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 factors (TRAFs) 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 interleukin 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.

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). The DND39 cells (2 × 10⁶ 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), 10 min before addition of sCD154. For treatment with anti-human IgM (Sigma Chemical Co., St. Louis, MO), 10 × 10⁶ 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 Hapes, 150 mM NaCl, pH 7.4, with protease inhibitors at 2 × 10⁷ 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 IgG-horseradish peroxidase (Bio-Rad, Hercules, CA) and detected...
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 (mlg) on human germinal center (GC) B cells prevented the CD40-induced upregulation of Fas. Similarly, culture of DND39 with anti-μm inhibited the upregulation of Fas induced by sCD154 (Fig. 3 E). To evaluate whether cross-linking of mlgM 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-μm. In contrast, there was a much less effect on the levels of sCD154-induced TRAF3 recruited to the CD40 complex in anti-μm-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-μm treatment also significantly reduced the amount of TRAF2 that could bind to the GST–CD40cyt fusion pro-
tein in the cell lysates (Fig. 3C). The most likely explanation is that the total TRAF2 protein expression was downregulated in response to anti-m treatment (data not shown). Measurement of the total TRAF2 and TRAF3 levels in the cells found that anti-m treatment reduced TRAF2 levels by 58%, whereas TRAF3 levels were reduced by 26% (Fig. 3D). As was observed with IL-4, anti-m altered the assembled receptor complex and also altered the biological response to CD40 signaling. FACS® analysis of CD40-induced Fas expression (Fig. 3E) found that anti-m 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-m-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 increases the amount of TRAF2 recruited to the ligand-induced complex; and (d) signals from the mlg 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 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 IκB (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
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.

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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