Anti–Immunoglobulin M Activates Nuclear Calcium/Calmodulin-dependent Protein Kinase II in Human B Lymphocytes

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Summary

We and others have previously shown that the nuclear protein, Ets-1, is phosphorylated in a calcium-dependent manner after ligation of immunoglobulin (Ig) M on B lymphocytes. As this phosphorylation was independent of protein kinase C activity, we tested whether a calcium/calmodulin-dependent protein kinase (CaM kinase) might phosphorylate the Ets-1 protein after elevation of intracellular free calcium concentrations. The dephosphorylated form of Ets-1 has been shown to bind to chromatin, suggesting that the operative kinase should be detectable in the nucleus. We prepared nuclear extracts from two human B cell lines in which increased intracellular free calcium levels correlated with increased phosphorylation of the Ets-1 protein. Activity of the CaM kinases was determined using a synthetic peptide substrate both in the absence and presence of an inhibitor specific for the CaM kinase family, KN-62. Stimulation of cells with anti-IgM led to increased activity of a nuclear kinase that could phosphorylate the peptide, and this activity was reduced by 10 μM KN-62. Kinase activity was reduced in lysates preadsorbed using an antibody specific for CaM kinase II. Two-dimensional phosphopeptide maps of the Ets-1 protein from cells incubated with ionomycin or anti-IgM contained two unique phosphopeptides that were absent in untreated cells. Incubation of isolated Ets-1 protein with purified CaM kinase II produced phosphorylation of peptides that migrated identically to those found in cells incubated with either anti-IgM or ionomycin. These data suggest a model of signal transduction by the antigen receptor on B lymphocytes in which increased intracellular free calcium can rapidly activate nuclear CaM kinase II, potentially resulting in phosphorylation and regulation of DNA-binding proteins.

The means by which ligation of surface proteins regulates the function of nuclear DNA-binding proteins is poorly understood. Among potential mechanisms that modulate the activity of these proteins, the best studied is phosphorylation (for review see reference 1). Several protein serine/threonine kinases are reported to function in the nucleus, including protein kinase C (PKC),1 casein kinase II, the cAMP-dependent protein kinase (PKA), and members of the family of calcium/calmodulin-dependent kinases (CaM kinases) (see reference 1). In addition, both microtubule-associated protein (MAP) kinase and p90^rsk have been reported to translocate to the nucleus after activation of HeLa cells (2). Once activated, nuclear kinases can phosphorylate specific DNA-binding proteins and alter the ability of these proteins to interact with DNA or other nuclear proteins with which they form functional complexes.

We previously reported that the nuclear protein Ets-1 is phosphorylated in B lymphocytes after ligation of the antigen receptor on B cells, surface IgM (3). Phosphorylation of the Ets-1 protein occurred in a calcium-dependent manner and did not appear to involve the calcium-regulated protein kinase, PKC (3). Observations that the Ets-1 protein associates with DNA only in its unphosphorylated state (4) and that calcium ionophore specifically increased Ets-1

1Abbreviations used in this paper: [Ca^{2+}]_i, intracellular free calcium; CaM kinase, calcium/calmodulin-dependent kinase; DTT, dithiothreitol; MLCK, myosin light chain kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKI, peptide inhibitor of PKA; PVDF, polyvinylidene difluoride.
phosphorylation on seryl and threonyl residues (5) predicted the presence a nuclear protein serine/threonine kinase that might be regulated directly by calcium. Previous work has demonstrated that at least two families of calcium-regulated kinases are present in the nucleus: the calcium-dependent isozymes of PKC and the CaM kinases, including myosin light chain kinase (MLCK) (for review see reference 6). To date, MLCK has been reported to use only myosin light chains as a physiologically significant substrate (7). Therefore, we tested whether a CaM kinase could be found in the nucleus of human B cells and whether ligation of surface IgM would activate that kinase.

**Materials and Methods**

**Cell Culture.** The human B cell lines Raji, T5-1, and Ramos were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM t-glutamine, nonessential amino acids, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were maintained in log phase before use in the experiment.

**Reagents.** The protease inhibitors PMSF, leupeptin, and apotinin were purchased from Boehringer Mannheim Corp. (Indianapolis, IN), and N-tosyl-l-phenylalanine chloromethyl ketone-treated trypsin was purchased from Worthington Biochemical Corp. (Freehold, NJ). Protein A-Sepharose was purchased from Calbiochem Corp. (La Jolla, CA), and cellulose TLC plates were obtained from Merck (Darmstadt, Germany). Antibodies specific for the Ets-1 protein were produced as previously described (3), and the control MOPC 21 antibody was obtained from American Type Culture Collection (Rockville, MD). A Fab(′)2 goat antihuman IgG and IgM was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA) and used for all anti-IgM stimulations. The antibody RU16 was made using a peptide corresponding to the β subunit residues 506-531 of CaM kinase II; this antibody detects both α and β/β' subunits on immunoblots of total rat forebrain homogenates (8). The peptide substrate, syntide (PLARTISVAGLPGBK) (9), PKI, a peptide inhibitor of PKA (TTYADFIASGRTGRRNAIHD) (10), and the control MOPC21 antibody was obtained from Sigma Chemical Co., St. Louis, MO; 30 mM p-nitrophenyl phosphate, 10 mM NaN3, 0.1 mM Na2VO4, 0.1 mM Na3MoO4, and 10 mM B-glycerophosphate. Samples were stored at -70°C or assayed the same day as prepared. Purity of the nuclear preparations was monitored by measuring for the presence of the cytosolic enzyme lactate dehydrogenase. Lactate dehydrogenase in the nuclear extracts represented 1-10% of the total enzymatic activity in the cells. Recovery of nuclear proteins from 5 × 107 cells was in the range of 0.5-0.7 mg.

**In Vitro Phosphorylation by Purified CaM Kinase II.** Ets-1 protein was immunoprecipitated as described above and kept on solid phase (protein A-Sepharose beads). The beads were resuspended in assay buffer consisting of 50 mM Hepes, pH 7.5, 10 mM MgCl2, 1 mM DTT, 50 μM cold ATP, 50 μM [γ-32P]ATP (~4,000 cpm/pmole), 1 μM cAMP, and 0.5 mM CaCl2. The reaction was started by addition of 75 ng/ml of rat forebrain CaM kinase II (specific activity = 2.4 μmol/min per mg protein using the syntide peptide as substrate). The reaction proceeded for 30 min at 30°C and was terminated by washing the pellet with ice-cold PBS and boiling in Laemmli sample buffer (15). Phosphoproteins were identified by SDS-PAGE and autoradiography.

**Protein Kinase Assays.** CaM kinase activity in the absence of in vitro activators was measured as previously described (17). Briefly, kinase assays were performed in assay buffer consisting of 20 mM Hepes, pH 7.5, 10 mM MgCl2, 0.02% Triton X-100, 2 mM DTT, 1 mM PKI peptide, 50 μM [γ-32P]ATP (~1,000 cpm/pmole), and protease inhibitors. When used, the peptide substrate syntide was present at 200 μM. Kinase assays were initiated by addition of enzyme samples to give a final volume of 50 μl. Reaction mixtures were incubated for 10-15 min at 30°C and transferred to p81 phosphocellulose paper, and the papers were washed extensively. Phosphorylation of peptide substrates was quantified by measure-
Ionomycin and anti-IgM were found to induce increased phosphorylation of Ets-1 in human B cells. The calcium-dependent phosphorylation of the nuclear Ets-1 protein in murine B cells (3) was observed. The deduced sequence of the Ets-1 protein predicts at least two consensus recognition sequences for CaM kinase II phosphorylation in the molecule (Thr-73 and Ser-156) (19). To pursue the mechanism of this phosphorylation, we identified two human B cell lines, T5-1 and Raji, that demonstrated increased phosphorylation of the Ets-1 protein (3.6-fold over control for anti-IgM) in response to increased calcium levels. Both cell lines respond with increased ([Ca^{2+}]) after incubation with anti-IgM (20 and Valentine, M. A., unpublished observations). To test whether CaM kinase II was responsible for the phosphorylation of Ets-1 in response to calcium, we incubated the Raji cells with medium alone, ionomycin, or anti-IgM (Fig. 1). Both ionomycin and anti-IgM induced increased phosphorylation of two distinct peptides that were phosphorylated in untreated cells (peptides 1 and 3). Peptide 2 was faintly distinguishable in the untreated control sample, accounting for 2% of the total radioactivity in the sample as quantified with the use of an autoradiography. The phosphorylation of this peptide was increased slightly (accounting for 5–9% of total cpm) in all other samples. When immunoprecipitated Ets-1 protein was incubated in vitro with purified CaM kinase II (Fig. 1 A, bottom right), the pattern of peptide phosphorylation appeared to be identical to that of Ets-1 peptides from cells stimulated with anti-IgM or ionomycin. None of the phosphopeptides comigrated with free phosphate or ATP (data not shown). The identity of the three peptides having increased phosphorylation was confirmed by removing them from the plates and mixing equal cpm from each peptide for each peptide were combined and reanalyzed in two dimensions. 

**Results**

**Ionomycin, Anti-IgM, and CaM kinase II Induce Increased Phosphorylation of Ets-1 Tryptic Peptides.** We previously reported the calcium-dependent phosphorylation of the nuclear Ets-1 protein in murine B cells (3). The deduced sequence of the Ets-1 protein predicts at least two consensus recognition sequences for CaM kinase II phosphorylation in the molecule (Thr-73 and Ser-156) (19). To pursue the mechanism of this phosphorylation, we identified two human B cell lines, T5-1 and Raji, that demonstrated increased phosphorylation of the Ets-1 protein (3.6-fold over control for anti-IgM) in response to increased calcium levels. Both cell lines respond with increased ([Ca^{2+}]) after incubation with anti-IgM (20 and Valentine, M. A., unpublished observations). To test whether CaM kinase II was responsible for the phosphorylation of Ets-1 in response to calcium, we incubated the Raji cells with medium alone, ionomycin, or anti-IgM (Fig. 1). Both ionomycin and anti-IgM induced increased phosphorylation of two distinct peptides that were not phosphorylated in untreated cells (peptides 1 and 3). Peptide 2 was faintly distinguishable in the untreated control sample, accounting for 2% of the total radioactivity in the sample as quantified with the use of an autoradiography. The phosphorylation of this peptide was increased slightly (accounting for 5–9% of total cpm) in all other samples. When immunoprecipitated Ets-1 protein was incubated in vitro with purified CaM kinase II (Fig. 1 A, bottom right), the pattern of peptide phosphorylation appeared to be identical to that of Ets-1 peptides from cells stimulated with anti-IgM or ionomycin. None of the phosphopeptides comigrated with free phosphate or ATP (data not shown). The identity of the three peptides having increased phosphorylation was confirmed by removing them from the plates and mixing equal cpm from each peptide for each peptide were combined and reanalyzed in two dimensions.

![Figure 1. Ionomycin and anti-IgM induce phosphorylation on the Ets-1 protein.](image-url)
tide as substrate, CaM kinase II uses syntide as substrate about 10× more efficiently than does CaM kinase IV (21). We incorporated into the assay the inhibitor KN-62, which is specific for the calmodulin-binding region of several members of the CaM kinase family. A second inhibitor, a peptide having the sequence corresponding to the autoinhibitory domain of CaM kinase II (α subunit residues 281–309) (11), was also used. In several experiments, addition of ionomycin (not shown) or anti-IgM to the cells stimulated the rate of phosphorylation of syntide by 1.4–2.5-fold above basal levels. This increase was reduced by both inhibitors at the early time points, suggesting that CaM kinase(s) was activated by these stimuli (Fig. 2). Similar results were obtained using the Raji cell line and resting (dense) or activated (buoyant) human tonsilar B cells. Anti-IgM induced a two-fold increase of syntide phosphorylation in the Raji cells and from two- to fourfold increases in tonsilar human tonsilar B cells. Anti-IgM induced a two-fold increase of syntide phosphorylation in the Raji cells and from two- to fourfold increases in tonsilar human tonsilar B cells. In other experiments, addition of calcium (0.5 mM) into the reaction mix resulted in only a slight increase in the activity of the anti-IgM- or ionomycin-treated samples. These results suggest that CaM kinase was rapidly activated by increased calcium levels at these early time points.

**Anti-IgM Increases a CaM Kinase Activity in the Nucleus.** As the data were consistent with anti-IgM–induced activation of CaM kinase in parallel with phosphorylation of Ets-1, we prepared nuclear extracts from Raji or T5-1 cells incubated with anti-IgM or medium and assayed for phosphorylation of syntide in the presence or absence of the inhibitor KN-62. Fig. 3 presents results for the Raji cells in which anti-IgM–induced phosphorylation of syntide was almost exclusively nuclear, and the nuclear kinase activity was inhibited by KN-62

![Figure 3](image-url)

**Figure 3.** Anti-IgM induces CaM kinase activity in the nucleus. Nuclear or cytosolic lysates were prepared from Raji cells, which were incubated for the times shown in the presence of medium alone or with 80 μg/ml anti-IgM. Phosphorylation of syntide was measured in the presence of 10 μM KN-62, and values are shown as the mean of triplicate assays from duplicate samples for each time point. Data are representative of at least three independent experiments for Raji (or T5-1) cells. –, nuclear-anti-μ, -Δ-, nuclear + KN-62, –, cytosol-anti-μ, -Δ-, cytosol + KN-62.

at the early time points. Cytosolic extracts routinely had one-third to one-eighth the activity of nuclear extracts, and activity changed minimally in extracts prepared from anti-IgM-stimulated cells. These results were similar to those obtained using the T5-1 cell line. In other experiments, increased syntide phosphorylation as induced by ionomycin or anti-IgM was reduced to unstimulated levels by preincubation (1 h) of cells with 10 μM KN-62 (data not shown). These data show that ligation of surface IgM can induce CaM kinase activity in the nucleus, and this activation is sensitive shortly after stimulus to an inhibitor that competes with calmodulin-binding sites.

**CaM Kinase II and the Ets-1 Proteins in the Nucleus in Raji Cells.** The above results were consistent with activation of one or more members of the family of CaM kinases in the nucleus of responsive cells. Delcayre et al. have previously reported the presence of calcium/calmodulin-dependent protein kinase activity in the nucleus of Raji cells (22). To identify which CaM kinase was activated, nuclear and cytosolic extracts used in Fig. 3 were probed using an antibody specific for CaM kinase II (Fig. 4 A). Equal amounts of proteins from the extracts were separated by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with the antibody. We found that CaM kinase II was present almost exclusively in the nucleus and migrated similarly to the 48- and 60-kD subunits of CaM kinase II isolated from rat brain (latter not shown). The identity of the higher molecular mass protein is unknown. Similar results were obtained using the T5-1 cell line. There is minimal information on the presence of the other CaM kinase family members in human B lymphocytes. CaM kinases I and III have been reported to be almost exclusively cytosolic; whereas significant levels of CaM kinase IV can be found in the nucleus, this isoform is not ex-
pressed in Raji cells (23). Blotting experiments were repeated using equal amounts of proteins from the cytosolic and nuclear extracts of Raji cells, probing for the presence of the Ets-1 proteins (Fig. 4 B). The 54-kD Ets-1 protein was found predominantly in the nucleus of the Raji cells. In contrast to previous results from murine B cells (3), migration of the Ets-1 protein (B) followed by 1111-protein A. Immunoreactive bands were visualized by autoradiography after 48-h exposure. Arrows indicate the migration of rat brain 48- and 60-kD CaM kinase II subunits and the 54-kD Ets-1 protein from cells incubated in medium or with anti-IgM (α-μ).

**Figure 4.** Both CaM kinase II and the Ets-1 proteins are predominantly localized in the nucleus in Raji cells. Equal amounts of protein from nuclear and cytosolic extracts used in the experiment shown in Fig. 3 were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with antibodies specific for either CaM kinase II (A) or the Ets-1 protein (B) followed by 1111-protein A. Immunoreactive bands were visualized by autoradiography after 48-h exposure. Arrows indicate the migration of rat brain 48- and 60-kD CaM kinase II subunits and the 54-kD Ets-1 protein from cells incubated in medium or with anti-IgM (α-μ).

**Discussion**

Activation of signaling pathways by ligation of the B cell antigen receptor is amplified by several protein serine/threonine kinases, some of which are dependent on second messengers for activation (for reviews see references 5, 24). Ligation of surface IgM leads to a rapid increase of the second messenger calcium, which, in turn, can activate numerous calcium-dependent enzymes, including the CaM kinases. In this report, we show that nuclear protein kinase activity is stimulated in response to anti-IgM or ionomycin in human B cells. CaM kinase II appears to account for the majority of the nuclear protein kinase activity that is stimulated by anti-IgM ligation as judged by (a) the presence of CaM kinase II in the nucleus; (b) induction of phosphorylation on syntide, which was inhibitable by KN-62; and, most compellingly, (c) reduction of nuclear protein kinase activity by preadsorption with anti-CaM kinase II and protein A-Sepharose beads.

The inhibitor KN-62 has no measurable activity on MLCK, PKC, or PKA type II (11, 25). Whereas KN-62 can inhibit the calcium/calmodulin activation of CaM kinase II, once the kinase is autophosphorylated and converted to a calcium-independent form (for review see reference 25), KN-62 has little effect (11). In the context of this mechanism, the rapid
anti-IgM-induced phosphorylation of syntide in both the Raji and T5-1 cells was almost totally ablated by addition of KN-62 to the extracts only at the early time points, implying that CaM kinase II was not autophosphorylated at these early time points. In contrast, anti-IgM-induced CaM kinase II activity was only partially inhibited by KN-62 after 10–15-min stimulation of the two cell lines and in normal tonsilar B cells (Valentine, M. A., unpublished results). As the induced protein kinase activity was adsorbed by anti-CaM kinase II antibodies at 10 min, this suggests that CaM kinase II became autophosphorylated (and resistant to inhibition by KN-62) later in stimulation.

A current model for CaM kinase II regulation proposes that kinase activation leads to autophosphorylation and entrapment of calmodulin (for reviews see references 26, 27). Our results provide evidence for a pathway initiated by ligation of surface IgM that increases calcium levels within the cell and rapidly activates nuclear CaM kinase II. Nuclear CaM kinase II then could phosphorylate the Ets-1 protein and negatively regulate its interaction with specific enhancer and promoter elements (5). Nuclear Ets proteins appear to exhibit cooperative effects in a variety of systems, including the induction of avian erythroleukemia through association with v-erb (28), transcriptional activation of the mim-1 promoter through association with myb (29), and stimulation of the Ets-1 promoter itself (30). At least two examples exist in which CaM kinases have been linked to phosphorylation of nuclear transcription factors in other cell types. Increased transcription was effected by the calcium-dependent phosphorylation of the cAMP response element–binding protein in transfected PC12 cells (31) and the C/EBP-β transcription factor in a transfected rat pituitary cell line (32). In each case, a member of the CaM kinase family was reported to be the active kinase. Our findings are consistent with these reports in that signals that increased [Ca^{2+}], rapidly activated CaM kinase II that was resident in the nucleus.

It will be of interest to determine the mechanisms that dictate the nuclear localization of protein kinases. The existence of nuclear proteins that function in a fashion similar to those responsible for the association of PKC to the cytoskeleton (33) or PKA to various cellular compartments (34) may be one mechanism to direct protein kinases to their appropriate nuclear substrates. Alternate splicing of the δ/β isoform of CaM kinases has been shown to result in nuclear targeting of the kinase (35). In this report we present evidence that both CaM kinase II and its potential substrate are located in the nuclear compartment. These data suggest a direct link between increases in [Ca^{2+}], that are initiated by membrane proteins and transcriptional control in the nucleus of human B lymphocytes. In view of recent reports of nuclear CaM kinases (36) and nuclear calmodulin–protein complexes (37), transcriptional regulation could involve direct control of calcium-dependent kinases resident in the nucleus.

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