Molecular Mechanisms of CD40 Signaling

GAIL A. BISHOP* and BRUCE S. HOSTAGER

Department of Microbiology, The University of Iowa, Iowa City, IA 52240, USA

Abstract. CD40, a member of the growing tumor necrosis factor receptor (TNF-R) family of molecules, functions as a transmembrane signal receptor in both hematopoietic and non-hematopoietic cell types, although its physiological roles are less well understood in the latter. Much has been learned over the past decade about the role of CD40 signaling in various cellular functions. In addition, some of the molecular events which occur subsequent to CD40 engagement have been characterized, although much remains to be understood. This review will summarize the known important biological roles of CD40, and discuss what is currently known about how CD40 signals.

Key words: CD40, lymphocyte activation, signal transduction, B cell; tumor necrosis family receptor family.

Biological Effects of CD40 Signaling

CD40, a 50 kDa transmembrane molecule, is expressed constitutively on B lymphocytes, dendritic cells, and monocytes/macrophages. CD40 signals to B lymphocytes are crucial to normal T cell-dependent B cell activation, isotype switching, and the development of a humoral memory response (reviewed in44, 83). The physiological importance of CD40-mediated signals was first appreciated with the discovery that lack of such signals (due to defective expression or binding of the ligand for CD40, CD154) is the underlying defect in the rare human immunodeficiency disease, X-linked hyper-IgM syndrome (reviewed in9). Subsequently, production and characterization of CD40 and CD154-deficient mice strengthened this causal link, as the phenotype of these mice closely mirrors the symptoms displayed by HIGM patients13, 87. In addition to a major direct role in B cell activation, CD40-mediated signals leading to the upregulation of adhesion and costimulatory molecules, as well as lymphokine production, are important for the activation of antigen presentation by dendritic cells11, 47, macrophages75, and B cells40, 45, 90. These CD40 signals to antigen-presenting cells (APC) have been shown to be critical for the activation of cytolytic T cells by cross priming6, 71, as well as stimulation of anti-tumor immunity in several experimental systems28, 58.

In addition to providing necessary signals for normal immune responses, CD40 can participate in autoimmune reactions3, 17, 23, 60, and contribute to transplant rejection16, 72. CD40 signals have been shown capable of both promoting8, 26, 48, 81 and inhibiting20, 61 the growth of malignant cells, so the potential utility of CD40 as a target for anti-tumor therapies is complex, and must be specifically determined for individual types of tumors. Interestingly, while constitutive CD40 expression is generally restricted to cells of the hematopoietic lineage, the molecule is also expressed on vascular endothelium29, 42, and its ligand CD154 is expressed on eosinophils22. Additionally, CD40 was

* Correspondence to: Gail A. Bishop, Ph.D., Professor of Microbiology and Internal Medicine, Director, Immunology Graduate Program, The University of Iowa, 3–570 Bowen Science Bldg., Iowa City, IA 52242, USA, tel.: +1 319 335 7945, fax: +1 319 33590 06, e-mail: gail-bishop@uiowa.edu
originally defined as a tumor antigen of bladder carcinoma\textsuperscript{43}, and CD40 expression has been reported on prostate, renal and cervical carcinomas, as well as melanoma\textsuperscript{44, 46, 68, 82}. Its endogenous or introduced expression on human prostate cancer cells correlates with increased resistance to growth inhibition via Fas or the TNF-R\textsuperscript{68}. It was also recently shown that treatment of cultured microglial cells with amyloid-β protein induces their expression of CD40. CD154 stimulation of these cells induced signs of activation and neuronal injury, and CD154 deficiency reduced the abnormal Tau phosphorylation seen in a transgenic mouse model of Alzheimer’s disease\textsuperscript{40}. Thus, it is clear that understanding how CD40 delivers its signals to cells is critical not only to understanding the pleiotropic effects of this signal receptor, but to rational design of therapies which seek to intervene in the CD40 signaling pathway.

**TRAF Molecules in CD40 Signaling**

Although it has been known for some time that engagement of CD40 has profound effects, a clear understanding of the steps leading from CD40 ligation to cell effector functions has not yet emerged. It is now appreciated that although CD40 may ultimately exploit some of the same intermediate signaling pathways as do receptors belonging to the Ig gene superfamily, the initial steps of the CD40 signal cascade do not mirror those used by more well-studied molecules such as the B cell antigen receptor or Fc receptors. First, CD40 itself has no intrinsic enzymatic activity, and neither the transmembrane (TM) nor cytoplasmic (CY) domains of CD40 contain any tyrosine-based motifs, which mediate direct binding to protein tyrosine kinases. However, analysis of protein-protein interactions using two-hybrid yeast and bacterial fusion protein techniques revealed that the CY domain of CD40 can directly associate with members of the family of CY proteins known as tumor necrosis factor receptor-associated factors (TRAFs). TRAFs are cytoplasmic proteins containing a TRAF domain, which associates with the receptor, a coiled-coil domain by which TRAFs are hypothesized to homo- and heterodimerize, a zinc RING (with the exception of TRAF1) and Zn finger domains. CD40, itself a member of the rapidly-growing TNF-R family, has to date been reported to bind to TRAFs 2, 3, 5 and 6\textsuperscript{13, 39, 40, 69}. However, understanding the specific roles of TRAFs in signaling by CD40 and other members of the TNF-R family is still incomplete.

One of the earliest approaches used to understand TRAF signaling was to overexpress wild-type (wt) and mutant TRAF constructs in an adenovirus-transformed human embryonic kidney cell line called 293, widely used for its ease of transient transfection and high expression of transfected molecules. Because 293 does not express CD40, interactions of CD40 and TRAFs were examined by also overexpressing transfected CD40 in these cells. As leukocytes themselves endogenously express rather modest amounts of TRAF proteins, use of this system greatly facilitated obtaining information on which TRAFs can bind CD40 in cells. However, the inherent and considerable artificiality of the system (irrelevant cell type, highly overexpressed proteins) has led to complications in interpretation, when used as a model for understanding CD40 signaling. A highly reproducible finding in both normal B cells and B cell lines is that CD40 ligation stimulates nuclear translocation and activation of nuclear factor κB (NF-κB)\textsuperscript{1, 48, 54}. To determine if TRAFs play a role in this process, a number of studies overexpressed CD40, TRAFs, and an NF-κB reporter gene transiently in 293 cells. Using this approach, it was concluded that TRAFs 2, 5, and 6 are required for CD40-mediated NF-κB activation\textsuperscript{39, 40, 69}. However, cells from mice made transgenic for truncated, “dominant-negative” (DN) TRAF2 (retaining the receptor-binding domain but lacking the Zn RING) are able to activate NF-κB in response to signals from TNF-R family members, including CD40\textsuperscript{52}. In a second study, embryonic cells from TRAF2-deficient mice (which die in utero or soon after birth), show the ability to activate NF-κB in response to TNF signals\textsuperscript{69}, in contrast to earlier conclusions based upon the 293 model\textsuperscript{69}. In the same month, it was shown in a study of CD40-mediated NF-κB activation in B cell lines that CD40 mutants unable to bind TRAF2 are still able to activate NF-κB\textsuperscript{35}. More recently, it was shown that CD40 mutants unable to bind TRAF6, but which retain the TRAF2 binding site, also activate NF-κB normally\textsuperscript{41}, in contrast to earlier experiments performed using the 293 model system\textsuperscript{69}. These findings suggest there may be cell type specificity in TRAF function, as well as potential redundancy between TRAFs in contributing to CD40-mediated NF-κB activation. These results also underscore the importance of studying TRAF functions in cells which normally express CD40, in order to obtain a compete understanding of how TRAFs participate in CD40 signaling \textit{in vivo}.

Another approach which has been informative in studying signaling proteins is overexpression of mutant forms of the protein which block the normal signal cascade and act as DN. A small number of studies have used this approach to examine TRAF roles in CD40
function in B lymphocytes. A complication to the use of this approach for studying TRAF function, however, is that it has proven difficult to achieve high levels of expression of DNTRAF molecules in B lymphocytes, as shown in 3 of the aforementioned studies; in the remaining study, levels of protein expression of the DNTRAF studied were not reported. It was possible to circumvent this problem to some degree, as well as preserve normal cell functions, by expressing DNTRAFs in an inducible manner. However, low expression of transfected TRAFs remains a technical challenge, and it is thus not advisable to draw conclusions regarding TRAF roles in particular CD40-mediated signaling events solely on the basis of results using DNTRAFs. A complementary approach has been B lymphocyte functional studies of mutant CD40 molecules whose binding of particular TRAF molecules has been characterized. An early study using agonistic anti-CD40 mAb as a stimulus found that the threonine in the TRAF2/3 binding motif was required for CD40-mediated growth inhibition in a mouse B cell line, M12, while a later study with the same cell line and soluble CD154 as a stimulus came to the opposite conclusion, although in the latter study the mutant CD40(T234A) molecule did not inhibit growth as effectively as wt CD40. It was subsequently shown that the T234A mutant is capable of binding a reduced amount of TRAF2, so the use of CD154 versus anti-CD40 as a stimulus may account for the discrepancy in results. Several studies showed that binding of TRAF2 is required for CD40-mediated B7 upregulation, as well as protection from antigen receptor-mediated growth arrest. It has also been shown that the binding site for TRAF2 is sufficient for CD40-mediated activation of c-Jun kinase (JNK), MAPKAP kinase 2, and the phosphorylation of IκBα, although additional studies showed that neither TRAF2 nor TRAF6 are absolutely required for the activation of NF-κB, and the two molecules may play redundant roles in this function. Structure-function studies have also revealed that the TRAF2/3 binding site is not required for CD40-mediated antibody production, but may play a role in cooperation between CD40 and the B cell antigen receptor, and that binding of TRAFs 2 and 3 is dispensable for CD40-mediated IL-6 production. To date, only a single structure-function analysis of the role of TRAF6 in CD40-mediated B cell functions has been reported; in this study it was shown that TRAF6 binding plays roles in antibody production, B7 upregulation, and IL-6 secretion, but is not required for the activation of JNK or NF-κB if the TRAF2 binding site of CD40 remains intact.

Over the past decade, advances in techniques for producing genetically modified mice have resulted in an explosion of the use of “knockout” mice, in which the expression of specific gene products has been disrupted, to study the role of a particular protein in the intact animal. While this powerful technology has led to many valuable new insights into the function of various signaling proteins, it becomes problematic when the protein in question plays multiple roles during development of the mouse. This appears to be the case for the TRAF molecules; mice made genetically deficient in TRAFs 2, 3, and 6 show very early lethality with multiple organ system abnormalities. Thus, although some attempts have been made to examine CD40 signaling in splenocytes from these mice, it is difficult to interpret such data, as there is no clear indication that numbers or phenotype of B cells developing in this environment are normal. Because macrophages from TRAF2−/− mice were found to produce elevated amounts of TNF, the TRAF2−/− mice were recently bred onto TNF knockout mice, which enhanced their survival. CD40-mediated proliferation and NF-κB activation were studied in the double knockouts, and found to be defective. However, it has been previously shown that CD40 ligation induces B cells to produce TNF-α, and TNF can induce B cells to produce antibody and upregulate costimulatory molecules. Additionally, it was shown that DNTRAF2 can inhibit CD40-induced B cell differentiation even when the CD40 molecule expressed is a mutant which does not bind TRAF2. Thus, CD40-mediated TNF production and subsequent signaling through the TNF-R may contribute to CD40 effector functions, which makes the results of the aforementioned study difficult to interpret clearly. In the case of TRAF6−/− mice, ~10% of the mice survive until birth-2 weeks, although none survive to adulthood. Splenocytes from these mice were pooled for experiments, in which decreased CD40-mediated proliferation and NF-κB activation was seen. However, the fact that so few of the mice even survive until birth suggests that any B cells present (defined in these experiments as what was left after depletion of CD4+ and Mac-1+ cells) developed in a very abnormal environment, and the phenotype of such B cells is unknown – they also show defective LPS responses. All TRAF3−/− mice die within 10 days of birth with severe progressive ruffling and massive loss of splenic cellularity. To study the function of their hematopoietic cells, fetal liver cells from these mice were transferred into irradiated recipients. Several months after reconstitution, analyses of immune function were performed. It was found that the B cells from mice that received
Traf3+- fetal liver proliferated normally in response to CD154, and also upregulated CD23 and B7-1. However, these mice showed defective T cell or antibody response to challenge with a T-dependent antigen16. It was concluded that TRAF3 plays no important role in CD40 function in B cells, in contradiction to results from the same lab which showed that expression of DNTRAF3 inhibits CD40-mediated B7 upregulation in B cells13. Thus, interpretation of data obtained using TRAF3+/- mice is complex, and does not provide definitive answers to the roles played by TRAFs in CD40 signaling to normal mature cells.

CD40 Signaling and Membrane Microdomains

In 293 cells, transfected TRAF molecules associate constitutively with transfected CD4031, but published studies presented a confusing and contradictory picture of the association of TRAF molecules with CD40 in B cells. Chaudhuri et al.12 concluded, based upon Western blotting of cell lysates, that TRAF2 associates with CD40 in resting B cells, and is released into the cytoplasm following CD40 engagement. Using similar techniques, Kühne et al.46 concluded exactly the opposite. To resolve this issue, and avoid any potential post-lysis artifacts which might complicate biochemical approaches, a fluorescently-labeled forms of TRAF2 was inducibly expressed in B cells32. Following induction of expression of this molecule, confocal immunofluorescence microscopy revealed that TRAF2 is dispersed across the cytoplasm in unstimulated B cells, but specifically excluded from the nucleus. When CD40 is ligated with either anti-CD40 mAb or cells expressing membrane CD154, TRAFs 2 and 3 rapidly traffic to the cell membrane and associate with CD40. It was discovered in the same study that CD40 and associated TRAF molecules traffic to membrane microdomains or “rafts” following CD40 ligation. In addition to requiring the CD40-binding TRAF domain, this recruitment to membrane rafts involves the Zn-binding domains of TRAFs, if they are absent, or if stimulation is performed in the presence of a membrane-permeable Zn chelator, TRAF recruitment to rafts is inhibited. Additionally, Zn chelation prevents the CD40-mediated activation of JNK. Taken together, these findings show that CD40 signaling results in the trafficking of both CD40 and TRAFs 2 and 3 to membrane microdomains, and that this recruitment is functionally important in CD40 signaling.

Kinase Activation by CD40

A number of signaling molecules have been proposed to participate in the proximal events in CD40 signal transduction. As outlined above, TRAF proteins appear to be key elements in signaling. Their amino acid sequences, together with available experimental results, suggest that TRAFs function primarily as adapter molecules. The relatively conserved carboxy-terminal TRAF-C domains of TRAFs mediate binding to CD4015, 36, 39, while both the TRAF-N and -C domains appear to mediate binding between TRAFs79. It is likely that aggregation of the TRAFs, initiated by the clustering of CD40 that occurs upon engagement by trimeric CD154, leads to their interaction with downstream signaling molecules. This aggregation may also be accomplished in a CD40-independent manner by gross overexpression of TRAFs in transiently transfected cell lines such as 293. At least three distinct CD40 signaling pathways appear to be linked to TRAF proteins. One leads to the activation of JNK, one to activation of a related stress-activated protein kinase, p38, and a third leads to NF-κB activation.

Germinai center kinase (GCK)91 and related enzymes73 have been shown to interact with the TRAF domain of TRAF2, and potentially mediate CD40-induced JNK activation. GCK can also bind MEKK1, a MAP3K which lies upstream of JNK14, 70, 88. Interestingly, MEKK1 may also interact with the N-terminal zinc-binding domain of TRAF24, suggesting that in an active signaling complex, MEKK1 contacts both GCK and TRAF2. TRAF2 may also interact in vivo with another MAP3K, ASK-1, which appears capable of mediating activation of both JNK and p3877. Activation of p38 may also occur via the interaction of TRAF2 with receptor-interacting protein (RIP) a death domain-containing serine/threonine kinase91.

The activation of NF-κB by TRAF2 and TRAF6 has been credited to NIK59, 77, a MAP3K that in turn can phosphorylate and activate the IkB kinase (IKK) complex responsible for phosphorylating and hence initiating the degradation of the inhibitor of NF-κB (IkB) proteins56. Although NIK can influence NF-κB activation in 293 epithelial cells, currently there is no definitive evidence that it specifically participates in CD40-mediated NF-κB activation in B lymphocytes. A lymphoplasia (aly) mice, which express a mutant form of NIK74, display defective B cell activation. However, this defect is not specific to CD40 responses, as B cells from aly mice also fail to respond normally to LPS or anti-IgM signals73. Thus, the role played by NIK in CD40 responses may be complex, and dependent upon
the activation state of the cell, as well as what additional signals it has received. Interestingly, MEKK1 has also been shown capable of phosphorylating and activating the IKK complex\(^31\), and may serve redundant functions with NIK in certain situations. It is also important to note that the interactions of TRAFs with MEKK1, NIK, and other kinases have almost exclusively been demonstrated under non-physiological conditions, in which both TRAFs and candidate kinases are transiently overexpressed in epithelial cell lines. It thus remains to be determined which, if any, of the potential TRAF-interacting kinases, when present at physiological levels, participate in the activation B lymphocytes and other antigen-presenting cells by CD40. It seems likely that additional kinases will be found to be involved in connecting CD40 ligation to the activation of NF-κB, as well as to additional signaling pathways.

In addition to the enzymes discussed above, several other kinases have been posited to contribute to CD40 signal transduction. CD40 engagement has been shown to activate the Src family kinase Lyn, and to induce the phosphorylation of both phosphatidylinositol-3-kinase and phospholipase C\(\gamma\) in human B cells\(^{35}\). However, it is not clear how these enzymes fit into the overall mechanism of CD40 signal transduction. Other components of the CD40 signaling apparatus undoubtedly await discovery. We previously demonstrated that a 22 amino acid truncation of CD40 (CD40Δ22), which abrogates the ability of CD40 to bind TRAFs 2 and 3, does not interfere with CD40-activated NF-κB activation nor antibody secretion in mouse B cell lines\(^{31,33,35}\). However, both these functions are nearly abolished by the removal of an additional ten amino acids in the mutant CD40Δ32\(^{33,35}\). Interestingly, CD40A32 retains the binding site for TRAF6 and shows normal ability to induce B cell IL-6 production\(^2\), a CD40 function which is TRAF6-dependent\(^{41}\). These findings suggest that a novel factor involved in the activation of both NF-κB and antibody production requires sequences between the TRAF6 and TRAF2/3/5 binding sites.

**CD40-Mediated Transcriptional Regulation**

CD40 signaling to B cells causes increased production of a number of proteins, including various surface molecules, lymphokines, and immunoglobulins. In addition, CD40 signals have been shown to be critical for normal isotype switching. In all of these cases, CD40 can affect transcriptional regulation of the involved genes. However, how this regulation is effected is not completely clear. Most of the studies of CD40-induced transcriptional regulation have focused upon activation of members of the NF-κB family of transcription factors, although it has also been reported that CD40 ligation can activate additional transcription factors, including BSAP\(^{34}\), AP-1, NF-AT\(^{18}\), Stat6\(^{55}\), Stat5\(^6\), and E2F\(^{50}\). However, the roles of each of these factors in CD40 signaling have not yet been explored. Most known TNF-R family members are able to activate NF-κB, and much work has been done in recent years on both the role of NF-κB activation in CD40 signaling, as well as how CD40 stimulates activation of this family of DNA binding proteins.

As described in the previous section, earlier studies of the link between CD40 and NF-κB showed that when CD40 is transiently overexpressed in 293 epithelial cells, NF-κB reporter genes become activated\(^{36}\); CD40 ligation is not required for this activation. However, as described above, this and other aspects of the CD40-NF-κB relationship defined by 293 cell experiments are not completely recapitulated in B cells. In addition, epithelial cells do not perform B cell effector functions, so it is not possible to test the cause and effect relationship between NF-κB activation and CD40-mediated physiologically relevant effector functions in 293 cells. To better understand the roles played by NF-κB *in vivo*, a number of mouse strains genetically deficient in single NF-κB subunits have been produced over the past 5 years. However, the participation of NF-κB dimers in the regulation of a large number of genes in mammals results in a complex phenotype for these mutant mouse strains. Mice deficient in RelA show embryonic lethality\(^3\), those deficient in RelB show marked abnormalities in hematopoietic development as well as widespread inflammation of multiple organs\(^{45}\). p52\(^{−/−}\) mice have defects in the organization of their lymphoid tissues\(^3\). It is thus difficult to obtain and/or interpret specific data on mature B cell function using these strains. Mice deficient in p50 or p65 subunits have less drastically altered phenotypes – both have been shown to have defects in humoral immune responses, but the status of CD40-mediated NF-κB activation has not been specifically reported in these mice\(^{76,92}\). Because NF-κB-mediated transcriptional regulation participates in so many cellular functions, B cells obtained from these knockout mice, having developed in an altered environment, may not be ideal as models for unraveling the importance or mechanism of CD40-mediated NF-κB activation in mature cells. As an alternative approach, Hsing and Bishopol\(^{34}\) inductively expressed a mutant form of the inhibitory protein IκB\(\alpha\) in two B cell lines. Induction of expression of this mutant IκB\(\alpha\) blocks CD40-mediated NF-κB activation.
Study of CD40 functions in these lines revealed that CD40-mediated NF-κB activation is critical for the some, but not all, CD40 effector functions\(^4\). Recent additional studies have shown that CD40-mediated IL-6 production requires TRAF6 association, but is independent of CD40-mediated increases in nuclear NF-κB\(^2\). It is thus clear that CD40-mediated transcriptional regulation involves transcription factors other than, additional to, and/or working cooperatively with NF-κB, and this deserves further study.

Conclusions

Information gained from studies of both humans and animals has emphasized the importance of CD40 signaling to the normal function of the immune response, and it is becoming clear that CD40 signaling also has an impact on non-immune cell function as well. A wide variety of general and cell-type-specific functions are induced and/or influenced by CD40 signal transduction. Work of many investigators has contributed to the current emerging picture of how CD40 signals, but much remains to be learned, particularly about the earliest steps of CD40-mediated signaling pathways, as well as how CD40 regulates transcriptional activation of a variety of genes. Acquisition of additional details about the CD40 signaling pathway will provide not only better understanding of the mechanisms of action of this biologically important and multi-functional receptor, but will also contribute to understanding the molecular mechanisms of signal transduction by other members of the TNF-R family.

References


Received in March 2000
Accepted in April 2000