CD154 Variants Associated with Hyper-IgM Syndrome Can Form Oligomers and Trigger CD40-mediated Signals*

(Received for publication, July 20, 1999)

Ellen Garber‡, Lihe Suš, Barbara Ehrenfels‡, Mike Karpusas‡, and Yen-Ming Hsu‡¶

From the §Department of Protein Engineering and ¶Department of Molecular Genetics, Biogen, Inc., Cambridge, Massachusetts 02142

X-linked hyper-IgM syndrome is a rare immunodeficiency disorder resulting from mutations in the gene encoding the CD40 ligand (CD154) molecule. These mutations are very heterogeneous, ranging from a single point mutation to a large deletion in the open reading frame. To investigate the molecular mechanisms that are responsible for the functional defect of these mutants, we examined the biochemical properties of 14 hyper-IgM-related CD154 mutant proteins produced by transient expression in COS7 cells. We show that deletion mutants lacking a significant portion of the tumor necrosis factor homologous domain cannot be stably produced. In contrast, point mutants can be detected as oligomers. Surprisingly, gene products of two point mutants, Thr-211 → Asp and Met-36 → Arg, can bind to the receptor, CD40. For Thr-211 → Asp variant, it is comparable to the wild-type protein in its surface expression level, biochemical structure, and functional activities. Thus, it appears that this mutation is a polymorphism of CD154 gene. For Met-36 → Arg variant, although it is interactive with CD40, it has a much lower surface expression level than wild-type protein. We propose that Met-36 → Arg mutant represents a prototype of a defective CD154 family whose low cell surface expression of intrinsically active protein is simply insufficient to trigger productive signals through CD40.

Hyper-IgM syndrome is a rare immune disorder characterized by low serum levels of IgG, IgA, and IgE accompanied by normal or elevated levels of IgM (1, 2). Mutations in the gene encoding CD154 have been found in a significant number (3–7), but not all, of the hyper-IgM patients indicating that in some individuals, this disorder can be non-X-linked as well (8–10). CD154 is a type II membrane glycoprotein expressed on the surface of activated T lymphocytes (11, 12) primarily as hetero-trimers (13). The C-terminal half of CD154 is highly homologous to the extracellular domain of TNFα (14) and is thought to be responsible for the trimerization of CD154 (3, 14–17). Indeed, the extracellular portion of CD154, consisting of essentially the TNF homologous (TNFH) region, can be produced as a homotrimERIC molecule by T lymphocytes as well as in various expression systems and is functionally capable of interacting with its receptor, CD40 (14, 18–21). Interaction of CD154 with CD40 expressed on the surface of B cells is necessary for the immunoglobulin isotype switching and for B cell differentiation (22–24). To date, more than 75 mutations of the CD154 gene have been identified (1, 25). As the mutations are very heterogeneous, the underlying mechanism responsible for the functional defect is likely to vary from patient to patient (25, 26). Some mutations cause defective splicing or early translational termination that result in protein products missing a major portion of the extracellular domain (3, 5, 27–31). In these cases, the absence of functional ligand is probably due to the fact that these proteins are prematurely terminated or misfolded and are likely to undergo prompt proteolysis after translation. In contrast, other mutations are more subtle and lead to one or two amino acid substitutions in the protein products (3–7, 27, 31, 32). How these point mutants lead to functional defects of CD154 is not clear. Several possibilities exist ranging from effects on folding or expression to subtle structural alterations that are sufficient to compromise the receptor binding activity of the mutant proteins. The ability to distinguish these possibilities for each of these point mutants is critical in the analysis of the receptor binding site(s) on CD154. Indeed, up to 20 point mutants identified in hyper-IgM patients were previously categorized into three classes based on the structural information from x-ray crystallography of CD154 (14) and on the TNF-β-based modeling of CD154 (33). Based on this categorization, class I and class II mutations, affecting the packing of core regions in monomers and the intersubunit interactions within trimers, respectively, were predicted to fail in the assembly of CD154 trimers. Class III mutations were thought to result in trimers that are properly assembled but are incapable of interacting with CD40 (33). To better understand the mechanism for functional defect in CD154 mutants, we expressed 14 hyper-IgM-related CD154 mutants, including 9 point mutants from the three classes categorized previously (33), in the COS7 cells and analyzed the biochemical properties of these proteins with respect to the following two questions: first, can these mutant proteins be produced and assembled into oligomers; and second, can these mutant proteins interact with the receptor, CD40?

Here we show that at least three different mechanisms account for lack of function of these mutants. The data provide further insights into how the genetic defects associated with hyper-IgM syndrome translate into functional defects.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Ig Fusion Protein—The BJAB, a human B cell line, was a gift from Dr. George Masialoa (Harvard Medical School, Boston, MA). The human B lymphocyte line, 2G6, was obtained from Drs. Seth Lederman and Len Chess (Columbia University, New York, NY). BJAB and 2G6 cells were maintained in RPMI supplemented with penicillin and streptomycin, 10% heat-inactivated fetal bovine serum, 4 mM glutamine. TRAP1 antibody was purchased from PharMingen (San Diego, CA). Procedures for engineering, producing,
and purifying CD40-Fc fusion protein, humanized 5c8, and rabbit polyclonal antibodies, rabbit anti-peptide antiserum, Rb779 and Rb784, were described previously (13).

Transient Expression of Wild-type and Mutant CD154 Proteins—We constructed a cDNA-derived plasmid carrying the full-length human CD154 cDNA flanked by minimal 5'- and 3'-untranslated sequences. Mutants of CD154 were made by unique site elimination mutagenesis of the wild-type CD154 plasmid template using a kit according to the manufacturer’s recommended protocol (Amersham Pharmacia Biotech). In designing the mutagenic primers, if a desired mutation did not produce a restriction site change, a silent mutation producing a restriction site change was introduced into an adjacent codon to facilitate identification of mutant clones following mutagenesis. To avoid aberrant codon usage, substituted codons were selected from those occurring at least once elsewhere in CD154 cDNA. Mutations were confirmed by DNA sequencing through restriction fragment analysis of the cDNA. Wild-type and mutant cDNAs were subcloned or reconstructed from restriction fragments with confirmed sequence into a unique SalI site in the plasmid pUC18. Each plasmid containing the introduced restriction site change was reconfirmed in the expression vector for each CD154 mutant. COS7 cells were transfected with supercoiled plasmid DNA using LipofectAMINE (Life Technologies, Inc.) following the manufacturer’s procedure. Plasmid DNA lacking the CD154 was used as a negative control, and a vector containing wild-type CD154 was used as a positive control in all experiments. Expression of CD154 and its variants were analyzed on transfected cells harvested 72 h after transfection. Metabolic labeling, biotinylation of cell surface proteins, immunoprecipitation, SDS-PAGE, and Western blotting analysis were performed using procedures described previously (13).

Preparation of Membrane Fractions—COS7 cells were collected 72 h after transfection by scraping with a rubber policeman and pelleted by centrifugation at 500 × g for 10 min. The cell pellet was suspended in homogenization buffer (1 mm EGTA, 20 mm HEPES, pH 6.8), and homogenized using a Dounce tissue grinder (Wheaton, Millville, NJ). Large debris was removed by centrifugation at 1,300 × g for 5 min in an SA600 centrifuge. Membranes were recovered from low-speed supernatant by centrifugation at 150,000 × g for 1 h in an SW41Ti rotor. The pellet was resuspended in homogenization buffer, layered on the top of an 8%/35% sucrose step gradient in homogenization buffer, and centrifuged at 150,000 × g for 1 h. Membrane fractions residing at the 8%/35% interface of the sucrose gradient were collected, diluted with two volumes of homogenization buffer, and pelleted at 200,000 × g for 30 min. The final membrane pellet was resuspended in PBS, pH 7.2, aliquoted, and stored at −80 °C.

NFκB Activation Assay—Approximately 2 × 10⁶ 293 cells in 0.3 ml of culture medium were incubated at 37 °C for 10 min with membrane preparations from transiently transfected COS7 cells. The 293 cells were pelleted by centrifugation at 1000 × g, resuspended in 0.4 ml of 10 mm HEPES, pH 7.9, 1.5 mm MgCl₂, 10 mm KCl, and incubated on ice for 10 min. The cells were then lysed by incubation in a Dounce homogenizer. Membranes were recovered from low-speed supernatant by centrifugation at 150,000 × g for 1 h in an SW41Ti rotor. The cell pellet was resuspended in homogenization buffer, layered on the top of an 8%/35% sucrose step gradient in homogenization buffer, and centrifuged at 150,000 × g for 1 h. Membrane fractions residing at the 8%/35% interface of the sucrose gradient were collected, diluted with two volumes of homogenization buffer, and pelleted at 200,000 × g for 30 min. The final membrane pellet was resuspended in PBS, pH 7.2, aliquoted, and stored at −80 °C.

NFκB Activation Assay—Approximately 2 × 10⁶ 293 cells in 0.3 ml of culture medium were incubated at 37 °C for 10 min with membrane preparations from transiently transfected COS7 cells. The 293 cells were pelleted by centrifugation at 1000 × g, resuspended in 0.4 ml of 10 mm HEPES, pH 7.9, 1.5 mm MgCl₂, 10 mm KCl, and incubated on ice for 10 min. The cells were then lysed by incubation in a Dounce homogenizer. Membranes were recovered from low-speed supernatant by centrifugation at 150,000 × g for 1 h in an SW41Ti rotor. The cell pellet was resuspended in homogenization buffer, layered on the top of an 8%/35% sucrose step gradient in homogenization buffer, and centrifuged at 150,000 × g for 1 h. Membrane fractions residing at the 8%/35% interface of the sucrose gradient were collected, diluted with two volumes of homogenization buffer, and pelleted at 200,000 × g for 30 min. The final membrane pellet was resuspended in PBS, pH 7.2, aliquoted, and stored at −80 °C.

Detection of Lypoxin-α by FACs Analysis—Membrane preparations from transfected COS7 cells were sterilized with UV light for 30 min prior to use in the assay. BJAB cells at 5 × 10⁵ cells/ml were incubated at 37 °C for 24 h with membrane preparations containing wild-type or variants of CD154. Cells were pelleted and washed with FACS buffer (1% fetal bovine serum with 1% sodium azide in PBS, pH 7.2) and resuspended in 200 μl of FACS buffer. Biotin-labeled anti-lyoxin-α, NC2, provided by Jeff Brown at Biogen (34), or a control antibody, MOPC21 (ATCC), was then added and the samples incubated at room temperature for 40 min. Cells were then washed, incubated with phycoerythrin-conjugated streptavidin (Becton Dickinson, San Jose, CA) in the dark for 40 min, washed, and fixed with 1% paraformaldehyde in PBS prior to analysis on a FACScan (Becton Dickinson) using CellQuest software (Becton Dickinson).

Crystallographic Analysis of T211D Mutant Protein—Crystals of the wild-type CD154 mutant T211D were grown in similar conditions and are isomorphous to those of the wild-type protein (14). X-ray diffraction data were collected to 1.95-Å resolution at −165 °C with an R-AXIS II detector (Molecular Structure Corp., Woodlands, TX). The data were processed with BioteX software (Molecular Structure Corp.), resulting in a reduced data set of 10,698 reflections with an R-merge of 6.2%.

RESULTS

To investigate the biochemical properties of wild-type and hyper-IgM variants of CD154, cDNAs encoding these proteins were engineered and transiently transfected into COS7 cells. The resulting protein products were analyzed using four reagents specific for CD154: a CD40-Fc fusion protein; an anti-CD154 mAb, 5c8; a rabbit polyclonal antiserum raised against an N-terminal peptide (residues 1–15) from CD154, Rb784; and a rabbit polyclonal antiserum raised against a C-terminal peptide (residues 247–261) from CD154, Rb779. The CD40-Fc fusion protein, consisting of the extracellular domain of CD40 and a human IgG Fc region, binds to its ligand, CD154 (35–38). 5c8 mAb binds to CD154 and blocks the interaction of CD154 with its receptor, CD40 (23). The Rb784 recognizes specifically the N-terminal 15 amino acid peptide (13) and the Rb779 specifically recognizes the C-terminal peptide of CD154.2 In this manner, the CD40-Fc fusion protein and 5c8 mAb provide readouts for functionally assembled CD154, whereas the anti-peptide antibodies provide non-function-based detection of protein products.

Immunoprecipitation of CD154 Variants—Fourteen CD154 mutants associated with hyper-IgM syndrome were investigated in these studies (Fig. 1). Among these, four are deletion mutants, and eight are point mutants including one double point mutant (S128R/E129G) from which two separate constructs, each containing S128R or E129G, were generated to dissect the contribution from each mutated residue. COS7 cells were transfected with cDNAs encoding the CD154 variants, metabolically labeled with [³⁵S]Met and [³⁵S]Cys, and subjected to immunoprecipitation studies. SDS-PAGE analysis of these immunoprecipitates is shown in Fig. 2. CD40-Fc, 5c8,
Hyper-IgM CD154 Variants

SDS-PAGE Analysis of CD154 Variants

Fig. 2. Immunoprecipitation of metabolically labeled CD154 variants. Cell lysates prepared from 35S-metabolically labeled transfected COS7 cells were immunoprecipitated with CD40-Fc (A), 5c8 (B), rabbit anti-C-terminal peptide antiserum, Rb779 (C), and rabbit anti-N-terminal peptide antiserum, Rb784 (D). Immunoprecipitates were analyzed by a 10–20% gradient SDS-polyacrylamide gel followed by autoradiography. Lane designations reflect construct numbers 1–17 as defined in Table I. M is for molecular weight markers.

Rb784, and Rb779 recognize the wild-type CD154 protein as shown in lane 2 of panels A, B, C, and D, respectively. Consistent with what we described previously (131), immunoprecipitated CD154 contains the full-length 33-kDa protein (p33) and the 31-kDa protein (p31). We also generated a CD154 variant in which the N-terminal 22 amino acids (cytoplasmic domain) were deleted. Lane 3 shows this protein, co-migrating with p31, is recognized by CD40-Fc (panel A), 5c8 (panel B), and anti-C-terminal peptide antiserum, Rb779 (panel D), but not the anti-N-terminal peptide antiserum, Rb784 (panel C). These results are consistent with our previous observation that at least some CD154 is made of heterotrimers containing the full-length p33 protein and the p31 protein, which lacks the cytoplasmic domain. In addition, the intensities of p33 and p31 are slightly less in CD40-Fc and 5c8 immunoprecipitates than those in Rb779 and Rb784 immunoprecipitates, suggesting that not all CD154 protein molecules were fully assembled as trimers.

Among the 14 mutants examined (Fig. 2, lanes 4–17), W140Amber (lane 9), 136Δ(10) (lane 11), and 136Δ(10) (lane 15) showed no detectable protein in any of the immunoprecipitates. Since these three mutations should result in early termination and a protein that is not detectable, while the variant with a modest deletion with the TNF homologous domain, up to 21 amino acid residues, can be produced, assembled, and processed like the wild-type protein.

Protein products of 10 point mutants tested were readily detected by both Rb784 and Rb779 showing that these variants are stably produced (Fig. 2, panels C and D, lanes 4–7, 10, 12–14, 16, and 17; see Table I for identity of mutations). Except for two variants (see below), all of these point mutants failed to interact with CD40-Fc fusion protein indicating that they are functionally inactive (Fig. 2, panels A and B). Since these mutants exhibit the wild-type heterotrimeric p33-p31 pattern and since Rb784 immunoprecipitates also contain the p31 protein (which lacks the N-terminal peptide recognized by Rb784), this result suggests that these variants also form oligomeric structures.

Results of some of the point mutants tested are unexpected. Based on the previous categorization, one may anticipate the class III mutants, Ser-128 → Arg/Glu-129 → Gly, Leu-155 → Pro, and Thr-211 → Asp, but not the class II mutants, Ala-123 → Gly and Ala-235 → Pro, to form properly assembled oligomers (33). Our results suggest that none of these point mutations cause an alteration in the structure of CD154 that is sufficient to disrupt oligomerization of the protein. These results imply that the structural requirement for trimerization of the TNF domain is less demanding than was anticipated previously (14, 33). Results of the immunoprecipitation analysis, also summarized in Table I, indicate that mutations causing minor deletions and one or two amino acid substitutions are likely to be accommodated and have no deleterious effect on the oligomerization of CD154. We infer that the functional defect of point mutants examined is not due to the failure to trimerize but

| Mutations | Total AA | CD40-Fc | 5c8 | Anti-N | Anti-C |
|-----------|---------|---------|-----|-------|-------|
| 1         | 261     | +       | +   | +     | +     |
| 2         | Wild-type | 261     | +   | +     | +     |
| 3         | Cyt(−)   | 239     | +   | +     | +     |
| 4         | A235P    | 261     | −   | +     | +     |
| 5         | G227V    | 261     | −   | +     | +     |
| 6         | E129G    | 261     | −   | +     | +     |
| 7         | L155P    | 261     | −   | +     | +     |
| 8         | Δ116–136 | 240     | −   | +     | +     |
| 9         | W140Amber | 139    | −   | −     | +     |
| 10        | W140G    | 261     | −   | −     | +     |
| 11        | 136Δ(9)  | 138     | −   | −     | +     |
| 12        | A123E    | 261     | −   | −     | +     |
| 13        | T211D    | 261     | +   | +     | +     |
| 14        | M36R     | 261     | +   | +     | +     |
| 15        | 136Δ(10) | 144     | −   | −     | −     |
| 16        | S128R    | 261     | −   | −     | +     |
| 17        | S128R/E129G | 241   | −   | −     | +     |

Numbers in the first column reflect lane designations from Fig. 2. The column marked “Total AA” indicates the total amino acid residues for the predicted translated products. Columns 4–7 summarize the expression data where “+” indicates that protein was detected and “−” that it was not detected in Fig. 2.
Hyper-IgM CD154 Variants

Biochemical Analysis of T211D and M36R CD154 Variants—We were surprised that the Thr-211 → Asp (T211D) and Met-36 → Arg (M36R) mutants were immunoprecipitated by CD40-Fc fusion protein and 5c8 (Fig. 2, lanes 13 and 14). The T211D variant was previously categorized as a class III mutant and was predicted to be inactive for receptor binding (33). The M36R variant, with the mutation occurring within the transmembrane region, was predicted not to be expressed on the cell surface (27). To further characterize these two variants, the T211D and M36R variants, along with the wild-type protein were transiently expressed in COS7 cells. Plasma membranes were prepared from these cells, designated as pmT211D, pmM36R, and pmWT, respectively, and were used for functional analysis. First, we checked the ability of these membranes to up-regulate the expression of lymphotoxin-α (39). As shown in Fig. 3 (panel A), both pmM36R and pmT211D up-regulate lymphotoxin-α as the wild-type CD154 does. Dose-response curves indicate that pmT211D and pmWT have similar specific activities and the pmM36R has a much lower one. Second, we tested the ability of these membranes to activate NFκB in a human B lymphocyte line, 2G6. Fig. 3 (panel B) shows that the pmWT and pmT211D have similar specific activities for activation of NFκB whereas the pmM36R exhibits a lower specific activity, approximately 10% of the pmWT. These results show that functionally, M36R and T211D variants are capable of interacting with CD40. However, pmM36R exhibits a lower specific activity than pmWT or pmT211D.

Biochemical Analysis of T211D and M36R CD154 Variants—Although pmM36R exhibits a lower specific activity in both NFκB and lymphotoxin-α up-regulation assays, the amount of protein products immunoprecipitated by both Rh784 and Rb779 were comparable to the wild-type protein (Fig. 2, panels C and D, lane 14 versus lane 2). Thus, we considered the possibility that the positive charge residue introduced into the transmembrane region may affect the efficiency in maturation of the translated proteins onto the cell surface. To test this possibility, we compared the abundance of CD154 on pmM36R with pmWT and pmT211D by Western blotting using the anti-C-terminal peptide antiserum, Rb779. Fig. 4 (panel A) shows that the pmM36R contains approximately one-tenth of CD154 protein as compared with that in pmWT and pmT211D. It is possible that M36R protein may be selectively lost during the processes of preparing membranes or that the observed M36R protein may reside in the endoplasmic reticulum that was co-fractionated with the plasma membranes. To rule out these possibilities, we performed a cell surface labeling using a non-permeable biotinylating reagent, followed by immunoprecipitation with 5c8, TRA1 (another anti-CD154 mAb), and CD40-Fc fusion protein. Fig. 4 (panel B) shows that the surface-labeled M36R variant is about 10% of the wild-type protein. This approximate quantitation is consistent with the estimate obtained from the Western blot analysis of plasma membranes, indicating that M36R variant indeed has a much lower surface expression. Together with the immunoprecipitation analysis using whole cell lysates, our results show that, although the introduction of the positive charge residue in the transmembrane region does not affect the production and the oligomeric assembly of M36R variant, a majority of the protein produced does not mature onto the cell surface.

For T211D variant, since it is functionally and biochemically comparable to the wild-type protein, we decided to examine its x-ray structure. A soluble form of this variant containing amino acid residues Gly-116 to the C-terminal Leu-261 was produced as described previously (14). The calculated Fobs − Fcalc difference electron density maps showed that there are no changes in the crystal structure of CD154 due to the T211D mutation (data not shown). The electron density for the side chain of the mutated residue is weak, but this is characteristic of the region of the molecule close to the EF loop. Together with the results

Functional assays for M36R and T211D variants. A, up-regulation of lymphotoxin-α. UV irradiated membranes from transfected COS7 cells were incubated with BJAB cells 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. B, NFκB activation assay for CD154 variants. Nuclear extracts were prepared from 2G6 cells treated with COS7 cell membrane expressing CD154, incubated with [32P]labeled double-stranded oligonucleotide probe, and subjected to PAGE on a 2–15% pre-cast Daiichi gel. The position of the retarded NFκB-oligonucleotide complex is indicated at right of the panel.
The biochemical and functional data of the M36R mutant protein are intriguing. As the introduction of positive charged amino acid residue into the transmembrane was thought to be a signal for degradation in the endoplasmic reticulum (40), the expression of this variant was predicted to be abrogated (27). In contrast to this prediction, our results from cell surface labeling experiment clearly show that the M36R mutant protein is present on the cell surface with an abundance approximately 10% of the wild-type protein. Two additional observations support the notion that the M36R mutation is likely to be responsible for the hyper-IgM disorder. First, this mutation has been identified in two unrelated individuals with distinct ethnic backgrounds (27, 31). Second, another CD154 variant carrying a similar mutation located within the transmembrane region, G38R, identified in another hyper-IgM patient (32), exhibits a similar low cell surface expression. Thus, the question becomes: how can a low surface expression of functional M36R variant lead to a hyper-IgM disorder? One possibility is that the density of CD154 on the cell surface may be critical for triggering a productive CD40-mediated signaling. While the M36R variant protein present on the cell surface is functionally capable of interacting with CD40, its lower surface expression may be insufficient to support productive signals. In this regard, M36R mutation can be categorized with a subgroup of common variable immunodeficiency disorders. Regardless of the heterogeneity of the underlying mechanisms, the primary defect within this group is a depressed CD154 expression (41). We cannot formally rule out the possibility that the mechanism of cell surface maturation of M36R variant may be quite different in the transient COS7 cells from that in activated T lymphocytes in vivo. Thus, it is possible that the M36R mutant protein may be completely absent from the surface of patients’ T lymphocytes. Alternatively, it was suggested that, in addition to triggering the CD40-mediated signals, CD154 may have an intrinsic signaling capacity as well (42–45). In this regard, it is conceivable that mutations in the transmembrane region may alter the signaling capacity of the cytoplasmic tail of CD154 and potentially lead to hyper-IgM disorder.

In summary, we show that for CD154, lack of a significant portion of the TNF region results in a lack of protein product, indicating that oligomerization via this region is critical for the stability of this protein. Importantly, all point mutants analyzed formed oligomers and were readily detected by antipeptide antisera. Thus, the functional defects in these variants are due to failure of the assembled proteins to interact with the receptor molecule. Further investigation is necessary to determine if each one of these variants indeed forms heterotrimers on the cell surface as the wild-type protein does. Interestingly, when mutations within the TNF homologous domain were highlighted in a three-dimensional structure of CD154, all, except for T211D, were mapped to the lower half of the trimeric CD154 (Fig. 5). While variants carrying mutations within the lower half of CD154 are not interactive with CD40, T211D variant, whose mutation is mapped to the upper half of the trimeric protein that is topologically quite distal from others mutations, appears normal in its ability to interact with CD40. Thus, the topological mapping of these mutations supports the notion that T211D mutation may not lead to a functional defect of CD154. Furthermore, the x-ray structure of this variant is virtually superimposable with that of the wild-type protein. Thus, T211D variant and wild-type proteins are similar in their surface expression levels, biochemical structures, and functional activities to signal through CD40. Together with the fact that this mutant has been identified in only one hyper-IgM patient and that B cells from this patient failed to undergo isotype switching upon stimulation with wild-type CD154 (7), we consider the possibility that T211D is a polymorphism of CD154 and is not responsible for the hyper-IgM disorder in this patient. Further studies using B and T lymphocytes from this patient will be needed to verify such a possibility.

ACKNOWLEDGMENTS—We thank Dr. George Masaiolas for B cells; Dr. Jeff Browning for the anti-Lymphotoxin-α mAb; and Drs. Linda Burksly, Teresa Cachero, Susan Kalled, and Blake Pepinsky for their critical reading of the manuscript. We also thank the DNA sequencing laboratory at Biogen.

---

Fig. 5. Three-dimensional representation of CD154 showing location of mutations associated with hyper-IgM syndrome. The figure shows the Co backbone of the homotrimeric TNF homologous domain (Gly-116 to Leu-261) of human CD154 crystal structure. Mutated residues are represented as spheres and marked in red, except for the T211D mutation, which is marked in blue. For clarity, the mutated residues are shown on green monomer only.

---

3 Y.-M. Hsu, manuscript in preparation.
REFERENCES

1. Notarangelo, L. D., and Peitsch, M. C. (1996) *Immunol. Today* 17, 511–516
2. Conley, M. E. (1992) *Annu. Rev. Immunol.* 10, 215–238
3. Aruffo, A., Farrington, M., Hollenbaugh, D., Li, X., Milatovich, A., Nonoyama, S., Noelle, R. J., Grosmaire, L. S., Stemkamp, R., Neuhauser, M., Roberts, R. L., Noelle, R. L., Francke, U., and Ochs, H. D. (1993) *Cell* 72, 291–300
4. Di Santo, J. P., Bonnefoy, J. Y., Gauchat, J. F., Fischer, A., and de Saint Basile, G. (1992) *Cell* 72, 5965–5967
5. Macchi, P., Villa, A., Strina, D., Sacco, M. G., Morali, F., Brugnoni, D., Giliani, S., Mantuano, E., Fasth, A., Andersson, B., Zegers, B. J. M., Cavagni, G., Reznick, I., Levy, J., van Bar, J., Parat, Y., Airo, P., Pilebian, A. Vezzoni, P., and Notarangelo, L. D. (1995) *Am. J. Hum. Genet.* 56, 898–906
6. Ramesh, N., Fuleihan, R., Ramesh, V., Lederman, S., Yellin, M. J., Sharma, S., and Noelle, R. J., Francke, U., and Ochs, H. D. (1993) *J. Clin. Invest.* 92, 259–266
7. Allen, R. C., Armitage, R. J., Conley, M. E., Rosenblatt, H., Jenkins, N. A., and Notarangelo, L. D. (1995) *Hum. Mol. Genet.* 4, 361, 725–728
8. Conley, M. E., Lucci, J., Su, L., Ehrenfels, B., Garber, E., and Thomas, D. (1997) *Science* 272, 911–915
9. Katz, F., Hinshaweld, S., Rutland, P., Jones, A., Kinnon, C., and Morgan, G. (1996) *Hum. Mol. Genet.* 8, 223–228
10. Bajorath, J., Seyama, K., Nonoyama, S., Ochs, H. D., and Aruffo, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 6550–6554
11. Armitage, R. J., Fanslow, W. C., Strockbine, L., Sato, T. A., Moff, M. B., Sato, T. A., Maliszewski, C. R., Clark, E. A., Smith, C. A., Grabstein, K. H., Gosman, D., and Spriggs, M. K. (1992) *Nature* 357, 80–82
12. Graf, D., Korthauer, U., Mages, H. W., Bieri, F., Padyakaychee, M., Malem, S., Uguazi, A. G., Notarangelo, L. D., Levinsky, R. J., and Krozcek, R. A. (1993) *Nature* 361, 539–541
13. Hasle, H., Kerndrpg, G., Jacobsen, B. B., Heegaard, E. D., Hornelath, E., Lilleveig, S. T. (1994) *Am. J. Pediatr. Hematol. Oncol.* 16, 329–333
14. Hollembaug, D., Wu, L. H., Ochs, H. D., Nonoyama, S., Grosmaire, L. S., Ledbetter, J. A., Noelle, R. J., Hill, E., and Aruffo, A. (1994) *J. Clin. Invest.* 94, 616–622
15. Villa, A., Strina, D., Macchi, P., Patrosso, M. C., Vezzoni, P., Tovo, P. A., Giliani, S., Ugaio, A. G., and Notarangelo, L. D. (1994) *Hum. Mutat.* 3, 73–75
16. Nonoyama, S., Shihmadu, M., Tsuru, T., Seyama, K., Nuno, H., Neuhauser, M., Kata, K., and Ochs, H. D. (1997) *Hum. Mutat.* 9, 624–627
17. Brown, J. L., Dougas, I., Ngam-ek, A., Bourdon, P. R., Ehrenfels, B. N., Miatkowski, K., Zafari, M., Yampaiglie, A. M., Lawton, P., Meier, W., Zafari, M., Yampaiglie, A. M., Lawton, P., Meier, W., Benjamin, C. P., and Hession, C. (1995) *J. Immunol.* 154, 33–46
18. Noelle, R. J., Roy, M., Shepherd, D. M., Stanemkovick, I., Ledbetter, J. A., and Aruffo, A. (1992) (1992) *Proe. Natl. Acad. Sci. U. S. A.* 94, 6550–6554
19. Aruffo, A., Fanslow, W. C., Strockbine, L., Sato, T. A., Clifford, K. N., Macduff, B. M., Andro, D. M., and Gimpel, S. D., Davis-Smith, T., Maliszewski, C. R., Clark, E. A., Smith, C. A., Grabstein, K. H., Gosman, D., and Spriggs, M. K. (1992) *Nature* 357, 80–82
20. Graf, D., Korthauer, U., Mages, H. W., Senger, G., and Krozcek, R. A. (1992) *J. Exp. Med.* 225, 3191–3194
21. Gauchat, J. F., Aubry, J. P., Mazzei, G., Life, P., Jomotte, T., Elson, G., and Noelle, R. J., Francke, U., and Ochs, H. D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 101, 11310–11320
22. Worm, M., and Geha, R. S. (1991) *Immunol. Today* 16, 1883–1890
23. Bonifacino, J. S., Cosson, P., Shah, N., and Klauser, R. D. (1991) *EMBO J.* 10, 2783–2793
24. Farrington, M., Grosmaire, L. S., Nonoyama, S., Fischer, A., Sato, T. A., Maliszewski, C. R., and Krozcek, R. A. (1995) *J. Exp. Med.* 155, 1749–1754
25. Natsu, G. J., Edgerton, M. D., Leborger, C., Lecanet-Henchoz, S., Graber, P., Durandu, J. P., Bernhard, J. Y., Bernard, A., Alt, and Bonnefoy, J. F. (1995) *J. Biol. Chem.* 270, 7025–7028
26. Pietravalg, F., Lecanet-Henchoz, S., Blaisey, H., Aubry, J. P., Elson, G., Edgerton, M. D., Bonnefoy, J. F., and Gauchat, J. F. (1996) *J. Biol. Chem.* 271, 5965–5967
27. Pietravalg, F., Lecanet-Henchoz, S., Aubry, J. P., Elson, G., Bonnefoy, J. Y., and Gauchat, J. F. (1996) *J. Exp. Med.* 176, 725–728
28. Noelle, R. J., and Ledbetter, J. A., and Aruffo, A. (1992) *Immunol. Today* 13, 431–433