BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 11–17 (1997)
ARTICLE NO. RC977415

The CD40 Ligand Directly Activates T-Lymphocytes via Tyrosine Phosphorylation Dependent PKC Activation

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Received August 17, 1997

The activation of B-lymphocytes depends critically on the interaction of the CD40 receptor with its ligand. Here, we provide evidence that the CD40 ligand (CD40L) also functions as a direct stimulatory molecule for T-lymphocytes. Activation of T-lymphocytes via CD40L induces tyrosine phosphorylation of cellular proteins including PLCγ. Tyrosine phosphorylation of PLCγ correlates with an IP3- and Ca^{2+}-release and an activation of PKC. Inhibition of src-like tyrosine kinases by Herbimycin A prevents these activation events suggesting a crucial role of tyrosine phosphorylation in T-lymphocyte activation via CD40L. © 1997 Academic Press

Key Words: **CD40 ligand; gp39; T-lymphocytes; tyrosine kinases; PKC.**

The cognate interaction of B-lymphocytes with T-lymphocytes is essential for proliferation, differentiation, isotype switching and survival of B-lymphocytes in germinal centers (1, 2). In particular, the interaction of the CD40 receptor on B-cells with the CD40 ligand (CD40L, gp39 or TRAP) expressed on T-lymphocytes seems to be very important for B-lymphocyte activation, since mutations of the CD40L result in the X-linked hyper-IgM syndrome, an immunodeficiency characterized by normal or elevated IgM levels and inefficient isotype switching due to impaired B-cell stimulation (3-8). Patients suffer from recurrent bacterial infections and a higher incidence of autoimmune diseases and malignancies. Molecular mechanisms of B-lymphocyte activation via the CD40 receptor have been studied extensively (for review 2).

However, there is some experimental evidence that binding to the CD40L also activates T-lymphocytes (3). In particular, it has been shown, that CD40L knock-out mice show a defect in the maturation of germinal centers, which can be corrected by injection of recombinant CD40-Fc proteins. This suggests that T-lymphocytes can be activated via the CD40L. The CD40L is a 39 kDa type II membrane glycoprotein expressed on T-lymphocytes but also in mast cells, basophils, NK cells, monocytes and even in B-lymphocytes (4-8). Human or mouse CD40L consists of 261 or 260 amino acids, respectively, with a 22 amino acid cytoplasmic amino-terminal tail lacking any known enzymatic activity (2). The cytoplasmic domains of the mouse and human CD40L are highly homologous with 82% identical residues (2) implying an important role of the cytoplasmic domain for the function of the CD40L. The CD40L exhibits significant homology to the TNF-, Fas or CD30-ligand (9).

In the present study, we tested the hypothesis that the CD40L functions as a stimulatory molecule for T-lymphocytes and we aimed to identify signalling events initiated by the CD40L. The results show a CD40L triggered tyrosine phosphorylation of several proteins including PLCγ. Tyrosine phosphorylation of PLCγ correlates with a release of IP3 and Ca^{2+} as well as with an activation of PKC upon CD40L triggering, which is inhibited by preincubation with Herbimycin A, a src-like tyrosine kinase inhibitor.

MATERIALS AND METHODS

Cells and stimulation. All reagents were purchased from Sigma, if not otherwise cited. Human leukemic Jurkat cells or murine CD40L positive or negative EL4 T-lymphocytes (10) were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 10 mM Hepes (pH 7.4), 2 mM L-glutamine, 100 μM non essential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies, Eggenstein, Germany) and 50 μM β-mercaptoethanol.

The src-like tyrosine kinase inhibitor Herbimycin A (Calbiochem, Bad-Soden, Germany) was added to the cells 12 h prior to any assay at a concentration of 10 μM. For activation, cells (2×10^6 or 20×10^6 per sample for total cell lysates or immunoprecipitations, respectively) were washed twice in sterile Hepes/Saline (H/S, 132 mM NaCl, 20 mM Hepes, 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, 0.8 mM $MgSO_4$) and stimulated at 37°C with 2 μg/ml monoclonal anti-human- or anti-mouse CD40L-antibody (Pharmingen, San Diego, CA, USA).

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0006-291X/97 $25.00

Immunoprecipitation and immunoblotting. Cell stimulation was terminated by lysis in 25 mM Hepes (pH 7.4), 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM NaCl, 10 mM each NaF, Na_3VO_4 and sodium pyrophosphate and 10 μg/ml of each aprotinin and leupeptin (RIPA-buffer). After lysis, DNA and cell debris were pelleted by centrifugation at 20.000xg for 15 min and the supernatants were added to 5xSDS-sample buffer and 5% β-mercaptoethanol (total cell lysates) or subjected to immunoprecipitation overnight at 4°C using 3 μg anti-PLCγ (UBI). For control immunoprecipitates, normal rabbit-Immunoglobulins were used as indicated. After addition of protein A/G-coupled agarose (Santa Cruz Inc., Santa Cruz, CA, USA) incubation was continued for at least 60 min. Immunocomplexes were washed 6-times in lysis buffer, applied to kinase-assays or resuspended in SDS-sample buffer (60 mM Tris (pH 6.8), 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol). Proteins were separated by SDS/PAGE, followed by electrophoretic transfer to Immobilon filters (Millipore, Eschborn, Germany). Blots were incubated overnight at 4°C with the monoclonal anti-phosphotyrosine antibody 4G10 (UBI) and developed by incubation with horseradish peroxidase-conjugated protein G (BioRad, Muenchen, Germany) and a chemoluminescence kit (Amersham, Braunschweig, Germany). Aliquots of the PLCγ immunoprecipitates were blotted with anti-PLCγ to analyze protein levels in all samples.

IP3 and Ca^{2+}-release, PKC-activity. Cells were metabolically labelled with [^{3}H]inositol (10 μCi/ml) for 2 h, stimulated via the CD40L and extracted with $CHCl_3$:CH_3OH:HCl (1:2:0.2, v/v/v). The aqueous phase was diluted with 5 mM disodium tetraborate and applied to Dowex1-X8 (BioRad) columns. IP3 was eluted with 1 M ammonium formate and 0.1 M formic acid after washing the columns with 60 mM sodium formate, 5 mM disodium tetraborate and 0.4 M ammonium formate, 0.1 M formic acid. IP3 formation was then analyzed by liquid scintillation counting of the column eluate.

Intracellular free Ca^{2+} was determined using fura-2 fluorescence ratios. Cells were attached to glass coverslips and loaded with fura-2-acetoxymethylester for 60 min at 37°C. Fluorescence image pairs using 340 and 380 nm excitation wavelength were recorded at 20°C on an inverted microscope (Zeiss Axiovert 135, Zeiss, Jena, Germany) using an intensified CCD camera (RL4, Proxitronic, Bensheim, Germany). 40-60 cells were analyzed in ratio images at 10 s intervals using a Digidata 2000 videograbber and Axon Imaging Workbench software (Axon Instruments, Foster City, CA). Control stimulations were performed with an isotype matched irrelevant antibody (an anti-integrin β7-antibody). Intracellular calibration was performed as described (11).

PKC-activity was measured by translocation of PKC from the Triton X-100 soluble fraction to the insoluble fraction upon stmulation as described earlier (12, 13). To this end, cells were stimulated via CD40L, washed with H/S and resuspended in 20 mM TrisHCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100 and 25 μg/ml aprotinin and leupeptin. Cells were homogenized by 10-15 strokes in a pre-cooled dounce homogenizer, 30 min incubated on ice and cellular debris was removed by 5 min centrifugation at 150.000xg. PKC was then partially purified by application of the post-centrifugation supernatants on DEAE cellulose (Whatman DE52) colums and elution in 20 mM TrisHCl (pH 7.5), 0.5 mM of each EDTA and EGTA, 10 mM β-mercaptoethanol and 200 mM NaCl. Enzyme eluates were added to a lipid preparation consisting of 280 μg/ml phosphatidylserine and 10 μM phorbol 12-myristate 13-acetate in Triton X-100. Addition of these co-factors induces optimal activity of PKC permitting the quantitative measurement of PKC activity in the cell lysates. Since translocation of PKC from the soluble to the insoluble fraction correlates with an activation of this enzyme, a decrease of the kinase activity in the lysates reflects PKC stimulation. To control the specificity of the reaction, a second aliquot of the samples was incubated with a specific PKC inhibitor, a pseudosubstrate composed of the amino acids 19-36 of PKC. Samples were incubated for 20 min at 20°C and the kinase reaction was initiated by addition of 25 μM PKC substrate peptide Ac-MBP (4-14), 10 mM ATP, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 20 mM TrisHCl (pH 7.5) and 10 μCi/sample [^{32}P]γATP. The samples were incubated for 5 min at 30°C and the reaction was terminated by filtration of aliquots through nitrocellulose filters (Life Technologies). Filters were washed twice in 1% H_3PO_4, twice in H_2O and radioactivity bound to the filters was determined by liquid scintillation counting. Binding of the phosphorylated substrate reflects the activity of the kinase. The results are expressed as % increase of kinase activity compared to unstimulated samples.

RESULTS

To test the hypothesis that the CD40L not only functions as a ligand for the CD40 receptor but also as a receptor transmitting signals in T-lymphocytes, we stimulated CD40L positive and CD40L negative mouse EL4 cells with monoclonal anti-CD40L-antibodies. Expression of CD40L was evaluated by flow cytometry. Activation of CD40L positive EL4 cells via the CD40L resulted in tyrosine phosphorylation of several cellular proteins (Fig. 1A). CD40L negative EL4 cells did not respond with an increase of cellular tyrosine phosphorylation (not shown) pointing to the specificity of the observed tyrosine phosphorylation mediated by the CD40L.

To identify target molecules regulated by CD40L, we measured tyrosine phosphorylation of PLCγ, formation of IP3, release of Ca^{2+} and activity of PKC upon stimulation with anti-CD40L-antibodies or purified CD40 receptor. PLCγ was immuno-precipitated from anti-CD40L stimulated EL4, and analyzed for tyrosine phosphorylation using 4G10 monoclonal anti-phosphotyrosine antibodies. CD40 ligand stimulation resulted in an approximately 7-fold increase of PLCγ tyrosine phosphorylation (Fig. 1B). Tyrosine phosphorylation of PLCγ upon CD40L triggering was confirmed in human leukemic Jurkat T-cells treated with anti-human CD40L-antibodies (Fig. 1B). Expression of CD40L on EL4 and Jurkat was similar as detected by FACS analysis (not shown).

PLCγ activation has been reported to regulate the release of IP3 and DAG from inositol phosphates. We therefore determined the release of IP3 from [^{3}H]-metabolically labelled cells upon CD40L triggering. The results show, that cellular stimulation via CD40L results in a ~4-fold increase of IP3-formation (Fig. 2A).

IP3 releases Ca^{2+} from intracellular stores and subsequently activates Ca^{2+}-influx (14). To determine the effect of CD40L triggering on Ca^{2+}-flux EL4 cells were loaded with fura-2 and intracellular free Ca^{2+}-concentration was analyzed by fluorescence ratio imaging. Stimulation with anti-CD40L induced an oscillating increase of intracellular free Ca^{2+} in 22 out of 62 cells (35%) (Fig. 2B). The oscillations were irregular in size and frequency. Times between Ca^{2+}-spikes ranged from seconds to several minutes. The typical response of a single cell is shown in Fig. 2B. This response is comparable with the Ca^{2+}-signal induced via the T cell receptor (15). Control experiments were performed on

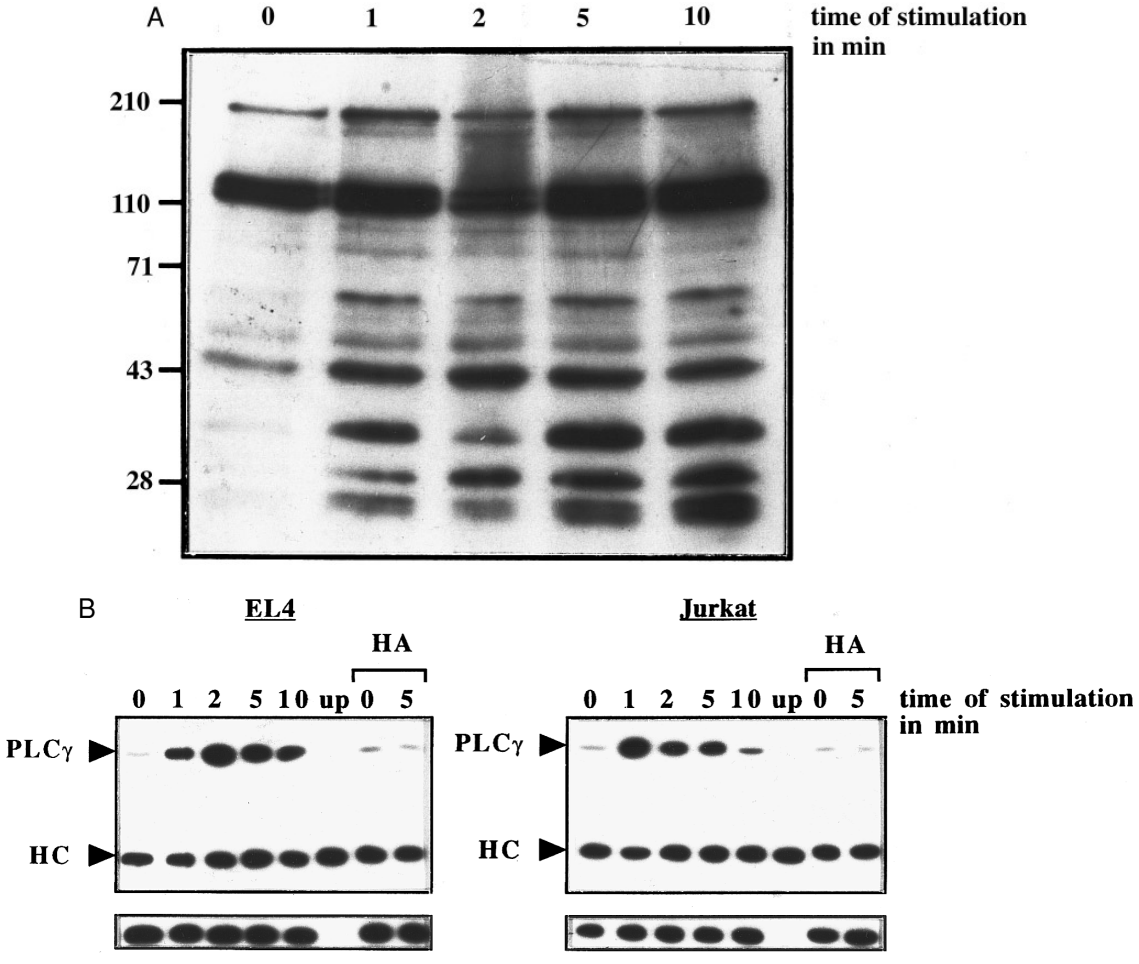

FIG. 1. Stimulation of EL4 cells via the CD40L results in tyrosine phosphorylation of several cellular proteins (A) including PLCγ (B). Tyrosine phosphorylation of PLCγ after CD40L stimulation is also observed in human leukemic Jurkat T-cells (B). Preincubation with the src-like tyrosine kinase inhibitor Herbimycin A prevents tyrosine phosphorylation of PLCγ (B). Murine EL4 or human Jurkat cells were stimulated with monoclonal anti-CD40L antibodies lysed and analyzed for cellular tyrosine phosphorylation by western blotting with the monoclonal anti-phosphotyrosine antibody 4G10 and ECL-development (A). Shown are representative blots of three independent experiments. PLCγ was immunoprecipitated using a polyclonal anti-PLCγ antibody. Immunoprecipitates were tested for tyrosine phosphorylation by incubation of the blots with the anti-phosphotyrosine antibody 4G10 followed by ECL-development. Similar amounts of PLCγ are present in all lanes revealed by blotting of aliquots of the PLCγ-immunoprecipitates with anti-PLCγ (small blots below). Up, unspecific immunoprecipitates with irrelevant rabbit antibodies; HC, heavy chain of the immunoprecipitating antibody. Shown are representative blots of two independent experiments.

34 cells stimulated with 2 μg/ml of an isotype matched antibody to β7 integrin. None of the cells showed a calcium increase after β7 integrin ligation (not shown).

The observed release of IP3 and Ca^{2+} points to a stimulation of PKC, which may transmit the signal initiated by the CD40L to further downstream molecules finally regulating gene transcription. Activity of PKC by CD40L triggering was determined by measuring the phosphorylation of a PKC-substrate peptide. Stimulation of EL4 cells via CD40L with anti-CD40L antibodies or purified CD40 receptor activated PKC 4-5-fold (Fig. 2C). A similar increase of PKC activity was also observed after stimulation of Jurkat cells via the CD40L indicating that the activation of the PKC pathway by the CD40L is not restricted to a certain cell line.

In order to investigate the significance of src-like tyrosine kinases for the observed activation events, we preincubated the cells with 10 μM Herbimycin A, a src-like tyrosine kinase inhibitor. Tyrosine phosphorylation of PLCγ, release of IP3 and Ca^{2+} as well as stimulation of PKC were almost completely prevented by pretreatment of the cells with Herbimycin A (Figs. 1B and 2A-C). The Ca^{2+} response was determined in 118 cells pretreated with Herbimycin A. None of the cells responded to CD40L triggering. However, all cells exhibited a sustained Ca^{2+}-increase after depletion of intracellular Ca^{2+} stores with 1 μM thapsigargin demonstrating a normal function of Ca^{2+} stores and membrane Ca^{2+}-channels. In summary, the results indicate a signalling cascade from the CD40L via PLCγ to IP3,

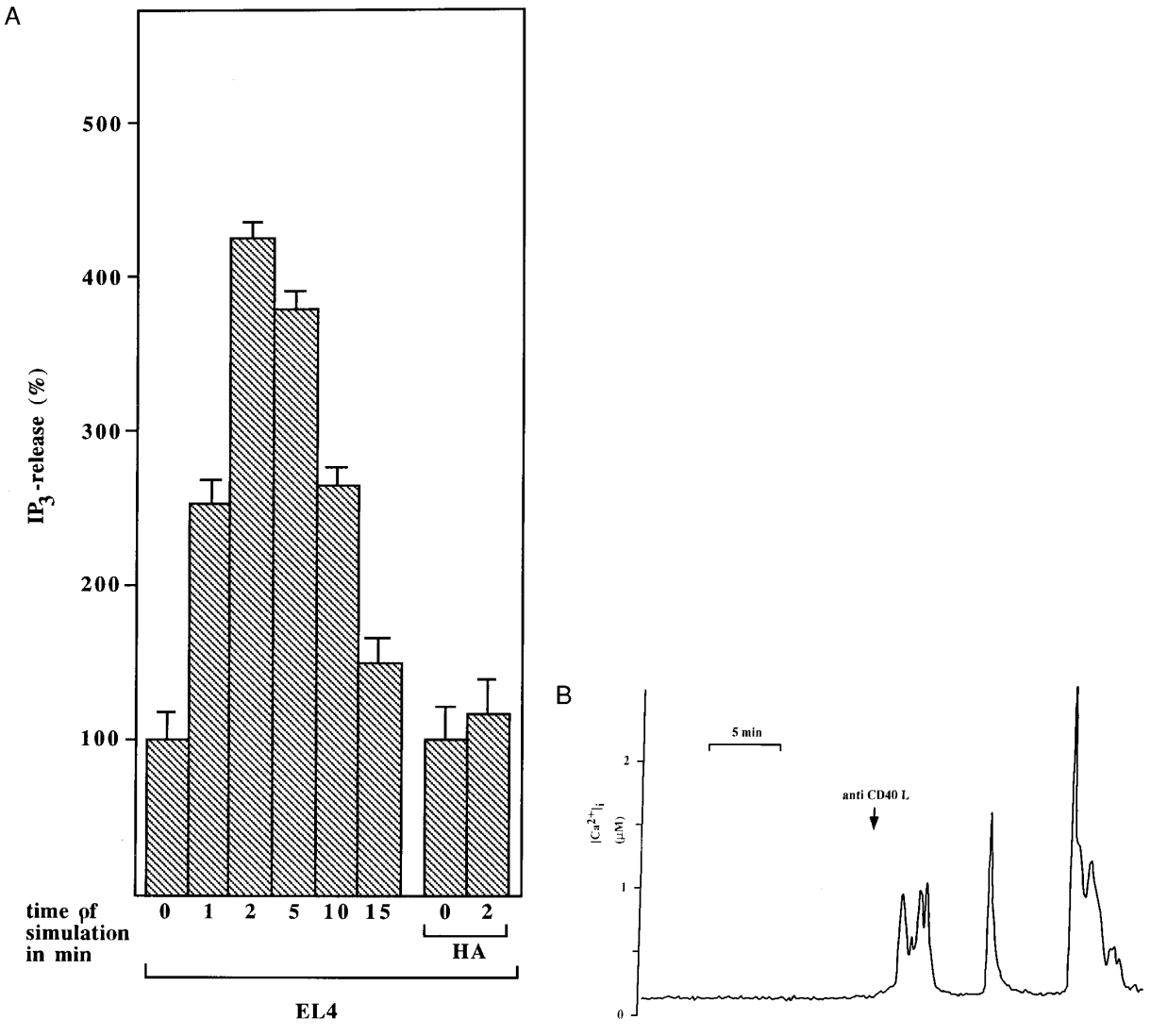

FIG. 2. Cellular activation via the CD40L induces formation of IP3 (A), release of Ca^{2+} (B), and an activation of PKC (C). Preincubation with Herbimycin A (HA) prevents these activation events (A-C). Activation of this pathway is not restricted to EL4 cells, since triggering of Jurkat T-lymphocytes via CD40L results in a similar stimulation of PKC (C). IP3-formation was determined from [^{3}H]inositol metabolically labelled cells by organic extraction, anion exchange chromatography, and liquid scintillation counting. CD40L induced Ca^{2+} release was analyzed in 84 Fura-2 loaded Jurkat cells by fluorescence microscopy. Control stimulations were performed with an anti-integrin β7-antibody. PKC-activity from stimulated or unstimulated cells was determined by phosphorylation of the substrate peptide Ac-MBP (aminoacids 4-14). Phosphorylation was measured by filtration of samples through nitro-cellulose filters, washing, and liquid scintillation counting of filter bound radioactivity. Results are expressed as percentage increase compared to unstimulated samples. Shown are means ± SD of two independent experiments, respectively.

Ca^{2+} and finally PKC-activation. The stimulation of this pathway seems to depend on the function of Herbimycin A sensitive protein tyrosine kinases.

DISCUSSION

The present study provides evidence for a novel role of the CD40L: In addition to its function as a ligand for the CD40 receptor it also seems to function as a signalling molecule on T-lymphocytes mediating T-cell activation. Therefore, the cognate interaction between T- and B-lymphocytes via the CD40L/CD40 receptor pair seems to result in the activation of both lymphocyte subsets.

Most of the described signals activated by CD40L are also triggered by the TCR/CD3-complex (16). Therefore, CD40L may partially function as a co-stimulatory receptor enhancing a signal delivered via the TCR/CD3-receptor. However, data from our laboratory show that the CD40L also activates the neutral sphingomyelinase as well as Jun N-terminal kinases and p38-K (17, 18). Since these signalling molecules are not or only very weakly

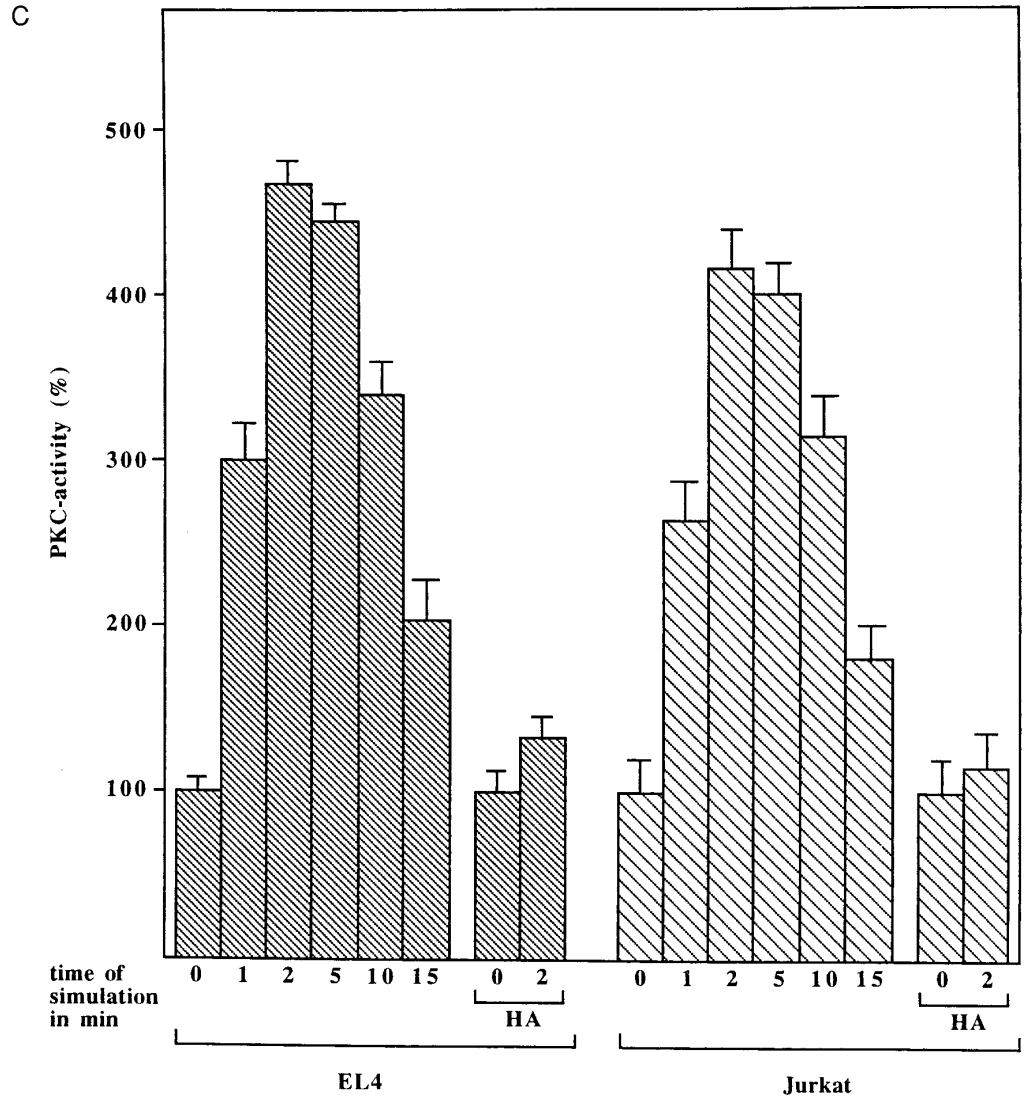

FIG. 2—*Continued*

activated by the TCR/CD3, it seems to be likely that the CD40L functions independently of the TCR/CD3-complex in the activation of T-lymphocytes.

Activation of PKC via Ca^{2+} and DAG has been shown to induce a variety of cellular changes including regulation of gene expression and seems to be crucial for cellular proliferation or differentiation. Therefore, it might be possible that the observed activation of PKC results in an increased NFAT or NFκB activity finally mediating cellular activation via the CD40L. This notion is supported by preliminary findings that CD40L triggering results in an IL-2-release in Jurkat T-lymphocytes.

Our results point to an important function of src-like tyrosine kinases for cell activation via CD40L. However, the inhibitor Herbimycin A is not an absolute specific inhibitor for src-like tyrosine kinases, therefore future experiments using genetic deficient models will be required to define the exact function of certain kinases for PLCγ/PKC activation by the CD40L. Unfortunately, the $p56^{lck}$ deficient Jurkat clone JCaM1.6 does not express CD40L and $p56^{lck}$ knock-out mice display a severe defect of thymocyte maturation and, therefore, are not suitable for studying signalling in mature, peripheral T-lymphocytes.

Activation of JNK/p38-K after CD40L triggering was observed in mouse and human T-lymphocytes. Since two different monoclonal antibodies were applied specific for the human or the mouse CD40L, respectively, it seems to be unlikely that the observed activation signals are mediated by an unspecific cross-reaction with other surface molecules. Furthermore, CD40L negative EL4 cells did not respond to stimulation with the monoclonal antibodies.

Several studies support our results, that the CD40L has a dual function resulting in activation of T- and B-lymphocytes upon binding to the CD40 receptor.

First, the best evidence for a stimulatory function of the CD40L for T-lymphocytes is provided by CD40 receptor knock-out mice (3). Although, these mice lack germinal centers, the formation of germinal centers can be initiated upon injection of the antigen DNP-KLH and co-injection of soluble CD40-Fc molecules. This implies that stimulation of T-lymphocytes via the CD40L has a crucial function in the initiation of germinal center formation.

Second, stimulation of activated peripheral T-cells with anti-CD40L enhances anti-TCR/CD3 triggered IL-4 synthesis and proliferation (19, 20). Thus, in this context the CD40L seems to function as a co-stimulatory molecule.

Third, a signalling function of the CD40L is further supported by the finding that other ligands of the TNF/NGF-receptor family exhibit similar dual functions (21, 22). CD30 ligand cross-linking by monoclonal antibodies or CD30-Fc-fusion proteins induced a synthesis of IL-8 and oxidative burst in neutrophils (22). Further, crosslinking of the CD30-ligand on mature T-lymphocytes increases proliferation and IL-6 production in these cells. Likewise, stimulation of B-lymphocytes via the OX40-ligand induced cell proliferation, m-RNA- and Ig-synthesis (21) pointing to a stimulatory function of these ligands in several cell types.

However, application of activating CD40 receptor-antibodies to CD40L knock-out mice restores cellular and humoral immune responses to adenoviral vectors via a B7.2-CD28 dependent mechanism (23). Thus, the CD40L may not be absolutely required for the stimulation of T-cells and the CD40L may function predominantly as a co-stimulatory molecule which can be replaced by other strong co-stimuli or by upregulation of other stimulatory receptors replacing the function of the CD40L in the CD40 receptor knock-out mice.

Thus, it might be posssible that the observed activation of T-lymphocytes via the CD40L functions predominantly in germinal centers as suggested by van Essen et al. (3), whereas peripheral T-lymphocytes do not require a signalling via the CD40L for stimulation during an immune response.

Alternatively, signalling via the CD40L may be involved in the regulation of the interaction between the CD40L and the CD40 receptor and may not result in stimulation of T-lymphocytes. CD40L/CD40 interactions might be influenced by internalization of the CD40L and/or cleavage of the CD40L by metalloproteinases. It might be possible that the observed activation of the PKC signalling pathway induces changes of the cytoskeleton finally leading to internalization of the CD40L. This hypothesis is supported by findings showing that the interaction of the CD40L with the CD40 receptor results in downregulation and internalization of the CD40L on the surface of T-lymphocytes. Furthermore, most members of the TNF/NGF-ligand family are cleaved on the surface by metalloproteinases. Thus, it might be also possible that PKC or other observed signalling events are involved in the regulation of CD40L internalization or metalloproteinase activity resulting in a termination of the stimulatory effect of T-lymphocytes on B-cells. Thus, the observed signalling events could be also involved in a negative regulation of B-lymphocyte activation by T-lymphocytes via the CD40L/CD40-system.

ACKNOWLEDGMENTS

The authors thank Dr. R. H. Zubler, Department of Medicine, University Hospital, Geneve, Switzerland, for valubale reagents and A. Behyl for excellent technical help. The study was supported by DFG Grant La 315-6/1 to E.G., F.L., and B.B.

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