Nuclear Localization in the Biology of the CD40 Receptor in Normal and Neoplastic Human B Lymphocytes

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CD40 is a tumor necrosis factor (TNF) receptor superfamily, (TNFR; TNFRSF-5) member, that initiates important signaling pathways mediating cell growth, survival, and differentiation in B-lymphocytes. Although CD40 has been extensively studied as a plasma membrane-associated growth factor receptor, we demonstrate here that CD40 is present not only in the plasma membrane and cytoplasm but also in the nucleus of normal and neoplastic B-lymphoid cells. Confocal microscopy showed that transfected CD40-green fluorescent protein (GFP) fusion protein entered B-cell nuclei. The CD40 protein contains a nuclear localization signal sequence that, when mutated, blocks entry of CD40 into the nucleus through the CD40 protein contains a nuclear localization signal sequence that, when mutated, blocks entry of CD40 into the nucleus through the nucleus. Nuclear fractionation studies revealed the presence of CD40 protein in the nucleoplasm fraction of activated B cells, and chromatin immunoprecipitation assays demonstrated that CD40 binds to and stimulates the BLyS/BAFF promoter, another TNF family member (TNFSF-13B) involved in cell survival in the B cell lineage. Like other nuclear growth factor receptors, CD40 appears to be a transcriptional regulator and is likely to play a larger and more complex role than previously demonstrated in regulating essential growth and survival pathways in B-lymphocytes.

The CD40 receptor is a 45–50-kDa cell surface-associated phosphorylated glycoprotein that is a member of the TNF receptor superfamily (TNFRSF-5) superfamily of cytokine receptors (1, 2). CD40 has a typical type I extracellular binding motif, a transmembrane domain, and other structural homologies to TNF members that anchor and initiate important signaling pathways in B lymphocytes (3, 4), dendritic cells (5), monocytes and macrophages (6), and a variety of nonhematopoietic cells. The binding of CD40 to its cognate ligand, CD154 (CD40L), is required for initiating thymus-dependent humoral responses in the immune system (7). Like other members of the TNF receptor superfamily, CD40 optimally signals as a dimeric or trimeric aggregate from its location in plasma membrane microdomains referred to as lipid rafts (8–10). In the immune system, this occurs primarily after cellular interaction, engagement, and binding of trimeric CD154 from activated CD4+ T lymphocytes or dendritic cells (3, 5, 11–13). The CD40 molecule can also bind HSP70 and the complement-associated regulatory protein C4BP (14, 15). Ligation and subsequent triggering of the CD40 plasma membrane receptor leads to a variety of immune and inflammatory responses (16).

A major CD40 signaling pathway emanating from CD40 ligand cognate binding is the canonical pathway to the pivotally important transcription factor family NF-kB, a family of genes primarily mediating immune and inflammatory responses (17, 18). The canonical signaling pathway to NF-kB involves the binding of multiple TNF receptor-associated factors (TRAFs), an important group of intracellular adaptor molecules that bind directly or indirectly to various members of the TNF receptor superfamily (19) and, in the case of CD40, form a macromolecular signaling complex called a signalosome (18). The CD40 signalosome pathway has been shown to be important in regulating cell proliferation involving the cell cycle and in modulating normal and neoplastic B-cell survival, primarily through the activation of Bcl-2 family members (e.g. bcl-xl, bfl-1, etc.). Agents that selectively block this pathway have been shown to inhibit cell growth and induce cellular apoptosis (20). A mechanism for both activities appears to be the down-regulation of constitutively activated NF-kB, a molecular signature characteristic of most aggressive B-cell lymphomas (21). Whereas CD40 has been shown to be involved in most crucial B-cell functional activities after binding its cognate ligand CD154 in the plasma membrane, the findings reported here indicate that the CD40 receptor is also present in the nucleus of both normal and neoplastic human B cells. Although nuclear localization has not been previously reported for CD40 or other TNF receptor family members, these findings are not unprecedented, since an increasing number of other plasma membrane-associated growth factor receptors (e.g. EGF receptor and fibroblast growth factor receptor) (22–24) have recently been shown to function in the cell nucleus through binding to various target gene promoters. Downstream cytoplasmic proteins, such as NF-kB regulatory components, the IKKs (25) (components of the CD40 signalosome), and particularly IKKα have also recently been shown to enter the nucleus and to bind to specific promoter regions in conjunction with CREB-binding protein, resulting in phosphorylation of histone H3 and the promotion of transcriptional activity (26, 27).

Our identification of the BLyS/BAFF promoter as a molecular target for nuclear CD40 suggests an important interaction between these TNF-TNF receptor family members. BLyS (B lymphocyte stimulator), also known as BAFF (B cell activation factor, TNFSF-13), is another member of the TNF ligand family of type II transmembrane proteins (28, 29) and is involved in the survival of both normal and neoplastic B cells. The mechanism(s) through which BLyS/BAFF maintains B-cell viability have only recently begun to be clarified (30–32).

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2 The abbreviations used are: TNF, tumor necrosis factor; ChIP, chromatin immunoprecipitation; EGFR, epidermal growth factor; EMSA, electrophoresis mobility shift assay; LBCL, large B-cell lymphoma; NLS, nuclear localization signal; sCD154, soluble CD154; TRAF, TNF receptor-associated factor; CREB, cAMP-response element-binding protein; NP, nucleoplasm; NE, nuclear envelope; ER, endoplasmic reticulum; PM, plasma membrane; GFP, green fluorescent protein; CD40-RE, CD40-response element; WT, wild type.
**Experimental Procedures**

**Cells and Reagents**—Human large B cell lymphoma (LBCL) cell lines (MS, McA, and FN) were established from fresh patient biopsy samples (33). Cells were cultured in RPMI (Invitrogen) containing 15% fetal calf serum (Hyclone, Logan, UT). HeLa cells (courtesy of Drs. R. Meyn and G. Zhou (M. D. Anderson Cancer Center, Houston, TX) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum. Normal human peripheral blood B lymphocyte cells were purified from the buffy coats of healthy donors using the human B cell enrichment serum. Normal human peripheral blood B cells were activated by incubation for 24 h with anti-IgM (35 μg/ml; ICN, Aurora, OH) and soluble CD40 L (1 μg/ml; PeproTech EC, Rocky Hill, NJ).

The following antibodies were used: polyclonal CD40 (C20), importin-α, importin-β, TRAF 5(H257), lamin B1, monoclonal Oct-1, and actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), calreticulin (Abcam Inc., Cambridge, MA), polyclonal BLYS (Upstate Biotechnology, Inc., Lake Placid, NY), G28.5 (hybridoma cell line), murine anti-CD40 monoclonal antibody (courtesy of Dr. E. Clark, University of Washington, Seattle, WA), and horseradish peroxidase-labeled secondary anti mouse Ig (Dako Cyto- Immunohistochemistry, Carpinteria, CA). Secondary antibodies labeled with Cy2, Cy3, or fluorescein isothiocyanate were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), and pDsRed2-Nu and pDsRed2-ER were purchased from BD Biosciences.

**Subcellular Fractionation of B Lymphoid Cells**—Nuclear and cytoplasmic extractions were performed as described previously (20). Subcellular fractionation was also performed according to methods described previously (34, 35), with minor modifications. Briefly, cells were homogenized in 1 ml of STM 0.25 (50 mM Tris/HCl, pH 7.4, 0.25 M sucrose, 5 mM MgSO₄, 2 mM dithiothreitol, 10 μg of leupeptin, 1 mM phenylmethylsulfon fluoride, 0.025% Nonidet P-40). Cell homogenates were adjusted to 1.4 M sucrose, layered between 0.5 ml of STM 2.1 and 1 ml of STM 0.8, and centrifuged at 100,000 g for 1 h. The pellet was collected as nuclei and suspended in 6 ml of TP buffer (10 mM Tris/HCl, pH 8.0, 10 mM Na₂HPO₄, 1 mM phenylmethylsulfon fluoride, 1.8 mg of heparin, 110 μg of DNase I), and incubated at 4 °C for 60 min. After sedimenting the mixture at 10,000 × g for 30 min, the nuclei- (NP) and nuclear envelopes (NE/ER) were obtained from the supernatant and pellet, respectively. The interface between 1.4 and 0.8 M sucrose layers was diluted with STM 0.25 and centrifuged at 5000 × g for 20 min to obtain the plasma membrane pellet (PM), and cytoplasmic supernatant. Calreticulin and lamin-B1 were used as an endoplasmic reticulum and nuclear marker, respectively.

**Co-immunoprecipitation**—Polyclonal CD40 antibody (C20) was cross-linked to Dynabeads protein A (Dynal Biotech, Oslo, Norway) according to the manufacturer’s protocol. Cell lysates were precleared with IgG Dynabeads–protein A for 10 min and then incubated with CD40–Dynabeads overnight at 4 °C. CD40-immunoprecipitated complexes were washed five times with immunoprecipitation buffer (10 mM Tris/HCl, pH 7.8, 1 mM EDTA, 150 mM NaCl, 1 mM NaF, 0.5% Nonidet P-40, 0.5% glutopyranoside, 1 μg/ml aprotinin, and 0.5 mM phenylmethylsulfon fluoride). Proteins were eluted by boiling in loading buffer and then processed for Western blot analysis.

**Electron Microscopy**—Cells were fixed on ice for 1 h in 0.1 M cacodylate buffer (0.1% glutaraldehyde and 1% paraformaldehyde, pH 7.4). Cells were dehydrated by two 5-min incubations in 50, 70, 95, and 100% dimethylformamide solutions in water (dimethylformamide/H₂O). Cell pellets were incubated in dimethylformamide/lowerc (1:1) for 30 min at room temperature. Sections (8 nm) were cut and mounted on 150- mesh copper grids, incubated with either primary or preimmune antibodies for 90 min, washed four times with phosphate-buffered saline, and labeled for 60 min with a secondary-6 nm gold-immunolabeled anti-rabbit antibody. Sections were washed with 2% uranyl acetate followed by 4% lead citrate and visualized using a JEM-1200EXII electron microscope (JEOL, Peabody, MA).

**Confocal Microscopy**—Confocal microscopy protocol was performed according to the methods of Pham et al. (18). The cells were visualized using an Olympus Fluoview PV 500 laser-scanning confocal microscope (Olympus, Japan). Images were captured using the ×60 objective with the appropriate filter sets.

[^35S]Methionine Metabolic Labeling—[^35S]Methionine labeling CD40 experiments were conducted as described previously (36). MS cells were washed and preincubated in methionine-free medium for 60 min and labeled with [³⁵S]methionine (50 μCi/ml) (PerkinElmer Life Sciences) at 37 °C for 30 min. Cells were collected, washed, and resuspended in complete medium containing 1 mg/ml of cold methionine for 0.5 and 2.5 h. Harvested cells were subfractionated into plasma membrane, cytoplasm, and nucleiolus fractions. Each fraction was immunoprecipitated with anti-CD40 antibody (C-20) as described previously, and ^35S-labeled CD40 protein was resolved by SDS-PAGE and autoradiography.

**Plasmid Construction and Site-directed Mutagenesis**—The pCDNA3-CD40 plasmid was constructed by ligating CD40, generated from a PIRAT h-CD40 cDNA (Open Biosystems, Huntsville, AL), into pCDNA3 at the KpnI and Apal sites. The pEGFP-CD40 plasmid was cloned by performing the PCR product generated using the template pIRAT h-CD40 cDNA with the oligonucleotide primers f' 5'-AAAGTCTTGTCTGCTGCTTTGAGCAG and 5'-GGGCCTTCACTGTCCTCCTCG into pEGFP at the HindIII and Apal sites. The pEGFP-CD40 mutant was constructed by substituting the putative nuclear localization signal (NLS) region from KKVAKK mutated to TTAVTT using the pEGFP-CD40 DNA template and the oligonucleotides f' 5'-AAAAAGTGGCACAACCAAGC-3' and 5'-CTTTATCAAAGCTGTCGCCAAACG-3' according to the protocols for the QuikChange multisite-directed mutagenesis kit (Strategene, La Jolla, CA). The pGL4-basic BLYS-Luc reporter plasmid was constructed as described previously (37). The pGL4-basic BLYS- (Mutant) Luc reporter plasmid was constructed by substituting TATT with GCCG on the CD40 putative binding site by using the QuikChange multisite-directed mutagenesis kit as described above. All expression vectors were verified by DNA sequencing.

**Transfection, Luciferase, and β-Galactosidase Assay and Real Time PCR Analysis**—Transient transfection of cultured LBCL cells and normal B-lymphocytes was conducted using the nucleofector protocol from Amaza Biosystems (Cologne, Germany). Luciferase and β-galactosidase assays in transfected LBCL cells were performed according to the manufacturer’s protocol (Promega). Luciferase activity values were normalized to transfection efficiency monitored by activity cotransfected with a β-galactosidase expression vector. Each sample in an experiment was repeated at least three times. Total RNA was isolated from transfected lymphoma cells (LBCL) using the NucleoSpin RNA II kit (Clontech). Real-time PCR analysis of BLYS mRNA was carried out in a 20-μl reaction mixture containing TaqMan MGB probes and primers (Assay ID H9251) (Applied Biosystems, Foster City, CA) with an ABI PRISM 7700 sequence detection system (Applied Biosystems).

**Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear protein extraction and EMSA were performed according to procedures described previously (20). Oligonucleotides containing the predicted CD40-binding site were synthesized and verified by DNA sequencing.
sequence (CD40 response element (CD40-RE)) in the BLyS promoter (nucleotides –555 to –576) were synthesized for EMSA. The upper strand sequences of these oligonucleotides were as follows: CD40 wild type (WT), 5′-TAAAAATATATTCACTTATAT-3′; CD40 mutant, 5′-TAAAAATAGCGGCACTTATAT-3′. Oligonucleotides were labeled with γ-32P and incubated with nuclear extract for 15 min, and the extracts were electrophoresed on 4.5% nondenaturing acrylamide gels. Corresponding unlabeled WT or mutant oligonucleotides were used for competition experiments. The gels were dried and analyzed by autoradiography.

Chromatin Immunoprecipitation Assay (ChIP)—Chromatin immunoprecipitation assays were performed using a ChIP assay kit (Upstate Biotechnology). Nuclei isolated from 1% formaldehyde-fixed LBCL cells were sonicated for three 10-s intervals. After centrifugation, precleared supernatant was incubated overnight with CD40 antibody and then incubated for another 2 h with protein A-conjugated magnetic dynabeads (Dynal Biotech Inc.). After the immune complexes were washed six times with washing buffer, DNA was reverse cross-linked by incubation at 65 °C overnight, and the extracted DNA was used for cloning or as a template for PCRs. The BLyS promoter region was amplified to produce a 306-bp PCR product using the primers 5′-AGGCGAGTTGACCTGTCCACCT-3′ and 5′-GGAAGGTGAAGTAAAGACTCAG-3′ located at –681 and –375, respectively. The primers 5′-AGCCTGGGT-
CTGGAGTTCTC-3' (scrambled sequence), and 5'-ACTGCATTG-CAGTCTGCTAT-3' (BLyS promoter upstream sequence) were used as controls. PCR was performed using 35 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min.

Cloning the Chromatin Immunoprecipitation DNA Fragment—
The immunoprecipitated DNA fragments were cloned into PCR-Blunt II Topo vectors as described previously (38). Briefly, the DNA was heated at 68 °C for 5 min and repaired in buffer containing 1 mM

FIGURE 2. Nuclear CD40 receptor in normal B lymphocytes. A, cytoplasmic (C) and nuclear (N) extracts (25 μg) from peripheral blood Go B cells and in vitro activated B cells (activated with anti-IgM (35 μg/ml) alone, sCD154 (1 μg/ml) alone, or the combination of anti-IgM (35 μg/ml) and sCD154 (1 μg/ml) for 24 h) were subjected to Western blot analysis for CD40, actin (cytoplasmic marker), and Oct1 (nuclear marker). B, Go B cells and activated B cells (as in A) were analyzed by confocal microscopy using CD40 antibody (red) and Topro-3 (blue). C, proteins (25 μg) from different subcellular fractions (PM, cytoplasm (C), NE/ER, and NP) from normal Go and activated B (anti-IgM and sCD154) cells were analyzed by Western blotting for CD40, lamin B1 (nuclear marker), and calreticulin (ER marker). Relative CD40 protein levels were analyzed by BioMaxID™ software and represented as percentage expression of CD40 in the PM fraction.
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dNTP and T4 DNA polymerase at 37 °C for 15 min. The DNA was dephosphorylated by calf intestine phosphatase and then cloned into PCR-Blunt II Topo vectors (Invitrogen). Each clone was subjected to DNA sequencing and subsequent Blast analysis to identify the DNA sequence from the National Institutes of Health Entrez Genome Project database.

RESULTS

Detection of CD40 in the Nucleus of LBCL Cells—We have previously identified a novel Signalsome structure involving the binding of ectopic CD40 ligand to CD40 receptor within cell membrane lipid rafts (18) that constitutes a novel Signalosome structure involving the binding of ectopic CD40 protein and mRNA (36), and this cell line was used as a negative pool of LBCL cells (Fig. 1) to detect CD40 protein expression in the nucleus as well as the cytoplasm.

CD40-transfected LBCL cell lines showed a marked increase in CD40 protein expression (Fig. 1C). The CD40 protein could be competed out by a CD40 peptide, conferring that this antibody is specific for the CD40 protein. Since the G28.5 mouse monoclonal antibody was an original antibody that was used to identify CD40 (1), we also used G28.5 to detect CD40 protein expression in the nucleus as well as the cytoplasm of LBCL cells (Fig. 1C). Since the HeLa cell line does not express CD40 protein and mRNA (36), the cell line was used as a negative control for CD40 detection. No detectable CD40 protein was found in either the nucleus or the cytoplasm in HeLa cells analyzed by Western blot and confocal microscopy with the C-20 polyclonal CD40 antibody (Fig. 1A and C). The C-20 polyclonal CD40 antibody showed specific CD40 binding with the highest binding affinity of multiple CD40 antibodies tested and was therefore chosen to conduct most experiments throughout this study.

To further assess the distribution of CD40 in LBCL cells, nuclear and cytoplasmic proteins were fractionated and analyzed by Western blotting. CD40 was observed in both the cytoplasmic and nuclear extracts of the LBCL cell lines MS and McAa (Fig. 1D). In addition, transmission electron microscopy utilizing ultrastructural immunohistochemistry revealed multiple punctate, dispersed CD40 protein accumulations in the nucleus after indirect staining with secondary immunoglobulin antibodies (Fig. 1E). Western blotting of subcellular fractions from LBCL cells confirmed that CD40 protein was differentially expressed in the plasma membrane, cytoplasm, and nucleus (Fig. 1A, bottom right). To verify this finding, we analyzed the distribution of CD40 using immunohistochemical staining of various human B-cell lymphoma cells. In addition to characteristic plasma membrane staining, we also detected CD40 expression in both the cytoplasm and the nucleus in a representative LBCL cell line (Fig. 1B).

To demonstrate the specificity of the C20 polyclonal CD40 antibody, we transfected a recombinant full-length CD40 into a LBCL cell line and then examined CD40 protein expression by Western blotting. The CD40-transfected LBCL cell lines showed a marked increase in CD40 protein expression (Fig. 1C). The CD40 protein could be competed out by a CD40 peptide, confirming that this antibody is specific for the CD40 protein. Since the G28.5 mouse monoclonal antibody was an original antibody that was used to identify CD40 (1), we also used G28.5 to detect CD40 protein expression in the nucleus as well as the cytoplasm of LBCL cells (Fig. 1C).

To investigate CD40 protein trafficking and distribution in vivo, a CD40 cDNA was fused to the coding sequence of a GFP expression vector and transfected into LBCL cells. CD40 was then observed by confocal microscopy in both the nucleus and cytoplasm 24 h after transfection (Fig. 4A). Co-transfection of LBCL cells with CD40-GFP and the expression vectors pDsRed2-Nu (nuclear marker) or pDsRed2-ER (ER marker) further demonstrated that CD40 was localized in the cytoplasm and nucleus of LBCL cells.

3 Y.-C. Lin-Lee, L. V. Pham, A. T. Tamayo, L. Fu, H.-J. Zhou, L. C. Yoshimura, G. L. Decker, and R. J. Ford, unpublished data.

4 Y. C. Lin-Lee and R. J. Ford, unpublished data.

FIGURE 3. 35S metabolic labeling for the newly synthesized CD40 protein in LBCL cells. A, LBCL cells (MS) (3 × 106) were grown in methionine-free culture medium containing labeled [35S]methionine for 20 min and then pulsed with cold methionine (1 µg/ml) at 0, 0.5, and 2.5 h. Newly synthesized CD40 protein was immunoprecipitated from nuclear, cytoplasmic, and plasma membrane cellular fractions and analyzed on SDS-PAGE and detected by autoradiography. B, CD40 competition experiments were performed by incubating a CD40 peptide (1 µg/ml) during immunoprecipitation of nuclear extracts from 2.5-h chase samples with CD40 (C-20) antibody.
FIGURE 4. CD40 receptors localize to the nuclear and cytoplasmic compartments in LBCL cells. At 24 h post-transfection of LBCL cells with either pCD40 (wild type NLS)-GFP (A) or pCD40 (mutant NLS)-GFP (C) plasmid, the cells were paraformaldehyde-fixed and analyzed by confocal microscopy. Co-localization of CD40 receptor proteins in different cellular compartments was accomplished by co-transfection of pCD40-GFP plasmid (green) with nuclear marker plasmid pDsRed2-Nu (red) or the endoplasmic reticulum marker plasmid pDsRed2-ER (red). Co-localization of both proteins in the same compartment reveals a yellow image. All experiments were performed at least twice with similar results. B, diagram of WT and mutant (Mut) CD40 NLS amino sequence (WT KKVAKK mutated to TTVATT). D, after transfection of LBCL cells with either wild type or mutant CD40-GFP NLS expression vectors as described in A and C, proteins (50 μg) from cytoplasmic and nuclear fractions were analyzed by Western blot for CD40, Oct 1 (nuclear marker), and actin (cytoplasmic marker). Since CD40 was expressed as CD40-GFP fusion protein, polyclonal GFP antibody was used to detect the fused protein. E, LBCL cells (MS) were fixed; stained for CD40 (red), importin-β (green), and Topro (blue); and analyzed by confocal microscopy. F, LBCL cells were stained for CD40 (red), importin-α (green), and Topro (blue). Confocal microscopic analysis was performed to visualize co-localization of CD40 with importin-β (F) or importin-α (F). G, association of CD40 with importin-α and importin-β karyopherins in LBCL cells. LBCL cellular extracts (500 mg) were used for immunoprecipitation with antibodies to CD40 (C20) or control rabbit IgG and subsequently analyzed by Western blot for CD40, importin-α, and importin-β. H, peripheral blood B cells activated by anti-IgM (35 μg/ml) and sCD154 (1 μg/ml) for 24 h were stained for CD40 (red), importin-β (green), and the nuclear marker Topro (blue). J, activated peripheral B cells were stained for CD40 (red), importin-α (green), and Topro (blue). Confocal microscopic analysis was performed to visualize co-localization of CD40 with importin-α and importin-β in activated B cells. J, CD40 protein interaction with importin-α and importin-β in activated B cells was detected by immunoprecipitation and Western blot analysis using polyclonal CD40, importin-α, and importin-β, as described previously.
In vivo (Fig. 4A). When the CD40 amino acid sequence was analyzed, we identified a putative NLS sequence KKVAKPTNKN starting at amino acid 216 that consists of a short cluster of positively charged lysine residues fulfilling the general criteria for an NLS (33) (Fig. 4B). We then constructed a CD40-GFP NLS mutant by substituting the amino acid threonine for lysines within the NLS sequence (40), LBCL cells were transfected with the mutant CD40-GFP plasmid, CD40-GFP protein was found to reside primarily in the cytoplasm, but not in the nucleus, when the cells were analyzed by confocal microscopy (Fig. 4C). Similar results were obtained by Western blot analysis, where nuclear CD40 protein was absent in cells transfected with mutant CD40-GFP. The cells transfected with WT CD40-GFP expressed both nuclear and cytoplasmic CD40 protein (Fig. 4D).

Interaction of Cytoplasmic CD40 Proteins with the Karyopherins (Importin-α/β) in Activated Normal B Lymphocytes and Lymphoma B Cells—To determine whether the heterodimeric karyopherin proteins (importin-α/β) recognize the CD40 NLS sequence (40), LBCL cells were analyzed by immunofluorescence staining with confocal microscopy, using antibodies to CD40 and either importin-α or importin-β. CD40 was found to be expressed in the cytoplasm and nuclei of the lymphoma cells, where it co-localized with importin-β (Fig. 4E, green) and importin-α (Fig. 4F, green). Co-localization of CD40 and the Importin proteins, appearing abundant around the nuclear membrane. Co-immunoprecipitation experiments confirmed that CD40 interacts with both importin-α and importin-β proteins in LBCL cell extracts (Fig. 4G). To determine whether the karyopherin-CD40 interactions also occur in normal B cells, we repeated the immunofluorescence experiments in (in vitro) activated normal B lymphocytes. Activation of normal B cells with anti-IgM and sCD154 stimulation resulted in co-localization of CD40 with importin-α or importin-β proteins in the cell cytoplasm, nucleus, and nuclear membrane (Fig. 4, H and I). The interaction of CD40 with both importin-α and importin-β in activated B cells was further confirmed by co-immunoprecipitation followed by Western blot analysis (Fig. 4J).

Binding of Nuclear CD40 Protein to the BLyS Promoter in LBCL Cells—Since the CD40 protein is distributed throughout the nucleus in B lymphoid cells, we next examined whether CD40 might have transactivation potential similar to that reported for the nuclear tyrosine kinase receptors ERB-2 and EGF receptor (22, 24). Initial preliminary studies showed that when the CD40 intracellular domain (CD40c) was fused with the Gal4 DNA-binding domain and transfected into LBCL cells, the chimeric protein showed increased trans-activation activity in luciferase assays. These results suggested that CD40 protein could function as a transcriptional regulator or modulator in the nuclei of B-cell lymphoma cells.

To test this hypothesis, we performed ChIP cloning and informatics analysis to identify potential target genes of nuclear CD40. LBCL cells were treated with formaldehyde to cross-link endogenous protein-DNA complexes that were subsequently immunoprecipitated by a specific antibody against CD40. A total of 104 immunoprecipitated DNA fragments were cloned and subsequently subjected to DNA sequencing (Fig. 5A). One of these clones was found to match the BLyS/BAFF pro-
moter sequence. This finding was verified by additional ChIP assays in which immunoprecipitation with CD40 antibodies was followed by PCR, using primers that amplified a 306-bp PCR product within the BLyS promoter. CD40 protein was found to bind to a specific sequence located within the −375 to −681 base pair span from the transcription start site of the BLyS promoter but did not bind to DNA precipitated with an IgG control (Fig. 5B). PCR analysis using primers upstream of the BLyS promoter also did not show CD40 binding, indicating that the CD40 protein binds to a specific region within the BLyS promoter.

To further assess the binding activity of CD40 to a more specific region of the BLyS promoter, we performed EMSA binding and supershift assays with nuclear extracts from LBCL cells by incubating the extracts with CD40 antibody or specific competitors. EMSA analysis subsequently defined a CD40 protein-DNA-binding complex within the −555 to −567 nucleotide sequence span of the BLyS promoter that could be blocked by competition with the wild type BLyS cold probe or antibody to CD40 protein but not by mutant BLyS nucleotide probes, a nonspecific (scrambled sequence) probe, or preimmune IgG (Fig. 5C). The labeled mutant probe also did not show a CD40-DNA-binding complex, indicating that CD40 binds to this specific region of the BLyS promoter (Fig. 5C). HeLa nuclear extracts were used as a negative control that did not show a CD40-DNA-binding complex due to the fact that HeLa cells lack CD40 expression. When a luciferase reporter construct (BLyS-Luc), containing either a wild type or mutant CD40 binding site (CD40-RE), was co-transfected with the CD40 expression vector into LBCL cells, the luciferase activity in the mutant BLys-Luc decreased significantly and had little effect on CD40 activation when compared with wild type BLys-Luc (Fig. 5D).

Up-Regulation of BLyS/BAFF Protein Expression by Nuclear CD40 Protein Binding—Overexpression of CD40 and BLyS proteins has been observed in LBCL cells in our laboratory and others (38, 41). To determine whether the BLyS promoter is responsive to CD40 activity, a luciferase reporter construct (BLyS-Luc) was co-transfected with a CD40 expression vector into LBCL cells. The reporter activity at 24 h post-transfection was 3.8 times higher in CD40-transfected cells than in cells transfected with an empty control plasmid (Fig. 6A). Real time PCR analysis also showed an increase in BLyS mRNA (2.8-fold) in CD40-transfected LBCL cells compared with the control transfected cells (Fig. 6B). Similarly, Western blot analysis showed that BLyS expression was 2.5 times higher in CD40-transfected LBCL cell extracts than in control extracts from cells transfected with the empty plasmid (Fig. 6C). We have shown previously (Fig. 4D) that CD40 proteins did not enter the nucleus when the NLS was mutated. When we co-transfected LBCL cells (FN) with the BLyS-Luc reporter plasmid along with either CD40 wild type or CD40 NLS mutant expression vectors, a 3.2-fold decrease of the BLyS promoter activity was observed in the CD40 NLS mutant-transfected cells (Fig. 6A). BLyS protein expression was also decreased when LBCL cells were transfected with the CD40 NLS mutant expression vectors (Fig. 6C).

**DISCUSSION**

Our finding that the TNF receptor, CD40, but not its cognate ligand, CD154 (CD40L), is found in the nucleus of normal activated human B lymphocytes and non-Hodgkin’s lymphoma B (LBCL) cells adds CD40 to the growing list of growth factor receptors, including EGF, fibroblast growth factors, and transforming growth factor-β, whose nuclear localization has been described in a variety of normal and neoplastic eukaryotic cell types (22, 24, 42–44). We have demonstrated the nuclear CD40 protein localization with multiple validated CD40 antibodies, including the definitive G28.5 monoclonal, to exclude the possibility that a co-expressed or co-purifying contaminant protein was being identified (1). CD40 receptor protein, having a nuclear residence, potentially opens an additional dimension involving critical TNF receptor family functions in normal as well as neoplastic B-lymphocytes. Although the concept, let alone a specific role for intranuclear growth factor receptors, has not been immediately intuitive (42, 45), the nuclear presence of these receptors suggests that TNF receptor such as CD40, probably have additional functional activities in the signaling pathway(s) that they trigger. In the case of the EGF, it has been shown not only to be present in the nucleus, but also the C terminus of the receptor protein has a transactivation domain that binds to the cyclin D1 promoter, suggesting that it plays a role in the transcriptional regulation of cell cycle genes and cell proliferation (22, 43). EGF receptor was recently reported to contain an NLS (47), involved in nuclear trans-migration, similar to our findings with...
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CD40. Our preliminary studies also showed similar transactivation activity for the cytoplasmic domain of CD40, and we have subsequently identified at least one important target gene promoter sequence that appears to be involved in CD40 nuclear transcriptional activity. The nuclear fractionation studies on isolated, purified normal and neoplastic B-cell nuclei indicate that nuclear CD40 protein is present primarily in the nucleoplasm fraction of the nucleus and also in the nucleus envelope/endoplasmic reticulum, again suggesting functional involvement of CD40 with chromatin, rather than possible involvement in nuclear (e.g. cytoskeletal) structural components or activities. The CD40 protein present in the nuclear envelope fraction appears to represent a trans-migrated form of the receptor linked to the karyopherins, importin-α and importin-β (Fig. 4), through a candidate NLS.

Other members of the CD40 signalosome have also been recently reported to be present in the nucleus (i.e. TRAFs, IKKs, NF-κB proteins) (25–27, 47, 48), suggesting that plasma membrane receptor signaling may be followed by nuclear migration of signaling pathway-component proteins. It has also been suggested that growth factor receptors that have migrated from the plasma membrane into the nucleus might be involved in “braking” or inhibiting proliferative signals from signaling pathways that they trigger in normal cells but may be ignored in cancer, as are other growth-limiting mechanisms (43, 48). Fig. 2 indicates that the binding of the CD40 ligand (CD154) to the CD40 receptor in the plasma membrane (lipid raft) is required for CD40 accumulation in the nucleus. When the cell surface receptor is bypassed in B cell activation (e.g. after calcium ionophore-phorbol ester treatment) in vitro, little, if any, CD40 is found in the nucleus.5

The [35S]methionine metabolic labeling studies also suggest that CD40 traverses the cytoplasm from its integral plasma membrane/lipid raft location, en route to entering the nucleus. Confocal and molecular studies indicated that the cellular traversal mechanism appears to involve the classic NLS-karyopherin pathway through the nuclear pore complex (40). In our transfection studies, confocal microscopy showed that a GFP-fused CD40 protein passed from the cytoplasm and entered the nucleus. When the cell surface receptor is bypassed in B cell activation (e.g. after calcium ionophore-phorbol ester treatment) in vitro, little, if any, CD40 is found in the nucleus.5

The nuclear CD40 protein by Reinhardt’s method also predicts nuclear localization of CD40 and importin-β, that have been shown to function in a similar capacity in the nuclear localization of fibroblast growth factor-1 (49). We have identified a candidate NLS in the CD40 protein molecule that follows the general NLS rules and appears to be required to functionally mediate the nuclear translocation of CD40 in transfected LBCL cells. Mutation of this putative NLS sequence abrogated the entry of CD40 into the nucleus, suggesting that nuclear transport of CD40 is an actively regulated process. Supporting these findings, computer analysis of CD40 receptor protein by Reinhardt’s method also predicts nuclear localization of the CD40 protein with a 70.6 reliability index (50). Since CD40 does not appear to have a protein structure characteristic of a classic transcription factor (e.g. zinc finger motif, etc.), its role in the nucleus is more likely to be that of a co-factor (e.g. co-activator) involved with gene transcription.

Identification of a BLYS/BAFF gene promoter sequence as a target of CD40 nuclear localization suggests that BLYS/BAFF is a downstream effector molecule of a CD40 pathway that mediates the B cell survival activities associated with CD40 stimulation (51). We and others have recently obtained further direct evidence that upon CD154 receptor binding, CD40 activates the BLYS promoter in normal B lymphocytes (38, 41). Nuclear CD40 appears to bind to the sequence TATT of BLYS CD40 RE promoter. This TATT sequence resembles the homeodomain target sequence, TAAT or TTA(T/C), of the HOX protein-binding site (52).

These studies demonstrate that at least some plasma membrane-associated growth factor receptors, such as CD40, can function in more than a single cellular location, possibly directly as well as indirectly influencing gene expression and involving cellular growth and survival pathways as well as other possible cellular functions.

Our studies raise a number of potentially important issues for further studies, regarding a mechanistic role of the CD40 protein in the complex transcriptional processes in target genes as well as in CD40 protein trafficking and cellular compartmentalization. These issues are currently being reassessed and redefined in a variety of other growth factor receptors in various eukaryotic cell types. Finally, the role of nuclear CD40 in neoplastic B cells is of particular interest here, and whereas we have identified CD40 in the nucleus of other types of neoplastic human B cells from the LBCL cells reported here, it is not clear at this time whether similar functional mechanisms are active or what role nuclear CD40 might play in the pathogenesis of these tumor cells.

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REFERENCES

1. Clark, E. A., and Ledbetter, J. A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4494–4498
2. Stamenkovic, I., Clark, E. A., and Seed, B. (1989) EMBO J. 8, 1403–1410
3. Noelle, R. J., Roy, M., Shepherd, D. M., Stamenkovic, I., Ledbetter, J. A., and Aruffo, A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6550–6554
4. Nonoyama, S., Hollenbaugh, D., Aruffo, A., Ledbetter, J. A., and Ocho, H. D. (1993) J. Exp. Med. 178, 1097–1102
5. Serra, P., Amrani, A., Yamanouchi, J., Han, B., Thiessen, S., Utsugi, S., Verdugan, L., and Santamarina, P. (2003) Immunity 19, 877–889
6. Inoue, Y., Otsuka, T., Niro, H., Naganou, S., Arinobu, Y., Ogami, E., Akahoshi, M., Miyake, K., Ninomiya, I., and Shimizu, S. (2000) J. Immunol. 174, 2147–2154
7. Foy, T. M., Shepherd, D. M., Durie, F. H., Aruffo, A., Ledbetter, J. A., and Noelie, R. J. (1993) J. Exp. Med. 178, 1567–1575
8. Pullen, S. S., Labadins, M. E., Ingraham, R. H., McWhirter, S. M., Everdeen, D. S., Alber, A., Crute, J. J., and Keely, M. B. (1999) Biochemistry 38, 10168–10177
9. Reyes-Moreno, C., Girouard, J., Lapointe, R., Darveau, A., and Mourad, W. (2004) J. Biol. Chem. 279, 7799–7806
10. Girouard, J., Reyes-Moreno, C., Darveau, A., Akoum, A., and Mourad, W. (2005) Mol. Immunol. 42, 773–780
11. Lane, P., Traunecker, A., Hubele, S., Inui, S., Lanzavecchia, A., and Gray, D. (1992) Eur. J. Immunol. 22, 2573–2578
12. Weil, R., and Israel, A. (2004) Curr. Opin. Immunol. 16, 374–381
13. Hans, B. A., Jiang, J., Singh, R. A. K., Song, W., Barry, M., Muns, M. H., Slawin, K. M., and Spencer, D. M. (2005) Nat. Med. 10, 1–8
14. Becker, T., Hartl, F. U., and Wieland, F. (2002) J. Cell Biol. 158, 1277–1285
15. Brodeur, S. R., Angelini, F., Bacharier, L. B., Blom, A. M., Mizoguchi, E., Fujiwara, H., Plebani, A., Notarangelo, L. D., Dahlback, B., Tsutskov, E., and Geha, R. S. (2003) Immunity 18, 837–848
16. Cheng, G., and Schoenberger, S. P. (2002) Curr. Dir. Autoimmun. 5, 51–61
17. Berberich, I., Shu, G. L., and Clark, E. A. (1994) J. Immunol. 153, 4357–4366
18. Pham, L. V., Tamayo, A. T., Yoshimura, L. C., Lo, P., Perry, N., Reid, P. S., and Ford, R. J. (2002) Immunity 16, 37–50
19. Rothe, M., Wong, S. C., Hanzel, W. J., and Goeddel, D. V. (1994) Cell 78, 681–692
20. Pham, L. V., Tamayo, A. T., Yoshimura, L. C., Lo, P., and Ford, R. J. (2003) J. Immunol. 171, 88–95
21. Gilmore, T. D. (2003) Cancer Treat. Res. 115, 241–265
22. Lin, S. Y., Makino, K., Xia, W., Matin, A., Wen, Y., Kwong, K. Y., Bourguignon, L., and Hung, M. C. (2001) Nat. Cell Biol. 3, 802–808
23. Peng, H., Myers, J., Fang, X., Stachowiak, E. K., Maher, P. A., Martins, G. G., Popescu, G., Berezney, R., and Stachowiak, M. K. (2002) J. Neurochem. 81, 506–524
24. Wang, S. C., Lien, H. C., Xia, W., Chen, I. F., Lo, H. W., Wang, Z., Al-Sayed, M., Lee, D., Bartholomeusz, G., Fu, O. Y., Giri, D. K., and Hung, M. C. (2004) Cancer Cell 6, 251–261
25. Verma, U. N., Yamamoto, Y., Prajapati, S., and Gaynor, R. B. (2004) J. Biol. Chem. 279, 3509–3515
26. Yamamoto, Y., Verma, U. N., Prajapati, S., Kwak, Y. T., and Gaynor, R. B. (2003) 5. L. V. Pham and R. J. Ford, unpublished data.
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Nature 423, 655–659
27. Anest, V., Hanson, J. L., Cogswell, P. C., Steinbrecher, K. A., Strahl, B. D., and Baldwin, A. S. (2003) Nature 423, 659–663
28. Mackay, F., and Ambrose, C. (2003) Cytokine Growth Factor Rev. 14, 311–324
29. Patke, A., Mecklenbrauker, I., and Tarakhovsky, A. T. (2004) Curr. Opin. Immunol. 16, 251–255
30. Huang, X., Di Liberto, M., Cunningham, A. F., Kang, L., Cheng, S., Ely, S., Liou, H. C., Macleman, I. C., and Chen, S. C. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 17789–17794
31. Mecklenbrauker, I., Kalled, S. L., Leitges, M., Mackay, F., and Tarakhovsky, A. (2004) Nature 431, 456–461
32. Castigli, E., Wilson, S. A., Scott, S., Dedeoglu, F., Xu, S., Lam, K. P., Bram, R. J., Jabara, H., and Geha, R. S. (2005) J. Exp. Med. 201, 35–39
33. Otto, H., Bucher, K., Beckmann, R., Hilbert, R., and Hucho, F. (1992) Neurochem. Int. 21, 409–414
34. Emig, S., Schmalz, D., Shabihou, M., and Buchner, K. (1995) J. Biol. Chem. 270, 13787–13793
35. Hakkarainen, T., Hemenoki, A., Pereboev, A. V., Barker, S. D., Asiedu, C. K., Strong, T. V., Kanerva, A., Wahlen, J., and Curiel, D. T. (2003) Clin. Cancer Res. 9, 619–624
36. Yang, M., Omura, S., Bonifacino, J. S., and Weissman, A. M. (1998) J. Exp. Med. 187, 835–846
37. Fu, L., Lin-Lee, Y. C., Pham, I. V., Tamayo, A., Yoshinura, L., and Ford, R. J. (2006) Blood, in press
38. Weinmann, A. S., and Farnham, P. J. (2002) Methods 26, 37–47
39. Chook, Y. M., and Blobel, G. (2001) Curr. Opin. Struct. Biol. 11, 703–715
40. He, B., Chadburn, A., Jou, E., Schottner, E. J., Knowles, D. M., and Cerutti, A. (2004) J. Immunol. 72, 3268–3279
41. Jans, D. A., and Hassan, G. (1998) Bioessays 20, 400–411
42. Wells, A., and Marti, U. (2002) Nat. Rev. Mol. Cell. Biol. 3, 697–702
43. Carpenter, G. (2003) Curr. Opin. Cell Biol. 15, 143–148
44. Lo, H. C., Hsu, S. C., Ali, S. C., Quinlivan, M., Xia, W., Weil, Y., Bartholomeusz, G., Aih, J. Y., and Hung, C. M. (2005) Cancer Cell. 7, 575–589
45. Min, W., Bradley, J. R., Gableta, I. J., Jones, S. J., Ledgerwood, E. C., and Pober, J. S. (1998) J. Immunol. 161, 319–324
46. Krolewski, J. (2005) Cell. Biochem. 95, 478–487
47. Reilly, J. F., and Mather, P. A. (2001) J. Cell. Biol. 152, 1307–1312
48. Reinhardt, A., and Hubbard, T. (1998) Nucleic Acids Res. 26, 2230–2236
49. Lee, H. H., Dadvorgaz, H., Cheng, O., Shi, J., and Cheng, G. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 9136–9141
50. McCabe, C., and Innis, J. W. (2005) Nucleic Acids Res. 33, 6782–6794