CD30 is a member of TNF receptor superfamily that comprises a group of cysteine-rich receptor proteins such as CD27, CD40, and Fas antigen (1–4). Biochemical studies of CD30 as well as functional studies of the ligand for CD30 (CD30L) provided strong evidence to support regulatory roles for CD30 in lymphocytes (5–7). CD30L induces various biological effects on human CD30-positive cell lines such as activation, proliferation, differentiation, and cell death, depending on cell type, stage of differentiation, transformation status, and the presence of other stimuli (7). Recently, it was reported that CD30-deficient mice showed impaired negative selection in the thymus (8) and that CD30 is involved in signaling TCR-mediated cell death of T-cell hybridoma (9). As for signal transduction of CD30, Ellis et al. reported the induction of Ca²⁺ influx by cross-linking CD30 on Jurkat cells (10), and signals mediated by CD30 were seen to regulate gene expression through activation of NFκB (11, 12).

Because the cytoplasmic tail of receptors of the TNF receptor family does not have intrinsic catalytic activity such as kinase activity, it was considered that molecules that associate with these receptors mediate signal transduction. Putative signal transducing proteins that associate with TNF receptor type II were cloned and named TNF receptor-associated factor (TRAF) (1). Subsequently, TRAF3 or CRAF1 (CD40bp, CAP1, LAP1) was identified as the CD40 signal transducing molecule (Refs. 14–17; reviewed in Ref. 18). We have recently cloned TRAF5 that associates with lymphotoxin β receptor and CD40 and mediates NFκB activating signals (19, 20).

We have found that an approximately 100-amino acid sequence of the C-terminal region of the CD30 cytoplasmic region was highly conserved among human, rat, and mouse CD30 protein, and in this region there are two subdomains with a higher conservation (D1 and D2) (21). Recently, the association of TRAF1, 2, and 3 with the cytoplasmic tail of CD30 has been reported, and the binding sites were characterized (22). However, it has remained to be determined whether and how these TRAF proteins are involved in NFκB activation by CD30. To better understand the CD30 signal transduction pathway that leads to NFκB activation, we asked whether TRAF5, as well as TRAF2, are involved in CD30 signaling. We found that TRAF5, as was shown for TRAF2, directly associates with the D2 subdomain of the CD30 cytoplasmic tail, and both TRAF2 and TRAF5 are involved in the signal transduction leading to NFκB activation.
TRAF5 and TRAF2 Mediate CD30 Signaling

hCD30(D1+D2), and phCD30(A132), respectively. These deletion mutants lack the D2 subdomain or both D1 and D2 subdomains at the C terminus (21), respectively. Those for mouse TRAF2 and TRAF5 tagged with FLAG were prepared using pME18S and PCR-amplified cDNA fragments encompassing the protein coding region. The resultant plasmids were named pMEFLAG-TRAF2 and pMEFLAG-TRAF5, respectively. The 293T cells were cotransfected with 1 μg each of expression vectors of these human CD30 and FLAG-tagged TRAF5 or TRAF2, using Lipofectin™ reagent (Life Technologies, Inc.).

RESULTS AND DISCUSSION

Signaling from CD30 was shown to activate NFκB; however, transducers of this signal have not been well characterized. On the other hand, two TRAF proteins, TRAF2 and TRAF5, were proven to have NFκB activating capacity. Therefore, study of the involvement of TRAF2 and TRAF5 in NFκB activation by CD30 is needed to better understand the biological functions of CD30. We reported that an approximately 100-amino acid sequence is highly conserved between rat and human CD30, and we have tentatively divided this region into two subdomains, D1 and D2 (21). These observations also held true for the mouse CD30 (23), thereby suggesting the functional importance of these subdomains (Fig. 1A). Moreover, it was shown that TRAF1, 2, and 3 interact with CD30 through binding sites in C-terminal 36 amino acids (22), which signifies that these TRAF proteins bind to the region containing the C-terminally located D2 subdomain. To determine if TRAF5 binds to CD30 and if so which of the subdomains are involved in the binding, we examined interactions of TRAF5, along with TRAF2, with CD30 in vitro and in vivo. First we examined in vitro binding using the CD30 cytoplasmic region fused to glutathione S-transferase and in vitro translated TRAF proteins. We constructed GST fusion proteins of CD30 having both or either one of them (Fig. 1A). GST fusion proteins with two subdomains were GST-hCD30(D1+D2) that has the C-terminal 136 amino acids of human CD30, and GST-mCD30(D1+D2) and GST-rCD30(D1+D2) that have C-terminal 110 amino acids of mouse and rat CD30, respectively. GST-rCD30 (D2) and GST-rCD30 (D1) have either of the subdomains of the rat CD30. Affinity purified GST fusion proteins were tested for their potential to precipitate 35S-labeled in vitro translated full-length mouse TRAF2 and TRAF5. As shown in Fig. 1B, both TRAF2 and TRAF5 were precipitated by GST-CD30 proteins having both D1 and D2 subdomains of human, mouse, or rat origin; they interacted with the CD30 GST fusion protein possessing the D2 subdomain but not with that having the D1 subdomain. Thus, it was suggested that TRAF5, as well as TRAF2, interacts with CD30 through the binding site(s) in the D2 subdomain.

To confirm this proposal, we next examined the interaction of CD30 with TRAF5 and TRAF2 by cotransfection and coimmunoprecipitation analysis. Human CD30 and deletion mutants were coexpressed with FLAG-tagged TRAF5 or TRAF2 in 293T cells. CD30 proteins were immunoprecipitated from cell lysates using the BerH2 anti-CD30 monoclonal antibody (DAKO), and the immune complexes were analyzed for the presence of FLAG-TRAF5 or FLAG-TRAF2 by immunoblotting using anti-FLAG M2 antibody (Eastman Kodak). The BerH2 anti-CD30 antibody co-immunoprecipitated TRAF2 and TRAF5 from the cells transfected with wild type CD30 cDNA. However, deletion of the D2 subdomain or both D1 and D2 subdomains abrogated the precipitation of either TRAF2 or TRAF5 (Fig. 2, upper panel). Conversely, the anti-FLAG M2 antibody co-immunopre-
The threonine residue at position 254 of CD40 was shown to be important for binding to TRAF3 (14) and for biological functions. We next examined the effect of amino acid substitution at 463 for interaction with TRAF2. Substitution of alanine for threonine deprived the mutant protein CD30A30A of the potential to interact with TRAF2 (Fig. 3B), suggesting that the threonine residue in the PEQET binding sequence is essential for biological activity of CD30 and that this threonine residue may have functional similarities with threonine 254 in CD40 (27).

Because we have previously demonstrated that TRAF5 can activate NFκB (19, 20), the results in the present study indicated that both the two TRAF proteins with NFκB activating capacity, TRAF2 and TRAF5, interact with CD30. Because signals from CD30 have been shown to activate NFκB and induce gene expression (11, 12), we asked if TRAF2 and TRAF5 are involved in NFκB activation. We performed a reporter gene assay using [κB]TK-CAT and [κBm]TK-CAT plasmids and expression vectors for CD30 and full size or N-terminally truncated TRAF2 or TRAF5 that lacks ring finger and zinc finger domains. We first confirmed that overexpression of TRAF2 or TRAF5 alone resulted in a 4.1- or 4.8-fold induction of CAT activity (Fig. 4A). On the other hand, the overexpression of CD30 alone showed about a 9-fold induction of CAT activity, also in a κB site-dependent manner (Fig. 4B). Cotransfection of CD30 and the TRAF domain of TRAF2 or TRAF5 suppressed CD30-mediated NFκB activation by 66 or 39%, respectively (Fig. 4B), indicating the dominant negative effect of TRAF domain overexpression. Simultaneous expression of TRAF domains of TRAF2 and TRAF5 further suppressed CD30-mediated NFκB activation by 80%, thereby
suggesting that both of these proteins are involved in signal transduction of CD30.

CD30 is expressed in activated T-cells as well as lymphocytes infected with human T-cell lymphotrophic virus-1 or EBV; therefore, we examined the expression of TRAF2 and TRAF5 in lymphocytes by Northern blot analysis. A panel of B- and T-lymphocyte cell lines, some of which are infected with human T-cell lymphotrophic virus-1 or EBV, were included in the study. We found that TRAF2 and TRAF5 transcripts were expressed in all the cell lines tested. CD30 mRNA was also expressed in these cell lines, except for EBV noninfected BJAB and Ramos cells (data not shown). Colocalization of expression provided further supportive evidence for the involvement of TRAF5 and TRAF2 in CD30 signal transduction.

These results demonstrate for the first time the involvement of TRAF2 and TRAF5 in NFκB activation by CD30. Direct interaction of TRAF5 with the C-terminal end of the CD30 protein and involvement of TRAF2 and TRAF5 in CD30-mediated NFκB activation underline the complexity of interplay between the TRAF family proteins in the signal transduction of CD30 and may explain the pleiotropic biological activity mediated by CD30-CD30L interaction. Stoichiometric studies of the interaction will be needed to analyze the interplay of TRAF proteins and determine the roles played by individual TRAF proteins during CD30 signal transduction.

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