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*J Immunol* 2003; 170:19-23; doi: 10.4049/jimmunol.170.1.19

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Cutting Edge: Induced Expression of a RhoA-Specific Guanine Nucleotide Exchange Factor, p190RhoGEF, Following CD40 Stimulation and WEHI 231 B Cell Activation

Jong Ran Lee,2∗ Yun Jung Ha,* and Hye Jin Kim*

Stimulation of the B cell surface receptor CD40 induces transcriptional activation and protein expression. To determine which proteins are required for the CD40-mediated B cell activation, we performed a two-dimensional gel electrophoresis of the WEHI 231 B cell lysates. We report in this study the identification of one protein in which the expression was remarkably induced following CD40 stimulation. It was the p190 Rho guanine nucleotide exchange factor (GEF), p190RhoGEF, a recently identified GEF that is specific for RhoA. Overexpression of either p190RhoGEF or RhoA (Q63L), a constitutively active form of RhoA, mimics the effects of CD40 stimulation, such as changes in cellular structure and NF-κB activation. These p190RhoGEF overexpression effects are abrogated when coexpressed with a dominant negative form of RhoA (T19N). We also provide evidence for the CD40-mediated cellular changes that are abrogated in cells that are overexpressed with the dominant negative form of either p190RhoGEF (Y1003A) or RhoA (T19N).

To identify the CD40-induced changes in the protein expression that might regulate the B cell activation, we performed a two-dimensional (2D)3 gel electrophoresis (6, 7) of the lysates from the WEHI 231 B cells, either CD40-activated or rested. We analyzed one protein spot on the 2D gels; it was remarkably increased in its expression following a 48-h CD40 stimulation. We identified the protein as the p190 Rho guanine nucleotide exchange factor (GEF), p190RhoGEF. For this identification, we used the peptide mass fingerprinting method (8) that used a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF)-mass spectrometry (MS).

Recently, p190RhoGEF was cloned and identified as specific for the RhoA small G protein (9, 10). In neuronal cells, the overexpression of this protein mimics activated RhoA in stimulating cytoskeletal contraction and preventing neurite outgrowth (9, 10). However, this GEF has never been reported to function in immune cells. We report in this study the p190RhoGEF role in B cell activation following CD40 stimulation. First, we demonstrate that the expression of this GEF is remarkably enhanced after the CD40 stimulation of the WEHI 231 B cells, which correlates with the cellular structural changes. We also show that the transient overexpression of p190RhoGEF mimics the function of activated RhoA. This modulates the filamentous actin (F-actin) activity that results in the size and shape changes of the cells, as well as activating the NF-κB. We also demonstrate that the transient overexpression of the dominant negative form of p190RhoGEF (Y1003A) or RhoA (T19N) blocks CD40-mediated activation effects. This indicates the crucial roles of these proteins in the CD40-mediated B cell activation.

Materials and Methods

Cell culture and treatment

Maintenance of the WEHI 231 mouse B lymphoma line and isolation of splenic B cells were described previously (11). When stimulated, the cells were cultured in a 100-mm dish containing 8 ml of RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37°C in 5% CO2. The stimulated cultures were harvested at various time points after the CD40 stimulation.

Received for publication July 31, 2002. Accepted for publication October 31, 2002.

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Ig: immunoglobulin; IgG: immunoglobulin G; IgM: immunoglobulin M; IgD: immunoglobulin D; IgA: immunoglobulin A; 2D: two-dimensional; F-actin: filamentous actin; GFP: green fluorescence protein; JNK: c-Jun N-terminal kinase; MALDI-TOF: matrix-assisted laser desorption/ionization-time of flight; MS: mass spectrometry; WT: wild type; GEF: guanine nucleotide exchange factor; SSC-H: side scatter; FL1: fluorescence intensity channel 1.

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3 Abbreviations used in this paper: 2D, two-dimensional; F-actin, filamentous actin; GFP, green fluorescence protein; JNK, c-Jun N-terminal kinase; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MS, mass spectrometry; WT, wild type; GEF, guanine nucleotide exchange factor; SSC-H, side scatter; FL1, fluorescence intensity channel 1.
Plasmids
The expression plasmid for p190RhoGEF (pcDNA3-HA) was kindly provided by Dr. W. Moolenaar (The Netherlands Cancer Institute, Amsterdam, The Netherlands). A single-point mutant, Y1003A, was prepared as described previously (9) using a QuickChange mutagenesis kit (Stratagene, La Jolla, CA). The expression plasmids for the wild-type (WT) RhoA (pRK5-myc), a constitutively active mutant (Q63L), and a dominant-negative mutant (T19N) were gifts from Dr. G. M. Bokoch (The Scripps Research Institute, La Jolla, CA). The reporter plasmids of NF-κB luciferase and CMV-β-gal were generous gifts from Dr. E. Clark (University of Washington, Seattle, WA) and Dr. G. MacGregor (Emory University, Atlanta, GA), respectively. The green fluorescence protein (GFP) plasmid was obtained from Clontech Laboratories (Palo Alto, CA).

Transient transfections and reporter gene assay
The cells (1 × 10^6) were resuspended in a 400 µl cytometer intracellular buffer (12) before electroporation at 200 V/65 ms with a BTX-T820 (Genetronics, San Diego, CA). The cells were transfected with 40 µg of the vector, or the indicated expression plasmid DNA, along with 5 µg of the GFP plasmid for immunochemistry. Reporter gene assays were performed as described previously (11) and luminescence was determined using Luminoskan TL Plus (Bio-Orbit, Turku, Finland).

RNA isolation and RT-PCR
Total RNA from the splenic B cells (5 × 10^6) that were either unstimulated or stimulated with anti-CD40 was isolated using the TRIsol reagent (Life Technologies, Rockville, MD). It was converted to cDNA with an oligo(dT) primer (Life Technologies). A fragment of either p190RhoGEF or β-actin was amplified and the PCR products were separated on a 1.2% agarose gel.

Immunocytochemistry
The cells were fixed with 3.7% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. After being blocked with 1% BSA for 1 h, the samples were incubated with anti-p190RhoGEF, 187. The bands shown are representative of three independent experiments.

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Flow cytometry
The cells were blocked with normal rabbit serum (The Jackson Laboratory, Bar Harbor, ME). This was followed by fixation/permeabilization with a Cytofix/Cytoperm solution (BD Pharmingen, San Diego, CA). The fixed/permeabilized cells were stained for p190RhoGEF using Ab 187 and FITC-conjugated secondary Ab (Kirkegaard & Perry Laboratories, Gaithersburg, MD) or anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. A 30-min incubation with FITC- or Texas Red-conjugated secondary Ab (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was performed and the PCR products were separated on a 1.2% agarose gel.

2D gel electrophoresis and MS
Immobiline DryStrips (pH 3–10 nonlinear, 7 cm; Amersham Pharmacia Biotech, Piscataway, NJ) were loaded with 1 mg of the whole-cell extracts prepared with a 4% Nonidet P-40 solution similarly as described previously (14). Isoelectric focusing was performed at room temperature using an IECphor electrophoresis unit (Amersham Pharmacia Biotech). The equilibrated strips were inserted onto SDS-7% PAGE gels for the second dimension. The 2D protein gels were then stained with Coomassie brilliant blue R-250 (Sigma–Aldrich). An individual protein spot was excised from 2D gels. Then the destaining procedure and in-gel digestion with trypsin followed. A mass analysis of the resultant peptide mixtures was performed using MALDI-TOF-MS (Voyager-DE STR; PerkinElmer, Wellesley, MA) that operated in the positive-ion reflector mode. Spectra were collected over the mass range of 800-3500 Da and calibrated with standard peptides. The protein identification was by peptide mass fingerprinting using MS-Fit.

Results and Discussion
CD40 is an important surface receptor that is involved in the activation and maturation process of B cells during the humoral immune response, as well as in the development of memory cells (1–5). To identify specific proteins that are expressed differentially during long-term activation by CD40 on B cells, we used the proteome approach. This has been rapidly and successfully applied to the analyses of many biological phenomena (14–16) by resolving the expressed proteins of the cell. Peptide sequencing and identification then followed.

The protein expression profile, which was resolved on 2D gels of lysates from the WEHI 231 B cells that were either rested (control) or stimulated with anti-CD40 for 48 h, revealed a dramatic change following CD40 ligation, especially at high molecular mass ranges (Fig. 1A). One protein spot (indicated by an arrowhead) showed remarkable size increase after the CD40 stimulation. A MALDI-TOF peptide mass analysis of the tryptic digest of this spot identified it as p190RhoGEF, a recently described GEF specific for the RhoA small G protein (9, 10). This RhoA-binding GEF of 190 kDa belongs to the Dbl family of GEFs that stimulate the exchange of GDP to GTP. The CD40-induced expression of p190RhoGEF was further confirmed by an immunoblot analysis with a specific Ab for p190RhoGEF, 187 (Fig. 1B), as well as at the message level by RT-PCR: the induction appears at 12 h, is greatest at 24 h, and diminishes 48 h after CD40 stimulation (Fig. 1C).

FIGURE 1. Induced p190RhoGEF expression after CD40 stimulation. A, Whole-cell extracts (1 mg) of the WEHI 231 B cells, which were either unstimulated proliferating (control) or stimulated for 48 h with anti-CD40 (1 μg/ml), were separated by 2D PAGE. The arrowhead indicates a protein spot that increases dramatically in its expression over the time course of CD40 stimulation. The gels shown are representative of two independent experiments. B, Whole-cell lysates of the mouse splenic B cells prepared as in A were separated on 2D PAGE and stained with Coomassie brilliant blue. The arrowhead indicates a protein spot that is not seen in the control. C, cDNA was prepared by PCR from the total RNA of the mouse splenic B cells that were either unstimulated (control) or stimulated for indicated times with anti-CD40 (1 μg/ml). The PCR products for p190RhoGEF and β-actin were separated as described in Materials and Methods. The data represent three independent experiments.
CD40-induced cellular changes and the colocalization of \( p190\text{RhoGEF} \) with RhoA

The enhanced \( p190\text{RhoGEF} \) expression after a 48-h CD40 stimulation was also shown by an indirect immunofluorescence for endogenous \( p190\text{RhoGEF} \) in WEHI 231 B cells (Fig. 2A, 50–60% induction). When stained together with RhoA, the colocalization of these two proteins was seen in the cytoplasm toward the plasma membrane after a 48-h CD40 ligation (Fig. 2A). In these cells, the CD40-induced cellular changes were also noted for their size and shape (60–80% response). Analyses of the CD40-stimulated cells using flow cytometry also quantitatively demonstrated cellular changes in size and shape (SSC-H) as well as an increase in the \( p190\text{RhoGEF} \) expression (FL1). As seen in Fig. 2B, the CD40-induced increase in FL1 and SSC-H (R1) was 5- and 6-fold, respectively, over the unstimulated control. A previous study reported that \( p190\text{RhoGEF} \) mimics activated RhoA in stimulating cytoskeletal contraction and preventing neurite outgrowth when overexpressed in a neuronal cell (9). However, this GEF does not function in lymphocytes. A remarkable increase in the \( p190\text{RhoGEF} \) expression after CD40 ligation strongly implicates the significant role of this protein on B cell activation. Our results, combined with previous reports, imply the possibility that at the colocalization site, the CD40-induced \( p190\text{RhoGEF} \) regulates RhoA activation. This causes cellular changes since these factors have been implicated in modulating cytoskeletal structures (17, 18).

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

**FIGURE 2.** Enhanced \( p190\text{RhoGEF} \) expression and cellular changes following CD40 stimulation. *A*, The WEHI 231 B cells, either unstimulated (control) or stimulated with anti-CD40 were doubly labeled for endogenous \( p190\text{RhoGEF} \) (left panels) and RhoA (middle panels) using anti-\( p190\text{RhoGEF} \), 187 and anti-RhoA, followed by the FITC- and Texas Red-conjugated secondary Ab, respectively. Fluorescence was visualized under a confocal microscope. The merged staining pattern is shown in the right panels. Three separate experiments were performed with similar results. *B*, Either rested (control) or CD40-stimulated WEHI 231 B cells were fixed and permeabilized. Endogenous \( p190\text{RhoGEF} \) was stained using Ab 187, followed by a FITC-conjugated secondary Ab. Standard flow cytometric analyses were performed for these cells. The results are presented as a dot plot, showing changes in the size and shape (SSC-H) vs fluorescence from \( p190\text{RhoGEF} \) staining (FL1). The dot plot shown is representative of three separate experiments.

Overexpression of \( p190\text{RhoGEF} \) mimics CD40-induced cellular changes

Because both the \( p190\text{RhoGEF} \) expression and cellular structure are changed after the CD40 stimulation, we tested to see if the \( p190\text{RhoGEF} \) overexpression alone (in the absence of CD40 stimulation) mimics the cellular changes that are mediated by CD40 ligation. We transiently transfected WEHI 231 B cells with a GFP plasmid for the transfection control, together with either a vector or a full-length \( p190\text{RhoGEF} \) plasmid. Consequent size and shape changes of these cells, if any, were visualized by staining F-actin using rhodamine-conjugated phalloidin in conjunction with a confocal microscopy. As seen in Fig. 3A, in the cells that were transfected with a vector plasmid that was determined by the GFP expression, CD40 stimulation induced cellular shape and size changes (~75%). The CD40-induced changes in the vector-transfected WEHI 231 B cells indicate that these cells are able to respond to CD40 ligation. Indeed, we confirmed the enhanced

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

**FIGURE 3.** Effects of the \( p190\text{RhoGEF} \) overexpression and RhoA activity on the changes of cell size and morphology. The WEHI 231 B cells that were transiently transfected with indicated vector(s) along with a GFP plasmid were either left unstimulated (control) or stimulated with anti-CD40 (1 μg/ml) for 48 h. These cells were stained for F-actin or \( p190\text{RhoGEF} \) using rhodamine-conjugated phalloidin or Ab 187. The cells with fluorescent green were analyzed under a confocal microscope. *A*, The changes in cell size and morphology, as well as the enhanced \( p190\text{RhoGEF} \) expression, were seen in the vector-transfected cells after CD40 stimulation. *B* and *C*, The cellular changes were analyzed in cells transiently transfected with either the \( p190\text{RhoGEF} \) or \( p190\text{RhoGEF} \) (Y1003A) plasmid. The effect of CD40 stimulation was also determined in the cells that were transfected with a plasmid of \( p190\text{RhoGEF} \) (Y1003A). *D* and *E*, The cellular changes were determined in the cells that were transiently transfected with RhoA (T19N) alone or together with \( p190\text{RhoGEF} \). The changes were also determined in the cells that were transfected with RhoA (T19N) after a 48-h CD40 stimulation. *F*, The changes in cell size and morphology were determined in cells transiently transfected with RhoA (Q63L). All of the data shown are representative of at least three independent experiments.
p190RhoGEF expression after the CD40 stimulation in these vector-transfected cells by staining them with anti-p190RhoGEF Ab, 187 (Fig. 3A). As expected, the cells that were transfected transiently with only the p190RhoGEF plasmid showed cellular changes in the absence of CD40 stimulation (Fig. 3B, ~70%). However, in the cells that were transfected with a dominant-negative form of p190RhoGEF (Y1003A), the observed cellular changes could not be determined either in the absence or presence of CD40 stimulation (Fig. 3C, >90%). These results strongly suggest that cellular changes after the CD40 stimulation are possibly mediated by the enhanced function of p190RhoGEF.

Because p190RhoGEF specifically activates the RhoA small G protein (9, 10), we then examined whether the changes that were shown in the cells that were transfected with the p190RhoGEF plasmid, or in cells that were stimulated with anti-CD40, may be a result of the increased RhoA activity. As shown in Fig. 3D, the WEHI 231 B cells that were transfected with the dominant negative of RhoA (T19N) showed no cellular changes, with or without CD40 stimulation (>90%). Moreover, the simultaneous overexpression of RhoA (T19N) and p190RhoGEF abrogated the cellular changes that were seen in the cells that were transfected with p190RhoGEF alone (Fig. 3E, ~70%). Also, distinct cellular changes were similarly observed in the WEHI 231 B cells that were transfected transiently with a constitutively active form of RhoA (Q63L), as seen in Fig. 3F (>90%). These results strongly imply that the enhanced activation of the RhoA small G protein mediates the p190RhoGEF function after the CD40 stimulation in WEHI 231 B cells.

**Overexpression of p190RhoGEF enhances the NF-κB activation**

Because the p190RhoGEF overexpression mimicked the same cellular changes that were induced by CD40 ligation, we examined whether the overexpression of this protein also induces the activation of NF-κB that is seen in the WEHI 231 B cells after the CD40 stimulation. Because our data demonstrated that p190RhoGEF functions through RhoA (the activity of which is known to induce changes in the cytoskeletal structure as well as gene transcription), we also examined whether the activity of RhoA plays a role in the activation of NF-κB by the p190RhoGEF overexpression. For these experiments, the WEHI 231 B cells were transiently transfected with the p190RhoGEF plasmid alone, or in combination with either a constitutively active or a dominant negative form of RhoA along with a reporter plasmid for NF-κB.

Compared with the reporter activity that is induced by the CD40 ligation in the cells that were transfected with the vector plasmid, the WEHI 231 B cells that were transiently transfected with only the WT RhoA showed similar activity. However, in the cells that were transfected with either p190RhoGEF or a constitutively active form of RhoA (Q63L), the NF-κB reporter activity was augmented ~2-fold or >4-fold. In contrast, the cells that were transfected with the dominant negative form of RhoA (T19N) showed a significant reduction in the reporter activity to the basal level, shown in the vector-transfected resting cells (Fig. 4). Additionally, the p190RhoGEF-induced reporter activity was further enhanced in the cells that were cotransfected with the constitutively active form of RhoA (Q63L), but returned to the basal in the cells that were cotransfected with the dominant-negative form of RhoA (T19N) (Fig. 4). Furthermore, in the cells that were transfected with the dominant negative of either p190RhoGEF (Y1003A) or RhoA (T19N), the NF-κB reporter activity was not induced by CD40 stimulation (data not shown).

These results show that similar to the cellular changes, the CD40-mediated NF-κB activation in WEHI 231 B cells requires the activities of RhoA and p190RhoGEF. In this study, the activity of RhoA is in part regulated by the enhanced p190RhoGEF expression after CD40 ligation. However, a recent study on neuronal cells also showed that p190RhoGEF can directly bind to the c-Jun N-terminal kinase (JNK)-interacting protein, JIP-1 (13), which is reported to serve as a substrate for JNK (19) and as a scaffolding protein for JNK activation (20). Therefore, further study will be required to determine the possibility that p190RhoGEF may control a wider range of cellular processes than those that are predicted by its GDP/GTP exchange activity.

In summary, we identified p190RhoGEF as a protein that is enhanced in its expression after CD40 stimulation in WEHI 231 B cells that use the proteome approach. We further demonstrated that this protein plays a significant role in the CD40-mediated B cell activation through the regulation of RhoA activity. However, it is yet to be determined which upstream signals that follow the CD40 stimulation control the expression and activity of p190RhoGEF. Further studies on the specific contribution of the enhanced expression and activity of p190RhoGEF to the CD40-mediated functional outcome

![Figure 4](http://www.jimmunol.org/)
would help us to characterize the molecular mechanisms for the B cell maturation and differentiation.

Acknowledgments
We thank Drs. Wouter Moolenaar, Gary Bokoch, and Ben Margolis for providing valuable reagents, and Dr. Gary Koretzky for a critical review of the manuscript, as well as his valuable comments.

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