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p56LCK PHOSPHORYLATION BY Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE TYPE II

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SUMMARY: Ca^{2+}/calmodulin-dependent protein kinases are implicated in regulating the Ca^{2+} signaling involved in T cell activation and in thymocyte selection. One of the earliest events in signaling through the T cell antigen receptor is activation of the protein tyrosine kinase p56lck. Following T cell activation or signaling through the IL-2 receptor, Ca^{2+}-mediated phosphorylation of p56lck occurs on serine/threonine residues. Isoforms of the multifunctional Ca^{2+}/calmodulin-dependent protein kinases, CaM kinase-II and CaM kinase-Gr are found in human T lymphocytes. CaM kinase-II, but not CaM kinase-Gr, phosphorylates the T cell tyrosine kinase p56lck *in vitro*. Tryptic phosphopeptide maps indicate that CaM kinase-II phosphorylates p56lck on multiple sites *in vitro*. Kinase assays of p56lck modified by CaM kinase-II indicate that CaM kinase-II modification does not appreciably affect p56lck phosphotransfer activity.

Ligand binding to the T cell antigen receptor/CD3 complex induces a cascade of events including activation of the tyrosine kinases p56lck and p59fyn, phosphorylation of phospholipase C-γ1 and hydrolysis of PIP_2. The release of IP_3-sensitive internal Ca^{2+} stores produces a transient increase in Ca^{2+} levels in the lymphocytes and translocation of protein kinase C (PKC). Subsequent opening of Ca^{2+} channels causes a sustained rise in Ca^{2+} levels that are required for T cell commitment to activation leading to cellular proliferation and cytokine production (1-4). p56lck, a member of the src family of tyrosine kinases, is expressed exclusively in lymphoid tissues, predominantly in thymocytes and peripheral T cells. It is physically associated through amino-terminal cysteine residues with the cytoplasmic domains of the CD4 and CD8 T cell surface glycoproteins and interacts with the β chain of the IL-2 receptor. p56lck is essential for normal signal transduction through the

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TCR during activation of mature T cells (reviewed in 5-8). Transgenic mice deficient in p56lck show a profound block in thymocyte development (9). p56lck is also implicated in growth transformation of B lymphocytes by Epstein Barr Virus (EBV) (10).

Upon signaling through the T cell receptor or following IL-2 stimulation of T lymphocytes, p56lck undergoes rapid phosphorylation that results in altered mobility on polyacrylamide gels. This conversion of p56lck to the p60 form is associated with serine, and possibly threonine, phosphorylation in the amino-terminal region (11-16). At least two serine protein kinase pathways are implicated in the modification of p56lck: p56lck phosphorylation can be induced by activators of PKC or through a PKC-independent pathway by treatment of T cells with calcium ionophores (13,16).

Ca^{2+} acts on multiple cellular targets. Because calmodulin is the primary receptor for Ca^{2+}, the participation of Ca^{2+}/calmodulin-dependent kinases in lymphocyte Ca^{2+} signaling has been proposed. The Ca^{2+}/calmodulin-dependent protein kinase type Gr, CaM kinase-Gr (also called CaM kinase-IV) is enriched in neurons and T lymphocytes, particularly immature thymocytes, and is present in EBV transformed B cells (17-19). In addition, isoforms of the abundant brain multifunctional Ca^{2+}/calmodulin-dependent protein kinase, CaM kinase-II have been identified in various tissues including spleen (20) and human lymphocytes (21). These kinases are potential targets for regulating the Ca^{2+}-signaling involved in T lymphocyte activation and thymic selection cascades.

MATERIALS AND METHODS

Enzyme purification. CaM kinase II was purified from forebrains of adult male Sprague-Dawley rats by ammonium sulfate fractionation, calmodulin agarose and gel permeation chromatography (19). Recombinant p56lck (GST-p56lck) was cloned and expressed as a glutathione S-transferase (GST) fusion protein in *E. coli* (22). Mutant p56lck contains a conservative lysine to arginine substitution at position 273, disrupting the nucleotide binding site and inactivating the enzyme (22). CaM kinase-Gr purified from human thymus was the kind gift of Dr. Talal Chatila (Children's Hospital, Boston).
CaM kinase assays. CaM kinase-II and CaM kinase-Gr assays were carried out at 30°C for 20 min. in 30 ml of 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 μM okadaic acid (Boehringer Mannheim), 1 μM DTT, 50 μM ATP with 5 μCi of [γ-^{32}P] ATP, ~0.4 μg of GST-LCK fusion protein or ~0.5 μg of GST *E. coli*-expressed protein was included as a substrate. Assays were performed in the presence of either 1 mM $CaCl_2$ and 600 nM calmodulin or 1 mM EGTA. Phosphorylated proteins were resolved by SDS-PAGE (23) on 8% acrylamide gels and visualized by autoradiography.
p56lck kinase assays. CaM kinase-II was autophosphorylated for 1 min. in the presence of Ca^{2+} and calmodulin under the conditions described above. ~50 ng recombinant active GST-p56lck was added to the appropriate reactions in a total volume of 20 μl and allowed to incubate for 10 min. at 30°C. The reactions were diluted into buffer containing 5 mM $MnCl_2$, additional Ca^{2+} and calmodulin and 0.5 mg/ml RR-SRC peptide substrate (Gibco-BRL) and incubated for 10 min. at 30°C. The reactions were stopped by adding SDS loading buffer and proteins were separated by electrophoresis on an 18% SDS-PAG followed

by imaging and quantitation on a Molecular Dynamics phosphorimager.

Phosphopeptide maps. CaM kinase-II phosphorylated mutant p56lck was resolved by SDS-PAGE, visualized by autoradiography and excised from the gel. Two-dimensional phosphopeptide maps of were obtained following digestion of dehydrated gel pieces with 100 μg/ml trypsin for 48 hrs. at 37°C and analysis of the released peptides by electrophoresis-chromatography (24).

Immunoblots. 1x 10^6 cells or 500 μg tissue was solubilized in 2X SDS gel loading buffer. Proteins were resolved by SDS-PAGE using 10% acrylamide and electroblotted onto nitrocellulose using standard procedures. The conformation-dependent CaM kinase-II mAb produced in our laboratory was incubated at 1:20 dilution of culture supernatant in the presence of 100 μM $CaCl_2$ and 5 μg/ml calmodulin (25). The blots were washed and probed with alkaline phosphatase conjugated goat anti-mouse IgG. Immunoreactive peptides were detected by the addition of appropriate substrates (Biorad). The SK-N-SH human neuroblastoma cell line was obtained from the American Type Culture Collection. Human Jurkat JE6-1 and rat G_1-TC T cell lines were provided by Talal Chatila, M.D. (Children's Hospital, Boston). Forebrains were obtained from adult Wistar rats.

RESULTS

Immunoblots using CaM kinase-II specific monoclonal antibodies indicate the presence of CaM kinase-II isoforms in rat and human T cell lines (Fig. 1). The rat T cell line G_1-TC contains only one immunoreactive peptide of $\sim M_r$ 55,000 which co-migrates with a minor band slightly larger than the α isoform from rat forebrain. The rat antisera cross-reacts with at least two isoforms in the human neuroblastoma cell line SK-N-SH; a polypeptide of M_r 54,000, similar in size to the rat neuronal α isoform, and a minor band of $\sim M_r$ 59,000, migrating close to the rat β isoform. In human Jurkat T cells, only a faint cross-reactive polypeptide of ~ 59 kDa is observed.

At least two Ca^{2+}/calmodulin-dependent protein kinases are found in T lymphocytes, CaM kinase-II and CaM kinase-Gr. *In vitro* phosphorylation assays indicate that the inactive mutant GST-p56lck fusion protein, but not GST alone, is phosphorylated by CaM kinase-II in a Ca^{2+}/calmodulin-dependent manner (Fig. 2). The altered mobility of the phosphorylated

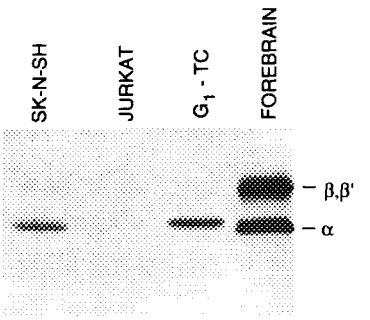

Fig. 1. Immunoblot analysis with the conformation-dependent rat CaM kinase-II mAb. Left to right: human neuroblastoma cell line SK-N-SH; human Jurkat T cell leukemia line, subclone JE6-1; rat G_1-TC T cell line; rat forebrain.

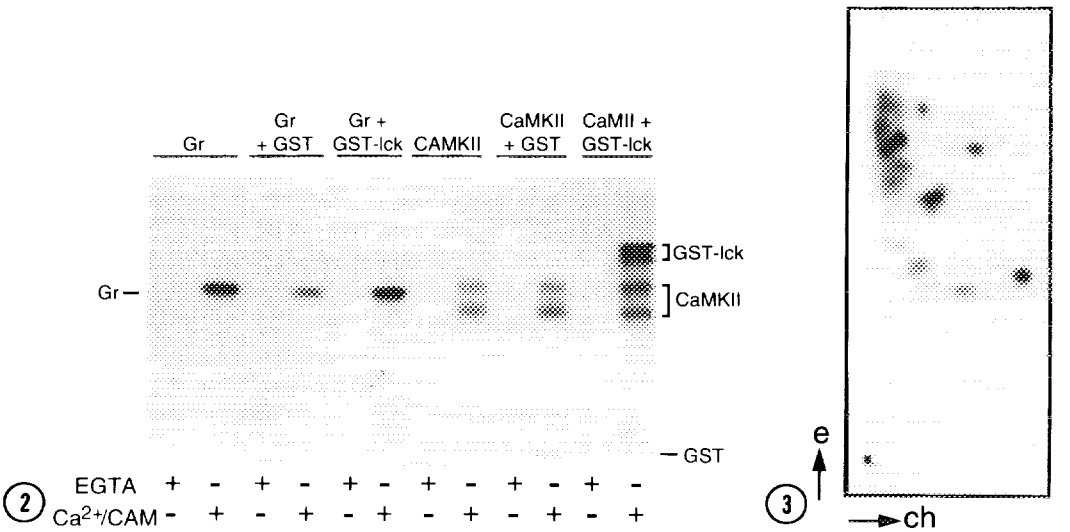

Fig. 2. In vitro Ca^{2+}/calmodulin-dependent phosphorylation of GST or inactive mutant GST-p56lck by CaM kinase-Gr or CaM kinase-II in the presence (+) or absence (-) of either EGTA or $CaCl_2$ and calmodulin (Ca^{2+}/CaM) as described in Materials and Methods.

Fig. 3. Phosphopeptide map of ^{32}P-labeled GST-p56LCK. *In vitro* CaM kinase-II phosphorylated GST-p56lck was excised from a dried SDS-gel, digested with trypsin and analyzed by electrophoresis (e)/chromatography (ch).

GST-p56lck, appearing as an upward smear on SDS gels, is consistent with phosphorylation at multiple sites.

In contrast, GST-p56lck is not an *in vitro* substrate for CaM kinase-Gr (Fig 2). CaM kinase-Gr is a multifunctional Ca^{2+}/calmodulin-dependent protein kinase enriched in neurons and thymocytes (17-19). The consensus phosphorylation site for CaM kinase-Gr, R-X-X-S, is similar to that established for CaM kinase-II (19, 26). This consensus site is found in p56lck at amino acids 39-42, suggesting that p56lck is a potential substrate for CaM kinase-Gr.

In order to characterize the sites of CaM kinase-II phosphorylation of p56lck, we performed two dimensional phosphopeptide mapping. Tryptic digestion of *in vitro* phosphorylated GST-p56lck gives rise to seven major spots and several minor spots (Fig. 3). Because the inactive mutant form of p56lck was used for these experiments, the peptides detected contain only phosphoserine or phosphothreonine. Analysis of the p56lck amino acid sequence reveals two peptides containing the CaM kinase-II consensus phosphorylation site of R-X-X-S/T. These sites are located at residues 39-42 and 207-210 in p56lck. CaM kinase-II can also use Lys in place of Arg on the N-terminal side of the phosphorylated residue (27). p56lck contains five peptides with the sequence K-X-X-S. Use of these sites by CaM kinase-II would account for the multiple phosphorylated peptides observed on the two dimensional maps. All of these sites occur within the amino-terminal two-thirds of the

p56lck polypeptide; five sites lie within the first 210 amino acids. This is consistent with findings that activation through the T cell receptor induces serine phosphorylation of p56lck in an amino-terminal 32 kDa fragment containing amino-acids 14-261 (14, 16).

To determine whether CaM kinase-II phosphorylation has an effect on p56lck tyrosine kinase activity, active recombinant GST-p56lck was added as a substrate to pre-phosphorylated CaM kinase-II under Ca^{2+}/calmodulin kinase phosphorylation conditions. After maximal phosphorylation, p56lck was diluted into tyrosine kinase assay buffer containing an exogenous peptide substrate specific for tyrosine kinases. Activity of the modified p56lck, as measured by incorporation of ^{32}P into the peptide substrate, was not appreciably altered compared with controls (data not shown).

DISCUSSION

The multifunctional CaM kinase-II has a broad tissue distribution and phosphorylates a variety of substrates including proteins involved in neurotransmitter release, lipid biosynthesis, carbohydrate metabolism, protein synthesis and cytoskeletal organization (reviewed in 27-31). The CaM kinase-II protein family shares complex biochemical and structural characteristics. Five isoforms, α, β, β', γ and δ, with molecular masses of 54-60 kDa have been identified from rat brain. Mammalian isoforms identified thus far differ mainly in the length and placement of inserts at the end of the calmodulin binding region.

CaM kinase-II from human lymphocytes phosphorylates a variety of substrates in response to stimulation with lectins or ionomycin and is induced to undergo autophosphorylation and subsequent characteristic Ca^{2+}-independent kinase activity upon stimulation through the TCR (4, unpublished data). CaM kinase-II isoforms from Jurkat T cells were recently identified by cDNA cloning. The human lymphocyte CaM kinase-II isoforms are unique, but show 95% sequence identity with the rat brain γ isoform, differing only in the variable inserts (21). The ~59 kDa protein detected in Jurkat cells by immunoblot in Fig. 1 may represent this isoform; however the immunoreactive polypeptide in the rat G_1-TC T cell line, migrating slightly above the brain a isoform, is probably a different form.

CaM kinase-II readily phosphorylates p56lck *in vitro*, suggesting a link between a Ca^{2+}/calmodulin signaling pathway and a tyrosine kinase known to be involved in T cell activation and thymocyte selection. The phosphorylation reaction is relatively selective: p56lck is not a substrate for the Ca^{2+}/calmodulin-dependent protein kinase type Gr from human thymus, although p56lck has a potential CaM kinase-Gr phosphorylation site. CaM kinase-II phosphorylates p56lck on multiple sites, causing retarded mobility in on SDS gels; however CaM kinase-II modification of p56lck does not produce the discrete p60 form

observed upon activation through the TCR or IL-2 receptor or treatment of the T cells with phorbol esters. Tryptic phosphopeptide analysis of p56lck isolated from Ca^{2+} ionophore-treated cells demonstrates Ca^{2+}-induced phosphorylation on serine of three amino-terminal peptides, although phosphorylation does not result in the shift to the p60 form of lck (16).

Electron microscopy and biochemical fractionation of CaM kinase-II in neurons indicates that the kinase is associated with plasma membrane, cytoskeleton, post synaptic densities and synaptic vesicles, as well as in the cytosol (27, 29). CaM kinase-II was recently shown to mediate the activation of a unique Cl^- channel in lymphocytes (32). Demonstration of CaM kinase-II association with the plasma membrane provides support for co-localization of CaM kinase-II with p56lck in the cell.

The function and significance of the serine/threonine phosphorylation of p56lck are unknown. Although readily phosphorylated on multiple sites, the activity of CaM kinase-II-modified p56lck on peptide substrates was unaffected. Using immune complex p56lck kinase assays of T cells treated with activators of PKC, Ca^{2+} ionophores, or TCR activation Veillette et al. (16) and Danielian et al. (12) were unable to detect any alteration in the ability of the various lck species to phosphorylate exogenous substrates. Because phosphorylation likely occurs in the N-terminal region of p56lck near the portion of the molecule involved in protein interactions (12,14), Ca^{2+}-dependent phosphorylation may be involved in regulating association with surface receptors or may alter substrate specificity of the protein tyrosine kinase. p56lck association with the CD4 or CD8 co-receptors is required for early T cell signaling events. Upon T cell activation CD4, CD8 and p56lck all undergo phosphorylation on serine (3,5-6). Activators of PKC induce dissociation of p56lck from CD4 and internalization of the co-receptor; however, CD8 dissociation from p56lck and internalization is not mediated by PKC (33), suggesting alternative regulation of the class I-restricted T cells, that may involve CaM kinase-II.

PKC-independent Ca^{2+}-mediated phosphorylation of p56lck will likely prove to be important in the regulation of the tyrosine kinase in T cell receptor and IL-2 receptor signaling. The *in vitro* phosphorylation data presented here, taken together with the co-localization of CaM kinase-II with p56lck in T lymphocytes, suggests CaM kinase-II involvement in the critical early events of lymphocyte activation and thymocyte maturation.

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REFERENCES

1. Gardner, P. (1989) Cell 59,15-20.
2. Mueller, D.L., Jenkins, M.K. and Schwartz, R.H. (1989) Ann. Rev. Immunol. 7, 445-480.
3. Perlmutter, R.M. (1993) Pediatr Res. 33 (suppl), S9-S15.
4. Premack, B.A. and Gardner, P. (1992) Am. J. Physiol. 263, C1119-C1140.
5. Bolen, J.B., Thompson, P.A., Eiseman, E. and Horak, I.D. (1991) Adv. Cancer Res. 57, 103-149.
6. Bolen, J.B., Rowley R.B., Spana, C. and Tsygankov, A.Y. (1992) FASEB J. 6, 3403-3409.
7. Samelson,L. and Klausner, R.D. (1992) J. Biol. Chem. 267, 24913-24916.
8. Veillette, A. and Davidson, D. (1992) Trends Genet. 8, 61-66.
9. Molina, T.J., Kishihara, K., Siderovski, D.P., van Ewijk, W., Narendran, A., Timms, E., Wakeman, A., Paige, C.J., Hartmann, K.-U., Veillette, A., Davidson,D. and Mak, T.W. (1992) Nature 357, 161-164.
10. Cheung, R.K. and Dosch, H.-M. (1991) J. Biol. Chem. 266, 8667-8670.
11. Carrera, A.C., Li, P. and Roberts, T.M. (1991) Internat. Immunol. 7, 673-682.
12. Danielian, S., Fagard, R., Alcover, A., Acuto, O. and Fischer, S. (1989) J. Immunol. 19, 2183-2189.
13. Horak, I.D., Gress, R.E., Lucas, P.J., Horak, E.M., Waldmann, T.A. and Bolen, J.B. (1991) Proc. Natl. Acad. Sci USA 88, 1996-2000.
14. Luo, K and Sefton B.M. (1990) Oncogene 5, 803-08.
15. Marth, J.D., Lewis, D.B., Cooke, M.P., Mellins, E.D., Gearn, M.E., Samelson, L.E., Wilson, C.B., Miller, A.D. and Perlmutter, R.M. (1989) J. Immunol. 142, 2430-2437.
16. Veillette, A., Horak, I.D., Horak, E.M., Bookman, M.A. and Bolen, J.B. (1988) Mol. Cell. Biol. 8, 4353-4361.
17. Frangakis, M.V., Chatila, T., Wood, E.R. and Sahyoun, N. (1991) J. Biol. Chem. 266, 17592-17596.
18. Hanissian, S., Frangakis, M.V., Bland, M.M., Jawahar, S. and Chatila, T.A. J. Biol Chem. (in press).
19. Ohmstede, C.-A., Jensen, K.F. and Sahyoun, N.E. (1989) J. Biol. Chem. 264, 5866-5875.
20. Fukunaga K., Goto, S. and Miyamoto, E. (1988) J. Neurochem. 51, 1070-1078.
21. Nghiem, P., Saati, S.M., Martens, C.L., Gardner, P. and Schulman, H. (1993) J. Biol. Chem. 268, 5471-5479.
22. Carrera, A.C., Alexandrov, K. and Roberts, T.M. (1993) Proc. Natl. Acad. Sci. USA 90, 442-446.
23. Laemmli, U.K. (1970) Nature 227, 680-685.
24. Jacobs, S. and Cuatrecasas, P. (1986) J. Biol. Chem. 261, 934-939.
25. LeVine, H., III, Su, J.-L. and Sahyoun, N.E. (1988) Biochem. 27, 6612-6617.
26. Frangakis, M.V., Ohmstede, C.-A and Sahyoun, N. (1991) J. Biol. Chem. 266, 11309-11316.
27. Hanson, P.I. and Schulman, H. (1992) Ann. Rev. Biochem. 61,559-601.
28. Kelly, P.T. (1991) Mol. Neurobiol. 5, 153-177.
29. Rostas, J.A.P. and Dunkley, P.R. (1992) J. Neurochem. 59, 1191-1202.
30. Schulman, H., Hanson, P.I. and Meyer, T. (1992) Cell Calcium 13, 401-411.
31. Schulman, H. and Lou, L.l. (1989) Trends Biochem. Sci. 14, 62-66.
32. Nishimoto, I., Wagner, J.A., Schulman, H. and Gardner, P. (1991) Neuron 6, 547-555.
33. Hurley, T.R., Luo, K. and Sefton, B.M. (1989) Science 245, 407-409.