Heteromultimeric Complexes of CD40 Ligand Are Present on the Cell Surface of Human T Lymphocytes*

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CD40 ligand (CD40L), a 33-kDa type II membrane glycoprotein expressed primarily on activated CD4+ T lymphocytes, is responsible for the helper function of T cells on resting B cells in a non-antigen-dependent, non-major histocompatibility complex-restricted fashion. Interaction of CD40L with its receptor CD40 induces proliferation of and isotype switching in B lymphocytes. Recently we solved the x-ray structure of recombinant soluble CD40L and showed that, similar to other members of the tumor necrosis factor family, CD40L indeed exists as a trimer. We now report that, under normal physiological conditions, CD40L molecules exist as heteromultimeric complexes. These CD40L complexes, made of the full length and smaller fragments of CD40L, are present on the cell surface of T lymphocytes and are capable of interacting with CD40 molecule. A prominent fragment with a mass of 31 kDa accounts for as much as half of the CD40L on the surface of Jurkat cells. N-terminal sequence data revealed that this fragment lacks the cytoplasmic tail. A minor 18-kDa fragment of CD40L was also characterized which lacks the cytoplasmic tail, transmembrane region, and stalk region of the extracellular domain. The presence of CD40L heteromultimeric variants implies an additional regulation of the functional activity of this ligand complex.

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* The abbreviations used are: CD40L, CD40 ligand; TNF, tumor necrosis factor; LT, lymphotoxin; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; PTH, phenylthiohydantoin; HRP, horseradish peroxidase.

The human CD40 cDNA was cloned by polymerase chain reaction from a λgt10 cDNA expression library made from human tonsil cells and subcloned into a pUC-derived vector. A soluble human CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutagenizing Ala-Leu 174→Val-Asp in the CD40-Fc fusion protein (CD40-Fc) was generated by first mutate...
CD40 extracellular domain to a cDNA encoding the hinge and Fc regions of a human IgG. A cytomegalovirus immediate-early (IE) promoter-driven expression vector (pSB132, Biogen) was constructed and transfected into COS7 cells. Transfected cells that secreted CD40-Fc were identified by both CD40-specific and human IgG Fc-specific antibodies. An adenovirus major late promoter-driven expression vector containing the dihydrofolate reductase selectable marker (pMDR901, Biogen) was constructed and transfected into CHO cells to produce stable cell lines. Clones were selected in 200 mM methotrexate, and those expressing CD40-Fc were identified by screening supernatants by enzyme-linked immunosorbent assay or Western blots using human IgG Fc-specific antibody. The best producing clone was adapted to suspension culture for large scale fermentation.

**Translational Expression of Wild-type and Mutant Forms of CD40L in COS Cells—** Mutant forms of CD40L were generated by unique site elimination mutagenesis using a kit following the manufacturer’s recommended protocol (Pharmacia Biotech Inc.). The target pUC-derived plasmid contained the wild-type CD40L cDNA (the CD40L open reading frame flanked by 21 base pairs of 5' and 109 base pairs of 3' untranslated region). The expression vector also contains an SV40 origin of replication for high copy expression in COS7 cells. COS7 cells were electroporated with supercoiled plasmid.

A cyttoplasmic domain-deleted mutant (cyt(-)) was engineered to encode a human CD40L of Met-21 to Leu-261 by removal of the coding region corresponding to amino acid residues 1–20. Wild-type or mutant CD40L cDNA was subcloned into an expression vector in which heterologous gene expression is driven by a cytomegalovirus immediate-early (IE) promoter. The vector contains an SV40 transcriptional termination and poly(A) addition site. The expression vector also contains an SV40 origin of replication for high copy expression in COS7 cells. COST7 cells were electroporated with supercoiled plasmid.

**Purification of Humanized 5c8 and CD40-Fc Fusion Protein—** Media from stably transfected cell lines were collected, concentrated 10-fold by Amicon ultrafiltration using YM30 spiral filtration cartridge (Amicon Inc., Beverly, MA), and purified using a protein A-Sepharose 4B column followed by passage through a Superdex 200 (Pharmacia) gel filtration column for buffer exchange with phosphate-buffered saline.

**Metabolic Labeling, Biotinylation of Cell Surface Proteins, Immunoprecipitation, and SDS-PAGE Analysis—** Metabolic radiolabeling was performed in Cys/Met-free RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum (FBS), 10 mM HEPES, pH 7.5, 5 mM glutamine, and 100 μCi/ml Tran35S-label (DuPont NEN) for 6 h. The cultures were then supplemented with a 10% volume of normal RPMI 1640 medium containing 10% non-dialyzed FBS for 15 h. For surface biotinylation, 1 × 107 cells were harvested by centrifugation, washed twice with phosphate-buffered saline at 4°C, after which sulfo-succinimidobiotin (Sulfo-NHS-biotin, catalogue number 21217, Pierce) was added to a final concentration of 500 μg/ml, incubated at room temperature for 30 min with agitation. The cells were then treated with 100 μg/ml glycine, pH 8.5, to quench the labeling reaction. Cells were lysed in lysing buffer containing 1% Brij 35, pH 7.2, 100 mM Tris-HCl, 1 mM EDTA, freshly supplemented with 2 mM phosphomethyl-sulfonyl fluoride. The lysates were cleared by centrifugation (14,000 × g, 10 min). The supernatants were precleared with protein A-Sepharose 4B beads at 4°C for 2 h and immunoprecipitated with 20 μl of protein A-Sepharose beads and 5 μg of the specific antibody as indicated. Immune complexes were collected on protein A beads which were then washed three times with lysis buffer and treated with Laemmli sample buffer before being resolved by SDS-polyacrylamide gel electrophoresis.

**Fingerprint Analysis by Endoproteinase-Glu-C (V8 Protease)—** Humanized 5c8-immunoprecipitated material was resolved by SDS-PAGE on a 10–20% gradient gel (Daiichi, Japan) under reducing conditions. Metabolic radiolabeling was performed in Cys/Met-free RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum (FBS), 10 mM HEPES, pH 7.5, 5 mM glutamine, and 100 μCi/ml Tran35S-label (DuPont NEN) for 6 h. The cultures were then supplemented with a 10% volume of normal RPMI 1640 medium containing 10% non-dialyzed FBS for 15 h. For surface biotinylation, 1 × 107 cells were harvested by centrifugation, washed twice with phosphate-buffered saline at 4°C, after which sulfo-succinimidobiotin (Sulfo-NHS-biotin, catalogue number 21217, Pierce) was added to a final concentration of 500 μg/ml, incubated at room temperature for 30 min with agitation. The cells were then treated with 100 μg/ml glycine, pH 8.5, to quench the labeling reaction. Cells were lysed in lysing buffer containing 1% Brij 35, pH 7.2, 100 mM Tris-HCl, 1 mM EDTA, freshly supplemented with 2 mM phosphomethyl-sulfonyl fluoride. The lysates were cleared by centrifugation (14,000 × g, 10 min). The supernatants were precleared with protein A-Sepharose 4B beads at 4°C for 2 h and immunoprecipitated with 20 μl of protein A-Sepharose beads and 5 μg of the specific antibody as indicated. Immune complexes were collected on protein A beads which were then washed three times with lysis buffer and treated with Laemmli sample buffer before being resolved by SDS-polyacrylamide gel electrophoresis.

**N-terminal Sequencing—** CD40L was immunoprecipitated from lysates of Jurkat D1.1 cells using humanized 5c8 antibody. The immunoprecipitate was resolved on a 10–20% gradient gel (Daichi, Japan) under reducing conditions. Protein fingerprints were generated by digestion with endoproteinase-Glu-C (V8 Protease) (Roche Diagnostics) and analyzed by tandem mass spectrometry (HPLC-DAD-MS/MS).

**Cell lysates prepared from 35S-metabolically labeled Jurkat D1.1 or CD40L-transfected COS7 cells were precleared and immunoprecipitated with CD40-Fc fusion protein, with antibodies indicated, and with control human (h.IgG) and rabbit (r.IgG) antibodies. The proteins were analyzed by a 10% SDS-polyacrylamide gel followed by autoradiography.

**RESULTS**

**Immunoprecipitation of the Metabolically Labeled CD40L—** To study the biochemical characteristics of CD40L, we chose the Jurkat D1.1 cell, a human T cell line on which the CD40L is constitutively expressed. Jurkat D1.1 cells were metabolically labeled and immunoprecipitated with reagents specific for CD40L humanized 5c8 (h.5c8) and CD40-Fc fusion protein (CD40-Fc). Murine 5c8 monoclonal antibody and CD40-Fc were shown previously to specifically recognize the CD40L protein (29, 30). Fig. 1, a and b, shows that CD40L with an apparent molecular mass of 33 kDa was observed in both D1.1 Jurkat cells and in transfected COS cells when h.5c8 or CD40-Fc was used for immunoprecipitation, but not with an isotype control human IgG1. A less abundant protein with an apparent molecular mass of 31 kDa (p31) was co-immunoprecipitated with 33-kDa CD40L. p31 was further characterized utilizing a rabbit anti-CD40L antisera, Rb784, which specifically recognizes the N-terminal domain (cytoplasmic domain, since this is a type II protein) of CD40L. As shown (Fig. 2) h.5c8 immunoprecipitated both the wild-type, and the cytoplasmic domain deleted mutant (cyt(-)) CD40L from cell lysates of metabolically labeled COS7 cells transiently expressing CD40L whereas Rb784 immunoprecipitated only the wild-type and not the cyt(-) CD40L from similar lysates. Interestingly, using lysates prepared from Jurkat D1.1 or COS7 cells expressing wild-type CD40L, Rb784 consistently immunoprecipitated the p31 protein (Fig. 1, a and b, and Fig. 2) indicating that expression of full-length CD40L is critical for the co-immunoprecipitation of p31 by Rb784 but not by h.5c8. These results suggested that the p31 is likely to be a fragment of CD40L and is missing the cytoplasmic domain that is recognized by Rb784. Alternatively, p31 is an intracellular protein associated with the cytoplasmic domain of CD40L.

**Immunoprecipitation of the Cell Surface-labeled CD40L—** To determine if p31 is an intracellular or extracellular protein, Jurkat D1.1 cells were labeled with Sulfo-NHS-biotin (a biotinylating reagent which is not membrane-permeable, hence capable of labeling only the cell surface proteins), lysed, and used for immunoprecipitation. Immunoprecipitates with h.5c8,
Cell lysates prepared from 35S-metabolically labeled COS7 cells transfected with mock plasmid or plasmid containing cDNA encoding either wild-type or cyt(-) CD40L were immunoprecipitated with h.5c8, Rb784, or CD40-Fc fusion protein. Proteins were analyzed by a 10–20% gradient SDS-polyacrylamide gel followed by autoradiography.

Fig. 2. Rabbit antiserum Rb784 recognizes the cytoplasmic tail of CD40L. Cell lysates prepared from 35S-metabolically labeled COS7 cells transfected with mock plasmid or plasmid containing cDNA encoding either wild-type or cyt(-) CD40L were immunoprecipitated with h.5c8, Rb784, or CD40-Fc fusion protein. Proteins were analyzed by a 10–20% gradient SDS-polyacrylamide gel followed by autoradiography.

Fig. 3. Immunoprecipitation of cell surface-labeled CD40L. Cell lysates prepared from biotin-labeled Jurkat D1.1 cells were pre-cleared and immunoprecipitated with CD40-Fc fusion protein, with antibodies indicated, and with control human (h.IgG1) and rabbit (r.IgG) antibodies. The proteins were analyzed by a 10–20% gradient SDS-polyacrylamide gel and transferred to nitrocellulose. The resolved proteins were probed with streptavidin-HRP and visualized by the ECL chemiluminescent method.

CD40-Fc, and Rb784 contained both CD40L and the p31 protein when visualized with streptavidin-HRP (Fig. 3). We also noticed that the abundance of the CD40L and the p31 proteins are comparable. This is in contrast to the results obtained from the metabolically labeled cell lysates in which the full-length 33-kDa CD40L is the predominant form. In addition, a doublet of minor bands with an apparent molecular mass of 18 kDa (p18) was also detected in immunoprecipitates with h.5c8, CD40-Fc, and Rb784, but not with control human IgG1 (Fig. 3). These results showed that both p31 and p18 are present on the cell surface, and each protein is either biochemically related to CD40L or is an extracellular protein associated with cell surface CD40L.

**The p31 Protein Is Biochemically Related to CD40L**—The p31 protein was further characterized by limited proteolysis. Lysates of biotin-labeled Jurkat cells were immunoprecipitated by h.5c8 and resolved by SDS-PAGE as before, and a limited proteolysis using the V8 protease was performed. Samples were then analyzed by two-dimensional SDS-PAGE electrophoresis. Fig. 4 shows that V8 protease treatment generated three fragments each from CD40L (spot 1) and p31 protein (spot 2) with apparent molecular masses of 27 kDa, 17 kDa, and 14 kDa (spot 1-1, 1-2, and 1-3), and 25 kDa, 15 kDa, and 12 kDa, (spot 2-1, 2-2, and 2-3), respectively. Thus, both full-length CD40L (spot 1) and p31 (spot 2) generate a similar pattern of cleavage products that differ in mass by 2 kDa. Such a parallel pattern indicates that p31 is likely to be a fragment of or a modified variant of CD40L. This inference was validated by sequence data described below. The intensity of cleavage product from p18 was too minor to interpret.

**p31 and p18 Are Fragments of the 33-kDa Full-length CD40L**—Definitive data on the relationship of p31 and p18 to CD40L was obtained by N-terminal sequencing. CD40L, p31, and p18 were immunoprecipitated from Jurkat cells by h.5c8, resolved by SDS-PAGE, transferred to a PVDF membrane, and subjected to N-terminal sequence analysis. Results from these analysis are shown in Fig. 5. N-terminal sequence data for 33-kDa CD40L matches the predicted translated protein sequence of CD40L (4, 24), indicating that Met-1 is preserved in the matured protein. The p31 protein sequence starts with Met-21 indicating that the difference between full-length CD40L and p31 is the result of N-terminal truncation (4, 24). The p18 protein starts with Gln-114 of the CD40L sequence and therefore is missing the entire cytoplasmic domain, the transmembrane region, and the extracellular stalk of the CD40L. These results unambiguously showed that both p31 and p18 are fragments of the full-length CD40L which lack the cytoplasmic tail. The fact that a peptide antibody, Rb784, specific for the cytoplasmic tail, is still capable of immunoprecipitating p31 and p18 indicates that p31 and p18 exist as heteromeric complexes with full-length CD40L.

**DISCUSSION**

We have used biochemical and immunological methods to investigate the nature of CD40L on the surface of T lymphocytes and transfected cells through which we revealed several important characteristics of CD40L. First, in addition to full-length CD40L, two fragments of CD40L, p31 and p18, are present on the surface of Jurkat D1.1 and CD40L-transfected COS7 cells. N-terminal sequence data indicate that p31 lacks the cytoplasmic tail while p18 lacks the cytoplasmic tail and transmembrane domain as well as the extracellular stalk. Second, full-length CD40L molecules, together with p31 or p18, form heteromultimers on the cell surface of both Jurkat D1.1 and transfected COS7 cells. The existence of CD40L heteromultimers was evidenced by the ability of antiserum Rb784,
which recognizes only the cytoplasmic domain of CD40L, to immunoprecipitate p31 and p18 along with full-length CD40L. Thus, p31 and p18 are physically associated with full-length CD40L on the surface of both T lymphocytes and transfected COS7 cells. We showed previously by x-ray data (17) that recombinant soluble CD40L molecules starting at Gly-116 exist as trimers indicating that the information for trimerization resides within the extracellular C-terminal 146 amino acid residues, the TNF-like domain. Thus, the heteromultimeric CD40L is likely to be trimeric. Third, cell surface CD40L complexes are primarily heteromultimeric, comprised of full-length CD40L and p31, while the intracellular CD40L complexes are primarily homomultimers of full-length molecules. This is based on the observation that comparable amounts of full-length CD40L and p31 were immunoprecipitated from lysates of surface-labeled cells while much less p31 was observed from lysates of metabolically labeled cells. The difference in full-length CD40L:p31 ratios implicates an intracellular CD40L pool. This is consistent with previously reported preformed intracellular CD40L in a subset of human CD4+ T lymphocytes (41). In addition, very little p18 was found in either lysate, indicating that only a minor fraction of heteromultimeric CD40L complexes contains p18. Finally, our results show that the full-length homomultimeric CD40L as well as surface heteromultimeric CD40L variants are capable of interacting with CD40 since they can be immunoprecipitated using CD40-Fc.

Our data also suggest a mechanism by which heterotrimetric CD40L variants are generated. Since p31 begins with methionine, the possibility exists that an alternative initiation site is used to translate p31 directly. Besides lack of a consensus Kozak sequence (31), the observation that metabolically labeled CD40L molecules are primarily full-length while cell surface molecules comprise nearly equal amounts of full-length CD40L and p31 suggests that p31 is proteolytically derived from full-length CD40L. We propose that full-length CD40L molecules assemble as homotrimers and, during surface-bound transport or once on the cell surface, undergo proteolysis to generate CD40L-p31 heterotrimers. A minor fraction of CD40L, homotrimetric full-length CD40L or CD40-p31 complexes, may undergo an additional proteolytic processing to generate heterotrimers containing p18. Importantly, our results indicate that cleavage at Arg-48/Arg-49 or Lys-106/Lys-107, identified in the extracellular domain of CD40L as potential proteolytic sites (30), is not responsible for generation of the described heterotrimetric variants.

Except for cell surface lymphotixin-αβ2 (LT-αβ2) (32), formation of heterotrimetric complexes has not been formally described for members of the TNF ligand family. Nevertheless, some evidence suggests that heterotrimetric forms of TNF-α and Fas ligand (FasL) may transiently exist (33–35). As heterotrimetric CD40L has never been reported previously, the generation of the heterotrimetric CD40L is quite unique in several aspects. First, at least two distinct proteases are involved: one located inside the cell cleaves the cytoplasmic tail off full-length CD40L to generate p31, another, likely located on the cell surface, cleaves at the carboxyl end of the extracellular stalk to produce p18. Expression of these proteases is not limited to lymphoid cells since the heterotrimetric complexes were also observed in transfected COS7 cells. Second, unlike heterotrimetric TNF-α and FasL, which appear as minor forms (34, 35), the heterotrimetric complexes represent the major form of CD40L expressed on the cell surface as nearly equal amounts of full-length CD40L and p31 were observed in surface-labeled cell lysate. Finally, in contrast to TNF-α and FasL heteromers which are present on cell surface transiently, the membrane-anchored CD40L heterotrimers appear to be quite stable. In a pulse-chase experiment, only a small amount of soluble CD40L was detected in the medium of cultured cells (data not shown). Thus, the heterotrimetric CD40L-p31 complex represents a kinetically stable form rather than merely a partially proteolyzed product prior to release from the cell surface.

It should be emphasized that heterotrimetric CD40L complexes are functionally active. We showed that both homotrimeric and heterotrimeric CD40L are capable of interacting with CD40 as CD40-Fc immunoprecipitates these variants. We also showed that CD40L molecules on Jurkat D1.1 cells, capable of stimulating B cells via CD40, exist primarily as heterotrimers. It is conceivable that differential post-translational proteolysis may result in functionally distinct forms of CD40L. Consistent with this notion is the observation that upon activation of T cells, there is a lag of functional activity of newly synthesized CD40L (37). Thus, a post-translational modification might be required for CD40L to deliver the contact-dependent signal(s) (37). If so, heterotrimetric CD40L-p31 complexes would be the functionally active CD40L.

Considering the proteolysis processing leads to conversion of homotrimeric full-length CD40L to heterotrimeric CD40L-p31 complex, it is possible that additional proteolysis may lead to the release of homotrimeric soluble CD40L from cell surface. Alternatively, membrane-anchored CD40L, either pre-existing intracellularly (41) or endocytosed after interaction with CD40 (43), may undergo a stimulation-dependent proteolytic process to generate the soluble CD40L in microsomes and subsequently released into the medium (42). The existence of biologically active soluble CD40L was first implicated in conditioned medium of a murine thymoma line (36) and later shown to be rapidly released by T lymphocytes upon activation (19). Consistently, recombinant soluble CD40L molecules were shown to be biologically active (17, 39). It should be noted that the p18 reported here and the soluble CD40L released from activated T lymphocytes (19) are different in their N termini by one amino acid. The reason for this is unclear. Nonetheless, soluble CD40L molecules differing in their N termini are biologically active (17, 19, 39) indicating that a specific N terminus (within the Glu-108 to Gly-116 region) is not critical for their functional activity. It is intriguing that although the soluble form of CD40L can activate B cell, direct cell contact with activated T cells is required for delivery of the CD40-mediated signal for B cell proliferation and differentiation. Thus, it is likely that signals transduced by CD40 via interaction with membrane-anchored heterotrimeric CD40L and that of the homotrimeric soluble CD40L are qualitatively different. We propose that interaction with CD40 may cause proteolysis of surface-anchored CD40L to generate soluble homotrimeric p18. Considering bi-directional signaling capacity of receptor-ligand interactions (38), generation of such soluble CD40L molecules would completely abrogate the signal-receiving capacity of T lymphocytes through CD40L.

The identification of heterotrimeric CD40L on the surface of T lymphocytes implicates an additional regulation of this protein during contact signaling between T and B cells. The post-
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translational modifications leading to expression of these heteromultimeric variants may provide a potential intervening step(s) for modulation of the functional activity of CD40L.

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