11.5), and centrifuged at 100,000 rpm for 30 min at 4 °C. The pellet was resuspended in 1× SDS sample buffer and denatured by boiling for 3–4 min. The supernatant was concentrated by column filtration (Microcon, Bedford, MA) to 1/10 of its original volume. The sample was prepared for SDS-PAGE by denaturation in 2× SDS sample buffer. All products were resolved by SDS-PAGE and visualized by autoradiography. Finally, inhibition of the signal peptidase was achieved by incubation of the translation mixture with 5 mM (final concentration) N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ke-

tone (Sigma) in 10% Me2SO for 1 h at 30 °C.

Proteinase K Digestions, Ultraacentrifugation, and Signal Peptide Inactivation—To distinguish in vitro translated trunctual CTLA-4 molecules inte-

grated in the ER membrane from those in the cytosolic phase, we used proteinase K digestion. Translation products were treated with 100 mM CaCl2, and incubated for 1 h with 20 μg/ml proteinase K (Sigma) with or without Triton X-100 (1%) at 0 °C. The reaction was terminated with phenylmethylsulfonyl fluoride (2 mM final concentration). As an addi-

tion to the SDS-PAGE analysis, inhibition of the signal peptidase was achieved by incubation of the translation mixture with 5 mM (final concentration) N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (Sigma) in 10% Me2SO for 1 h at 30 °C.
Fig. 2. CTLAAla17 is inefficiently processed in the endoplasmic reticulum. A, CTLAAla17 and CTLAThr17 constructs were in vitro transcribed and translated under conditions outlined under “Experimental Procedures.” Where indicated, ER-containing microsomal membranes were added. In the presence of membranes an intermediate is apparent in the CTLAAla17 reaction at ~25 kDa, but virtually absent in the CTLAThr17 reaction. This suggests incomplete ER processing. A control reaction that contains no mRNA is shown in lane 5. B, we quantified the % total unprocessed by measuring densities of the 29-kDa band (considered processed) and the 25 kDa (considered unprocessed) within each lane for CTLAAla17 and CTLAThr17. All densities were measured with NIH Image on scanned autoradiographs. Background was subtracted for each band and represented are the mean ± S.E. of 11 independent experiments.

Fig. 3. Signal peptide cleavage of the major products of CTLAAla17 and CTLAThr17 is unaffected. We assessed signal peptide cleavage by performing the translation reactions for each allele in the presence of the signal peptidase inhibitor, N-methoxy succinyl-Ala-Ala-Pro-Val-chloromethyl ketone. Inhibition of cleavage results in a 4-kDa increase in molecular weight (band shift to 33,000) as expected for the gain of the 37-amino acid signal peptide. This indicates that the major translation products of both alleles are cleaved and that the additional 25-kDa band is a monoglycosylated protein and migrates at 22 kDa. The 25-kDa intermediate observed in lanes 2–5 represents a monoglycosylated protein and migrates with the same apparent molecular mass as the intermediate observed in the CTLAAla17 reaction (lane 6). A, in vitro translated CTLAAla17 and CTLAThr17, in the presence of microsomal membranes, were subjected to overnight digestion with EndoH resulting in a major digestion product at 22 kDa (lanes 2 and 3) abrogating the difference between the two alleles. This confirms that the 25-kDa precursor observed following microsomal processing of the CTLAAla17 represents a cleaved but improperly glycosylated CTLA-4.

Fig. 4. Deglycosylation abolishes allelic differences in processing efficiency. A, CTLAThr17 and CTLAAla17 were in vitro translated in the presence of microsomal membranes and subjected to partial EndoH digestions for 1 or 2 h at 37 °C. A ladder of intermediates was observed representing the deglycosylated proteins. The smaller digestion product obtained in lanes 2–5 corresponds to a completely deglycosylated protein and migrates at 22 kDa. The 25-kDa intermediate observed in lanes 2–5 represents a monoglycosylated protein and migrates with the same apparent molecular mass as the intermediate observed in the CTLAAla17 reaction (lane 6). B, in vitro translated CTLAAla17 and CTLAThr17, in the presence of microsomal membranes, were subjected to overnight digestion with EndoH resulting in a major digestion product at 22 kDa (lanes 2 and 3) abrogating the difference between the two alleles. This confirms that the 25-kDa precursor observed following microsomal processing of the CTLAAla17 represents a cleaved but improperly glycosylated CTLA-4.

gave rise to a full-length intermediate of ~33 kDa resulting from a gain of 4 kDa by the 29-kDa intermediate, corresponding to a gain of the 37-amino acid signal peptide (Fig. 3). Thus the major bands in the translated products of both CTLAAla17 and CTLAThr17 represent cleaved intermediates. The cleavage status of the additional CTLAAla17 25-kDa band was less clear because of band overlap (Fig. 3), as inhibition was not complete at the maximum effective concentration of inhibitor (higher concentrations inhibited translation). Thus the major bands for both alleles must represent fully glycosylated and fully cleaved product.

CTLA-4 Has Two N-linked Glycosylation Sites—CTLA-4, a cell surface receptor, has two predicted N-linked glycosylation sites located in the extracellular domain, at Asn113 and Asn145, thought to be important for structural integrity (46). Although not all instances of the consensus sequon Asn-X-Thr/Ser (X = any amino acid) are necessarily glycosylated (47, 48), the observed size of the major band clearly indicated full glycosylation of most of the molecules for either allele. This was further confirmed with partial digestion of the in vitro translation products with EndoH that, in glycoproteins with several glycosylation sites, produces a ladder of partially digested molecules differing by only one N-linked chain. Two additional major bands with an estimated molecular weights of 25,000 and 22,000 appeared after 1–2 h of digestion (Fig. 4A, lanes 2–5) representing cleavage of, respectively, one or two N-linked glycosylation moieties from the 29-kDa form that was still visible. Further digestion for up to 16 h never produced smaller
products. The 25-kDa band was indistinguishable in size from the aberrant intermediate seen with CTLAAla17. This allowed us to conclude firmly that, in addition to being cleaved, the 29-kDa product was glycosylated at both sites and, tentatively, that the aberrant CTLAAla17 product represents cleaved, monoglycosylated protein.

This was further confirmed with complete EndoH deglycosylation. As expected, prolonged digestion for 16 h with EndoH resulted in a major band of 22 kDa for both alleles, with no extra band for CTLAAla17 (Fig. 4B). Thus complete deglycosylation abolished any difference between allelic forms of the protein, allowing us to conclude that the extra band seen after microsomal processing of nascent CTLAAla17 represents cleaved but aberrantly glycosylated CTLA-4.

**Differential Processing of the Two Alleles Depends on Glycosylation**—The deglycosylation results regarding the nature of the unprocessed intermediate observed in CTLAAla17 were further corroborated by site-specific mutagenesis designed to abrogate glycosylation by removing each glycosylation site separately and both together in each allele. Asn113 and Asn145, the predicted N-linked glycosylation sites, were replaced with Asp residues at the same positions (Fig. 5). The resulting constructs were in vitro transcribed and translated, as described above.

All glycosylation mutants translated in the absence of microsomal membranes behaved as the wild-type alleles and a 26-kDa translation product was detected. In the presence of ER-containing membranes, the double glycosylation mutant migrated at 22 kDa, as expected of an unglycosylated CTLA-4 with a cleaved signal peptide. No extra band corresponding to the differentially processed CTLAAla17 band was detectable (Fig. 5, lanes 10 and 11), clearly indicating that the allelic difference in processing requires the presence of glycosylation sites. The uniform 25-kDa electrophoretic mobility of the single-site mutants CTLAThr17(N113D) and CTLAAla17(N113D), CTLAThr17(N145D), and CTLAAla17(N145D) (Fig. 5, lanes 2, 3, and 6, 7) is consistent with loss of the signal peptide (4 kDa) and gain of a single high mannose glycosylation site (2–3 kDa). Again there was no difference between alleles, indicating that differential processing occurs only in the presence of both glycosylation sites. These results also confirm with certainty that both CTLA-4 signal peptide alleles are ER-translocation competent and can be cleaved, because a completely deglycosylated product migrates at 22 kDa, 4-kDa less than the monomeric CTLA-4 product translated in the absence of membranes.

**The Partial Glycosylation Intermediate Is Not Integrated in the ER Membrane**—We next addressed the question of whether the cleaved but aberrantly glycosylated CTLAAla17 intermediate, representing almost one-third of all molecules of the autoimmune predisposing allele in the ER, can be correctly targeted to the cell surface. Misprocessed proteins are retained in the ER bound to chaperones such as calnexin and calreticulin and eventually translocated back to the cytoplasmic phase where they undergo ubiquitin-driven, proteasome-dependent degradation (49). Partition of the aberrantly glycosylated CTLAAla17 between cytosolic phase and ER membrane was evaluated by two independent assays. Protection from proteinase K digestion and resistance to extraction by alkaline high salt are both indicators of integration into the microsomal membranes. Because CTLA-4 is a type I transmembrane glycoprotein, its 36-amino acid COOH tail is exposed in the cytoplasm and will be digested, resulting in a 4-kDa loss.

Indeed, proteinase K digestion resulted in the expected 4-kDa reduction of the 29-kDa major bands seen with both alleles (Fig. 6A, lanes 2 and 5). A band corresponding to a 4-kDa reduction in the size of the 25-kDa intermediate seen with CTLAAla17 is also seen (Fig. 6A, lane 5) but this form is considerably less intense relative to the upper band, indicating partial sensitivity to proteinase K digestion. By densitometric quantification, prior to digestion it constitutes 34.5% ± 0.6 of total CTLAAla17 product, but only 18.5% ± 0.8 after digestion (p = 0.0001, n = 3 independent experiments) (Fig. 6B, in reference to lanes 5 and 6 in Fig. 6A). The corresponding percentages for CTLAThr17 are 10.8 ± 1.0 and 9.9 ± 1.8, respectively (NS, n = 3) (Fig. 6B, in reference to Fig. 6A, lanes 1 and 2). Thus, the allelic difference in the abundance of the incompletely processed form can be accounted for almost entirely by molecules located outside the ER.

Strikingly similar results were obtained upon treatment of translation reactions with high Na2CO3, which releases soluble and peripheral membrane proteins while transmembrane proteins remain inserted in the ER lipid bilayer, followed by separation of pellet (P) and supernatant (S) fractions by ultracentrifugation. The major 29-kDa bands were largely recovered in the pellet (Fig. 6C, lanes 5 and 6) for CTLAThr17 and CTLAAla17. This supports the results found in the protease sensitivity assay and confirms that CTLAAla17 and CTLAThr17 are both translocation competent and integral ER membrane proteins. It was apparent, however, that significantly less incompletely glycosylated CTLAAla17 intermediate was recovered in the pellet. By densitometric quantification it was found to be 36.2% ± 1.0 of the total prior to separation by ultracentrifugation and 24.9% ± 0.4 in the pellet (p = 0.0078, n = 3) (Fig. 6D). The corresponding values obtained for the CTLAThr17 intermediate were 8.9% ± 2.7 prior to ultracentrifugation and 10.0% ± 1.2 recovered in the pellet (NS, n = 3). This remarkable concordance between two independent methods demonstrates that roughly half of the aberrantly processed
CTLAA17 is in the cytoplasmic phase. Because it has undergone signal peptide cleavage and glycosylation, this fraction does not represent failure of translocation to the ER but rather retrotranslocation back to the cytoplasmic phase for proteasomal degradation.

CTLAAla17 and CTLAThr17 Do Not Co-localize in Co-transfected COS-1 Cells—To determine whether the differential processing observed in the cell-free system would translate into cell surface expression differences, we devised a dual-transfection system in COS-1 cells with fusion proteins of CFP and YFP downstream of otherwise identical CTLAAla17 and CTLAThr17. In these experiments reciprocal constructs were always tested to exclude possible effects because of the different properties of the fluorescent proteins. In addition, we directly demonstrated that co-transfections with the same allele tagged with each of the two FPs (e.g. CFP-CTLAThr17 and YFP-CTLAAla17) showed that the same allele did not behave differently as a result of the different fluorescent protein fusion and was targeted and expressed in the same compartments (Fig. 7A, panels a–c). Similar results were observed for CFP-CTLAA17 and YFP-CTLAA17 co-transfections (Fig. 7A, panels d–f).

CTLA-4 is known to localize to endosomal compartments in T-cells as well as in non-T cell systems (43, 50). To demonstrate that the fusions of CTLA-4 resulted in a properly targeted protein, each allele was transfected independently and the cells were stained with an antibody to the transferrin receptor followed by detection by a Cy-5 conjugate. The transferrin receptor has been previously shown to co-localize with CTLA-4 in post-Golgi compartments as was the case for our fusion proteins (data not shown).

When the two alleles, now labeled with different fluorescent proteins (CFP-CTLAThr17 and YFP-CTLAA17) were introduced into COS-1 cells clear differences in targeting were seen, with distinct regions where only one allele could be found (Fig. 7B, panels a–c, see arrows). Similar observations were made for reciprocal transfections of YFP-CTLAThr17 and CFP-CTLAA17 (Fig. 7B, panels d–f). These results are consistent with our in vitro observations of cytoplasmic retrotranslocation of CTLAA17. The expected resulting difference in targeting to the cell surface was thus investigated.

Cell Surface Levels of CTLAA17 in COS-1 Cells Are Lower Than CTLAThr17—Using the same dual transfection system described above, CTLA-4 constructs with signal peptide allelic variants were tested for quantitative differences in expression at the cell surface of COS-1 cells. Surface expression is specific for activated T-lymphocytes, but truncating the last 22 amino acids of the carboxyl tail allows partial cell surface expression in a non-T cell system (43). By co-expressing fluorescent fusion proteins of the COOH-truncated CTLA-4 alleles in one single cell, it was possible to simultaneously measure fluorescence intensity at the cell surface for both alleles and compare it with intracellular fluorescence (Fig. 7C, panels a–f). Ratios of cell surface/intracellular mean fluorescence were quantified in single cells for both CTLA-4 alleles and compared with a paired, two-tailed Student’s t test. Mean cell surface/intracellular fluorescence intensity ratios are 9.4 ± 2.2 for YFP-GlyThr17 and 1.5 ± 0.8 for YFP-GlyAla17 (p = 5 × 10−4, n = 9 cells, example illustrated in Fig. 7C). The average ratios of cell surface/intracellular fluorescence intensity for YFP-GlyAla17 and CFP-GlyThr17 co-transfections were 6.0 ± 2.4 and 10.0 ± 2.8, respectively (p = 0.01) (Fig. 7C). The ratio varied somewhat from cell to cell, accounting for the relatively large mean ± S.E. but, because the comparisons were paired within the same cell, the difference was highly significant statistically. The results presented here were calculated from at least 9 different cells obtained in at least two independent transfections for each allele. Cells were selected for expressing the two colors at
roughly equal intensities, and their selection was finalized prior to any knowledge of the quantitative results. By choosing to measure ratios within the same cell for both alleles correction for transfection efficiency differences is inherent as well as correction for any differences in fluorophore efficiency. Again, in this case same-allele co-transfections were performed and similar calculations were done but no statistical significance was found (p > 0.05). This allowed us to conclude that, for any given level of expression, there is significantly less CTLA GlyAla17 expressed at the cell surface of COS-1 cells than CTLA GlyThr17.

DISCUSSION

Our data demonstrate that a common amino acid polymorphism in a signal peptide can have measurable consequences on the efficiency of processing the protein and ultimately on its expression level at the cell surface. In the case of CTLA-4, a molecule involved in the inhibitory regulation of the immune response, this mechanism offers an attractive explanation for the higher frequency of diabetes and other autoimmune diseases in individuals homozygous for the Ala allele (19, 20, 22, 23, 25, 26, 33). Signal peptide mutations completely abrogating proper targeting of a protein have been described in Mendelian disorders (51–54) but, to our knowledge, this is the first demonstration of a subtler effect in the context of susceptibility to a common complex disease. However subtle, such effects are extremely important toward composite molecular prediction and better understanding of the mechanism leading to effective prevention/treatment.

To summarize our findings: by a widely used model of in vitro reconstitution of translation and ER processing, we have shown that both alleles of the signal peptide are capable of translocation to the ER and both are completely and correctly cleaved. The difference appears to lie in the fact that up to one-third of CTLA Ala17 molecules are glycosylated on only one of the two possible sites.

The absence of an effect on translocation or cleavage was not surprising, as the polymorphism does not alter any of the known consensus elements required for translocation or the signal peptidase cleavage site. Somewhat less expected was the effect on glycosylation, as this modification is not ordinarily thought of as depending on the signal peptide. However, evidence is arising that in the time interval between entry of the amino-terminal of the nascent polypeptide into the ER and signal peptidase cleavage, the signal peptide may participate in the process of protein folding and alter its interactions with ER chaperone proteins and modifying enzymes (42, 55). Thus, the highly inefficient processing of the human immunodeficiency virus glycoprotein 120 was shown to be due strictly to its signal peptide, as the exact same mature-protein sequence is processed efficiently with a control signal peptide (42). The native human immunodeficiency virus glycoprotein 120 signal sequence is translocation-competent, but affects processing by prolonging glycoprotein 120 association with ER chaperones and results in incorrect folding, which causes ER retention (42).

Moreover, it is now clear that although the oligosaccharyltransferase complex is tethered to the ER membrane, transfer of N-linked high mannose moieties to proteins is not synchronous with translocation to ER, but may follow folding of the protein and is profoundly influenced by it (56). It is known that not all consensus Asn-X-Ser/Thr N-linked glycosylation sites are equally glycosylated or glycosylated at all. A proline in position X (or immediately after Ser/Thr) eliminates glycosylation (47, 57), whereas Trp, Asp, Glu, and Leu decrease glycosylation efficiency (57). By introducing glycosylation sequons into different positions of the Saccharomyces carboxypeptidase Y, Holst et al. (56) showed dependence of glycosylation on
protein folding and the position of the sequon in the context of the folded protein, results that led them to conclude that glycosylation does not necessarily precede folding, and can be affected by it. In addition, deletion of downstream sequences was shown to abrogate glycosylation of the hepatitis C virus E1 protein (58). Taken together, these observations suggest a mechanism whereby a signal peptide variant may affect the glycosylation efficiency through altered chaperone association and folding.

We performed the single mutant experiments to define which of the two sites remained unglycosylated in the partially processed CTLAAla17 fraction. Assuming that each mutation did not otherwise change CTLA-4 processing, we had expected that elimination of the specific site that remained unglycosylated in a fraction of CTLAAla17 would result in a single monoglycosylated band for both alleles, whereas elimination of the site fully glycosylated in both alleles would give a monoglycosylated band in both alleles, plus an additional minor unglycosylated band in CTLAAla17. Instead we found a single monoglycosylated band in both alleles, regardless of which site was mutated. The simplest explanation for this is that abolition of the consistently glycosylated site leads to alterations in protein folding and/or interactions with ER chaperones that abolish the glycosylation inefficiency seen with CTLAAla17. Alternatively, eliminating one of two glycosylation sites through mutagenesis may relieve competition between them, although a mechanism for such competition is not obvious and no previous paradigm exists.

Our cell surface targeting studies used a truncated CTLA-4 mutant that by-passes the activated lymphocyte-specific regulatory mechanism of translocation from Golgi to the cell surface. We believe that this does not detract from the validity of our conclusions, as passage from Golgi to the cell surface is a post-ER event, occurring after cleavage of the signal peptide and its efficiency should not differ between the Ala17 and Thr17. Given the allelic differences in early processing we found in the in vitro system of isolated ER, the obvious explanation is that less of the Ala17 makes it to the Golgi, and therefore less is translocated to the cell surface.

Throughout this discussion we have assumed that doubly glycosylated CTLA-4, the major band in both alleles, represents correct ER processing leading to functional expression at the cell surface: it is practically the only form seen with the Thr17 allele, homozygotes for which are common in the general population and healthy. Extrapolating from our in vitro assay, we propose that Ala homozygotes express one-third less CTLA-4 on the surface of their T-cells than Thr homozygotes, which might tip the balance in favor of immune response and predispose them to autoimmune disease. One might speculate that survival of this allele in evolution despite this disadvantage is because of the counterbalancing effect of better defense against infectious diseases.

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