CD154 Variant Lacking Tumor Necrosis Factor Homologous Domain Inhibits Cell Surface Expression of Wild-type Protein*

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X-linked hyper-IgM (XHIM) syndrome is an immunological disorder resulting from mutations in the CD154 gene. Some mutations occur in splicing sites and result in transcripts encoding wild-type and mutant proteins. These mutants lack the tumor necrosis factor homologous (TNFH) domain and consequently fail to trimerize. Given that the TNFH domain is responsible for trimerization, one may predict that the TNFH mutant cannot participate in the assembly of wild-type CD154. Thus, it was puzzling why these patients exhibit XHIM phenotype, presumably resulting from a lack of functional CD154. One possibility is that the TNFH mutant exhibits a dominant negative effect over the wild-type protein. To investigate this, we coexpressed the wild-type protein and a TNFH mutant and examined the biochemical and functional properties of the resulting CD154 products. We demonstrate that despite the lack of the TNFH domain, the TNFH mutant can associate with the wild-type protein. Furthermore, such an association compromises the ability of the wild-type protein to mature onto the cell surface. These results provide a mechanism for the defect of CD154 in XHIM patients producing both wild-type and TNFH variants and suggest that besides the TNFH domain, the stalk region participates in the assembly of CD154 trimers.

CD40 ligand (CD154) is a type II membrane protein expressed primarily on activated T cells. The interaction of CD154 with its receptor, CD40, is critical for the functions of T helper cells to induce differentiation, proliferation, and Ig isotype switching in B cells (for review see Refs. 1 and 2). The CD154 gene, located at Xq2.6–2.7, spans over 12 kilobase pairs and consists of five exons (3). The first exon encodes the cytoplasmic region, the transmembrane domain, and 6 amino acids of the extracellular domain. The second and third exons encode the extracellular stalk region. The fourth and fifth exons encode the C-terminal 147 amino acids (3), which share a limited homology with other members of the TNF family and is called the TNF homologous (TNFH) domain. The x-ray structure of the CD154 TNFH domain reveals that it contains a sandwich-like fold of two β sheets with jelly roll or Greek key topology and forms a trimer similar to that seen in TNF and lymphotixin-α (4). Given the facts that for CD154, the TNFH domain alone is capable of forming trimers (4, 5) and that deletion mutants missing a major portion of this domain did not trimerize (6), it appeared that the TNFH domain is necessary and sufficient for the assembly of trimeric CD154 protein.

Mutations in the CD154 gene can prevent the expression of functional CD154 protein, which lead to an immunodeficiency characterized by an elevated IgM and low IgG and IgA levels in serum called X-linked hyper-IgM (XHIM) syndrome (7–11). Over 70 unique mutations in the CD154 gene have been identified in more than 100 patients (12). These mutations are very heterogeneous and include insertions, deletions, and point mutations. Thus, it is conceivable that the underlying mechanisms responsible for the functional defects of CD154 protein in these patients are quite different (6, 13). Because the CD154 gene is X-linked, each cell from normal individuals or XHIM patients makes a single species of CD154-encoding transcript. However, in some XHIM patients mutations in the donor splicing sites (see Ref. 13 and references therein) or the acceptor splicing sites (14) lead to generation of multiple species of mRNAs consisting of the wild-type and one or more aberrantly spliced transcripts within a single cell. In each of these cases, the mis-spliced mRNA transcript encodes a variant of CD154 protein lacking either a major portion of, or the entire, TNFH domain (13, 14). Because the TNFH domain appears to be responsible for the assembly of trimeric CD154 protein, variants lacking the TNFH domain (TNFH(−) mutant) were predicted not to participate in the assembly of CD154 trimers and therefore not affect the functional activity of the wild-type protein. Thus, it is unclear why these patients exhibit the XHIM syndrome, presumably resulting from a lack of functional CD154 protein. One possibility, which may explain this seemingly conflicting observation, is that the TNFH(−) mutant may interact with the wild-type protein and affect the assembly of the functional trimeric wild-type protein. To directly test the effect of a TNFH(−) mutant on CD154 function, we coexpressed wild-type protein and a TNFH(−) mutant and analyzed the biochemical and functional properties of the CD154 protein products. Our data revealed that the mutant protein still interacts with the wild-type protein despite the lack of the TNFH domain, thus defining a previously unrecognized structural element that contributes to CD154 trimer assembly.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Ig Fusion Protein—BJAB, a human B cell line, was a gift from Dr. George Mosialos (Harvard Medical School) and was maintained in RPMI 1640 medium supplemented with 50 units/ml penicillin and 50 μg/ml streptomycin, 10% heat-inactivated fetal bovine serum, and 4 mM glutamine. 9E10 monoclonal antibody was produced by guest on July 24, 2018 from http://www.jbc.org/ Downloaded on July 24, 2018

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§ The abbreviations used are: TNF, tumor necrosis factor; TNFH, TNF homologous; XHIM, X-linked hyper-IgM; PAGE, polyacrylamide gel electrophoresis; FACS, fluorescence-activated cell sorter.

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TNFH(−) CD154 Variant

RESULTS

TNFH(−) Mutant Is Associated with Wild-type CD154—To investigate the effect of TNFH(−) mutant on the expression of wild-type protein, lysates of metabolically labeled COS7 cells cotransfected with cDNAs encoding these proteins were analyzed by immunoprecipitation using CD154-specific antibodies. Fig. 1 shows that when wild-type CD154 was expressed alone, immunoprecipitates of anti-N-terminal peptide antiserum, Rb784 (lane 3), and anti-CD154 mAb, 5c8 (lane 4), contained primarily the full-length p33 protein, some p31 protein, and a small amount of p18. This is consistent with our previous observation (15). These components were not observed in the immunoprecipitates of anti-Myc mAb, 9E10 (lane 1), or control rabbit antiserum (lane 2). When the Myc-tagged TNFH(−) mutant was expressed by itself, a p17 component, corresponding to the mutant protein, was immunoprecipitated by 9E10 and Rb784 but not by 5c8 or control rabbit antiserum (Fig. 1, lanes 5–8). When mutant and wild-type proteins were coexpressed, immunoprecipitates of 9E10 contained not only the p17 Myc-tagged mutant protein but also the p33, p31, and p18 wild-type proteins (lanes 9). Similar protein patterns were found in immunoprecipitates of Rb784 and 5c8 but not for the control serum (lanes 10–12). These results reveal three important findings. First, the mutant protein is stably produced and can be readily detected. Second, the mutant protein, missing the TNFH domain, can associate with the wild-type CD154. Third, at least some of the wild-type protein, while associated with the mutant form, can still interact with 5c8.

Association of TNFH(−) Mutant Protein Reduces the Receptor-binding Activity of the Wild-type Protein in a Dose-dependent Fashion—Because the TNFH(−) mutant lacks the receptor-interacting domain, it is conceivable that the wild-type protein associated with the mutant protein may have a reduced receptor-interacting activity. To examine this in detail, we coexpressed a constant amount of wild-type protein in the presence of a varying amount of the TNFH(−) mutant. Wild-type proteins immunoprecipitated by Rb784 were quite comparable irrespective of the amounts of TNFH(−) DNA used for transfection (Fig. 2, bottom panel) indicating that, under the transfection conditions used, the total amount of the wild-type protein expressed is not affected by the introduction of TNFH(−) mutant. In contrast, the amount of wild-type protein recognized by CD40-Fc fusion protein (Fig. 2, upper panel) or by 5c8 (Fig. 2, middle panel) is inversely related to the amount of TNFH(−) DNA used for transfection. Only a small portion of the total TNFH(−) protein expressed (Fig. 2, bottom panel) was immunoprecipitated by either CD40-Fc (Fig. 2, upper panel) or 5c8 (Fig. 2, middle panel). These results show that the expression of the TNFH(−) mutant affects the receptor- and 5c8-interacting activities of the wild-type CD154 in a dose-dependent fashion.

Effect of TNFH(−) Mutant Protein on the Function of Wild-type CD154—To determine whether the association of TNFH(−) mutant with the wild-type protein affects the ability of the wild-type protein to trigger CD40-mediated functions, BJAB cells were treated with membranes prepared from COS7 cells cotransfected with cDNAs encoding the wild-type and/or
were incubated with 5 x 10^5 BJAB cells in 0.3 ml of culture medium for 24 h. Cells were stained with biotin-labeled antilymphotoxin-α mutant protein reduced the lymphotoxin-α-dependent signaling. Fig. 3 shows that the coexpressed TNFH(−) mutant protein at the ratio indicated (Fig. 3). FACS analysis was used to determine the up-regulated lymphotoxin-α on the surface of BJAB cells, a downstream response to CD154-dependent signaling. Fig. 3 shows that the coexpressed TNFH(−) mutant protein reduced the lymphotoxin-α up-regulated by the wild-type CD154 protein in a dose-dependent manner. When the amount of wild-type cDNA used for transfection is one-third of the mutant cDNA, the functional activity of CD154 was reduced by 80%. We also quantified the CD154 in these membrane fractions by Western blotting analysis using a rabbit antiserum that specifically recognizes the C-terminal peptide of CD154. Results show that 25 to 50% of wild-type CD154 protein is lost when mutant protein was coexpressed (Fig. 3, lower table). These results indicate that the TNFH(−) mutant reduces the functional activity of the coexpressed wild-type, and this effect is dose-dependent for the mutant protein expressed. Because total wild-type CD154 produced is not affected by the coexpressed TNFH(−) mutant (Fig. 2), the reduction of wild-type protein in membrane fractions from cotransfected cells (Fig. 3, lower table) suggests that mutant protein may affect the surface maturation of the wild-type CD154.

**TNFH(−) Mutant Does Not Express on the Cell Surface**—To rule out the possibility that the reduction of functional CD154 in membrane fractions may be because of a selective loss of the wild-type protein caused by the coexpressed TNFH(−) mutant protein during membrane fractionation processes, we examined CD154 proteins on the surface of intact cells. COS7 cells cotransfected with cDNA encoding for wild-type and/or TNFH(−) mutant were rinsed with phosphate-buffered saline to remove the cell debris and unattached cells and were labeled with biotin in situ. Cells were then lysed, centrifuged to clarify particulate, and immunoprecipitated (i.p.) with CD40-Fc, Rb779, Rb784, or 9E10. Immunoprecipitates were subjected to a 10–20% SDS-PAGE, transferred to a nitrocellulose sheet, and probed with horseradish peroxidase-conjugated streptavidin.

**Fig. 3. Up-regulation of lymphotoxin-α.** UV-irradiated membranes from transfected COS7 cells containing 65 μg of total protein, along with varied amounts of CD154 (Fig. 3, column 4, lower table), were incubated with 5 x 10^5 BJAB cells in 0.3 ml of culture medium for 24 h. Cells were stained with biotin-labeled anti-lymphotoxin-α mAb NC2, followed by phycoerythrin-labeled streptavidin, and fixed with 1% paraformaldehyde prior to FACS analysis. Ratios of cDNAs used for transfections are indicated in the table. Mean fluorescence intensities (MFI) are plotted.

**Fig. 4. Cell surface expression of CD154 proteins.** COS7 cultures transfected with cDNA(s) encoding for wild-type and/or TNFH(−) mutant were rinsed with phosphate-buffered saline to remove the cell debris and unattached cells and were labeled with biotin in situ. Cells were then lysed, centrifuged to clarify particulate, and immunoprecipitated (i.p.) with CD40-Fc, Rb779, Rb784, or 9E10. Immunoprecipitates were subjected to a 10–20% SDS-PAGE, transferred to a nitrocellulose sheet, and probed with horseradish peroxidase-conjugated streptavidin.

**Fig. 5. Schematic for how surface maturation of wild-type CD154 is compromised by the coexpressed TNFH(−) mutant.** When wild-type proteins (in blue) and the TNFH(−) proteins (in red) are coexpressed, trimeric wild-type protein can be found associated with or without the TNFH(−) mutant. Wild-type protein not complexed with TNFH(−) mutant are transported to the cell surface, and some are in heterotrimeric forms (15). Wild-type CD154 associated with mutant protein are retained inside the cell. Although the stoichiometry of TNFH(−) mutant to wild-type CD154 may be varied in these complexes, it is clear that at least some of the TNFH(−) mutant-associated wild-type CD154 proteins retain their receptor-interacting activity.
DISCUSSION

To study the effects of the TNFH(−) mutant on the wild-type CD154, we coexpressed a truncated form of CD154 lacking the TNFH domain and wild-type CD154 in COS cells and examined their biochemical and functional properties. Our results indicate that the mutant and wild-type proteins are expressed efficiently, but by forming a complex with the wild-type protein, the mutant protein prevents the cell surface expression of the wild-type CD154. This observation is significant in at least two aspects. First, it indicates that whereas patient’s cells are capable of making both wild-type and TNFH(−) mutant CD154 protein, they may fail to produce sufficient functional CD154 on the surface of activated T cells, consequently leading to the hyper-IgM syndrome. Second, it suggests that, in addition to the TNFH domain, other region(s) of the CD154 structure may contribute to the assembly of CD154 trimers.

Because CD154 is transiently expressed on the cell surface of activated T lymphocytes, it is conceivable that the reduced level of the functional CD154 expressed on the surface, caused by association with the TNFH(−) mutant protein, may be too low to trigger a productive signaling through CD40 and consequently lead to hyper-IgM syndrome. As the inhibitory effect of the TNFH(−) mutant requires the physical association of the wild-type CD154 with the mutant protein, the extent of the inhibitory effect is directly dependent on the stoichiometry of the mutant versus wild-type proteins. This is in line with the observation that patients carrying this mutation seem to have varied clinical syndromes. The low surface expression of functional CD154 protein is reminiscent of the biochemical properties of two other CD154 mutations associated with XHIM, the M36R and G38R, reported recently (6). Thus, these splicing mutations, along with the M36R and G38R, can be categorized as a subgroup of XHIM. Mutations in this subgroup all lead to low or no cell surface expression of the otherwise functional proteins, and the inability to produce sufficient functional CD154 protein on the surface of activated T lymphocytes ultimately leads to the hyper-IgM syndrome.

Importantly, our results clearly show that the TNFH(−) mutant is capable of association with the wild-type CD154 protein, and by doing so it blocks the latter from being expressed on the cell surface. It should be mentioned that this TNFH(−) CD154 variant was reported to reduce the functional activity of the coexpressed wild-type protein by forming complexes with the latter (17). However, it was concluded that the functional defect is because of a reduced receptor-interacting ability of the mutant-wild-type CD154 complexes expressed on the cell surface (17). In contrast, we show that the mutant-wild-type complexes are retained inside the cell and therefore do not participate in any functional CD154 activity. Because only wild-type protein not associated with mutant protein can be expressed on the cell surface we reason that the reduced CD154 functional activity is simply due to a reduced abundance of the wild-type CD154 protein present on the surface for interaction with CD40. One possibility for the discrepancy between our results and the results published earlier is related to the experimental procedures for biotin labeling of cell surface proteins. Rather than detaching the transfected cells and performing the surface biotin labeling in cell suspensions, we maintained the transfected cells on the culture plate throughout the labeling process. It is conceivable that the detaching step may break some cells and result in biotinylation of intracellular components.

In addition, our observation indicates that the stalk may participate in the assembly of CD154. Thus, for CD154 there are at least two structural elements, the TNFH domain and the extracellular stalk region, that can contribute to subunit oligomerization. Although our data suggests that the interactions between the stalk regions may lead to formation of higher order oligomers of wild-type TNFH(−) mutant complexes (Fig. 5), it is not clear how, during the assembly of the wild-type CD154 in a normal individual, the TNFH-TNFH interaction coordinates with the stalk-stalk interaction to produce uniform trimers and to avoid formation of larger aggregates. Because the stalk regions vary greatly in size and in amino acid sequences among the members of TNF ligand family, it would be important to determine whether the interaction between stalk regions also occurs for other family members. This is particularly relevant to other TNF family members, because their genes are located in somatic chromosomes, and the coexpression of mutant and wild-type proteins is expected within a single cell from heterozygous individuals. It is not clear why the wild-type CD154 protein complexed with the TNFH(−) variant(s) fails to mature onto the cell surface. Although more extensive studies are warranted to understand the molecular mechanism of the intracellular retention, our observation implicates an ectopic introduction of the non-TNFH region of CD154 as a therapeutic opportunity to effectively block the expression of functional CD154 on the cell surface.

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