Brief Definitive Report

Assembly and Regulation of the CD40 Receptor Complex in Human B Cells

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Summary

CD40 is a member of the tumor necrosis factor (TNF) receptor superfamily. Studies with human B cells show that the binding of CD154 (gp39, CD40L) to CD40 recruits TNF receptor–associated factor 2 (TRAF2) and TRAF3 to the receptor complex, induces the downregulation of the nonreceptor-associated TRAFs in the cell and induces an increased expression of Fas on the cell surface. Combined signaling through the interluekin 4 receptor and CD40 induces an increased expression of Fas with a commensurate increase in the level of TRAF2, but not TRAF3, that is recruited to the receptor complex. In contrast, engagement of the membrane immunoglobulin and CD40 limits Fas upregulation and reduces the recruitment of TRAF2, relative to TRAF3, to the CD40 receptor complex. These studies show that the TRAF composition of the CD40 receptor complex can be altered by signals that influence B cell differentiation.

CD40 is a 50-kD cell surface receptor found on a wide spectrum of cell types, including B cells and antigen-presenting cells (1, 2). Interaction of CD40 with its cognate ligand CD154 (gp39, CD40L), which is expressed predominantly on activated $CD4^+$ T cells, has profound effects on both humoral and cellular immunity (1, 3–5). Although the biological effects of disruption of the CD40–CD154 interaction have been well described, the nature of the biochemical signals generated as a consequence of ligand binding remain ill-defined. The lack of an enzymatic domain in the cytoplasmic region of CD40 suggests that signaling is achieved through receptor-associated proteins. The TNF receptor–associated factors (TRAFs) have been identified as candidate CD40 signaling proteins (6–9). The TRAF family members TRAF2 and TRAF3 have been shown to bind to CD40 through their COOH-terminal TRAF domains, whereas truncation of the NH_2-terminal RING and zinc fingers of TRAF2 has been shown to block CD40-mediated NF-κB activation (7). Presently, it is not known whether these molecules constitutively associate with CD40 or are induced to assemble upon stimulation with CD40 ligand. The studies presented evaluate the dynamics of CD40 receptor assembly initiated by CD154 binding. It is shown that ligand binding to CD40 induces the recruitment of both TRAF2 and TRAF3 to the receptor complex and triggers the rapid downregulation of the remaining nonreceptor-associated TRAF2 and TRAF3. Furthermore, it is shown that engagement of the B cell receptor Ig complex or triggering of the IL-4 receptor can modify the TRAF composition of CD40 receptor complex as well as alter the biological response to CD40 cross-linking.

Materials and Methods

Cell Culture. The DND39 cell line, a human, EBV^- Burkitt lymphoma cell line (10) was cultured in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 2 mM glutamine and 10% FBS (Hyclone, Logan, Utah).

Receptor Assembly Assays. The DND39 cells (2×10^6 cells/ml) were cultured for 15 min with and without 4 nM sCD154 (CD8–CD154; reference 11). IL-4–treated cells received 2 ng/ml huIL-4 (Peprotech, Rocky Hill, NJ) 10 min before addition of sCD154. For treatment with anti-human IgM (Sigma Chemical Co., St. Louis, MO), 1×10^7 cells were incubated with 10 μg/ml anti-IgM for 24 h, followed by sCD154 for 15 min. After stimulation, the cells were lysed in 1% Digitonin, 50 mM Hepes, 150 mM NaCl, pH 7.4, with protease inhibitors at 2×10^7 cells/ml. Cleared lysate was immunoprecipitated for 2 h at 4°C with 10 μg/ml anti-hCD40 monoclonal antibody, either BE-1 (Ancell, Minneapolis, MN) or S2C6 (gift of S. Paulie, Stockholm University, Sweden) and Protein G–Sepharose (Sigma). Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and coprecipitated TRAF molecules detected with polyclonal rabbit anti–human TRAF2 (C20) (Santa Cruz Biotechnologies, Santa Cruz, CA) or anti–human TRAF3 (N) produced against a peptide corresponding to residues 9–32 of the human TRAF3 sequence. Bound antibodies were detected with goat anti–rabbit Ig–horseradish peroxidase (Bio-Rad, Hercules, CA) and detected

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with SuperSignal Substrate (Pierce Corp., Chicago, IL). CD40-cleared lysates were treated with 20 μg/ml glutathione-S-transferase (GST)–CD40cyt and glutathione–agarose for 2 h at 4°C and TRAF2 and three were detected as described above. Total TRAF2 and TRAF3 immunoprecipitations were performed on lysates from untreated and sCD154-treated DND39 cells using anti-human TRAF2 antibodies produced against a peptide corresponding to residues 249–266 of the human TRAF2 sequence or the anti-TRAF3 antibodies described above.

Flow Cytometry. Following 24-h stimulation, DND39 cells were incubated for 20 min at 4°C with either a control mouse IgG1–biotin or mouse anti–human CD95–biotin (10 μg/ml; PharMingen, San Diego, CA). After washes in PBS with FCS, antibody binding was detected with streptavidin–PE (2 μg/ml; Southern Biotechnology Associates, Birmingham, AL). Cells were analyzed on a Becton Dickinson FACScan® flow cytometer. A minimum of 10,000 cells were collected for each sample. Residual dead cells and cell aggregates were excluded from analysis by low angle and orthogonal light scatter.

Densitometric Analysis of TRAF Content. Autoradiographs of the Western blots from the anti-IgM experiments were scanned using Ofoto® 2.0. The TRAF peaks were quantitated using NIH Image 1.60.

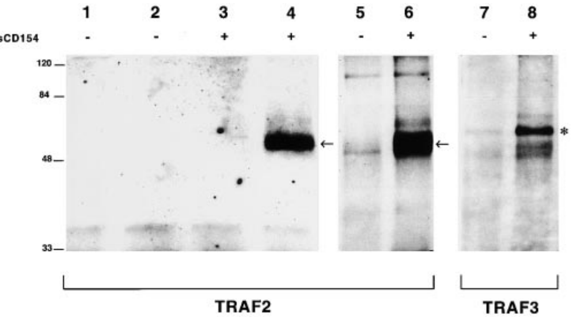

Figure 1. Stimulation with sCD154 induces recruitment of TRAF2 and TRAF3 to CD40. (*A*) DND39 cells (2×10^7 cells/lane) were either left unstimulated (lanes *1*, *2*, *5*, and *7*), or stimulated with sCD154 (4 nM) (lanes *3*, *4*, *6*, and *8*) for 15 min before lysis and immunoprecipitation with either irrelevant mouse IgG1 (lanes *1*, *3*) and or with anti-human CD40 mouse IgG1 monoclonal BE-1 (*2*, *4*) or S2C6 (lanes *5*, *6*, and *7*, *8*), respectively. The immunoprecipitated samples were immunoblotted for TRAF2 (*arrowhead*) (lanes *1–6*) or for TRAF3 (indicated by asterisk) (lanes *7*, *8*). This experiment is representative of 12 experiments.

Results and Discussion

DND39 is a CD40-responsive, human Burkitt B cell lymphoma that has been shown to increase sterile transcripts from the IgE promoter (10) and be rescued from growth inhibition by cross-linking of CD40 (12). Within 15 min of addition of a soluble, multimeric form of CD154 (sCD154) both TRAF2 and TRAF3 could be coimmunoprecipitated with CD40. Immunoprecipitation of CD40 from nonactivated DND39s did not reveal constitutively associated TRAF2 or TRAF3 (Fig. 1). Association of TRAFs was maximal at a 4 nM concentration of ligand and found to peak after 15 min of ligand addition (data not shown). The association of the TRAF molecules with CD40 was mirrored by a decrease in the cytosolic pool of TRAF2 and TRAF3, which could be precipitated with a fusion protein consisting of GST and the CD40 cytoplasmic domain (GST–CD40cyt; reference 6) (see Fig. 3, *C* and *D*). However, the reduction in the cytosolic TRAF2 and TRAF3 could only be partially accounted for by recruitment of these molecules to the receptor complex. When total cellular TRAF2 or TRAF3 was immunoprecipitated with anti-TRAF2 or TRAF3 antibodies, a reduction in TRAF content was observed following engagement of CD40 (see Fig. 4). Therefore, in addition to the recruitment of TRAF2 and TRAF3 to CD40, a significant amount of the cellular TRAF2 and TRAF3 is lost from the detergent-soluble fraction. Whether this loss is due to movement of the TRAF molecules to another subcellular location or to degradation of the TRAFs is currently being studied.

The biological response of B cells to CD40 signaling can be enhanced or inhibited by the engagement of other receptors on B cells. For example, IL-4 and CD40 engagement synergize to induce B cell growth and immunoglobulin isotype switching (13–15). In DND39 cells, cross-linking of CD40 along with IL-4 can synergistically upregulate the synthesis of the germline epsilon transcripts (10) and the expression of Fas (see Fig. 2 *C*). Studies were performed to determine whether at least some of the agonistic effects of IL-4 on CD40 signaling could be due to changes in the protein components of the CD40 receptor complex. As shown in Fig. 2 *A*, a 10-min pretreatment of DND39 cells with IL-4 increased the amount of TRAF2 recruited to the CD40 complex in response to sCD154. The amount of TRAF3 recruited to CD40 in response to sCD154 was unchanged by the inclusion of IL-4 (Fig. 2 *B*). As shown in Fig. 2 *C*, engagement of CD40 induced upregulation of Fas, which was enhanced by the coadministration of IL-4. Cells cultured in IL-4 alone expressed low levels of Fas. Thus, short-term pretreatment of the cells with IL-4 selectively increased the association of TRAF2 with the CD40 receptor complex and increased Fas expression.

Cross-linking of the B cell receptor Ig complex in B cells has been shown to exert both agonistic (16, 17) and antagonistic (18) effects on CD40 signals. The latter study showed that the cross-linking of membrane immunoglobulin (mIg) on human germinal center (GC) B cells prevented the CD40-induced upregulation of Fas. Similarly, culture of DND39 with anti-μ inhibited the upregulation of Fas induced by sCD154 (Fig. 3 *E*). To evaluate whether cross-linking of mIgM altered the assembly of the CD40 receptor complex, sCD154-induced TRAF association was investigated. As shown in Fig. 3, the association of TRAF2 with CD40 was strongly downregulated in cells that were precultured with anti-μ. In contrast, there was much less effect on the levels of sCD154-induced TRAF3 recruited to the CD40 complex in anti-μ–treated B cells. The mean of three experiments found that the level of TRAF2 recruited to the receptor was reduced by 52%, whereas the level of TRAF3 recruitment was only reduced by 19%. Anti-μ treatment also significantly reduced the amount of TRAF2 that could bind to the GST–CD40cyt fusion pro-

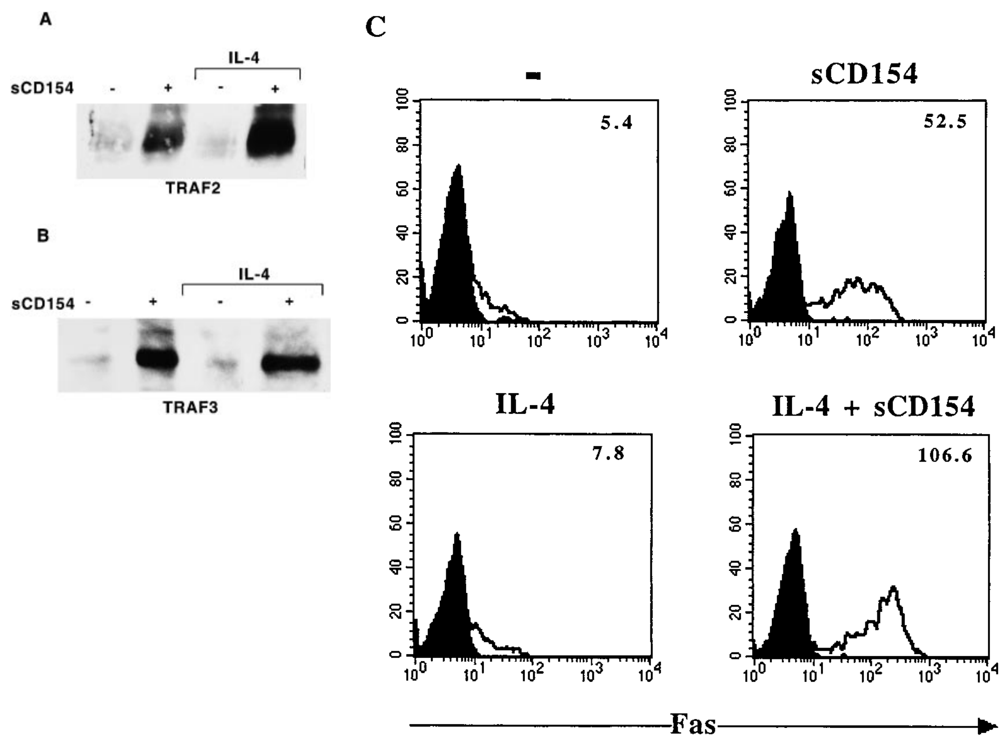

Figure 2. Pretreatment with IL-4 increases TRAF2, but not TRAF3, recruited to CD40 and increases cell surface Fas expression. DND39 cells (2 × 10^7 cells/lane) were either left untreated or pretreated for 10 min with human (h)IL-4 (2 ng/ml) (Genzyme, Cambridge, MA); and both groups were either left unstimulated (*minus*) or stimulated with sCD154 (4 nM) (*plus*) for 15 min. Lysates were immunoprecipitated for CD40 and immunoblotted for TRAF2 (*A*) or TRAF3 (*B*). (*C*) Fluorescence-activated cell sorting analysis of DND39 cells after treatment with hIL-4 and sCD154. DND39 cells were incubated for 24 h with media (*minus*), 2 ng/ml hIL-4, sCD154, or both (as indicated). Fas expression was detected with anti-CD95 monoclonal antibody (*open profile*) or a control mouse IgG1 monoclonal (*closed profile*). The mean fluorescent intensity of the cells is indicated in the upper righthand corner. This experiment is representative of four experiments.

tein in the cell lysates (Fig. 3 *C*). The most likely explanation is that the total TRAF2 protein expression was downregulated in response to anti-μ treatment (data not shown). Measurement of the total TRAF2 and TRAF3 levels in the cells found that anti-μ treatment reduced TRAF2 levels by 58%, whereas TRAF3 levels were reduced by 26% (Fig. 3 *D*). As was observed with IL-4, anti-μ altered the assembled receptor complex and also altered the biological response to CD40 signaling. FACS® analysis of CD40-induced Fas expression (Fig. 3 *E*) found that anti-μ treatment reduced Fas upregulation by approximately three- to fourfold. FACS® analysis of the level of cell surface CD40 revealed no difference between untreated and anti-μ–treated cells (data not shown).

The data presented in this study suggest that (*a*) the binding of CD154 is necessary and sufficient for the recruitment of both TRAF2 and TRAF3 to the CD40 receptor complex in human B cells; (*b*) a majority of the TRAF2 and TRAF3 molecules are depleted from the cytoplasmic pool upon ligand binding, some of which is recruited to the receptor with the remainder lost to either the detergent insoluble fraction or degraded; (*c*) IL-4, a cytokine that can enhance biological signals by CD40, selectively increased the amount of TRAF2 recruited to the ligand-induced complex; and (*d*) signals from the mIg complex exerted a selective effect on reducing the amount of TRAF2 versus TRAF3 that can be recruited to the CD40 complex upon ligand binding.

The molecular basis for why TRAF2 and TRAF3 are recruited to the receptor complex after CD154 binding is unknown. However, it is likely that receptor oligomerization plays an important role. Goeddel and coworkers have recently shown that the binding of TNF-α to TNF-R1 induced the recruitment of TRAF2 to the receptor complex (19). Molecular modeling studies based on similarities to TNF-α and TNF-β, have suggested that CD154 forms trimers and these trimers bind to three CD40 molecules (20, 21). It is also evident from functional studies with recombinant CD154 that membrane bound or multimerized CD154 possesses much better biological activity than monomeric CD154 (22). Finally, the fact that the GST–CD40cyt protein binds TRAF molecules also suggests that a high density matrix of the CD40 cytoplasmic domain may mimic an aggregated receptor and create sites for high affinity TRAF binding. Thus, taken together, it may be proposed that aggregation of TNF-R family members is a critical event in TRAF recruitment.

At the present time, the mechanisms responsible for the the rapid and extensive reduction of TRAF2 and TRAF3 after CD40 engagement are unknown. It is possible that upon receptor engagement the TRAF molecules are rapidly ubiquitinated and degraded, in a fashion similar to I-κB (23). Alternatively, the CD40 signal may result in movement of the TRAFs to a subcellular location that is not captured after detergent solubilization. The fact that the majority of the TRAF2 and TRAF3 was lost from the cell after addition of ligand has implications for signaling via the other members of the TNF-R family, which also bind TRAF2 (i.e., TNF-R2, LT-βR, and CD30) or TRAF3 (CD30 and LT-βR). One might anticipate that within an

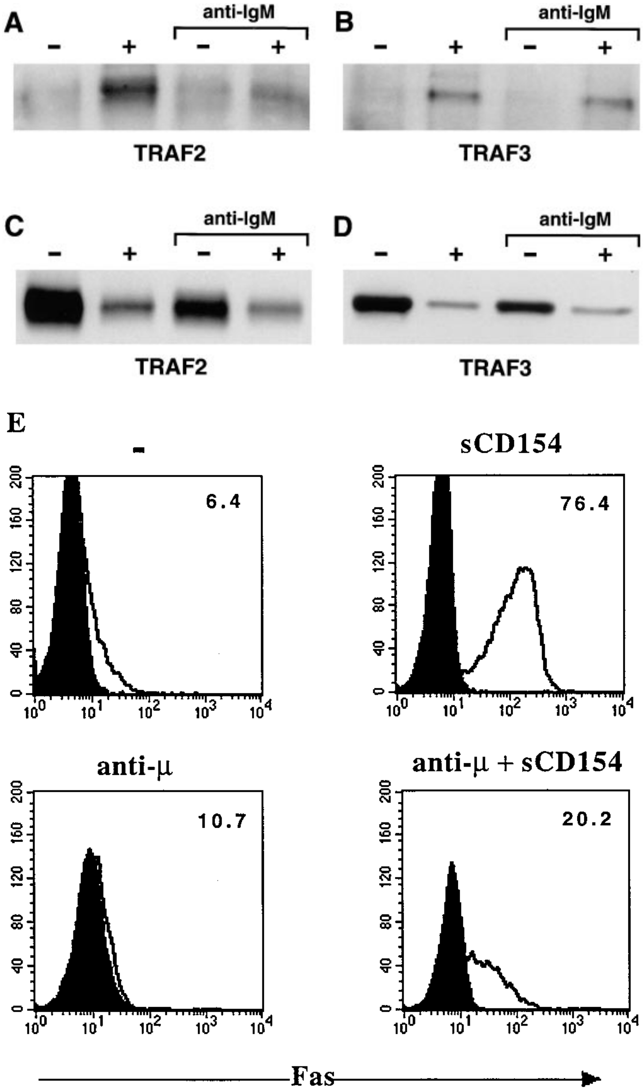

Figure 3. Anti-μ cross-linking reduces TRAF2 association and Fas upregulation induced by CD40 signaling. DND39 cells (2×10^7 cells/lane) were incubated in media or with 10 μg/ml goat anti–human IgM for 24 h and both groups were either left unstimulated (*minus*) or stimulated with sCD154 (4 nM) (*plus*) for 15 min. Lysates were immunoprecipitated for CD40 and immunoblotted for TRAF2 (*A*) or TRAF3 (*B*). (*C* and *D*) Immunoblot analysis of cytosolic TRAF content after GST–CD40cyt immunoprecipitations. The lysates used for the anti-CD40 precipitations described in *A* and *B* were further precipitated with GST–CD40cyt. 5×10^6 cells/lane were loaded for the TRAF2 blot, while the TRAF3 blot received 2×10^7 cell equivalents per lane. (*E*) Fluorescence-activated cell sorting analysis of DND39 cells after treatment with anti-IgM and sCD154. DND39 cells were incubated for 24 h with media (*minus*), 10 μg/ml anti-IgM, sCD154, or both (as indicated). Fas expression was detected with anti-CD95 monoclonal antibody (*open profile*) or a control mouse IgG1 monoclonal (*closed profile*). The mean fluorescent intensity of the cells is indicated in the upper righthand corner. This experiment is representative of four experiments.

individual cell, the engagement of one TNF-R family member might cause elimination of the majority of the available TRAF molecules, leading to the desensitization of signalling through other receptor family members.

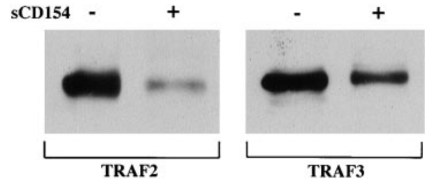

Figure 4. Engagement of CD40 results in the loss of immunoprecipitable TRAF2 and TRAF3 from DND39 cells. DND39 cells (2×10^6 cells/ml) were left untreated (*minus*) or treated with 4 nM sCD154 (*plus*) for 15 min. After treatment, lysates were immunoprecipitated with either anti-TRAF2 or anti-TRAF3 antibodies. 5×10^6 cell equivalents were loaded per lane.

The ligand-induced assembly of the CD40–TRAF2–TRAF3 receptor complex in resting B cells may be triggered by the release of TRAF2 and TRAF3 complexes that are retained in the cytosol through interactions with the recently identified Tank/I-TRAF (9, 24). By analogy to studies with TNF-R2 (24), it may be that upon stimulation with CD154, CD40 oligomerizes and creates a higher affinity binding site for the TRAF molecules than found on Tank/I-TRAF. Accumulating evidence suggests TRAF2, perhaps through NF-κB activation, plays a dominant role in the early responses of resting B cells to CD40 signaling. Early events in murine B cells are likely to be mediated by TRAF2, because B cells from TRAF3 knockout mice responded as wild-type B cells for the upregulation of such activation antigens as CD23 and B7-1 as well as proliferation, yet were deficient in Ig isotype switching (25). Correspondingly, the DND39 cells lost their capacity to upregulate Fas when the cytosolic pool of TRAF2 was diminished. This loss in the ability to upregulate Fas may be due to the decreased amount of available TRAF2, but more importantly, the altered ratio of TRAF2/TRAF3. Similar imbalances in TRAF2/TRAF3 were observed in HEK 293 cells, where overexpression of TRAF3, relative to TRAF2, blocked the NF-κB activation via CD40 (7). Therefore, signals that alter the abundance of TRAF molecules or the ratio of TRAF molecules may qualitatively change the biological signals through CD40.

As stimulated B cells differentiate to GC B cells, memory B cells, and plasma cells, the function of CD40 changes. In immature B cells, CD40 engagement rescues from apoptosis (26, 27), in mature B cells it induces proliferation and differentiation (2), in GC B cells it induces Fas expression (18, 28, 29), and in some lymphomas it induces apoptosis (30, 31). It appears that the CD40 receptor can be rewired. The fact that biological mediators such as IL-4 and anti-μ treatment can both modify the recruitment of TRAF2 to the receptor complex and alter the biological readout suggests that the TRAF composition of the CD40 receptor may contribute to the molecular basis for the rewiring. Currently, we are attempting to establish a causal relationship between the TRAF composition of the CD40 receptor complex and the functional signals delivered by CD40 engagement.

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