Role of TNF Receptor-Associated Factor 3 in the CD40 Signaling by Production of Reactive Oxygen Species through Association with p40phox, a Cytosolic Subunit of Nicotinamide Adenine Dinucleotide Phosphate Oxidase

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Role of TNF Receptor-Associated Factor 3 in the CD40 Signaling by Production of Reactive Oxygen Species through Association with p40\textsuperscript{phox}, a Cytosolic Subunit of Nicotinamide Adenine Dinucleotide Phosphate Oxidase\textsuperscript{1}

Yun Jung Ha* and Jong Ran Lee*†

To extend our previous report, which showed the production of the reactive oxygen species (ROS) after the CD40 ligation in the B cells, we further examined the possible mechanisms for ROS production and the involvement of CD40-induced ROS in p38 activation. Our research shows that the stimulation of WEHI 231 B lymphomas with anti-CD40 induced ROS production and p38 activation. An antioxidant N-acetyl-L-cysteine or an inhibitor for NADPH oxidase blocked both of these, but the inhibitors for 5-lipoxygenase did not. We also show that the treatment of cells with inhibitors for the phosphatidylinositol 3-kinase (PI3-K) interfered with the CD40-induced ROS production and p38 activation. In addition, when overexpressed with a dominant negative form of either Rac1 (N17Rac1) or the TNFR-associated factor (TRAF)3, the WEHI 231 B cells did not show a full response to CD40 stimulation to produce ROS. Molecular association studies further revealed that the TRAF3 association with p40\textsuperscript{phox}, a cytosolic subunit of NADPH oxidase and p85 (a subunit of PI3-K), may possibly be responsible for the production of ROS by CD40 stimulation in WEHI 231 B cells. Collectively, these data suggest that the CD40-induced ROS production by NADPH oxidase in WEHI 231 requires the role of TRAF3, as well as activities of PI3-K and Rac1. The Journal of Immunology, 2004, 172: 231–239.

The TNF superfamily member, CD40, is expressed on a variety of cells. These include B cells, dendritic cells, and epithelial cells. In B cells, the ligation of CD40 delivers activation signals that lead to proliferation, differentiation, germinal center formation, isotype switching of Ig genes, and cytokine secretion (1, 2). As in the case of many members of the TNFR superfamily, CD40 has several cysteine-rich extracellular domains and a short cytoplasmic tail, without enzymatic activity. Signaling via CD40 results in the activation of several enzymes and transcription factors (3–12). Activation of these effector molecules by CD40 is primarily initiated by the interaction of the cytoplasmic region of CD40 with specific TNFR-associated factor (TRAF)\textsuperscript{3} molecules (13–16).

Among members of the TRAF family, TRAF2, 3, 5, and 6 were the most extensively studied as mediators in CD40-activated signal transduction. The CD40 cytoplasmic domain contains two linear TRAF binding sites. They are the membrane proximal site that binds TRAF6 and the membrane distal site that directly binds TRAF2 and TRAF3 (13, 14). The conserved C-terminal half of the TRAF proteins, designated the TRAF domain, mediates interactions with multimerized receptor cytoplasmic domains (13). For TRAF5, however, the indirect recruitment to the CD40 cytoplasmic domain as a hetero-oligomer with TRAF3 through their predicted isoleucine zippers has also been shown (15, 16).

Several studies have shown that the reactive oxygen species (ROS), which are produced after the ligation of TNFR superfamily members, act as second messengers in the signal transduction pathways, which link TNF family receptors to the activation of c-Jun NH\textsubscript{2}-terminal kinase (JNK) and NF-κB (17, 18). Previously, we also showed that ROS that are produced by CD40 ligation are important in the downstream signaling events that lead to the activation of JNK and NF-κB, as well as IL-6 secretion in murine primary B cells and a B cell line, WEHI 231 (19). Furthermore, a transient increase in the ROS intracellular levels was reported in a variety of nonphagocytic cells in response to growth factors or certain cytokines (20–22). These highly reactive ROS are known to regulate many important cellular events. These include the transcription factor activation, cell proliferation, and apoptosis.

Mechanisms for ROS production in nonphagocytic cells that follow cytokine or growth factor treatment are still largely unknown. The 5-lipoxygenase (5-LO), the activity that requires an activating protein for 5-LO (FLAP) (23–26), is known to catalyze the production of leukotrienes and ROS from arachidonic acid (27, 28). The activity of 5-LO is required for ROS production by CD28 stimulation in T lymphocytes (29) and for NF-κB-dependent transcription that is induced by IL-1β in lymphoid or endothelial cells.

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Abbreviations used in this paper: TRAF, TNFR-associated factor; ROS, reactive oxygen species; JNK, c-Jun NH\textsubscript{2}-terminal kinase; 5-LO, 5-lipoxygenase; FLAP, 5-LO activating protein; PI3-K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; NAC, N-acetyl-L-cysteine; DPI, diphenyleneiodonium chloride; ETYA, eicosatetraynoic acid; DCFDA, 2',7'-dichloro-dihydrofluorescein diacetate; DNTRAF, dominant negative TRAF; HA, hemagglutinin; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry.
ROLE OF TRAF3 IN CD40 SIGNALING BY ROS

Materials and Methods

Abs and reagents

Rat anti-mouse CD40 mAb (clone IC10) from R&D Systems (Minneapolis, MN) and hamster anti-mouse CD40 mAb (HM40-3) from BD PharMingen (San Diego, CA) were used for the cell stimulation. For the isolation of splenic B cells, anti-mouse Thy 1.2 mAb from Sigma-Aldrich (St. Louis, MO) and baby rabbit complement from Pel-Freeze Clinical Systems (Brown Deer, WI) were used. Anti-CD40 rabbit polyclonal Ab from Santa Cruz Biotechnology (Santa Cruz, CA) was used for immunoprecipitation and Western blotting. Anti-p38 rabbit polyclonal Ab and anti-hemagglutinin (HA) mAb were also obtained from Santa Cruz Biotechnology. Ab against the phosphorylated form of p38 and a secondary Ab, HRP-conjugated IgG, were purchased from New England Biolabs (Beverly, MA). We also used anti-p85 rabbit polyclonal Ab, anti-myc mAb (9E10) from Upstate Biotechnology (Lake Placid, NY) and anti-flag mAb (M2) from Sigma-Aldrich. A secondary Ab, HRP-conjugated anti-mouse IgG, was purchased from Bio-Rad ( Hercules, CA).

L-Y294002 and MK-886 were obtained from Biomol (Plymouth Meeting, PA). N-acetyl-cysteine (NAC), diphermylplasmin (DPI), eosinatyoaninc (ETYA), wortmannin, and D-sorbitol were purchased from Sigma-Aldrich. The 2',7'-dichloro-dihydro fluorescin di-acetate (DCFDA) was obtained from Molecular Probes (Eugene, OR). ECL reagents, GammaBind G-Sepharose, immobilized pH gradient (IPG) buffer, and Immobiline DryStrips were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). The TRIZol reagent and oligo(dT) 12-18 primer were obtained from Life Technologies (Grand Island, NY).

Plasmids

Mammalian expression vectors that encode N17Rac1, the p85 subunit of PI3-K (myc), and 5-LO (HA) were kindly provided by Drs. T. Finkel (National Institutes of Health, Bethesda, MD), A. Toker (Harvard Medical School, Boston, MA), and O. Rådmark (Karolinska Institute, Stockholm, Sweden), respectively. The plasmids of TRAF3, TRAF5, and a dominant negative (DN) form of TRAF3 (DNTRAF3) are generous gifts from Dr. D. Goeddel (Tularak, South San Francisco, CA). The mammalian DsRed expression plasmid, pCMVDsRed, was constructed by inserting the CMV promoter sequence into pCMV5 (Invitrogen, Carlsbad, CA) into the pDsRed1-1 vector (Clontech Laboratories, Palo Alto, CA) at sites of the enzyme ByII and HindIII. The mouse CD40 cDNA was generated by RT-PCR from WEHI 231 B cells, using oligonucleotides 5'-ggcggccgcgtcctcgctgcag-3' and 5'-ggcgtcggaggccacggccatgtctgcag-3', and cloned into pCDNA3 (Invitrogen). The wild-type TRAF2 and TRAF6 were prepared by the PCR amplification of cDNA from WEHI 231 B cells, using primer pairs 5'-gattgatcagacagtgctgg-3' and 5'-gattgatcagacagtgctgg-3'. DNTRAF2 used the 5'-gattgatcagacagtgctgg-3' and 5'-gattgatcagacagtgctgg-3' primers. DNTRAF6 used the 5'-gattgatcagacagtgctgg-3' and 5'-gattgatcagacagtgctgg-3' primers. Am- plification of p38 was ligation of a primed template (as previously indicated) that was annealed into the BglII and XhoI sites. DNA sequencing confirmed that all of the PCR products were completely free of undesired mutations.

RNA isolation and RT-PCR

The total RNA from WEHI 231 B cells (1 × 10^7) was isolated using the TRIzol reagent and converted to cDNA with a oligo(dT) primer (Life Technologies). For PCR, a pair of primers (as previously indicated) was used to amplify each desired cDNA. The PCR products were separated on agarose gels and used for cloning.

Cell culture and isolation of splenic B cells

The mouse B lymphoma cell line, WEHI 231, was cultured in a RPMI 1640 medium that contained 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM glutamine, and 5 × 10^-5 M 2-ME. A hybrid embryonic kidney cell line (293T) was cultured in DMEM that was supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM glutamine. The cell lines were maintained at 37°C with 5% CO_2/95% O_2. Splenic B cells were isolated from adult female BALB/c mice (4- to 8-week-old) as previously described (19). To obtain T cell-depleted B cells, whole spleen cells were depleted of T cells by incubating with anti-mouse Thy 1.2 mAb and a rabbit complement. When the WEHI 231 and splenic B cells were treated with pharmacological inhibitors, they were preincubated for at least 1 h before the experiment at a density of 1 × 10^6 cells/ml. The experiments were performed in the continuous presence of inhibitors.

Transient expression of plasmid DNA

The WEHI 231 B cells were washed twice with PBS and resuspended at 10 or 20 × 10^6 cells in a 400 μl intracellular buffer (cytomix, Ref. 38). The vector, or an indicated expression plasmid DNA (20 μg), was added with the DsRed expression plasmid DNA (5 μg). Electroporation was performed using BTX-T820 (Genetronics, San Diego, CA) at 200 V and 65 ms. For transfecting HEK 293T cells, the cells were seeded the day before transfection at a density of 1 × 10^6 cells/60 mm culture dish. The cells were then transduced with 5 μg each of various expression plasmids, or DNACaM, for 1 h, followed by phosphate precipitation. The transfected cells were cultured in the growth medium for 24 h before the experiments.

Immunoprecipitation and Western blotting

Activation of p38 MAPK after CD40 ligation was determined by the phosphorylation degree of the enzyme. WEHI 231 and/or splenic B cells were incubated in the medium alone, or in the medium that contained anti-CD40 (10 μg/ml), H_2O_2 (300 μM), or 0.6 M D-sorbitol at 37°C for various periods of time. The cell lysates were prepared in a 1% Nonidet P-40 lysis buffer that contained inhibitors for proteases and phosphatases, as previously described (19). For Western blot analysis, the lysates from cells (1 × 10^6 for WEHI 231, 2 × 10^6 for splenic B cells) were mixed with a 2× Laemmli’s sample buffer, boiled, and subjected to SDS-10% PAGE. For immunoprecipitation, WEHI 231 B cells (10 × 10^6) were stimulated with control Ig or anti-CD40 Ab for 10 min, or the HEK 293T cells were transfected with various expression plasmids. The lysates that were prepared from these cells were tumbled with GammaBind G-Sepharose beads that were conjugated with each indicated Ab. The immune complexes were then subjected to SDS-PAGE, which was followed by Western blotting.

Fluorescence measurement of intracellular ROS

ROS production that followed CD40 ligation was measured using a fluorescent probe, DCFDA (19). The cells (1 × 10^6) were incubated for 15 min with 20 μM DCFDA in a loading buffer (138 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl_2, 1.2 mM MgCl_2, 10 mM glucose in 10 mM PBS, pH 7.4). After 15 min of loading, the cells were washed and plated on a culture dish. The culture dishes were then transferred to a Zeiss Axioven 100 M inverted microscope that was equipped with a ×40 Achroplan LD objective and a...
Zeiss LSM510 confocal attachment. The cells were stimulated with either the loading buffer with control Ig (control), or the buffer that contained anti-CD40 mAb (10 μg/ml). Fluorescence intensities were measured under excitation settings at 488 nm and emission at 515–540 nm every 30 s for 30 min. Because the dye fluoresces only in the reaction with ROS, background fluorescence levels in cells that were loaded with the dye were minimal. The settings of the confocal microscopy that were corrected from any background fluorescence were used for the fluorescence measurements after the stimulation of CD40. Fluorescence cell intensities that were collected with identical parameters, such as contrast and brightness, were compared between the control and CD40 stimulation.

Two-dimensional gel electrophoresis

Lysates from 1 × 10⁸ cells that were stimulated either with control Ig or anti-CD40 for 10 min were tumbled with GammaBind G-Sepharose that was conjugated with anti-CD40 Ab. The immune complexes were processed to prepare the samples for two-dimensional PAGE. The precipitated protein samples were mixed with a rehydration buffer that contained 9 M urea, 20 mM DTT, and a 0.5% IPG buffer (pH 3–10). They were then loaded onto Immobiline DryStrips (7 cm, pH 3–10 nonlinear gradient; Amersham Pharmacia Biotech). Before the second dimension, the strips were incubated in room temperature using an IPGphor electrophoresis unit (Amersham Pharmacia Biotech). Isoelectric focusing was performed at room temperature using an ICGphor electrophoresis unit (Amersham Pharmacia Biotech). Before the second dimension, the strips were incubated in an equilibration buffer (6 M urea, 2% SDS, 50 mM Tris-Cl; pH 8.8, 30% glycerol). The equilibrated strips were inserted onto SDS-PAGE gels. The two-dimensional protein gels were then stained with Coomassie brilliant blue R-250.

Mass spectrometry and protein identification

Individual protein spots were excised from two-dimensional gels, which was followed by a destaining procedure and in-gel digestion with trypsin. The recovered peptides were desalted and concentrated using C₁₈ ZipTips (Millipore, Bedford, MA), eluting peptides in 50% (v/v) acetonitrile/water. The samples were co-crystallized with matrix (α-cyano-4-hydroxy-trans-cinnamic acid; Hewlett-Packard, Meriden, CT) on gold-coated sample plates. A mass analysis of the resultant peptide mixtures was performed using a Voyager DE-STR (Perkin-Elmer Applied Biosystems, Foster City, CA) matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) that operates in the positive-ion reflector mode. MALDI-TOF mass spectra were collected over the mass range from 800 to 3500 Da and calibrated with standard peptides. The proteins were identified by peptide mass fingerprinting (using MS-Fit) that searched against a mammalian subset in the SWISS-PROT, or in the National Center for Biotechnology Information nonredundant protein database.

Statistical analysis

The paired Student t test was used to determine the statistical significance of the data. Values of p < 0.01 were considered significant.

Results

ROS were produced after CD40 ligation through the activities of NADPH oxidase and PI3-K in WEHI 231 B cells

ROS are involved with signaling events that are mediated by members of the TNFR superfamily. We showed the role of ROS that are produced by CD40 ligation on downstream signaling events in both primary B cells and the B cell line, WEHI 231. In this study, we extended our study on the role of ROS as signaling intermediates that follow CD40 ligation in WEHI 231 B cells. First, we investigated the CD40-induced production of intracellular ROS in cells that are incubated continuously with a redox-sensitive fluorescent probe, DCFDA. The representative fluorescence images of cells after a 10-min stimulation with either the control Ig or anti-CD40 are shown in Fig. 1A; the fluorescence intensity that is produced by CD40-driven ROS is compared quantitatively in Fig. 1G. As previously shown by flow cytometry (19), the CD40-mediated response in the WEHI 231 cells appears at 2 min and persists up to 30 min (data not shown).

The sources of ROS, which are generated after receptor ligation in various cell types among nonphagocytic cells, are yet to be determined. They may include 5-LO and NADPH oxidase. Because the NADPH oxidase system is known to function in B cells (35), we first investigated whether an inhibitor for NADPH oxidase, DPI, could block ROS production in anti-CD40-stimulated WEHI 231 B cells. As seen in Fig. 1, B and G, the preincubation of cells with 15 μM DPI for 1 h inhibited the anti-CD40-induced ROS production in WEHI 231. Next, we investigated the possible role of 5-LO on ROS production after the CD40 ligation in the WEHI 231 B cells. The cells were preincubated for 1 h, either with 35 μM ETYA (an inhibitor for 5-LO) or with 0.5 μM MK-886 (an inhibitor for FLAP). This was followed by stimulation with anti-CD40. As shown by representative fluorescence images (Fig. 1, C and D) and relative fluorescence intensities (Fig. 1G), either ETYA or MK-886 failed to block ROS production by CD40 ligation in WEHI 231. This demonstrates that 5-LO is not responsible for the
CD40-induced ROS production to the basal level (Fig. 1, pretreatment with either compound strongly inhibited the CD40-induced ROS production in WEHI 231 B cells. To examine the effects of PI3-K antagonists, the cells were preincubated with 10 μM LY294002 or 0.1 μM wortmannin for 1 h before stimulation with anti-CD40. In the WEHI 231 cells, preincubation with either compound strongly inhibited the CD40-mediated ROS production to the basal level (Fig. 1, E–G). The effect of PI3-K inhibitors on the ROS production may be due to the direct effect of the inhibitors on the PI3-K-dependent events that are required for the oxidase assembly, as well as interruption of the CD40-induced PI3-K activity. These results, thus, indicate that ROS production by the activity of NADPH oxidase after CD40 stimulation is under the control of PI3-K activity in the WEHI 231 B cells.

Activation of p38 MAPK after CD40 ligation in WEHI 231 B cells requires ROS production

Based on a recent study, which showed that the low level of oxidative stress selectively activates p38 (39), we next determined whether the p38 activation is mediated by ROS that are produced in response to CD40 ligation in WEHI 231 B cells. Stimulation of the cells with anti-CD40 resulted in the rapid activation of p38 MAPK, which lasted up to 30 min before diminishing toward the baseline (Fig. 2). As shown in Fig. 2A, preincubation with 30 mM NAC (a pharmacological antioxidant) strongly diminished the CD40-mediated p38 activation. Treatment with 300 μM H2O2 also activated p38, as assessed by the strong induction of p38 MAPK phosphorylation in WEHI 231 B cells.

To discover whether the activity of NADPH oxidase is involved in p38 activation by CD40, the cells were preincubated with DPI before the detection of p38 MAPK activation after stimulation with anti-CD40. As expected, DPI inhibited the CD40-induced activation of p38 MAPK in WEHI 231 (Fig. 2B). This indicates that NADPH oxidase activity is possibly required for the CD40 signalization pathway that leads to p38 activation in these cells. However, the p38 activation that is induced by CD40 ligation in WEHI 231 was not modified (Fig. 2C) when the cells were preincubated with an inhibitor for 5-LO, ETYA, or an inhibitor for FLAP (MK-886). Similarly, in primary mouse splenic B cells as shown in Fig. 3, the activity of NADPH oxidase, but not 5-LO, was also involved in the CD40-induced p38 activation (Fig. 3D).

PI3-K is involved in the CD40-induced ROS production in WEHI 231 B cells, as we have shown in Fig. 1, E–G. We, therefore, examined the effects of PI3-K antagonists on p38 activation after CD40 stimulation in these cells. The cells were preincubated with 10 μM LY294002 or 0.1 μM wortmannin for 1 h before stimulation with anti-CD40 or 0.6 M sorbitol (an osmotic inducer of p38 MAPK). Similarly, preincubation with either compound inhibited the CD40-mediated p38 response, but did not interfere with the sorbitol-induced p38 activation in the WEHI 231 B cells (Fig. 2D). These results, therefore, further indicate that ROS generation may play an important role in signaling events that are mediated by CD40 in these cells.

Rac is a component in the cascade of CD40-mediated ROS production

Rac, a small GTP-binding protein, is required for the activation of NADPH oxidase (33, 34). It also plays a role in mediating TNF-α-induced ROS production to the basal level (Fig. 1, pretreatment with either compound strongly inhibited the CD40-induced ROS production in these cells. In similar experiments (see Fig. 3, A–C), we have also found in mouse splenic B cells that the NADPH oxidase pathway is a major source for the ROS production after the ligation of CD40.

An increasing number of studies have demonstrated that the role of PI3-K is essential for ROS production after stimulation with cytokines or growth factors. We, therefore, investigated whether PI3-K is involved in the CD40-induced ROS production in WEHI 231 B cells. To examine the effects of PI3-K antagonists, the cells were preincubated with 10 μM LY294002 or 0.1 μM wortmannin for 1 h before stimulation with anti-CD40. In the WEHI 231 cells, preincubation with either compound strongly inhibited the CD40-mediated ROS production to the basal level (Fig. 1, E–G). The effect of PI3-K inhibitors on the ROS production may be due to the direct effect of the inhibitors on the PI3-K-dependent events that are required for the oxidase assembly, as well as interruption of the CD40-induced PI3-K activity. These results, thus, indicate that ROS production by the activity of NADPH oxidase after CD40 stimulation is under the control of PI3-K activity in the WEHI 231 B cells.

Correlation of ROS production and p38 MAPK activation after CD40 ligation. A, The effects of CD40-induced ROS production on p38 activation. The cells (5 × 10⁶) that were either left untreated or pretreated with 30 mM NAC for 1 h were stimulated with the medium containing control Ig (control), or 10 μg/ml anti-CD40 for various times. Some of the untreated cells (5 × 10⁶) were also incubated with 300 μM H2O2, for the indicated times as positive controls for ROS-mediated p38 activation. The lysates from the 1 × 10⁶ cells were subjected to SDS-PAGE and a phospho-p38 (P-p38) Western blot analysis. The same blot was stripped and re-probed with anti-p38 Ab to insure equal loading of the cell lysates in each lane. B–D, The effects of the inhibitor treatment on p38 activation mediated by CD40-induced ROS production. The cells were either left untreated or pretreated with 15 μM DPI (B), 35 μM ETYA, or 0.5 μM MK886 (C), 0.1 μM wortmannin, or 10 μM LY294002 (D) before the CD40 ligation. The cells were also treated with 0.6 M D-sorbitol for 30 min as a positive control for the p38 activation. The data shown represent three separate experiments.
α-induced intracellular ROS production by 5-LO-linked cascade (31). We also investigated whether Rac signaling helped in the production of ROS after CD40 ligation in WEHI 231 B cells. For these experiments, we transiently transfected the WEHI 231 cells with an expression plasmid that encodes a dominant negative mutant of Rac1 (N17Rac1), along with an expression plasmid that encodes a red fluorescent protein (pCMVDsRed). We then determined the intracellular ROS production after stimulation with anti-CD40 in these cells. The cells that were transfected with either a vector or N17Rac1 were visualized by red fluorescence from the DsRed protein; they may have been expressed together. When stimulated with anti-CD40, the majority of the vector-transfected cells, but not the N17Rac1-transfected cells, responded by producing ROS. Representative fluorescence images of the transfected cells with red fluorescence and ROS-induced fluorescence changes in these cells are shown in Fig. 4A. These results are also shown as relative fluorescence intensities in the cells that were transfected with the control or N17Rac1 plasmid (Fig. 4B). Therefore, it is likely that NADPH oxidase is the major downstream mediator of Rac signaling for the production of ROS after CD40 stimulation in WEHI 231 B cells.

TRAF3 is required for the production of ROS after CD40 ligation

Because CD40-mediated signal transduction is primarily initiated by the recruitment of specific TRAF molecules (13–16, 36, 37), and because the CD40 cytoplasmic domain contains binding sites that directly bind TRAF2, 3, and 6 (13, 14), we examined the role of these TRAFs on CD40-induced ROS production. To distinguish the effect of each TRAF protein on ROS production after CD40 ligation, if any, we transiently expressed the dominant negative form of each TRAF protein, along with a red fluorescent protein, DsRed, in the WEHI 231 B cells. We then incubated these transfected cells with a DCFDA probe and determined the intracellular ROS production after stimulation with anti-CD40. The cells that were transfected with either a vector or each DNTRAF were visualized by red fluorescence from the coexpressed DsRed protein. Fluorescence changes from red to yellow (due to the green fluorescence from intracellular ROS that was produced after CD40 stimulation) were compared between the cells that were transfected with vector and those with each DNTRAF. When stimulated with anti-CD40, cells that were transfected with DNTRAF2 or DNTRAF6 responded by producing ROS at the same level as the vector-transfected cells. In contrast, the WEHI 231 B cells that were transfected with DNTRAF3 responded significantly less to ROS production after stimulation with anti-CD40 in these cells was measured (described in Fig. 1). The effects of CD40-induced ROS production on p38 activation. The cells were either left untreated or pretreated with 15 μM DPI or 0.5 μM MK886 before the CD40 ligation. As in Fig. 2, the lysates from the 2 × 10⁶ cells were subjected to SDS-PAGE and a P-p38 Western blot analysis. The same blot was stripped and re-probed with anti-p38 Ab to insure equal loading of the cell lysates in each lane. The data shown represent three separate experiments.

![Image](http://www.jimmunol.org/)

**FIGURE 3.** ROS production and p38 MAPK activation after CD40 ligation in mouse splenic B cells. A–C, Fluorescence images show CD40-mediated ROS production and the treatment effects with inhibitors for NADPH oxidase or 5-LO. As in Fig. 1, the intracellular ROS production after CD40 ligation for 25 min in cells that are either untreated (A) or treated with 15 μM DPI (B) and 35 μM ETYA (C) was measured. Aa–Cc, Present fluorescence of the cells after stimulation with control Ig. Fluorescence after CD40 ligation is shown (Aa–Cc). Data represent three independent experiments. D, The effects of CD40-induced ROS production on p38 activation. The cells were either left untreated or pretreated with 15 μM DPI or 0.5 μM MK886 before the CD40 ligation. As in Fig. 2, the lysates from the 2 × 10⁶ cells were subjected to SDS-PAGE and a P-p38 Western blot analysis. The same blot was stripped and re-probed with anti-p38 Ab to insure equal loading of the cell lysates in each lane. The data shown represent three separate experiments.

**FIGURE 4.** Effects of N17Rac1 expression on CD40-induced ROS production. A, The fluorescence images show ROS production after CD40 ligation for 15 min in cells that were transfected with either a vector or N17Rac1 plasmid. The cells were transiently transfected with expression plasmids that encode N17Rac1, a dominant negative mutant of Rac1, and a red fluorescent protein, DsRed. The intracellular ROS production after stimulation with anti-CD40 in these cells was measured (described in Fig. 1). Aa and Ac, Red fluorescence from the transfected cells that are stimulated with control Ig. Ab and Ad, Changes in the fluorescence after CD40 ligation. Changes in the fluorescence to yellow, due to the green fluorescence from CD40-induced ROS production, are clearly shown in cells that were doubly transfected with the vector and DsRed plasmids (Ab). In the case of the concurrent expression of DsRed and N17Rac1, however, not all of the cells responded to the CD40 stimulation that led to fluorescence changes (Ad). B, The fluorescence intensity after CD40 ligation relative to the control is shown in cells that were transfected either with a vector or with the N17Rac1 plasmid. For each experiment, the fluorescence intensity from the whole cell that was fluoresced red from DsRed expression was digitized as arbitrary units in the stimulation with either control Ig or anti-CD40, and the average fluorescence intensity from at least 50 cells were calculated. Data shown are pooled fluorescence intensities from three experiments and are presented as the average ± SD; *, p < 0.001.
produce ROS after CD40 stimulation. Representative fluorescence images of the transfected cells with red fluorescence and ROS-induced fluorescence changes in these cells are shown in Fig. 5A. These results are also presented as relative intensities of the fluorescence in cells that were transfected with the control or each DNTRAF plasmid (Fig. 5B). Therefore, it is likely that TRAF3 is selectively required for mediating CD40 ligation in the ROS production of WEHI 231 B cells.

CD40 associates directly with TRAF3 or p85, a regulatory subunit of PI3-K, but not with p40phox, a subunit of NADPH oxidase

We investigated the possible CD40 signaling complexes that might be responsible for ROS production in WEHI 231 B cells. To determine the molecular associations, if any, the molecules that are involved in the pathway for CD40-induced ROS production (including CD40, TRAF3, PI3-K, and NADPH oxidase) were expressed in HEK 293T cells. Because p85 is a regulatory subunit of PI3-K, and because p40phox (a cytosolic component of NADPH oxidase) is known to be crucial for the activity of this enzyme (34), CD40 was expressed along with TRAF3, p85, or p40phox in HEK 293T cells. Analyses of the immune complex by anti-CD40 showed that CD40 associates directly with TRAF3, regardless of the stimulation with anti-CD40 (Fig. 6A). Interestingly, CD40 also associates with p85, but not with p40phox (Fig. 6A). Analyses of the immune complex by anti-p85, however, failed to demonstrate the association of p85 with CD40 (data not shown). This suggests the possibility that the p85 association with CD40 only occurs in receptor ligation.

TRAF3 mediates the CD40 signal to NADPH oxidase by direct associations with p40phox and p85

Because we showed that the DNTRAF3 expression inhibits CD40-induced ROS production in WEHI 231 B cells (Fig. 5), and because CD40 does not associate directly with p40phox (Fig. 6A), we, therefore, investigated the role of TRAF3 in mediating the CD40 signal to either p40phox or p85. We first determined whether TRAF3 selectively associates with p40phox or p85. In HEK 293T cells that are transfected with empty vector or each plasmid of TRAF2, 3, 5, or 6, along with p40phox or p85, the immune complex that was formed by Ab against p40phox or p85 was analyzed for the association with each TRAF protein. Results from these experiments showed that p85 primarily associates with TRAF3 (Fig. 6B), although it associates weakly with TRAF5 (data not shown), and p40phox associates with TRAF3 only (Fig. 6C). These results strongly support the role of TRAF3 in mediating the CD40 signal to p85 and p40phox. Consequently, this results in the production of ROS after CD40 ligation.

Because both PI3-K and NADPH oxidase are required for CD40-induced ROS production, and because p85 associates with CD40 in receptor cross-linking, we continued to investigate whether p85 directly associates with p40phox. When p85 was expressed with p40phox in HEK 293T cells, an examination of the immune complex by Ab against p40phox or p85 revealed that these two proteins do not associate with each other (Fig. 6D). These results further indicate the role of TRAF3 in mediating the CD40 signal to p40phox, because neither CD40 nor p85 directly associates with p40phox.

Additionally, the CD40 immune complex in cells that were stimulated either with control Ig (Fig. 7A) or with anti-CD40 (Fig. 7B) was separated by two-dimensional PAGE. The protein spots were analyzed by peptide mass fingerprinting methods using MALDI-TOF MS. As shown in Fig. 7B, TRAF3 and p40phox proteins were recruited to the CD40 receptor complex after CD40 ligation. These data further indicate that CD40 ligation activates NADPH oxidase, in part by recruiting cytosolic p40phox to the membrane compartment through TRAF3 association. This results in ROS production in WEHI 231 B cells. Direct molecular associations were also confirmed from the CD40 immune complexes in WEHI 231 cells that were stimulated with anti-CD40. The association of CD40 with p85 was demonstrated to be specific to CD40 engagement by direct coimmunoprecipitation experiments followed by Western blotting (Fig. 7C).

Discussion

An increasing number of studies has demonstrated that ROS are generated by receptor ligation and play a significant role in activating downstream signaling events in mammalian cells. CD40, a member of the TNFR superfamily, plays a key role in the interaction between B and T cells that express the CD40 ligand (CD40L, gp39) (1, 2). Recent studies suggest that CD40 ligation induces the activation of several signaling intermediates, including JNK, p38 MAPK, and NF-κB (3–12). Studies also reported that PI3-K is activated by CD40 stimulation and plays a crucial role for CD40-induced proliferation and NF-κB activation in B cells (40–42). We previously suggested that one way in which the CD40 ligation may be linked to JNK, NF-κB activation, and subsequent IL-6 production is via the production of ROS (19).

In the present study, we investigated the role of various effector molecules on ROS production after CD40 ligation in WEHI 231 B cells. These effector molecules include NADPH oxidase, 5-LO, PI3-K, and Rac. All of these are reported to play roles in receptor-mediated ROS production in other systems. The NADPH oxidase

![Figure 5](http://www.jimmunol.org/DownloadedFrom/10.4049/jimmunol.18-0861-f05)

**FIGURE 5.** Effects of the DNTRAF2, 3, or 6 expression on CD40-induced ROS production. A, The fluorescence images show ROS production after CD40 ligation for 15 min in cells that were transfected with either a vector or each DNTRAF plasmid. As in Fig. 3, the cells were transiently transfected, then the CD40-induced ROS production was measured. Aa, e, and g, Red fluorescence of the DsRed protein from the transfected cells are shown. Ab, d, f, and h, CD40-induced fluorescence changes in the transfected cells. Fluorescence changes by CD40 stimulation were very little, if any, in the cells that were transfected with DNTRAF3 (e and f). To present fluorescence changes in more transfected cells, the cells shown in these panels are slightly reduced. B, The relative fluorescence intensity from CD40-induced ROS production is shown in cells that were transfected with either a vector or with each DNTRAF as a ratio of control fluorescence. Data obtained as in Fig. 3 are pooled fluorescence intensities from three experiments and are presented as the average ± SD; *, p < 0.001.
system works in B cells (35) and the 5-LO system also works for the ROS generation by IL-1/H9252 in Raji B cells (43). When we compared the p67phox (a component of NADPH oxidase) and 5-LO expression level by immunoblotting the cell lysates, WEHI 231 expressed a significant amount of p67phox but a minimal level of 5-LO when compared with these protein expressions in Raji cells (data not shown). These observations coincide with the results that are shown in Fig. 1. There the pharmacological inhibitor(s) for NADPH oxidase, but not for 5-LO, selectively inhibited CD40-induced ROS production in WEHI 231 B cells. Similar results were also shown in primary mouse splenic B cells (Fig. 3). In several receptor systems, the PI3-K and Rac roles were also demonstrated in ROS generation pathways using NADPH oxidase (32, 33, 44). Our study, using pharmacological and molecular tools, also demonstrated that PI3-K and Rac act as effector molecules that are possibly required for ROS production after CD40 ligation in WEHI 231 B cells.

Recently, quite a few data suggest that diverse receptors stimulate ROS production. These are not cytotoxic, but are rather required for the MAPK family member activation, gene expression, and/or cell proliferation (17–22, 45–47). CD40 stimulation on B cells preferentially activates JNK or p38 MAPK (7, 8, 11, 12); ROS stimulate the activity of these MAPK enzymes (17, 19, 39, 45). We also showed in this study a correlation of ROS production with p38 MAPK activation after CD40 stimulation in WEHI 231 and mouse splenic B cells (Figs. 2 and 3). These results, combined with our previous finding (19), suggest that the CD40-stimulated activation of JNK and p38 partly depends upon ROS production as second messengers. These data, therefore, suggest the possibility of the kinase cascade as a primary transducer of intracellular oxidative signals to the nucleus for relevant gene expression to determine cellular fates.

From our study with inhibitors, we know that NAC does not globally interfere with receptor-mediated activation in all of the MAPK family members in B cells. We previously showed that this antioxidant does not block the anti-IgM stimulation of ERK in WEHI 231 B cells (9). Additionally, we examined the NAC effect...
FIGURE 7. Recruitment of TRAF3, p40<sub>phox</sub>, and p85 to CD40 receptor complex after stimulation in WEHI 231 B cells. A and B, The lysates were prepared either from the cells that were stimulated with control Ig (A), or from the cells that were stimulated with anti-CD40 for 10 min (B), and were incubated with beads that were conjugated with anti-CD40 Ab. The precipitated proteins were resolved by two-dimensional PAGE, followed by staining with Coomassie brilliant blue R-250 (described in Materials and Methods). The identification of protein spots by peptide mass fingerprinting using MALDI-TOF MS indicates that TRAF3 (spot 2; 5/16, 31% masses matched) and p40<sub>phox</sub> (spot 3; 5/11, 22% masses matched) are recruited to CD40 receptor complex after stimulation. γ-Actin (spot 1; 9/28, 32% masses matched) exists in the complex from both control and stimulated cells. These results were confirmed in a separate independent experiment. C, The lysates from 10<sup>6</sup> WEHI 231 cells that were stimulated either with a control Ig or anti-CD40 for 10 min were tumbled with GammaBind G-Sepharose beads that were conjugated with either rabbit Ig or anti-CD40 Ab. The immune complexes were then subjected to SDS-PAGE, which was followed by Western blot analysis with anti-p85 Ab. The data shown represent three independent experiments.

on p38 activation after anti-IgM stimulation, because p38 activation is also shown by B cell receptor ligation (8, 10). In the cells that were preincubated with NAC, the anti-IgM-stimulated p38 activation was not modified in WEHI 231 B cells (data not shown), compared with the inhibition of the CD40-mediated p38 activation. Similarly, we also observed that anti-IgM stimulation did not result in ROS generation in these cells (data not shown). This indicates the existence of different mechanisms for p38 activation by these two receptors in the B cells.

Our data collectively demonstrate that the CD40 ligation in B cells possibly uses NADPH oxidase for the ROS production, which then mediates downstream p38 MAPK activation. Our data also show that the CD40-induced ROS production via NADPH oxidase requires the activity of PI3-K and Rac1. We continued our investigation to further identify the molecules that associate with CD40 to activate either PI3-K or NADPH oxidase. Among the TRAF proteins that are known to directly bind with the cytoplasmic domains of CD40, we found that TRAF3 is specifically required for ROS production after CD40 ligation. We also found that CD40 associates indirectly through TRAF3 with p85, as well as p40<sub>phox</sub>.

As diagramed in Fig. 8, CD40 ligation is signaled through TRAF3 to recruit p40<sub>phox</sub> and p85 to the receptor complex. Therefore, via the p40<sub>phox</sub> recruitment, the cytosolic subunits (p40-p47-p67<sub>phox</sub>) of NADPH oxidase may migrate to the membrane. There they bind to cytochrome b558 (gp91) to assemble the active oxidase (34). Because the phosphoinositides that are produced by PI3-K activation also regulate the oxidase complex by binding to the PX domain of p40<sub>phox</sub> (48–51), then the TRAF3-mediated recruitment of both p40<sub>phox</sub> and p85 would enhance the oxidase activation. PI3-K activation may be a separate event from the TRAF3-dependent pathway, depending upon the cellular context that would include the expression levels of the signaling molecules and activation states of the receptor. Further studies are underway in our laboratory to identify the CD40 signaling complexes that lead to ROS production in B cells, as well as to map the binding sites in the association of molecules. These studies will assist us in our endeavor to clearly elucidate the mechanisms in CD40-induced ROS signaling.

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