T-DNA insertion is within 1.3 map units of the *Era1* gene.

11. Genomic Southern blots of *era1-1* DNA digested with Eco RI and probed with right-border (RB) T-DNA produced three bands (13, 7.0, and 8 kb) that could be accounted for by a contiguous double insertion of T-DNA in which one of the copies has been inverted to give two flanking RB regions attached to plant genomic DNA. Subsequent analysis with other restriction enzymes verified that the 7- and 8-kb bands contained the insertion points of T-DNA and flanking plant DNA. DNA pools enriched for these fragments were ligated to the λ-ZAPII arms according to the manufacturer's instructions (Stratagene) and five positive plaques were identified that hybridized with the RB probe. Two plasmids (pSC10 and pSC11), each containing about 1.2 kb of T-DNA attached to different flanking regions, detected a single Eco RI 1.5-kb fragment in wild-type DNA by Southern analysis. Both clones therefore contain genomic sequences that are contiguous in wild-type DNA, indicating that the RB fragments in the *era1-1* mutant represent the ends of a single-site T-DNA insertion. pSC10 was used as a probe to screen an *Arabidopsis* cDNA library, PRL2 λ-ZipLox (ABRC, stock CD4-7); and five positive cDNAs were identified and sequenced. The longest cDNA, pZL51 (1.45 kb), was used to screen Columbia (λ-ZAPII, Stratagene) and Lansberg genomic (λ-FIX, ABRC stock CD4-8) libraries and four positive clones were identified. One clone spanning 6 kb and encompassing the entire pZL51 clone was completely sequenced. A larger genomic insert (14 kb) was used to size deletions in the fast-neutron mutants (*era1-2* and *era1-3*).
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15. To assay farnesylation, 1 g (fresh weight) of wild-type or mutant flower buds was homogenized in extraction buffer [50 mM Hepes (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 5 mM dithiothreitol (DTT), leupeptin (2 μg/ml) aprotinin (2 μg/ml), and 1 mM phenylmethylsulfonyl fluoride]. The synthetic heptapeptides GGCCAIM (CAIM) and GGCCAIL(CAIL) (*19*) were prepared as described [B. He *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11373 (1991)]. Target peptide sequences were chosen according to activities measured in tobacco culture [S. K. Randall *et al.*, *Plant Cell* **5**, 433 (1993)]. Extracts were clarified at 4°C at 10,000*g* for 10 min and then at 100,000*g* for 30 min. Soluble protein extract (100 μg) was incubated at 30°C for 40 min in 25 μl of reaction buffer [50 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 5 mM DTT, 50 μM peptide, and 0.5 μM [^{3}H]farnesyl diphosphate (FPP) (17.0 Ci/mmol; Amersham)]. Reactions were terminated with EDTA (at a final concentration of 50 mM), spotted onto Silica Gel 60 thin-layer chromatography plates (Millipore), and developed with *n*-propanol and water (7:3 v/v) for 4 to 5 hours. The plates were dried, sprayed with En^{3}Hance (New England Nuclear), and exposed to Kodak X-OMAT AR film at −70°C for 4 days.
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19. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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TECHNICAL COMMENTS

CD2: An Exception to the Immunoglobulin Superfamily Concept?

A central tenet of the immunoglobulin superfamily (IgSF) concept is that the evolutionary success of IgSF domains derives from their ability to form stable protein modules at the cell surface (*1*, *2*), and this is entirely consistent with the high degree of conservation of framework residues in IgSF domains revealed by structural studies (*3*). The recent proposal by Daniel F. Wyss *et al.* (*4*) that some IgSF domains, such as the ligand binding domain of the human T lymphocyte antigen, CD2, "maintain their stable conformation as a result of dynamic interactions between the polypeptide and its attached glycan," is incompatible with the IgSF concept and therefore warrants careful consideration.

CD2 is an integral membrane glycoprotein that has two extracellular IgSF domains N-glycosylated at three sites [reviewed in (*5*)]. The ligand- and antibody-binding properties of a soluble form of CD2 consisting of both IgSF domains are indistinguishable before and after reduction of the N-linked glycans to single N-acetylglucosamine residues by endoglycosidase H treatment (*6*). After complete removal of the glycans with N-glycanase, the protein also binds ligand with wild-type affinity, although the binding of some antibodies is reduced in accord with the reduction in protein solubility observed after complete deglycosylation of CD2 (*6*). These results suggest that the N-linked glycans do not stabilize the folded conformation of two-domain CD2, but may enhance its overall solubility. In contrast, the proteolytic fragment consisting of domain 1 of CD2, on which Wyss *et al.* base much of their analysis, appears to be metastable because N-glycanase treatment of this fragment completely abrogates ligand and antibody binding (*4*, *7*). These differences in the behavior of the single- and two-domain forms of CD2 undermine the biological significance of the effects observed by Wyss *et al.* as there is no counterpart in nature for the single domain form of CD2. It is also relevant that protein stability artifacts have been encountered ever since the earliest attempts were made to produce truncated soluble forms of IgSF molecules by recombinant DNA methods [see, for example, (*8*) and (*9*)]. In the light of these discrepancies and technical uncertainties, it seems inappropriate to conclude that CD2 is a clear exception to the IgSF concept.

These observations do not rule out the possibility that the domain 1 glycan has some other role in CD2 expression and this is clearly implied by the mutational data of Wyss *et al.* The problem with the mutational approach, however, is that it does not necessarily distinguish between the potential effects of glycosylation on (i) protein folding, (ii) post-folding conformational stability, or (iii) protein trafficking to or beyond the cell surface. In view of the conformational stability of deglycosylated two-domain CD2 (*6*, *10*) and the unimpaired trafficking of unglycosylated mutant forms of CD2 to the cell surface (*4*, *7*), it would appear that the domain 1 glycan influences the initial folding of CD2 rather than determining, as proposed by Wyss *et al.*, its post-folding stability. A fluorescence energy transfer study of peptides used as model folding intermediates (*11*), which suggests that N-linked glycans alter the conformational space available to β-turn glycopeptides that are analogous to the glycosylated DE loop of CD2 domain 1, is consistent with this possibility.

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Response: In our report, we stated that the functional form of human CD2 (hCD2) is maintained by the stabilizing effect of its N-linked glycan in domain 1. Earlier we had observed that (i) whereas soluble nonglycosylated human CD2 domain 1 (hsCD2d1) expressed in *Escherichia coli* was not functional, Chinese hamster ovary cell derived glycosylated hsCD2d1 was fully active, and (ii) complete removal of the single N-glycan in domain 1 of hCD2, either by N-glycanase treatment of hsCD2d1 or by mutation of the Asn65-Xxx-Thr67 sequence of hCD2, completely abrogated CD58 and anti-CD2 monoclonal antibody binding (*1*, *2*). Our recent structural studies on glycosylated hsCD2d1 (*3*, *4*) revealed that this N-glycan is opposite to the CD58 binding site in hCD2 and is not involved in CD58 recognition. To determine why this N-glycan is nevertheless critical for hCD2 adhesion function, we studied both enzymatically treated hsCD2d1 and a series of full-length hCD2 mutants (*4*). From these studies we concluded that the N-glycan crucially stabilizes the folded protein structure, and showed that the first N-acetylglucosamine residue (GlcNAc-1) is sufficient to keep the glycoprotein folded (*4*). Several recent studies on glycoproteins have shown that carbohydrates globally stabilize the polypeptide fold (*5*–*11*) and in two cases, an increase in the stability of about 1.2 kcal/mol was measured (*6*, *8*). In two examples, this stabilizing function was achieved by a single sugar unit like in hCD2 (*5*, *6*). However, the mechanism of this stabilization is not clear yet. On the basis of our mutations, we have not been able to identify a particular stabilizing interaction between glycan and protein in the folded state (*4*). On the other hand, the presence of the bulky, conformationally restricted GlcNAc-1 ring is likely to lower the entropy of the unfolded state favoring the folded form. The marginal stability of hCD2 has been shown to be due to the unfavorable clustering of five lysines centered around Lys-61 (*4*). Compared to hCD2, rat CD2—which does not require a glycan for maintenance of its stable fold—contains a glutamic acid at the corresponding position of Lys-61. A nonglycosylated hCD2 mutant containing a K61E mutation is stable and shows full CD58 binding activity. This suggests that in this hCD2 mutant, Glu-61 removes the unfavorable clustering of positive charges and forms a stabilizing salt bridge with Lys-69, eliminating the need for a stabilizing glycan (*4*).

Davis and van der Merwe have done independent work on CD58 and anti-CD2 monoclonal antibody binding of enzymatically treated hsCD2d1d2 using surface plasmon resonance (SPR) analysis (*12*).

First, they state that the N-linked glycans do not stabilize the folded conformation of hsCD2d1, but may enhance its overall solubility. However, it is unlikely that the stabilizing effect demonstrated for hsCD2d1 should not contribute to the stability of the two-domain fragment, in particular because similar stabilization effects of glycans have been found in a number of other glycoproteins (*5*–*11*). Consistent with our view, they observed a marked reduction of binding activity for several anti-CD2 monoclonal antibodies compared to the results of endoglycosidase H treatment, when in addition, the GlcNAc-1 of the three N-glycans of hsCD2d1d2 were removed with N-glycanase treatment (*12*). Furthermore, with N-glycanase treatment, hsCD2d1d2 needs to be used in their SPR analysis of CD58 binding activity immediately to avoid protein aggregation. Exposure of hydrophobic groups on removal of the three GlcNAc-1 of hsCD2d1d2 per se is unlikely in this case, given the nature of the hsCD2d1d2 surface defined by the crystal structure in the vicinity of the three N-glycans (*13*). The observed aggregation is more likely a consequence of partial unfolding, a common phenomenon, as hydrophobic protein core interiors will be exposed. Although N-glycanase treated hsCD2d1d2 binds CD58 with wild-type affinity, this does not necessarily mean that it is equally stable as wild-type hCD2. The fact that N-glycanase–treated hsCD2d1d2 binds several conformationally-sensitive antibodies to a much lower degree than wild-type hCD2 and its high tendency to aggregate clearly show that this molecule is no longer fully native-like, although it may still be able to provide an intact CD58 binding site for some amount of time.

Second, Davis and van der Merwe suggest that the N-glycan, within the adhesion domain of hCD2, influences the initial folding of hCD2 rather than determining its post-folding stability. However, the CD2 copy number of nonglycosylated hCD2 mutants on the cell surface is around 50% or greater of wild-type hCD2. Misfolded proteins are usually recognized and degraded by the quality control system in the endoplasmic reticulum, and hence are expressed at very low levels relative to wild-type proteins (*14*). Nevertheless, besides its role in post-folding stability, the N-glycan may also play an important role in the conformational maturation of hCD2.

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