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Identification of the glycosaminoglycan-attachment site of mouse invariant-chain proteoglycan core protein by site-directed mutagenesis

(Ia antigens/major histocompatibility complex/chondroitin sulfate/xylosyltransferase/transferrin receptor)

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ABSTRACT **The invariant chain (Ii), a nonpolymorphic glycoprotein that associates with the immunoregulatory Ia proteins encoded by the major histocompatibility complex, has a proteoglycan form (Ii-CS) that bears a chondroitin sulfate glycosaminoglycan. In this proteoglycan form, Ii may remain associated with Ia at the cell surface. Inhibitors that prevent the addition of glycosaminoglycan to Ii have been found to depress antigen-presenting function. Ii does not have multiple candidate glycosaminoglycan-attachment sites, and we used site-directed mutagenesis to replace a candidate serine glycosaminoglycan-acceptor site with alanine at position 201 in the murine Ii protein. Transfection of the normal or altered gene into Ii-negative COS-7 cells showed that equivalent amounts of core Ii protein and its acidic, terminally glycosylated forms were synthesized, but the Ala-201 mutant Ii did not give rise to Ii-CS. The mutant protein had apparently normal transport through the Golgi compartment and associated stably with Ia molecules. Thus, this mutation directly identifies the site of glycosaminoglycan addition and shows that it can be eliminated without adversely affecting the overall biosynthesis of Ii.**

The class II (Ia) gene products of the major histocompatibility complex are molecules whose structural variation influences the ability of helper T cells to recognize processed exogenous antigen (1, 2). These immunoregulatory molecules are composed of α and β chains encoded by the major histocompatibility complex. In addition, the α and β chains are intracellularly associated with a nonpolymorphic glycoprotein called invariant chain (Ii) (3), which is encoded by a single gene (4, 5). The most abundant form of Ii is a M_r 31,000 protein, though several other polypeptide forms of the Ii protein are found because of production of two differentially spliced mRNAs (6, 7) and because translation can be initiated at two different start codons (6, 8).

The association between the Ia $\alpha\beta$ heterodimer and the M_r 31,000 Ii begins in the endoplasmic reticulum, where an excess of Ii is made. All three chains in the $\alpha\beta$Ii complex undergo coordinate posttranslational processing events. In addition to N- and O-linked oligosaccharides, some Ii chains receive the unusual addition of a chondroitin sulfate glycosaminoglycan (GAG) during Golgi processing (9, 10), increasing their size to M_r 45,000–70,000. M_r 31,000 Ii dissociates from the Ia heterodimer sometime between the trans-Golgi compartment and insertion of Ia into the plasma membrane, but some surface Ia continues to be associated with the GAG-bearing form of Ii (Ii-CS) (11).

The specific association of Ii and Ia during biosynthesis (12, 13) led to the suggestion that Ii has a role in intracellular transport of Ia. However, Ia can be transported to the cell surface in Ii-negative (Ii$^-$) transfectants carrying only Ia cDNA clones (14, 15). Thus, the function of Ii has not been determined. Treatment of antigen-presenting cells with inhibitors of GAG addition (xylosides) interferes with their ability to present antigen to T cells, suggesting that Ii-CS may have a specialized role in helper-T-cell recognition (16, 17). In order to examine the role of Ii-CS more specifically, and to understand the biochemistry of Ii biosynthesis and GAG addition, we made a site-specific mutation in the predicted site of GAG addition in Ii. The biosyntheses of wild-type and mutant Ii were compared following transfection into Ia$^+$ or Ia$^-$ cell lines.

MATERIALS AND METHODS

Reagents. Monoclonal antibody reagents were In-1, a rat anti-mouse Ii reagent (18) obtained from G. Hämmerling (Deutches Krebsforschungszentrum, Heidelberg); MAR-18, a mouse anti-rat κ-chain antibody (ATCC TIB 219) (19) used to bind In-1 immune complexes to protein A; and MKD6, a mouse anti-I-A^d reagent (20) derived by P. Marrack and J. Kappler (National Jewish Hospital, Denver).

Site-Directed Mutagenesis. The mismatched-primer-extension method (21) of *in vitro* mutagenesis was performed on a 1.3-kilobase full-length cDNA insert from pcEXV-mIi3 (A. Sant, R. Germain, and J.M., unpublished work) subcloned into phage vector M13mp9 (22). After second-strand synthesis and ligation, nuclease S1 digestion of unprimed template DNA, and transfection into *Escherichia coli* JM101, plaques were screened by hybridization to the mutagenic oligonucleotide. Mutated phage were replated and plaque-purified. The DNA sequence of the entire Ii mutant coding region was determined (23). The insert was transferred into the cDNA expression vector pcEXV-gpt (F. Ronchese and R. Germain, unpublished), a modification of pcEXV-1 that contains the *E. coli* xanthine (guanine) phosphoribosyltransferase gene.

DNA-Mediated Gene Transfer. Transient transfection into COS-7 cells (10^6 cells per 100-mm dish) was performed with plasmid DNA (2 μg/ml) and DEAE-dextran (500 μg/ml), followed after 3–4 hr by 20% glycerol shock (24). Transiently transfected cells were harvested after 48–60 hr and either solubilized directly for immunoblotting or radiolabeled and solubilized for immunoprecipitation. In previous studies (14, 25), flow microfluorimetry had shown that typically 10–20% of COS-7 cells expressed the surface products of transfected genes.

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Abbreviations: GAG, glycosaminoglycan; Ii, invariant chain; Ii-CS, invariant chain bearing chondroitin sulfate; NEPHGE, nonequilibrium pH gradient electrophoresis.
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Stable transfection of BALB/c mouse 3T3 cells was carried out using calcium phosphate-precipitated DNA (26). A BALB/c 3T3 cell transfectant, BADH3.1, originally called BdH3.1 (14), that expressed cell surface I-A^d α and β chains but not endogenous Ii was transfected by overnight incubation with precipitated purified plasmid DNA (10 μg/ml) containing the wild-type or the mutated Ii cDNA clones inserted into pcEXV-gpt. After incubation for 24 hr in fresh medium, the cells were selected in medium containing mycophenolic acid (6 μg/ml), hypoxanthine (15 μg/ml), and xanthine (250 μg/ml). Clones were isolated and screened for Ii expression by RNA blot hybridization and immunoblot analysis. Stable transfected clones bearing wild-type Ii (BADH3.1Ii1.4) or mutated Ii (BADH3.1Ii-ala201-1.2) were obtained. A previously obtained transfected cell line (BIi3.7) bearing only the wild-type Ii gene and not $\alpha\beta$ was used for some controls.

Radiolabeling and Gel Electrophoresis. Transfected cells were metabolically labeled with [^{3}H]leucine or [^{35}S]methionine by standard techniques. Radiolabeling with $^{35}SO_4^{2-}$ employed sulfate-free medium as described (9). Lysates of radiolabeled or unlabeled cells were prepared and treated as previously noted (9). Protein blotting from 10% acrylamide gels was performed (27), and Ii protein was identified on blots by means of the monoclonal anti-Ii reagent In-1. Two-dimensional electrophoretic analysis (28) was performed using a 3:1:1 ratio of pH 5–8, pH 8–9.5, and pH 3–10 Ampholines (LKB) for nonequilibrium pH-gradient electrophoresis (NEPHGE). First-dimension NEPHGE gels were run at 400 V for 4 hr, and $NaDodSO_4$/polyacrylamide gels were used for the second dimension.

RESULTS

All chondroitin sulfate GAGs thus far described are attached to protein by an O-glycosidic linkage between xylose and serine (29–31). Analysis of synthetic peptides containing putative GAG-acceptor sites established that xylosyltransferase preferentially recognizes Ser-Gly sequences (32). Attachment of GAG in several proteins has been localized to Ser-Gly sites by direct evidence (33, 34). Examination of the murine Ii DNA sequence (35, 36) encoding the predominant 215 amino acid (M_r 31,000) form of Ii showed two Ser-Gly sites at Ser-132 and Ser-201. Since the Ii protein has reverse polarity in cell membranes (37), both sites are in the presumed extracellular domain of the molecule. The Ser-132 site is close to the sites for attachment of N-linked oligosaccharides (Asn-113 and Asn-119) and is not conserved in the human Ii cDNA sequence (38). Because the human Ii-CS has molecular properties comparable to those of murine Ii-CS (39), lack of conservation made the Ser-132 site a less attractive acceptor candidate. We concluded that Ser-201 was the most likely candidate for GAG addition.

To directly test whether Ser-201 was the initiation site for the GAG in the murine Ii chain, a single conservative mutation (serine to alanine) was introduced into the Ii cDNA clone pcEXV-mIi3 (Fig. 1). Because this cDNA clone had been found to direct the biosynthesis of both Ii and Ii-CS after DNA transfer into an Ia^-Ii^- cell line (A. Sant, R. Germain and J.M., unpublished work), we expected to analyze the effect of the mutation by transfecting it into mammalian cells. The mutant Ii construct was therefore transiently transfected into COS-7 cells in parallel with a wild-type construct. Immunoblot analysis of the lysates from the transfected cells indicated that the mutation did not reduce the synthesis of the Ii core protein (Fig. 2*A*). However, when the cells were radiolabeled with [^{35}S]sulfate to detect the addition of the sulfated GAG, immunoprecipitable Ii-CS was detected only in the cells transfected with the wild-type construct and was completely absent in cells transfected with the mutant construct (Fig. 2*B*). Because DNA sequence analysis of the mutant had revealed only the single nucleotide change predicted, this result indicated that the Ser-201 residue is necessary for the addition of the GAG side chain.

It was possible that the mutation caused a structural change in Ii resulting in its retention in the endoplasmic reticulum. In this event, GAG addition, a Golgi process, could not occur even if the site of GAG addition were intact. To determine whether the mutant form of Ii was transported normally through the Golgi, [^{3}H]leucine-labeled Ii precipitates from transfected COS-7 cells were analyzed by two-dimensional electrophoresis. Fig. 3 demonstrates that Ii produced by both the wild-type and mutant constructs is processed to the same level of charge heterogeneity. This suggests that processing of oligosaccharides, terminal sialylation, and any other modifications conferring charge heterogeneity are equivalent for both the Ser-201 and Ala-201

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Human      -   -   Pro -   -   -   -   -   -   -
Mouse      Glu Asp Leu Ser Ser Gly Leu Gly Val Thr
           GAA GAC CTA TCT TCT GGC CTG GGA GTG ACC
               [--- --- --- G-- --- ---]
Ala-201    -   -   -   -   Ala -   -   -   -   -
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FIG. 1. Mutagenesis of the candidate GAG-acceptor site in mouse Ii. The mouse nucleotide sequence encoding amino acids 197–206 is shown on the center line with the corresponding mouse protein sequence and the conserved human protein sequence above it. The synthetic oligonucleotide utilized in the mutagenesis is shown bracketed below the nucleotide sequence. The resulting amino acid sequence of the Ii Ala-201 mutation is displayed on the bottom line.

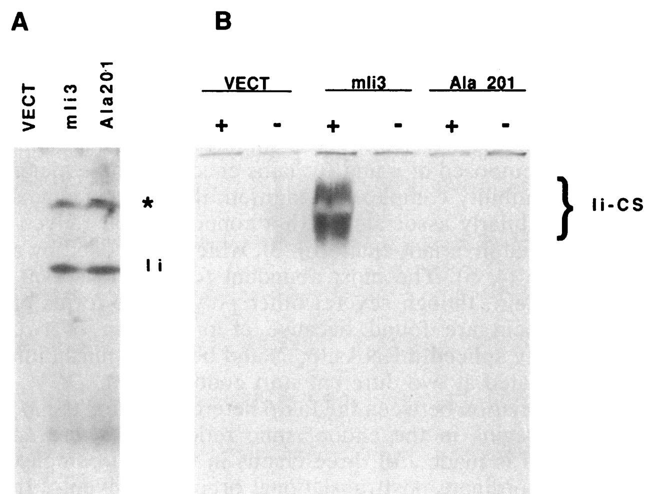

FIG. 2. Analysis of Ii and Ii-CS synthesis in COS-7 cells transiently transfected with wild-type or mutant Ii genes. (*A*) Immunoblot analysis of lysates of COS-7 cells transiently transfected with the pcEXV-3 vector (VECT), the wild type Ii cDNA clone pcEXV-mIi3 (mIi3), or the site-specific mutant pcEXV-mIi3-Ala201 (Ala201). The M_r 31,000 Ii core protein (Ii) is present in equivalent amounts in cells transfected with the wild-type and mutant Ii cDNA clones and not present in cells containing the vector alone. The asterisk marks the position of the Ii dimer typically seen in the absence of reduction. (*B*) Immunoprecipitates made from lysates of [^{35}S]sulfate-radiolabeled COS-7 cells transfected with vector alone (VECT) or with wild-type (mIi3) or mutant (Ala201) Ii cDNA. Immunoprecipitates were made with the anti-Ii reagent In-1 (+) or with a control irrelevant (anti-dinitrophenyl) monoclonal reagent (−). The proteoglycan form of Ii (Ii-CS) was only present in cells transfected with the wild-type cDNA clone.

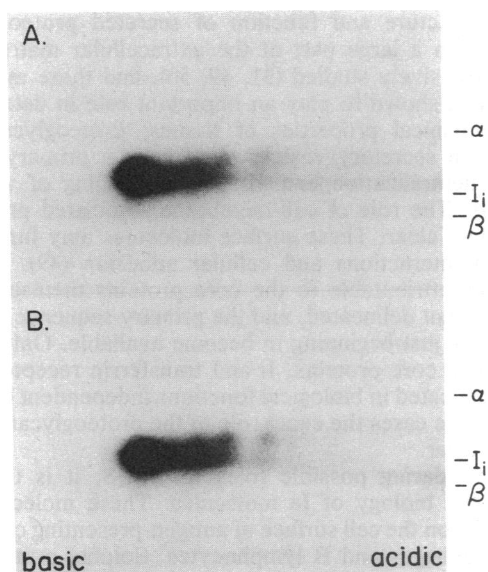

FIG. 3. Two-dimensional (NEPHGE–NaDodSO$_4$/PAGE) analysis of Ii precipitates made from COS-7 cells transfected with wild-type or mutant Ii cDNA. The wild-type (*A*) and mutant (*B*) Ii gene products are equivalently heterogeneous in charge. The presence of the processed forms of Ii implies that both gene products are transported and processed in the Golgi. The marked migration positions of α, Ii, and β were obtained from a control immunoprecipitate run side-by-side in the second-dimension gel.

forms of the Ii protein and, hence, that both forms move through the Golgi and undergo similar processing except for GAG addition.

To examine the ability of the mutant Ii to interact with Ia molecules, stable transfectants of BALB/c 3T3 cells expressing Ia α and β chains but not Ii (BADH3.1) (14) were supertransfected with the wild-type and mutant constructs. As in the COS-7 system, precipitation with the monoclonal anti-Ii reagent (Fig. 4, lanes 1, 3, and 5) indicated that Ii protein was produced in transfectants containing either wild-type or mutant Ii chain (Fig. 4*A*) but that the Ii-CS form of Ii was generated only in the cells expressing the wild-type Ii chain (Fig. 4*B*). Immunoprecipitates made with the anti-Ia monoclonal reagent (Fig. 4*A*, lanes 2, 4, and 6) contained coprecipitated Ii core protein when either the wild-type or the mutant Ii gene was present. As would then be expected, Ii-CS was coprecipitated with Ia only in cells expressing the GAG-bearing, wild-type Ii (Fig. 4*B*). Two-dimensional electrophoretic analysis of immunoprecipitates (Fig. 4*C*) showed that the coprecipitating M_r 31,000 protein is truly Ii and corroborates the finding (Fig. 3) that both wild-type and mutant Ii proteins are equivalently transported through the Golgi apparatus. These two-dimensional gels also show that both the mutant and wild-type Ii molecules give rise to the M_r 25,000 Ii fragment (arrowheads), and further support the conclusion that these products are equivalently processed by the cell. The data indicate that the Ii Ala-201 mutation is specific for GAG attachment and that it does not appear to otherwise affect the processing of Ii or its assembly with Ia.

DISCUSSION

It was predicted that Ii core-protein sequence would contain a single acceptor site for chondroitin sulfate GAG. To specifically and directly identify the site for GAG attachment, we constructed a site-directed mutation in the mouse Ii cDNA that changed the candidate Ser-201 acceptor site to an alanine. Comparison of the expression of the mutant and wild-type Ii proteins after transfection into COS-7 or BALB/c 3T3 cells showed that the mutation completely eliminated the addition of GAG to Ii. If the other Ser-Gly at positions 132–133 also had been used as an attachment site for GAG, we would have expected to find an intermediate-size form of Ii-CS produced in the cells transfected with the

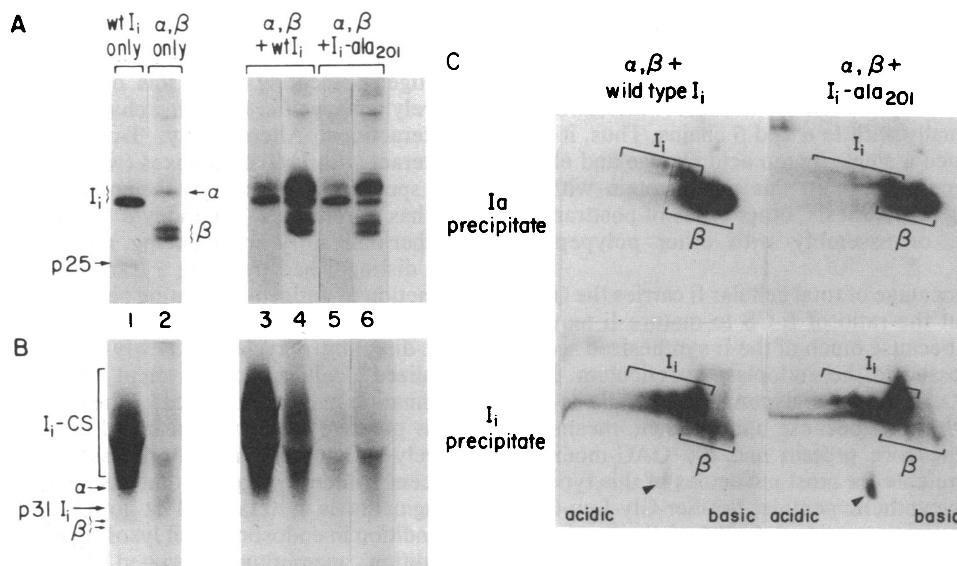

FIG. 4. Analysis of mutant and wild-type Ii assembly with Ia in stably transfected cells. Immunoprecipitates were derived from [^{35}S]methionine- (*A*) or [^{35}S]sulfate- (*B*) labeled cell lysates. BALB/c 3T3 cells transfected with wild-type Ii alone (BIi3.7, lanes 1) or with Ia αβ alone (BADH3.1, lanes 2) are shown as controls. The test cells are Ia αβ transfectants supertransfected with wild-type Ii cDNA (BADH3.1Ii-1.4, lanes 3 and 4) or mutant Ii cDNA (BADH3.1Ii-ala201-1.2, lanes 5 and 6). Precipitates made with anti-Ii (lanes 1, 3, and 5) show that [^{35}S]methionine-labeled Ii is equivalently precipitated from cells carrying wild-type or mutant cDNA but that the GAG-bearing Ii-CS form is precipitated only from the [^{35}S]sulfate-labeled cells carrying the wild-type Ii. Precipitates made with anti-Ia (lanes 2, 4, and 6) show that the mutant Ii chain associates with αβ as well as the wild-type does. (*C*) NEPHGE analysis of immunoprecipitates comparable to those of *A* verifies that the M_r 31,000 band seen on the one-dimensional gels is Ii and is processed to acidic forms. Under the NEPHGE conditions chosen, α is not resolved. Arrowheads indicate the M_r 25,000 polypeptide that coprecipitates with Ii and may be a cleavage product of the M_r 31,000 form of Ii.

mutant cDNA clone. These data bear out the prediction that mouse Ii contains only a single GAG-attachment site, at Ser-201.

Studies that employed partially purified xylosyltransferase and synthetic peptide acceptors (32) had suggested that the most-favored acceptor amino acid for xylose, the GAG-initiating monosaccharide, is serine followed in sequence by a glycine. The Ser-Gly acceptor site has been verified directly in two instances, for a heparin sulfate proteoglycan (33) and a dermatan sulfate proteoglycan (34). At least one proteoglycan with a high GAG/protein ratio contains multiple contiguous attachment sites and has a repeating Gly-Ser sequence (40, 41). Other proteoglycans with a low GAG/protein ratio are paucisubstituted by GAG, including fibroblast chondroitin sulfate/dermatan sulfate proteoglycan of M_r 40,000 (42), a melanoma-associated chondroitin sulfate proteoglycan (43), the heparin sulfate proteoglycan form of the transferrin receptor (44), and the chondroitin sulfate proteoglycan form of Ii. Their core-protein structure, where it is known, shows a limited number of noncontiguous Ser-Gly sites that are potential acceptors for GAG. Comparison of neighboring amino acids around potential Ser-Gly addition sites has generated several hypotheses about the characteristics of GAG-acceptor sequences (40, 45). Experimental evidence for the importance of various neighboring residues in the region of Ser-Gly acceptor sites has been obtained by analysis of *in vitro* xylosyltransferase activity (46) on synthetic peptide substrates (32, 45). The neighboring sequence in the site we identified in murine Ii is consistent with the data identifying relevant residues in acceptor peptides.

The maturation of N-linked oligosaccharides of Ii (13, 47) and the addition of O-linked oligosaccharides (48) produces increasingly more acidic forms of M_r 31,000 Ii. The pattern of these acidic forms of Ii in the transfectants showed that, despite the failure of GAG addition, processing conferring charge heterogeneity on mutant Ii paralleled that found in the wild-type protein. This suggests that the failure of Ii Ala-201 to produce Ii-CS was not due to the inability of the mutant protein to be transported through the Golgi apparatus. In addition, analysis of the stable Ia$^+$ transfectants demonstrated that this mutation did not affect the ability of Ii to complex normally with Ia α and β chains. Thus, it was possible to introduce a single amino acid change and eliminate the GAG acceptor site in this core protein without severely disturbing its synthesis, other types of posttranslational processing, or assembly with other polypeptide chains.

Only a small percentage of total cellular Ii carries the GAG side-chain (11), but the ratio of Ii-CS to mature Ii may be somewhat higher, because much of the Ii synthesized seems to remain unprocessed in the endoplasmic reticulum. It is not clear whether a low proteoglycan/protein ratio is unusual for proteoglycans, because independent means of identifying both the core protein and the GAG-modified product are not available for most molecules of this type. It is interesting that a synthetic peptide, Ser-Ser-Gly-Leu-Gly, that corresponds to amino acids 200–204 of the murine Ii sequence and contains the GAG-addition site, is only marginally active as an acceptor peptide in the xylosyltransferase assay (M. Klinger, L. Rodén, and S.E.C., unpublished data). These data could indicate that addition of xylose to Ii is somewhat inefficient and that the ratio of Is-CS to Ii is low partly for this reason. Alternatively, GAG addition may be the terminal event in the processing of all Ii molecules, and the small amount of Ii-CS present in cells may result from a short half-life for this form (11). Detailed analysis of the transfectants containing the wild-type and mutant Ii genes may distinguish between these possibilities.

The structure and function of secreted proteoglycans, which form a large part of the extracellular matrix, have been extensively studied (31, 49, 50), and these molecules have been shown to play an important role in determining the mechanical properties of tissues. Proteoglycans that localize in secretory vesicles may have a primary role in charge neutralization and efficient packaging of vesicular contents. The role of cell-membrane-associated proteoglycan is less clear. These surface molecules may function in cell–cell interactions and cellular adhesion (49). Specific functions attributable to the core proteins themselves are generally not delineated, and the primary sequence of these proteins is just beginning to become available. Only two of the known core proteins, Ii and transferrin receptor, have been implicated in biological functions independent of GAG, and in these cases the exact role of the proteoglycan form is not yet clear.

In considering possible roles for Ii-CS, it is useful to review the biology of Ia molecules. These molecules are expressed on the cell surface of antigen-presenting cells such as macrophages and B lymphocytes. Soluble protein antigens taken up by these cells are internalized, denatured and/or degraded into peptides (processed), and displayed (presented) on the cell surface in the context of the Ia molecules. Recent studies (51-54) indicate that a physical complex of Ia and peptide is formed. This complex of surface-expressed processed antigen and Ia is bound by the T-cell antigen receptor, leading to helper-T-lymphocyte activation and release of soluble lymphokines from the T cell. The observations that Ii-CS is associated with Ia during terminal biosynthesis (9-11) and at the cell surface (11) and that interference with the synthesis of Ii-CS is accompanied by loss of antigen-presenting function (16, 17) suggest some possible roles for Ii-CS in Ia biology. The Ii-CS molecule may enhance the binding between antigen-presenting cell and T cell, or it may affect Ia trafficking in ways that influence the association of Ia with processed antigen. In considering either model, it is important to recall that only a small fraction of Ia molecules may be associated with Ii-CS at any one time.

If Ii-CS enhances cellular adhesion between T cells and antigen-presenting cells, this enhancement could be relatively nonspecific, involving charge properties or lectin-type interactions. Alternatively, T-cell surface molecules that interact with Ia/Ii complexes (with or without antigen) may be specifically affected by the presence of the glycan on Ii. If Ii has a role in T-lymphocyte binding, this role could be either obligatory or enhancing, and these alternatives could be distinguished by using a transfection system to test the function of antigen-presenting cells in a quantitative fashion.

Another possibility is that Ii-CS may influence Ia function by directing recycled or newly synthesized Ia to a "specialized" cellular compartment where Ia/antigen complex formation may be influenced. The microenvironment within this putative compartment could either positively or negatively affect Ia binding to processed antigen. For example, recent evidence suggests that association of Ia with antigen fragments is not favored at low pH (54), the prevailing condition in endosomal and lysosomal compartments, and in addition, membrane-associated Ia is known to be very protease-resistant. Thus, Ia may recycle through an acidic and/or proteolytic compartment to clear protein fragments from internalized Ia and provide an opportunity for new associations to take place.

Recycling of Ia has not been well documented, however, although some Ia molecules have been localized to intracellular compartments (55, 56). Quantitative assessment of Ia internalization has shown that, unlike transferrin-receptor recycling, internalization of Ia is slow and affects only 20% or less of surface Ia molecules, and return of the molecules

to the surface has not been verified (J.A.H. and S.E.C., unpublished data). Recycling of Ii itself has not been studied because surface-binding monoclonal reagents are not available. The idea that Ia/Ii-CS may be involved in recycling remains provocative because transferrin receptor, whose recycling properties are well established, shares with Ii the features of reverse membrane polarity and an alternative proteoglycan form. Transferrin receptor is known to recycle rapidly through a monensin-sensitive pathway (57) in which iron is unloaded in an intracellular acidic compartment. Though transferrin receptor has a proteoglycan form, the role of GAG in transferrin receptor recycling has not been investigated. It is possible that the proteoglycan forms of transferrin receptor or Ii traffic differently through the cells than their core-protein forms. It is unknown whether Ia internalization requires Ii or Ii-CS association, or whether a putative subsequent step, return of Ia (with or without new antigen fragments) to the surface of the antigen-presenting cell, might depend on its association with either Ii or extant Ii-CS or on its repassage with Ii through the Golgi. Even if such pathways are minor, they may be important to the function of antigen-presenting cells.

The concept that GAG is useful in antigen-presenting-cell function is consistent with the observation that xylosides, which inhibit proteoglycan synthesis (58), diminish the capacity of spleen cells to serve as antigen-presenting cells (16, 17). However, although the xyloside inhibition data show that proteoglycans play an important role in antigen processing and/or presentation, it is not clear that this finding is due to a specific effect on Ii-CS. The specificity of the mutation that we have described may provide a system to distinguish between the roles of Ii and Ii-CS in the immunobiology of Ia–antigen interactions and T-cell activation and/or in the intracellular transport or recycling of Ia molecules. In addition, this system may provide some insights into the biosynthesis and function of proteoglycan molecules and into specific roles played by GAG in various proteoglycan core proteins that can be engineered to prevent GAG attachment.

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