CD40 ligand (CD40L) is a 33-kDa type II membrane glycoprotein mainly expressed on activated CD4+ T cells in trimeric form. When it is mutated, the clinical consequences are X-linked hyper-IgM syndrome (XHIM), a primary immunodeficiency disorder characterized by low levels of IgG, IgA, and elevated or normal levels of IgM. Mutated CD40L can no longer bind CD40 nor provide signals for B cells to proliferate and to switch from IgM to other immunoglobulin isotypes. When considering gene therapy for XHIM, it is important to address the possibility that the mutated CD40L associates with transduced wild type CD40L, and as a consequence, immune reconstitution is not attained. In this study, we demonstrate that the various mutated CD40L species we have identified in patients with XHIM, including both full-length and truncated mutants, associate with wild type CD40L on the cell surface of co-transfected COS cells. The association between wild type and mutated CD40L was also observed in CD4+ T cell lines established from XHIM patients with leaky splice site mutations. The clinical phenotype of these patients suggests that this association between wild type and mutated CD40L species may result in less efficient cross-linking of CD40.

CD40 ligand (CD40L)1 (CD154, gp39, or TRAP) is a type II membrane glycoprotein, consisting of 261 amino acid residues, and is expressed mainly on activated CD4+ T cells in trimeric form. When it is mutated, the clinical consequences are X-linked hyper-IgM syndrome (XHIM), a primary immunodeficiency disorder characterized by low levels of IgG, IgA, and elevated or normal levels of IgM. Mutated CD40L can no longer bind CD40 nor provide signals for B cells to proliferate and to switch from IgM to other immunoglobulin isotypes. When considering gene therapy for XHIM, it is important to address the possibility that the mutated CD40L associates with transduced wild type CD40L, and as a consequence, immune reconstitution is not attained. In this study, we demonstrate that the various mutated CD40L species we have identified in patients with XHIM, including both full-length and truncated mutants, associate with wild type CD40L on the cell surface of co-transfected COS cells. The association between wild type and mutated CD40L was also observed in CD4+ T cell lines established from XHIM patients with leaky splice site mutations. The clinical phenotype of these patients suggests that this association between wild type and mutated CD40L species may result in less efficient cross-linking of CD40.

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3 The abbreviations used are: CD40L, CD40 ligand; AP, antisenese primer; CD30L, CD30 ligand; DMEM, Dulbecco’s modified Eagle’s medium; FasL, Fas ligand; FCS, fetal calf serum; mAb, monoclonal antibody; MFI, mean fluorescence intensity; nt, nucleotide number; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcription-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SP, sense primer; TNF, tumor necrosis factor; XHIM, X-linked hyper-IgM syndrome.

iso-type switching and to escape apoptosis (3, 4). In addition, CD40L-CD40 interaction influences many aspects of T cell-mediated inflammatory responses, such as up-regulation of adhesion molecules, cell extravasation, production of inflammatory cytokines and chemokines, as well as activation of macrophage effector function (3).

The physiologic significance of the CD40L-CD40 interaction has been underscored by the observation that mutations of the CD40L gene cause X-linked hyper-IgM syndrome (XHIM) (5-9), a primary immunodeficiency disorder characterized by low or absence of IgG, IgA, and IgE and normal or elevated IgM. Mutations of the CD40L gene identified in XHIM patients are highly heterogeneous. They include missense, nonsense, and splice site mutations, and insertions or deletions (10, 11), and are distributed throughout the CD40L gene which consists of 5 exons and 4 introns and spreads over 12 kilobase pairs in genomic DNA (12, 13). More than 75 unique mutations have been reported to date (10, 11). In most instances, the mutated CD40L on the cell surface of activated T cells is undetectable if anti-CD40L monoclonal antibodies (mAb) or CD40-Ig, a fusion protein consisting of the extracellular domain of CD40 and the Fc portion of human immunoglobulin G, are used. However, if a polyclonal anti-CD40L antiserum is used, the expression of mutated CD40L by activated T cells is detected in the majority of XHIM patients (11).

CD40L is a member of the TNF superfamily that includes TNF-α, TNF-β, Fas ligand (FasL), and CD30 ligand (CD30L) (14). Based on x-ray crystallographic structures available for TNF-α (15, 16), TNF-β (17, 18), and CD40L (19), it appears that members of the TNF superfamily characteristically form homotrimers consisting of three monomers, each folding with a “jelly roll” topology (15, 16, 19). Most of the TNF superfamily members exist in both membrane-anchored and soluble forms due to proteolytic cleavage (20-22), except for the TNF-β homotrimer which is expressed only as soluble form. Heterotrimer formation is known only among lymphotoxin β and TNF-β (23, 24). Each trimer, e.g. TNF-β and CD40L, can bind three molecules of the counter-receptor along the surface groove between two adjacent subunits (18, 25, 26), leading to receptor clustering or aggregation required for activation signaling into the target cells (18, 27, 28).

When considering gene therapy for XHIM patients, the possibility of an association between the transduced wild type CD40L and the patient’s mutated CD40L resulting in heterotrimer formation has to be addressed. Since two CD40L monomers contribute to form one functional CD40-binding site (25, 26), the association of wild type CD40L monomer with mutated CD40L monomer is expected to generate decreased numbers of CD40-binding sites and, consequently, render the heterotrimer less efficient in clustering CD40. In this study, the association of wild type CD40L with various mutated CD40L species isolated from XHIM patients is demonstrated in transfectcd COS
cells and in activated T cell lines established from XHIM patients with different splice site mutations.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Antibodies—** COS cells, murine IgG, anti-human CD40L monoclonal antibody 106 (mAb 106), and biotinylated mAb 106 (bio-106), rabbit anti-human CD40L antiserum, and the CD40-Ig construct consisting of the extracellular domain of CD40 fused with the Fc region of human IgG1 were provided by Dr. Diane Hollenbaugh (Bristol-Myers Squibb). COS cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (FCS) (HyClone, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml of streptomycin. Murine anti-CD40L mAb 5c8 (IgG2a) and Rb784, a rabbit antiserum (27) recognizing the N-terminal 15 amino acids of CD40L, were provided by Biogen Inc. (Cambridge, MA). Murine anti-Flag mAbs, M5 (IgG1), and biotinylated M5 (bio-M5), were purchased from Eastman Kodak Co.). Murine anti-human FasL mAb NOK1 (IgG1) (28) was a generous gift from Dr. Hideo Yagita (Juntendo University, Tokyo, Japan) and murine anti-human CD30L mAbs, M80 (IgG1), M81 (IgG2a), and M82 (IgG2b) were provided by Immunex Corp. (Seattle, WA). Interleukin-2-dependent CD4+ T cell lines (>90% CD4+) were prepared from T cell lines established from XHIM patients using a magnetic cell sorting system (Miltenyi Biotec Inc., Auburn, CA) and maintained in Yssel’s medium (Genetical Products, Calabasas, CA) supplemented with 8% heat-inactivated FCS, 2% heat-inactivated human AB serum (Genetical Products), 100 units/ml penicillin, and 100 μg/ml streptomycin according to standard methods.

**Construction of Expression Plasmid—** A schematic representation of the protein structures we expressed in COS cells and the arbitrary designation of each plasmid and corresponding protein are shown in Fig. 1 and Table I, respectively. All of the expression plasmids were constructed by reverse transcription-polymerase chain reaction (RT-PCR) with Pho DNA polymerase (Stratagene, La Jolla, CA) using cDNA isolated from activated peripheral blood mononuclear cells derived from a healthy volunteer or from selected XHIM patients (11). The human CD40L cDNA consisting of nt 1–807 (nucleotide numbering is based on the sequence data of Diane Hollenbaugh et al. (1)) was amplified by RT-PCR using sense primer (SP) 1 (5’CGCCGATCCATTTCAACTTTA-ACACAGC3) and antisense primer (AP) 1 (5’GCCGTGAGTTCAAGTTTTATAGTAAGCCAAGG3') and subsequently cloned into BamHI and XhoI sites of pcDNA3.1(+) (Invitrogen, Carlsbad, CA). Naturally occurring mutant cDNAs, exon 2-skipped cDNA, 19 nucleotides of intron 2-inserted cDNA, and exon 3-skipped cDNA, were similarly cloned into pcDNA3.1(+) using cDNA generated from the appropriate XHIM patients. The expression vector of the CD40L lacking the cytoplasmic domain (CytDel, Met21-Leu261) was generated using SP2 (5’CGCCGATCCATTTCAACTTTAACACAGC3) and AP1, cloned into the same vector; Flag peptides consisting of DKYDDDDL were inserted between Met1 and the N-terminal Flag peptide (E3skip and F-E3skip, respectively), resulting from the translation of the mRNA transcripts carrying the 19 nucleotides’ insertion of intron 2 and identified in XHIM patients with intron 2 splice donor site mutation; 11 and 12, a truncated mutant CD40L without or with the N terminus Flag peptide (E2ins and F-E2ins, respectively), representing F-W140X and F-Q186X, respectively; 8, mutant CD40L with in-frame deletion of 44 amino acids encoded by exon 2 and identified in XHIM patients with intron 2 splice donor site mutation; 13, Flag-tagged control protein construct (F-HybFasL or F-HybCD30L), representing the hybrid protein in which the extracellular region of the human FasL (from Gln 103 to Leu281) or of human CD30L (from Gln63 to Asp234) was fused to the IC and TM, respectively; 7, Flag-tagged mutant CD40Ls with premature termination selected from patients with XHIM, representing F-W140X and F-Q186X, respectively; 3, naturally occurring mutant CD40Ls, and 12, a truncated mutant CD40L without or with the N terminus Flag peptide (E3skip and F-E3skip, respectively), resulting from the translation of exon 3-skipped mRNA transcripts identified in XHIM patients with intron 3 splice donor site mutation; 13, Flag-tagged control protein construct (F-HybFasL or F-HybCD30L), representing the hybrid protein in which the extracellular region of the human FasL (from Gln103 to Leu281) or of human CD40L (from Gln63 to Asp234) was fused to the IC and TM of CD40L. Both F-HybFasL and F-HybCD30L contain three extra amino acids, RDF, at the fusion site. See also Table I; the number of each cartoon shown here corresponds to that in Table I.

**Fig. 1.** Schematic presentation of the constructs including wild type CD40L, naturally occurring mutant CD40Ls, and control constructs used in this study. Shown are the contribution of each exon of the CD40L gene to the domain structure: IC, intracellular tail; TM, transmembrane domain; ECU, extracellular unique region; and TNFH, TNF homology domain. The number on the right side of the protein structure represents the amino acid number where each domain starts or the one where each protein or truncated ECU domain ends. In order to discriminate wild type CD40L both by protein size and by antigenicity from various mutant CD40Ls, a Flag peptide (DYKDDDDL) was inserted at the N terminus between the first amino acid methionine and the second amino acid isoleucine of wild type CD40L. Flag-tagged proteins are represented by the ‘F-’ preceding each protein name. The constructs designed for this study included the following: cartoon 1, wild type CD40L; 2, CD40L lacking the intracellular tail and starting at Met21 (CytDel); 3, Flag-tagged wild type CD40L (F-Wild); 4, Flag-tagged CD40L with the extracellular part of CD40L consisting of Gln114-Leu261 (soluble CD40L) cleaved off (F-Stalk); 5, Flag-tagged mutant CD40Ls with one or two amino acid substitutions in TNFH selected from patients with XHIM, including F-DM (double mutations resulting in S128R/E129G), F-T147N, F-Y170C, F-G227V, F-A235P, F-T254M, and F-L258S; 6 and 7, Flag-tagged mutant CD40Ls with premature termination selected from patients with XHIM, representing F-W140X and F-Q186X, respectively; 8, mutant CD40L with in-frame deletion of 44 amino acids encoded by exon 2 and identified in XHIM patients with intron 2 splice donor site mutation; 11 and 12, a truncated mutant CD40L without or with the N terminus Flag peptide (E3skip and F-E3skip, respectively), resulting from the translation of exon 3-skipped mRNA transcripts identified in XHIM patients with intron 3 splice donor site mutation; 13, Flag-tagged control protein construct (F-HybFasL or F-HybCD30L), representing the hybrid protein in which the extracellular region of the human FasL (from Gln103 to Leu281) or of human CD40L (from Gln63 to Asp234) was fused to the IC and TM of CD40L. Both F-HybFasL and F-HybCD30L contain three extra amino acids, RDF, at the fusion site. See also Table I; the number of each cartoon shown here corresponds to that in Table I.
Expression plasmids and proteins expressed by them when transfected to COS cells were named arbitrarily for convenience on explaining the experimental results. See also Fig. 1 since the numbers in the left column correspond to those of Fig. 1 in which structure of protein is shown schematically.

| Plasmid   | Flag tag | Expressed protein                  | Structure and feature of protein* |
|-----------|----------|------------------------------------|----------------------------------|
| pWild     | –        | Wild type CD40L                    | Met1–Leu286 of wild type CD40L   |
| pcYtDel   | –        | CytDel                             | Met1–Leu286 of wild type CD40L   |
| pF-Wild   | +        | F-Wild                             | N-Flag + wild type CD40L (Ile3–Leu86) |
| pF-Stalk  | +        | F-Stalk                            | N-Flag + Ile3–Met13 of wild type CD40L |
| pF-DM     | +        | F-DM                               | N-Flag + CD40L with S128R and E129G |
| pF-T147N  | +        | F-T147N                            | N-Flag + CD40L with T147N        |
| pF-Y170C  | +        | F-Y170C                            | N-Flag + CD40L with Y170C        |
| pF-G227V  | +        | F-G227V                            | N-Flag + CD40L with G227V        |
| pF-A235P  | +        | F-A235P                            | N-Flag + CD40L with A235P        |
| pF-T254M  | +        | F-T254M                            | N-Flag + CD40L with T254M        |
| pF-L258S  | +        | F-L258S                            | N-Flag + CD40L with L258S        |
| pF-W140X  | +        | F-W140X                            | N-Flag + CD40L with W140X        |
| pF-Q186X  | +        | F-Q186X                            | N-Flag + CD40L with Q186X        |
| pE2skip   | +        | E2skip                             | Met1–Lys97 of CD40L + 2 extra amino acids |
| pE2ins    | +        | E2ins                              | Met1–Lys97 of CD40L + 21 extra amino acids |
| pF-E2ins  | +        | F-E2ins                            | N-Flag + Ile3–Lys97 of CD40L + 21 extra amino acids |
| pE3skip   | +        | E3skip                             | Met1–Lys97 of CD40L + 11 extra amino acids |
| pE3ins    | +        | F-E3ins                            | N-Flag + Ile3–Lys97 of CD40L + 11 extra amino acids |
| pF-HybFasL| +        | F-HybFasL                          | N-Flag + Hev-Leu97 of FasL        |
| pF-HybCD30L| +      | F-HybCD30L                         | N-Flag + Hev-Leu97 of CD40L + Gin113–Leu281 of FasL|

* N-Flag means that Flag peptide (DYKDDDDK) was added at the N terminus of protein and preceded by initiation methionine.

## TABLE I

**Association of CD40L Mutants with Wild Type CD40L**

### Metabolic Labeling, Immunoprecipitation, and SDS-PAGE Analysis—Metabolic radio-labeling of activated interleukin-2-dependent CD4+ T cell lines was performed in Met/Cys-free RPMI 1640 medium (Sigma) supplemented with 10% dialyzed heat-inactivated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. CD4+ T cells were suspended at 5×10^6 cells/ml in labeling medium containing 0.2 mCi/ml [3H]methionine. The labeling mixture was incubated for 18 h at 37°C and the reaction mixture was then treated with 12 μl of 6X Laemmli’s sample buffer containing β-mercaptoethanol for 5 min in boiling water and resolved by SDS-PAGE.

### Detection of Expressed Proteins by Western Blotting—Following immunoprecipitation from the lysate of transfected COS cells, proteins were resuspended on SDS-PAGE and transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA). The blotted membrane was incubated in a blocking solution containing 5% blocking non-fat milk (Bio-Rad) in 20 mm Tris-HCl (pH 7.5), 150 mm NaCl, 0.025% NaN3, and 0.1% Tween 20 (TBS-T), for 2 h at room temperature or overnight at 4°C and then probed with a specific antibody to β-mercaptoethanol for 1 h at 4°C. After centrifugation, the supernatant was mixed with or without 5 units of protease-free N-glycosidase F (Roche Molecular Biochemicals) in a total volume of 60 μl containing 50 μm sodium phosphate buffer (pH 7.2), 100 μM iodoacetamide (Roche Molecular Biochemicals) and incubated for 18 h at 37°C. The reaction mixture was then treated with 12 μl of 6X Laemmli’s sample buffer containing β-mercaptoethanol for 5 min in boiling water and resolved by SDS-PAGE.
ionomycin and 10 ng/ml phorbol 12-myristate 13-acetate (Sigma) for 4 h at 37 °C. Radiolabeled cells were lysed at 5 × 10⁶ cells/ml in lysis buffer. The resultant lysate was precleared and immunoprecipitated as described above. Proteins were resolved by 13% SDS-PAGE, and fluorography was performed using ENTENSIFY (NEB) according to the manufacturer’s instruction. For metabolic labeling, Cos transfection, the cells were washed 36 h after transfection with PBS and incubated in Met/Cys-free DMEM (Sigma), supplemented with 5% dialyzed heat-inactivated FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.2 μCi/ml EXPRE³⁵S³⁵ for 4 h and processed similarly.

RESULTS

Surface Expression of Transduced Protein—Surface expression of wild type CD40L, mutant CD40L species, and control hybrid proteins (F-HybFasL and F-HybCD30L) by transfected COS cells was confirmed by flow cytometry (data not shown). Wild type CD40L and Flag-tagged wild type CD40L (F-Wild) were expressed abundantly by transfected COS cells and detected by mAb 5c8 as well as CD40-Ig construct, suggesting that the Flag peptide at the N terminus does not affect the structure and function of CD40L. Two unique truncated mutants, CytDel and E2skip, both found in XHIM patients (11), were detected in transfected COS cells using mAb 5c8 and the CD40-Ig construct, respectively. The expression of other mutated CD40L species, including mutants with amino acid substitutions and truncated mutants, was confirmed by the binding of a polyclonal anti-CD40L antiserum. Similarly, both F-HybFasL and F-HybCD30L expressed by transfected COS cells were detected well by mAb NOK1 and three different anti-CD30L mAbs preparations (M80, M81, and M82), respectively. Since NOK1 recognizes the antigenic epitope consisting of two FasL monomers, it seems likely that F-HybFasL retains the inherent structural integrity of FasL and forms a trimer. The finding that F-HybCD30L expression was detectable by three different anti-CD30L mAbs suggests that this hybrid construct also retains the inherent structural integrity of CD30L and forms a trimer.

Biochemical Characterization of Wild Type CD40L Transiently Expressed by COS Cells—The biochemical characteris-
ing different Flag-tagged mutant CD40L species were co-transfected. To test the system, we co-transfected COS cells with pWild and either pF-Wild, pF-HybFasL, or pF-HybCD30L and examined their association. As shown in Fig. 3A, F-Wild was detected by bio-M5 in CD40-Ig-generated immunoprecipitates of lysates from pWild + pF-Wild co-transfected cells, but nei-
indicated as kDa on the left.

12% SDS-PAGE, transferred onto Immobilon membrane. mAb M5 was used for detection. The sizes of the molecular mass standard proteins are indicated as kDa on the left of each panel. Note that all Flag-tagged truncated mutant CD40L tested could be detected in the immunoprecipitates obtained with Mab M5 but not in those obtained with CD40-Ig. C, the lysates of COS cells co-transfected with pWild and either pF-Wild or a plasmid expressing a truncated mutant CD40L were subjected to immunoprecipitation with CD40-Ig. The immunoprecipitates were resolved by 12% SDS-PAGE, transferred onto Immobilon membrane, and probed with mAb bio-M5. The sizes of the molecular mass standard proteins are indicated as kDa on the left of each panel. Note that all truncated mutant CD40Ls tested could be detected in the immunoprecipitates obtained with CD40-Ig when co-expressed with wild type CD40L (C), although they could not be immunoprecipitated with CD40-Ig when expressed alone in COS cells (see B).

By using the same technique, naturally occurring mutants of CD40L were co-transfected with wild type CD40L to test whether mutants can associate with wild type CD40L. Seven mutants with a single or two amino acid substitutions were studied (Table I). F-DM which has two amino acid substitutions (S1128R/E129G) and F-T147N, which has a larger molecular mass than F-Wild and other Flag-tagged missense mutants due to the generation of a cryptic N-glycosylation site by the amino acid substitution, was the only mutant detected in the CD40-Ig-generated immunoprecipitate, although only as an extremely faint band (Fig. 4B). In contrast, all mutants with amino acid substitutions studied were detected (Fig. 4C) in immunoprecipitates obtained with CD40-Ig if COS cells were co-transfected with pWild and expression plasmids of Flag-tagged missense mutants. Similarly, wild type CD40L was readily demonstrated in immunoprecipitates obtained with Mab M5 from the lysate of each co-transfectant (data not shown). These findings clearly demonstrate that all mutants with amino acid substitutions studied associate with wild type CD40L and that the resultant complexes, although they include mutants that cannot bind CD40-Ig by themselves, can bind CD40-Ig. The inability of these mutants to bind CD40-Ig appears not to be due to their inability to form complexes by themselves; when plasmids carrying Flag-tagged mutants and those carrying corresponding wild mutants were co-transfected to COS cells, both Flag-tagged and plain mutants were detected in the immunoprecipitates obtained with Mab M5 using an anti-CD40L polyclonal antiserum (data not shown). This observation implies that these mutants can form complexes themselves but fail to generate binding sites for CD40.

To investigate if truncated CD40L can associate with wild type CD40L, we transfected COS cells with the following Flag-tagged truncated mutants (Table I): F-Stalk; F-W140X, the most commonly identified nonsense mutant in XHIM patients failed to be immunoprecipitated with CD40-Ig (Fig. 4B) as expected since these mutants were isolated from patients with XHIM. F-T147N, which has a larger molecular mass than F-Wild and other Flag-tagged missense mutants due to the generation of a cryptic N-glycosylation site by the amino acid substitution, was the only mutant detected in the CD40-Ig-generated immunoprecipitate, although only as an extremely faint band (Fig. 4B). In contrast, all mutants with amino acid substitutions studied were detected (Fig. 4C) in immunoprecipitates obtained with CD40-Ig if COS cells were co-transfected with pWild and expression plasmids of Flag-tagged missense mutants. Similarly, wild type CD40L was readily demonstrated in immunoprecipitates obtained with Mab M5 from the lysates of each co-transfectant (data not shown). These findings clearly demonstrate that all mutants with amino acid substitutions studied associate with wild type CD40L and that the resultant complexes, although they include mutants that cannot bind CD40-Ig by themselves, can bind CD40-Ig. The inability of these mutants to bind CD40-Ig appears not to be due to their inability to form complexes by themselves; when plasmids carrying Flag-tagged mutants and those carrying corresponding wild mutants were co-transfected to COS cells, both Flag-tagged and plain mutants were detected in the immunoprecipitates obtained with Mab M5 using an anti-CD40L polyclonal antiserum (data not shown). This observation implies that these mutants can form complexes themselves but fail to generate binding sites for CD40.

To investigate if truncated CD40L can associate with wild type CD40L, we transfected COS cells with the following Flag-tagged truncated mutants (Table I): F-Stalk; F-W140X, the most commonly identified nonsense mutant in XHIM patients.
could be immunoprecipitated with CD40-Ig (Fig. 5C). Similarly, if COS cells were co-transfected with pE2skip and pF-E2ins, followed by the immunoprecipitate with mAb M5, E2skip could not be detected by mAb bio-106 (data not shown). These observations suggest that the stalk region, which is mainly encoded by exon 2 of the CD40L gene, as well as the TNFH domain, play an important role in the association of CD40L monomers. This conclusion is further supported by the observation that the amount of E2skip found in the mAb M5-generated immunoprecipitate from a pF-Wild + pE2skip co-transfectant was very low (Fig. 6, right lane in right panel) when compared with the amount of E2skip found in the immunoprecipitate obtained with CD40-Ig by which wild type CD40L and E2skip are independently immunoprecipitated regardless of association (Fig. 6, left lane in right panel).

Another interesting feature of the E2skip mutant is the fact that the efficiency of E2skip to transduce a signal through CD40 appears to be less than that of wild type CD40L if E2skip is anchored on cell surface, although the binding of E2skip and CD40-Ig in the cell lysate is normal. Although E2skip is efficiently recovered in CD40-Ig-generated immunoprecipitates from transfected COS cells (Fig. 6, middle panel and left lane in right panel), the staining intensity of pE2skip-transfected COS cells with CD40-Ig (as shown by flow cytometry) is much lower than that of pWild-transfected cells: whereas the mean fluorescence intensity (MFI) of E2skip-expressing COS cells was 60.4 when detected by CD40-Ig (immunostained histograms obtained by flow cytometry not shown). On the other hand, similar MFI were obtained for E2skip- and wild type CD40L-expressing COS cells when mAb 5c8 was used for detection (421.7 and 406.8, respectively). We hypothesize that the weaker intensity of CD40-Ig binding by membrane-expressed E2skip, when compared with the bright staining with mAb 5c8, is due to steric hindrance resulting from the loss of the extracellular stalk which is encoded mainly by exon 2.

**Association Occurs When Mutant and Wild Type CD40L Are Co-expressed and the Resultant Complex Is Present on the Cell Surface**—To demonstrate that the association of mutated CD40L with wild type CD40L occurs exclusively when they are co-expressed, we compared the immunoprecipitation of lysates from co-transfectants with that of lysate mixtures prepared from corresponding single transfectants. We selected F-T147N and F-W140X, representing a CD40L with an amino acid substitution and a CD40L that is truncated, respectively, since both are expressed well when transfected into COS cells. Following preclearance of lysate mixture with protein G-Sepharose beads overnight at 4 °C and further incubation for 1 h at either 4 or 37 °C, immunoprecipitation with mAb M5 was performed. Since wild type, F-Wild, and F-T147N are equally well recognized by mAb 106 (11) and expressed by COS cells in glycosylated and unglycosylated form, the discrimination between wild type, F-Wild, and the mutant is difficult. We therefore treated the immunoprecipitates with N-glycosidase F before resolving with SDS-PAGE. When compared with the immunoprecipitate of COS cells co-transfected with pWild and pF-Wild, only a trace (at 4 °C) or a very low amount (at 37 °C) of wild type CD40L was recovered from the immunoprecipitate of the mixture of the lysates of each single transfectant (Fig. 7A), suggesting that association of wild type CD40L and F-Wild occurs during co-expression by COS cells but not during the preclearing or immunoprecipitation process. However, a very small amount of wild type CD40L did associate with F-Wild at 37 °C (3rd lane from the left in Fig. 7A). To test the association of mutants with wild type CD40L, similar experiments were performed using F-T147N and F-W140X. Similar to the obser-
Association of CD40L Mutants with Wild Type CD40L

Fig. 7. Lysates from co-transfected COS cells but not mixtures of lysates from singly transfected COS cells show an association between wild type CD40L and mutants. A, comparing immunoprecipitates using expression plasmid pWild and pF-Wild. COS cells were either co-transfected with pWild and pF-Wild, singly transfected with pWild, or singly transfected with pF-Wild, and lysates from each transfectant were prepared (see “Experimental Procedures”). The lysates from co-transfected COS cells were preclarified overnight with protein G-Sepharose at 4 °C and subsequently immunoprecipitated (IP) with mAb M5. The lysates from COS cells transfected with either pWild or pF-Wild were mixed, preclarified overnight at 4 °C, and the supernatant further incubated either at 4 °C for 1 h (2nd lane from the left) or at 37 °C for 1 h (3rd lane), and immunoprecipitated with mAb M5. To indicate the position of wild type CD40L and F-Wild, respectively, on SDS-PAGE, lysates of COS cells transfected with pWild and with pF-Wild were immunoprecipitated with mAb 106 and M5, respectively. All immunoprecipitates were subsequently treated with N-glycosidase F to simplify the identification of wild type CD40L and F-Wild, resolved by 12% SDS-PAGE, and detected by mAb bio-106. Note that wild type CD40L could barely be detected in the mixture of lysates from each corresponding single transfectant when incubated at 4 °C, whereas a small amount of wild type CD40L could be identified when incubated at 37 °C; in contrast a significant amount of wild type CD40L was immunoprecipitated together with F-Wild in lysates from the co-transfectant. B, comparing immunoprecipitates using expression plasmid pWild and pF-T147N. This experiment is similar to that shown in A. We used pF-T147N instead of F-Wild to represent a CD40L mutant with an amino acid substitution. The preparation and treatment of cell lysates was the same as those described in A. Note that wild type CD40L could barely be detected in the mixture of lysates from each corresponding single transfectant when incubated at 4 °C, although a small amount of wild type CD40L was found when incubated at 37 °C. In contrast, larger amount of wild type CD40L was immunoprecipitated together with F-T147N in lysates from the co-transfectant. C, comparing immunoprecipitates using expression plasmid pWild and pF-W140X. This experiment is similar to those shown in A and B. We used pF-W140X as the representative for a truncated CD40L caused by a nonsense mutation. The preparation and treatment of cell lysates were the same as those in A and B except that N-glycosidase F treatment was not performed. Although wild type CD40L could be detected in lysates from the co-transfectant, there was no wild type CD40L demonstrable in the mixture of lysates from each corresponding single transfectant even when incubated at 37 °C.

FIG. 7.

viation made with wild type CD40L and F-Wild, a strong association between wild type CD40L and F-T147N occurred only in co-transfected cells but not if lysates of singly transfected COS cells were mixed in vitro at 4 °C, and only at very small quantities when mixed at 37 °C (Fig. 7B). No association between wild type CD40L and a truncated mutant F-W140X was observed if mixed in vitro at 4 °C and at 37 °C, respectively (Fig. 7C).

To demonstrate that the complexes formed between wild type and mutated CD40L are anchored in the cell membrane, surface biotinylation of the transfectant was performed using membrane-impermeable Sulfo-NHS-biotin, followed by immunoprecipitation and Western blotting (Fig. 8). An association between wild type CD40L and F-T147N or F-W140X, respectively, was observed on the cell surface in both CD40-1g- and mAb M5-generated immunoprecipitates (Fig. 8, A and B, respectively) from surface-biotinylated co-transfected COS cells. Association of Mutant and Wild Type CD40L Occurs in CD4⁺ T Cell Lines Established from XHIM Patients with Leaky Splice Site Mutations—The association of wild type CD40L with E3skip were further confirmed in CD4⁺ T cell lines established from two XHIM patients with different intron 3 splice donor site mutations, nt 367G→A in one patient (Fig. 9A, lane 3) and nt 367 + 5g→a in the other (Fig. 9A, lane 4). CD4⁺ T cells from both patients are able to generate normally spliced and exon 3-skipped mRNA (11). When metabolically labeled activated cultured CD4⁺ T cells were lysed and immunoprecipitated with CD40-1g, E3skip was clearly detected in lysates from both patients (Fig. 9A, lanes 3 and 4) and in a lysate of pWild + pE3skip co-transfected COS cells (Fig. 9A, lane 7). On the other hand, no association of E2skip with E2ins was observed in a CD4⁺ T cell line from an XHIM patient with the intron 2 splice donor site mutation nt 309 + 2t→a (11) (Fig. 9B), as expected from the finding in co-transfected COS cells (Fig. 8). E2ins could not be detected in the immunoprecipitate obtained with CD40-1g from the metabolically labeled lysate of the patient’s CD4⁺ T cells (Fig. 9B, lane 2) in the lysate of pE2skip + pE2ins co-transfected COS cells (Fig. 9B, lane 6). In contrast, the immunoprecipitates obtained with Rb784 from the patient’s CD4⁺ T cell line (Fig. 9B, lane 4) and from pE2skip + pE2ins co-transfected COS cells (Fig. 9B, lane 8) demonstrated that E2ins was expressed but did not co-immunoprecipitate with E2skip; the short protein band in Fig. 9B, lanes 4 and 8, has the same position as the single band shown in the immunoprecipitate obtained with Rb784 from pE2ins-transfected COS cells (Fig. 9B, lane 10).

DISCUSSION

CD40L, a member of the TNF superfamily, is expressed on the cell surface as a trimer (5, 19) similar to most other members of this family, including TNF, CD30L, and FasL (14). In addition to the full-length wild type CD40L, other derivatives of CD40L participate in forming a heteromultimeric complex. In this study of activated CD4⁺ T cells and CD40L-transfected COS cells, we have demonstrated that this heterocomplex con-
that F-T147N and F-W140X were probed with streptavidin-horseradish peroxidase conjugates. Note biotinylated using Sulfo-NHS-biotin which is membrane-impermeable when co-transfected with pWild (A)
cell surface of transfected COS cells. (B)
sociation on the cell surface. By immunoprecipitation and Western blotting demonstrates as-
approximately 18 kDa in size (the
of CD40L takes place in three grooves formed by the three functional groove for CD40 binding. A homotrimer consisting of one wild type CD40L is expected to bind three CD40 molecules and form a complex on the cell surface if simultaneously trans-
CD40L, although we have no direct evidence. The proteolytic cleavage leading to a CD40L trimer containing significant molecules of “Stalk” may play a role in attenuating the CD40-CD40 interaction in vivo by decreasing the binding site for CD40 and, as a consequence, make CD40 clustering less efficient. This may be an important strategy to limit CD40L activity under physiologic conditions.

Several lines of evidence suggest that the extracellular TNFH domain is of importance for trimer formation. A soluble form of CD40L exists as a trimer (19, 21) and was found to be biologically active in stimulating B cell proliferation and immunoglobulin class switch (21, 33). Based on x-ray crystallography (19) and computer-based structural analysis (31), several amino acid residues in the TNFH domain have been shown to be of importance for trimer formation. Mutations occurring at those residues have been identified in patients with XHIM (10, 11). It is of interest that CD40L mutants that have amino acid substitutions at positions Y170C and G227V, known to be important for trimer formation, were still able to associate with wild type CD40L if transfected together with wild type CD40L into COS cells. Furthermore, our data suggest that the Stalk region (His\textsuperscript{47}-Met\textsuperscript{113}), which is mainly encoded by exon 2 of the CD40L gene, must also be important for complex formation since all mutants with intact extracellular Stalk region tested were able to associate with wild type CD40L, even if a mutant lacks the entire TNFH domain (Stalk), has a truncated TNFH domain (F-W140X and F-Q186C), or has cryptic amino acid residues after Lys\textsuperscript{360} (F-E2ins and F-E3skip). The mutated CD40L lacking the exon 2-encoded Stalk due to an in-frame deletion from Ile\textsuperscript{62} to Lys\textsuperscript{360} (E2skip) was able to associate with wild type CD40L, although much less efficiently compared with the complex formation between wild type CD40L and either Flag-tagged wild type CD40L or Flag-tagged mutants carrying amino acid substitutions. Thus, the integrity of the entire extracellular part of CD40L, including the extracellular unique region as well as the TNFH domain, is of importance for efficient complex formation of CD40L.

Similar to the mechanism involved in TNF receptor activation by TNF-\textalpha and TNF-\beta, ligand-induced receptor clustering or aggregation has been associated with CD40 signaling. Several observations support this hypothesis as follows: (i) both agonistic and non-agonistic anti-CD40 mAbs have been identified (34–36); (ii) non-agonistic mAb can be rendered bioactive when cross-linked with a secondary antibody (34, 35); (iii) agonistic activity of a mAb is not dependent on the epitope to which it binds, and mAbs bound to different epitopes of CD40 can elicit strong CD40 signaling (35); (iv) anti-CD40 mAb BL-C4, a pentameric IgM mAb, has been reported to generate a strong signal that induces monocytes to produce interleukin-1 (37), whereas the anti-CD40 mAb G28-5, known to be agonistic to stimulating B cells (36), did not.

The generation of expression plasmids from cDNA derived from XHIM patients with diverse, informative mutations of CD40L provided us with the opportunity to co-transfect COS cells with wild type and mutated CD40L to investigate heterotrimer formation. We found that various naturally occurring CD40L mutants can physically associate with wild type CD40L and form a complex on the cell surface if simultaneously translated. It is intriguing to consider the possible physiologic function of these complexes, although the stoichiometry of these heterotrimers has not been determined in this study. Binding of CD40 takes place in three grooves formed by the three monomers (25, 26), and each monomer contributes to form the functional groove for CD40 binding. A homotrimer consisting of wild type CD40L is expected to bind three CD40 molecules resulting in the clustering of CD40 and signal transduction. In contrast, a heterotrimer consisting of two wild type CD40L molecules and one mutated CD40L molecule is expected to bind only one CD40 molecule. A heterotrimer consisting of one wild type and two mutated CD40L molecules can no longer bind CD40. Thus, heterotrimer formation is expected to decrease the number of binding sites for CD40 and may no longer be able to ligate CD40 sufficiently to initiate signal transduction in CD40-expressing cells such as B lymphocytes, monocytes, and
Fig. 9. Immunoprecipitation of metabolically labeled CD4*T cell lines carrying intron 2 or intron 3 splice site mutations. A, association of a truncated E3skip mutant with wild type CD40L, both being co-expressed in CD4*T cell lines from XHIM patients with intron 3 splice site mutations. Activated CD4*T cell lines were metabolically labeled and lysed (see “Experimental Procedures”). Similarly, COS cells were transfected with either pWild, pE3skip, or pWild + pE3skip and metabolically labeled. Cell lysates are as follows: lanes 1 and 2, CD4*T cell line from a normal control; lane 3, CD4*T cell line from a patient with the mutation nt 367G → A; lane 4, CD4*T cell line from a patient with the mutation nt 367 + 5g → a; lane 5, COS cells transfected with pWild; lane 6, COS cells transfected with pE3skip; and lane 7, COS cells co-transfected with pWild and pE3skip. CD40-Ig was used for the immunoprecipitation in lanes 2–7, whereas human IgG1 was used in lane 1 as a control reagent. The sizes of the molecular mass standard proteins are indicated as kDa on the left. B, a truncated E2skip mutant does not associate with a truncated E2ins mutant in a CD4*T cell line from an XHIM patient with intron 2 splice site mutation (nt 309 + 2t → a). An activated CD4*T cell line derived from the patient with the mutation nt 309 + 2t → a was metabolically labeled and then lysed. Similarly, COS cells were either co-transfected with pE2skip and pE2ins or transfected with pE2ins, metabolically labeled, and then lysed as a control experiment. Cell lysates are as follows: lanes 1, 2, 3, and 4, CD4*T cell line from the XHIM patient with nt 309 + 2t → a; lanes 5, 6, 7, and 8, COS cells co-transfected with pE2skip and pE2ins; lanes 9 and 10, COS cells transfected with pE2ins. As indicated, control human IgG1 was used for the immunoprecipitation in lanes 1 and 5, CD40-Ig in lanes 2 and 6, and control rabbit sera in lanes 3, 7, and 9, and Rb784 in lanes 4, 8, and 10. The sizes of the molecular mass standard proteins are indicated as kDa on the left. In contrast to A which shows that wild type CD40L and E3skip can associate with each other and be immunoprecipitated with CD40-Ig, E2skip, a truncated mutant which can still bind CD40-Ig, does not associate with E2ins; CD40-Ig was able to immunoprecipitate only E2skip although E2ins was co-expressed by CD4*T cells effectively as demonstrated by the immunoprecipitation with Rb784, an antisemur recognizing the intracellular domain of CD40L.

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