# 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

[1] *Abbreviations 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.

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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 isoforms 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 L-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 aprotinin 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 F(ab')2 goat anti–human 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 (PLARTLSVAGLPGKK) (9), PKI, a peptide inhibitor of PKA (TTYADFIASGRTGRRNAIHD) (10), and the CaM kinase II inhibitor peptide α subunit residues (281–309) (MHRQETVDCLKKFNARRKLKGAILTTMLA) (11) were synthesized at the Howard Hughes Chemical Synthesis Facility (University of Washington, Seattle, WA). Purified CaM kinase II was either isolated from rat forebrain (12) or was generously supplied by Dr. Natalie Ahn. The CaM kinase inhibitor KN-62 was synthesized as previously reported (13).

*Immunoprecipitation.* For metabolic labeling studies, cells were incubated with [$^{32}$P]orthophosphate for 3–5 h at 37°C as previously described (14). Cells were washed in PBS/0.1% azide and lysed, and the lysates were precleared twice before the addition of the appropriate antibodies and protein A–Sepharose (14). Adherent complexes were eluted in Laemmli sample buffer (15), and proteins were separated on 12.5% polyacrylamide by SDS-PAGE.

*Preparation of Cell Extracts.* For kinase assays of whole-cell extracts, duplicate cell suspensions ($10^7$ cells per assay point) were incubated with medium, ionomycin (1 μM), or 50 μg/ml of anti-IgM for various times at 37°C. The cells were pelleted for 10 s (13,000 *g*), and the stimulation was stopped by addition of ice-cold PBS/0.01% azide. The cellular pellet was resuspended in 0.3 ml of lysis buffer (20 mM Hepes, pH 7.5, 10 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol [DTT], and 80 mM β-glycerophosphate). This buffer and all other buffers contained the following protease inhibitors, added just before use: 1 mM PMSF, 50 μg/ml aprotinin, 2 μg/ml peptstatin, and 1 μg/ml leupeptin. The cells were lysed by three 1-s bursts using a probe sonicator (Bo Braun Biotech Intl., Allentown, PA). Soluble and particulate fractions were separated by centrifugation for 20 min (100,000 *g*, 4°C) using a centrifuge (model TL-100; Beckman Instruments, Inc., Fullerton, CA). Supernatants were assayed the same day or stored at −70°C until assay.

Extracts from both nuclear and cytosolic compartments were prepared as described by Dignam et al. (16). Cells (1–5 × $10^7$) were washed once with 1 ml PBS and once with 1 ml lysis buffer (10 mm Hepes, 10 mM KCl, and 1.5 mM $MgCl_2$, pH 7.9, 4°C). Cells were lysed by suspending the cell pellet in lysis buffer (20 μl/$10^7$ cells) containing 0.1% NP-40 for 10 min on ice. To isolate nuclei, the lysate was vigorously mixed and microcentrifuged (10 min at 12,000 rpm, 4°C), and the nuclear pellet was washed once with 1 ml lysis buffer without NP-40. Nuclear proteins were extracted by resuspending the nuclear pellet in extraction buffer (20 μl/$10^7$ cells) (420 mM NaCl, 20 mM Hepes, 1.5 $MgCl_2$, 0.2 mM EDTA, and 25% glycerol, pH 7.9) for 10 min at 4°C. After vigorous mixing, the nuclear suspension was microcentrifuged (10 min, 4°C), and the pellet was discarded. The supernatant was microcentrifuged as before (5 min, 4°C). Supernatant fluid from the second centrifugation was diluted (30 μl/$10^7$ cells) in dilution buffer (50 mM KCl, 20 mM Hepes, 0.2 mM EDTA, and 20% glycerol, pH 7.9), and the protein concentration was measured (Micro BCA protein assay; Pierce Chemical Co., Rockford, IL). DTT (0.5 mM), PMSF (0.5 mM), and leupeptin (10 μg/ml) were added to both lysis and extraction buffers just before use. The diluting buffer contained the same amounts of DTT and leupeptin, but only 0.2 mM PMSF. All buffers contained the following phosphatase inhibitors (all from Sigma Chemical Co., St. Louis, MO): 30 mM *p*-nitrophenyl phosphate, 10 mM NaF, 0.1 mM $Na_3VO_4$, 0.1 mM $Na_2MoO_4$, and 10 mM β-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 × $10^7$ 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 $MgCl_2$, 1 mM DTT, 50 μM cold ATP, 50 μM [γ-$^{32}$P]ATP (∼1,000 cpm/pmol), 1 μM calmodulin, and 0.5 mM $CaCl_2$. The reaction was started by addition of 75 ng/ml of rat forebrain CaM kinase II (sp act = 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 $MgCl_2$, 0.02% Triton X-100, 2 mM DTT, 1 mM PKI peptide, 50 μM [γ-$^{32}$P]ATP (∼1,000 cpm/pmol), 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-

ment of Cerenkov radiation using a beta counter as previously described (17). Activity in the absence of substrate was subtracted from the mean of duplicate or triplicate samples in the presence of substrate to yield net kinase activity. Assay results were standardized to the protein concentration of the samples as determined using BCA reagent (Pierce Chemical Co.) with BSA as standard. The incubation times and the amount of protein used in all reactions were previously established to be linear for syntide phosphorylation. Typically, extracts were diluted to 1:5 or 1:10 in these assays.

*Immunoblotting.* Equal amounts of protein from cell lysates, nuclear, or cytosolic preparations were loaded onto a 10% polyacrylamide gel, subjected to electrophoresis, and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore Corp., Bedford, MA). The membrane was blocked for 30 min using BLOTTO (2% dried milk/PBS/0.005% Tween-100) and incubated overnight with the appropriate antibody. After two 10-min washes with BLOTTO, the membranes were incubated with a 1:100 dilution of $^{125}$I-labeled protein A, and the proteins were visualized by autoradiography or by use of a phosphorimager.

*Phosphopeptide Mapping.* Phosphopeptide mapping was performed as previously described (18). Briefly, the protein band of interest was excised from dried gels, washed extensively, and incubated overnight at 37°C with 150 μg of TPCK-trypsin. Peptides were dried, redissolved in electrophoresis buffer (acetic acid/formic acid/water 8:2:90, pH 1.9) and spotted on a TLC plate before electrophoresis. The plate was dried overnight and the peptides were allowed to separate by ascending chromatography for 6 h before autoradiography. Confirmation of peptide identity after various treatments was performed by removing labeled peptide from the plates, measuring the radioactivity of each sample by Cerenkov counting, and subsequent mixing of equal cpm from the samples. These mixtures were then reanalyzed by the two-dimensional analysis detailed above.

## 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 phophorylation 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 ($[Ca^{2+}]_i$) 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 elevation of $[Ca^{2+}]_i$, Raji cells were incubated 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 the phosphorimager. Phosphorylation of this peptide was increased slightly (accounting for 5–9% of total cpm) in all other samples. When

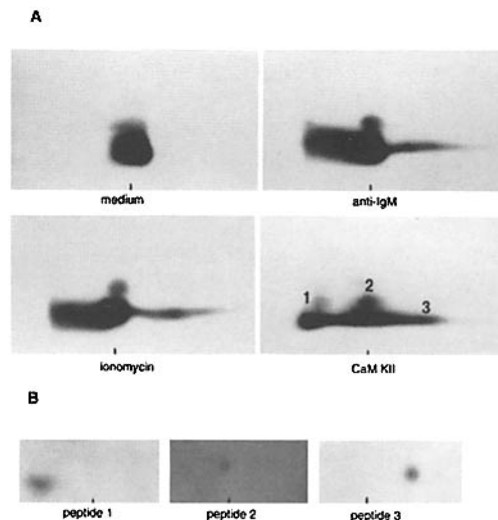


**Figure 1.** Ionomycin and anti-IgM induce phosphorylation on the Ets-1 protein. The human B cell line Raji was metabolically labeled with [$^{32}$P]orthophosphate and then incubated with medium, 1 μM ionomycin, or anti-IgM (50 μg/ml; anti-μ) for 5 min before being washed, lysed, and immunoprecipitated with anti–Ets-1. Equal amounts of proteins were analyzed by SDS-PAGE (10% gels, reducing conditions). Ets-1 proteins were excised from the gels and analyzed by two-dimensional phosphopeptide mapping as described in Materials and Methods (*A*). For comparison, purified CaM kinase II was incubated with unlabeled Ets-1 proteins bound to Sepharose beads using phosphorylating conditions; the proteins were eluted from the solid phase and analyzed as described above. In all cases, the position of the origin is marked. Increased phosphorylation of three peptides was quantified using a phosphorimager. Phosphopeptide 1 was present only in stimulated cells or after in vitro phosphorylation of Ets-1 by purified CaM kinase II and comprised 11–30% of total counts in the protein. Peptide 2 phosphorylation contributed 4–9% of total counts in Ets-1 from stimulated cells; peptide 3 had 35, 19, and 34% of total label incorporated into Ets-1 from anti-IgM–, ionomycin-, or CaM kinase II–treated samples, respectively. (*B*) Peptides 1–3 were removed from the plates of proteins from anti-IgM–, ionomycin-, or CaM kinase II–treated cells. After Cerenkov counting, equal cpm from each treatment for each peptide were combined and reanalyzed in two dimensions.

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 of each peptide from the anti-IgM, ionomycin, or CaM kinase II plates (Fig. 1 *B*). In all three instances, the mixtures migrated as a single phosphopeptide. These results demonstrate that the Ets-1 protein can serve as a substrate for CaM kinase II and that the pattern of phosphopeptides produced by in vitro phosphorylation with the purified enzyme is identical to that induced by those stimuli that increase levels of $[Ca^{2+}]_i$ in intact cells.

*Anti-IgM Induces CaM Kinase Activity in Human B Cells.* To test for anti-IgM–induced activation of a CaM kinase, we prepared cell extracts from the Raji or T5-1 cells and measured phosphorylation of the peptide substrate, syntide. Whereas both CaM kinase II and CaM kinase IV can use syn-

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 four-fold increases in tonsilar human 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

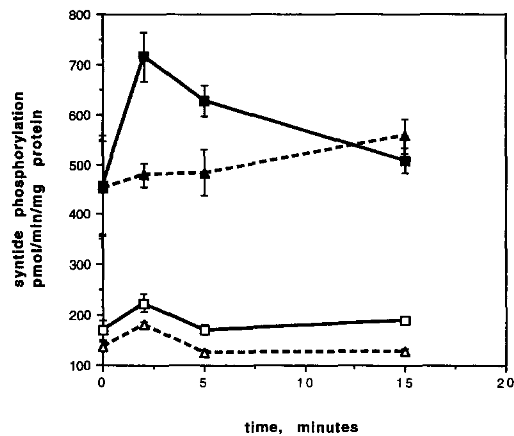


**Figure 3.** Anti-IgM induces CaM kinase activity in the nucleus. Nuclear or cytosolic lysates were prepared from Raji cells, which were incubated for the times shown in the presence of medium alone or with 50 μg/ml anti-IgM. Phosphorylation of syntide was measured in the presence of 10 μM KN-62, and 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, -▲- , nuclear + KN62, –□–, cytosol-anti-μ, -△-, cytosol + KN62.

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-

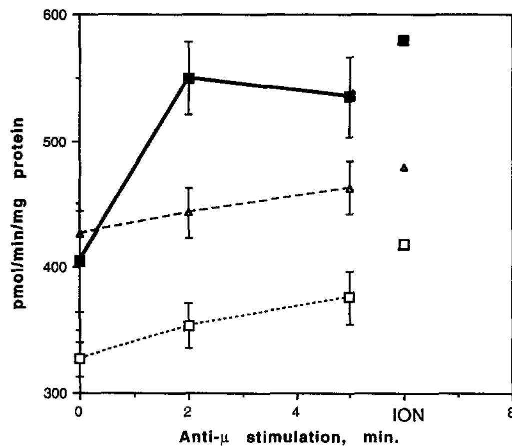


**Figure 2.** Anti-IgM–induced phosphorylation of syntide is inhibited by KN-62. T5-1 cells were incubated with medium or with anti-IgM for the times shown. Other T5-1 cells were also incubated with ionomycin (1 μM) for 5 min. Total cellular lysates were prepared and assayed for syntide phosphorylation in the presence or absence of 20 μM inhibitor peptide or 10 μM KN-62. The time and amount of extract used for T5-1 were determined to be linear for syntide phosphorylation by previous assay. Data are representative of results from four to six replicate experiments, and phosphate incorporation is given as picomoles/minute per milligram of total protein. Similar results were obtained using Raji cells (not shown). –■–, anti-μ, -△-, peptide, ···□···, KN-62.

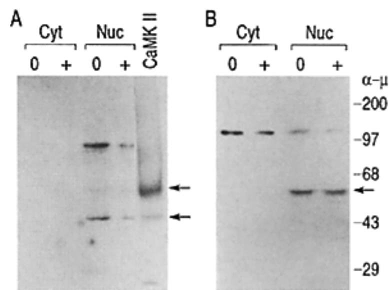


**Figure 4.** Both CaM kinase II and the Ets-1 proteins are predominantly localized in the nucleus in Raji cells. Equal amounts of protein from nuclear and cytosolic extracts used in the experiment shown in Fig. 3 were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with antibodies specific for either CaM kinase II (*A*) or the Ets-1 protein (*B*) followed by $^{125}$I-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).

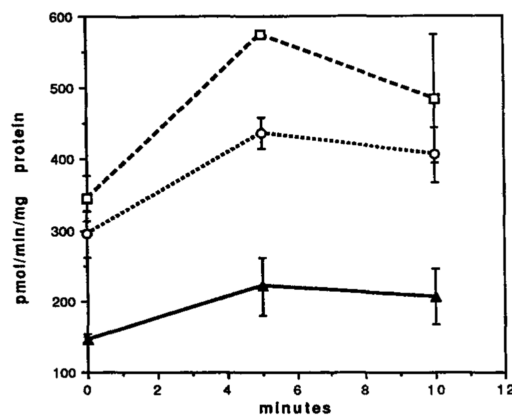


**Figure 5.** Adsorption of CaM kinase II activity from nuclear extracts. Raji cells were incubated with medium or anti-IgM for 5 or 10 min, nuclear extracts were prepared, and the extracts were aliquoted for the following treatments: (*a*) no treatment; (*b*) incubation with anti-CaM kinase II (6 μg) and protein A–Sepharose (4°C, 30 min); or (*c*) incubation with BSA (6 μg) and protein A–Sepharose. After incubation, the Sepharose beads were pelleted and the supernatants were assayed for syntide phosphorylation. Data are presented as percent cpm for 0 min (untreated = 23,941 cpm), 5 min with anti-IgM (untreated = 39,124 cpm), and 10 min with anti-IgM (untreated = 37,219 cpm) samples. Similar results were obtained in each of three replicate experiments. --□--, untreated, —▲—, CaM KII adsorbed, ···○···, BSA/Sepharose.

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 in these two cell lines was not markedly changed. These results indicate that both CaM kinase II and the Ets-1 protein, a putative substrate, are localized to the nuclear compartment in these cells.

*Preadsorption with Anti-CaM Kinase II Antibodies Reduces Anti-IgM–induced Syntide Phosphorylation in Nuclear Extracts.* To demonstrate formally that the kinase activated in the nuclear extracts of anti-IgM–treated cells is CaM kinase II, we incubated the nuclear extracts with anti-CaM kinase II antibody before assaying for syntide phosphorylation. Extracts were aliquoted to receive no treatment (untreated), the anti-CaM kinase II antibody and protein A–Sepharose (CaM kinase II adsorbed), or BSA and protein A–Sepharose (BSA/Sepharose) (Fig. 5). The Sepharose beads were removed from the samples, and extracts were then assayed for syntide phosphorylation. We found ∼5–14% reduction of syntide phosphorylation in extracts incubated with BSA/Sepharose, consistent with a dilutional effect. In contrast, adsorption with the anti-CaM kinase II antibody resulted in 46–66% decrease of anti-IgM–induced phosphorylation. Immunoadsorption also decreased a portion of the basal activity in untreated cells. These results demonstrate anti-IgM induction of nuclear CaM kinase II activity in human B lymphocytes. As KN-62 treatment of cells could inhibit CaM kinase activity only at the early time points after anti-IgM, our results are consistent with a calcium-independent form of the kinase in untreated cells and in cells exposed to anti-IgM at the later time points. In other experiments, after adsorption of CaM kinase II to the Sepharose beads, we assayed for kinase activity of the immunoprecipitated protein. Whereas we found both 48- and 60-kD proteins present by immunoblotting, we were never able to recover kinase activity in the immunoprecipitates either in the absence or presence of calcium/calmodulin. Similar to our experience with several antibodies directed against some PKC isoforms, however, the inability of the antibody to maintain enzymatic activity may be due to irreversible denaturation induced during immunoprecipitation.

## 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-$\beta$ 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 $[Ca^{2+}]_i$ 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 $\delta/\beta$ 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 $[Ca^{2+}]_i$ that are initiated by membrane proteins and transcriptional control in the nucleus of human B lymphocytes. In view of recent reports of nuclear calcium transport (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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