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MURINE T-CELL DIFFERENTIATION ANTIGEN CD8 IS A DIRECT SUBSTRATE OF PROTEIN KINASE C

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SUMMARY: Murine T cell differentiation antigen CD8α (Lyt-2) is phosphorylated in vivo after phorbol 12-myristate 13-acetate (PMA) treatment of cells. Concanavalin A, dibutyryl cAMP and calcium ionophore are unable to stimulate phosphate incorporation into CD8α. Depletion of cellular protein kinase C (PKC) by prolonged PMA treatment abolished this phosphorylation, suggesting that PKC is required for this effect. Using the amino acid sequence derived from cloning CD8α, peptides encompassing both possible intracellular phosphorylation sites were made and used to test the ability of various kinases to phosphorylate CD8α sequences. Only the proximal serine peptide was a kinase substrate, and of PKC, cAMP-dependent kinase and the multifunctional calcium/calmodulin-dependent kinase, only PKC was able to phosphorylate this peptide. These studies provide the first definitive evidence that CD8α is a direct substrate of PKC.

In mature peripheral T cells CD8α (Lyt-2), in association with CD8β (Lyt-3), is expressed on cytotoxic and suppressor T cell subpopulations. CD8 is believed to interact with class I major histocompatibility complex antigen (MHC) during cell-cell interactions (1,2,3). This molecule appears to have an auxilliary role in the antigen recognition process in T cells, increasing the affinity of binding to the nonpolymorphic region of MHC molecules.

Recently, we and others have cloned cDNAs which encode the CD8α molecule (4, 5). CD8α was found to have two forms, a 38 kDa form and a 34 kDa form. These two proteins arise by developmentally regulated alternative splicing of mRNA from a single gene (5, 6) and are identical in their extracellular domains. Only the 38 kDa form has a significant intracellular domain. Immature T cells contain equal amounts of 38 kDa and 34 kDa mRNA and express both proteins on the cell surface. Mature T cells express only the larger form on the cell surface even though they have 34 kDa mRNA (6).

Protein kinase C, which is activated by calcium, phospholipid and diacylglycerol may play a central role akin to that of cAMP-dependent and the calcium/calmodulin-dependent kinases (7,

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ABBREVIATIONS

kDa, kilodaltons; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; EDTA, ethylenediamine tetraacetic acid; DMSO, dimethyl sulfoxide; MHC, major histocompatibility complex.

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8). PI turnover has been observed in association with lymphocyte activation by antigen and anti-CD3 antibody (9, 10). CD8 has been shown to be a phosphoprotein, although the kinase(s) responsible have not been specifically identified (11,12). This increase in interest in phosphorylation and its role in regulation of the immune response has prompted us to examine the phosphorylation of the T cell antigen CD8. Phosphorylation of many receptor molecules including the insulin receptor, the EGF receptor and the β-adrenergic receptor is thought to have regulatory importance (for a review, see 13). In this study we demonstrate that CD8 is phosphorylated in vivo and that this phosphorylation is directly mediated by protein kinase C.

MATERIALS AND METHODS

BALB/c thymocytes or L cells to be labeled were incubated in phosphate-free Dulbecco's Modified Eagle Medium (Gibco Laboratories, NY) for 2 h and then labeled with 0.5 mCi/ml [^{32}P]-orthophosphate (ICN Radiochemicals, Irvine, CA) for a further 2 h. Labeled cells were stimulated with various agents for 10 min then harvested for immunoprecipitation.

Labeled cells for immunoprecipitation were solubilized in phosphate buffered saline containing 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 25 mM sodium fluoride, 25 mM sodium pyrophosphate, and 10 μM zinc chloride. Cell lysates were preabsorbed with Staphylococcus aureus Cowan I strain and immunoprecipitated with monoclonal anti-Lyt-2 antibody 53-6.7 (14). Immune complexes were precipitated with protein A-sepharose bound monoclonal mouse anti-rat kappa chain antibody MAR 18.5 (15). Samples were analysed by polyacrylamide gel electrophoresis under reducing conditions (16).

To down regulate PKC, L cells were cultured with 1 μM PMA for 10 h. Protein kinase C activity against histone substrate in whole cell extracts was found to be undetectable (results not shown). Cells were labeled and treated as described above.

In vitro phosphorylation of CD8 was done with membranes prepared from L cell cDNA transfectants by homogenization in 5 mM HEPES pH 7.4, 1 mM magnesium chloride, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged to remove nuclei after addition of sucrose to 0.25 M. Supernatants were centrifuged at 100,000 x g for 1 h. The pellet was resuspended in 20 mM HEPES pH 7.4 with 0.2% NP-40 and used as a substrate in phosphorylation reactions.

Phosphorylation reactions using purified PKC (generously provided by Cynthia Csernansky) were carried out in 20 mM Tris pH 7.5, 5 mM magnesium acetate, 0.04 mg/ml phosphatidyl serine, 0.1 ug/ml diolein, 1 mM calcium chloride, 20μM[γ-^{32}P]ATP (1-10 Ci/mmole) and 0.2% NP-40. Reactions were done at 30°C for 10 min and stopped with 20 mM EDTA.

Peptide phosphorylation was carried out as above. After addition of EDTA, an equal volume of 20% trichloroacetic acid was added and the tubes centrifuged. Supernatants were spotted onto phosphocellulose paper which was washed in running water for 20 min. Papers were dried and Cerenkov radiation measured. Peptides were generously synthesized by Dr. John J. Nestor Jr., Institute of Bio-organic Chemistry, Syntex.

To determine phosphorylation stoichiometry, CD8 immunoprecipitates from control and PMA treated cells which had been labeled overnight with [^{35}S]-methionine were analysed by two dimensional gels as described (17). Isoelectric forms of CD8α were individually cut from the gel and [^{35}S] content determined in a Beckman LS 3801 scintillation counter. Phosphorylation stoichiometry was determined by measuring the net shift into more negative isoelectric forms of CD8α protein with PMA treatment.

RESULTS AND DISCUSSION

To determine if CD8 is phosphorylated in vivo, BALB/c thymocytes were labeled with [^{32}P]-orthophosphate and stimulated with various agents. Figure 1 shows immunoprecipitated

CD8 from control cells and cell treated with concanavalin A, PMA, calcium ionophore A23187 and dibutyryl cAMP. Lane 1 shows CD8α 38 kDa form precipitated from [^{32}P]-labeled L cell transfectants. Lanes 2-11 are immunoprecipitated from [^{32}P]-labeled cells treated with the indicated agents. CD8α is not phosphorylated under basal conditions. The 38 kDa form becomes highly phosphorylated when cells are stimulated with PMA, but not when concanavalin A or agents which activate calcium-dependent kinases or the cAMP-dependent kinase are applied. CD8β and the 34 kDa form of CD8α are not phosphorylated in vivo under any of the tested conditions.

While the expression of CD8 is limited to lymphoid cells, the mechanism which is responsible for phosphorylation of this molecule is not exclusive to this type of cell. L cells which have been transfected with CD8α 38 kDa or 34 kDa sequences express these proteins on their cell surfaces at high levels (6). These L cell transfectants were labeled to determine whether CD8 could be phosphorylated in a non-lymphocyte system. Figure 2 demonstrates that even in this non-immune cell line the 38 kDa form of CD8α can be phosphorylated in response to activators of PKC. L cells transfected with the 34 kDa form of CD8α show no phosphorylation either in the control or the PMA treated conditions.

To further define the role of PKC in this phosphorylation, cells were pretreated with PMA to decrease the amount of cellular PKC activity. Long-term phorbol ester treatment has been shown in several cell types (16, 18) to decrease the amount of activatable PKC without affecting the activity of other protein kinases. In L cell transfectants pretreated in this manner PMA no longer stimulates the phosphorylation of CD8 (Fig. 2). This data strongly suggests that PKC is required for the in vivo phosphorylation of CD8.

To assess the significance of the in vivo phosphorylation of CD8, we wished to determine the percentage of CD8 molecules phosphorylated in vivo in response to PMA treatment. To be an important regulatory mechanism, phosphorylation usually must occur on a large fraction of the substrate molecules. To measure this we labeled cells with [^{35}S]-methionine and treated with PMA or with control medium. CD8 was immunoprecipitated and analysed in a two-dimensional gel system. Charge differences caused by phosphorylation or glycosylation allow separation of multiple isoelectric forms. CD8 from control cells shows one major and several minor isoelectric forms. Since this CD8 has no phosphate, these isoelectric varients are probably due to differences in glycosylation. Upon PMA stimulation, there is a pronounced shift of the CD8 protein into more acidic forms, indicating that PMA treatment increases the negative charge of the CD8 molecule. To quantitate this shift, each isoelectric form was cut out of the gel and the ^{35}S measured by liquid scintillation counting. The net charges of the control and PMA-treated CD8 were calculated. The difference is equal to the percentage of molecules in the population which were phosphorylated in response to PMA. By this method, 56% of the CD8 molecules in the cell were phosphorylated by a 10 min PMA treatment.

The observation that only the 38 kDa form of CD8α is phosphorylated in vivo is consistant with sequence data from CD8α cDNA clones (Table 1). The 38 kDa and 34 kDa forms share a common N-terminal sequences of 193 amino acid residues. The sequences of the two proteins diverge at the point which according to hydrophobicity analysis (4) represents the end of the transmembrane domain and the beginning of the cytoplasmic portion of the CD8 molecule. The 34

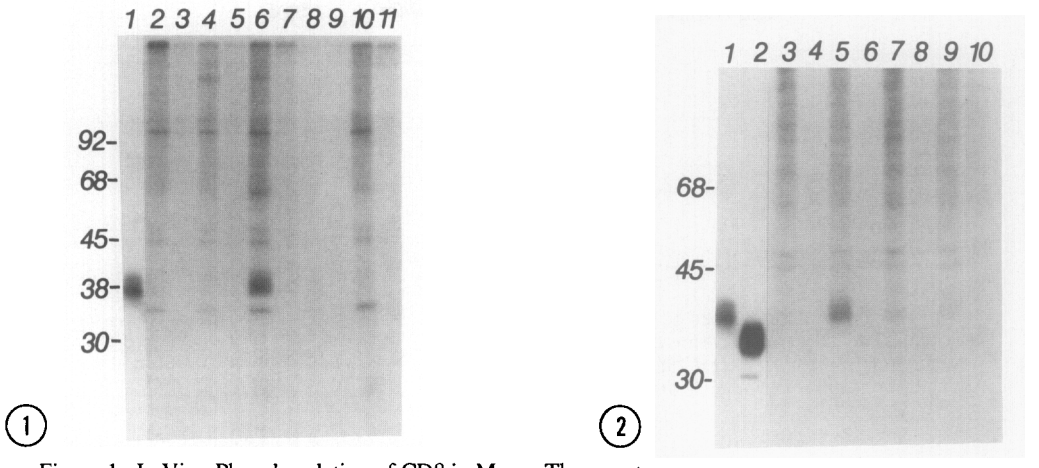

Figure 1. In Vivo Phosphorylation of CD8 in Mouse Thymocytes

BALB/C thymocytes were labeled with [^{32}P]-orthophosphate as described in Materials and Methods. Cells were stimulated with 10 μg/ml concanavilin A (lanes 4 and 5), 1 μM PMA (lanes 6 and 7), 10 μM calcium ionophore A23187 (lanes 8 and 9) or 2 mM dibutyryl cAMP (lanes 10 and 11). Lanes 2 and 3 represent unstimulated thymocytes. Lane 1 shows the 38 kDa form of CD8α immunoprecipitated from L cell 38 CDα transfectants as a marker. After 10 min., cells were lysed and immunoprecipitated as described with anti-Lyt-2 antibody (lanes 1,2,4,6,8 and 10) or with anti-Thy-1 antibody as a control (lanes 3,5,7,9 and 11). Immunoprecipitates were run on 10% acrylamide gels and proteins visualized by autoradiography.

Figure 2. Phosphorylation of CD8α in PKC-deficient L Cell Transfectants

L cells transfected with either the 38 kDa form (lanes 1,3,5,7 and 9) or the 34 kDa form (lanes 2,4,6,8 and 10) of CD8α (6) were pretreated for 10 h with either 1 μM PMA (lanes 7-10) or with its solvent, DMSO, as a control (lanes 3-6). Cells were then labeled as described with either [^{35}S]-methionine for markers (lanes 1 and 2) or with [^{32}P]-orthophosphate (lanes 3-10). Labeled cells were stimulated with 10 μM PMA (lanes 5,6,9 and 10) for 10 min or treated with DMSO as a control (lanes 3,4,7 and 8). Cells were lysed and CD8 immunoprecipitated as described. Proteins were separated on 10% acrylamide gels and visualized by autoradiography.

kDa cytoplasmic region is composed of only two amino acid residues, a serine and an arginine. The 38 kDa form has a cytoplasmic region comprised of 27 amino acids with two serines both of which are distal to basic amino acids, making them potential phosphorylation sites. Neither site offers a classic Arg-Arg-X-Ser site defined for the cAMP-dependent kinase. Both sites have basic residues distal to the serine. This arrangement has been shown with peptide substrates to be non-optimal for cAMP-dependent kinase activity while not adversely affecting the ability of PKC to phosphorylate such a serine (7, 19).

In order to investigate the exact nature of CD8 phosphorylation, the ability of PKC to phosphorylate CD8 in vitro was studied. Figure 3 demonstrates that exogenously added rat brain PKC can phosphorylate the 38 kDa form of CD8α in membranes purified from L cell transfectants. Membranes from control L cells show no similar substrate. Radioactive ATP alone did not cause phosphate incorporation into CD8, indicating that the membrane preparations were not responsible for the observed phosphorylation of CD8 (data not shown).

This experiment, while demonstrating that CD8 is a substrate for PKC, does not address the question of which site or sites on CD8 are phosphorylated. To answer this question, peptides containing ser-195 and ser-216 were synthesized (Table 1). Both peptides contain the adjacent basic residues which could potentially affect the ability of the peptide to be phosphorylated.

Table 1. Amino Acid Sequences of CD8α 34 kDa and 38 kDa Forms and Synthetic Peptides

CD8α 34 kDa	--CYHSR
CD8α 38 kDa	--CYHRSRKRVCKCPRPLVRQEGKPRPSEKIV
Peptide ser-195	AYHRSRKRVA
Peptide ser-216	GKPRPSEKIV

C-terminal sequences of the two forms of CD8α deduced from cDNA clones (5, 6) are shown. The two forms differ only in their cytoplasmic domains. The remaining, identical, transmembrane and extracellular portions are not shown. Synthetic peptides continuing the two potential phosphorylation sites are shown.

Phosphorylation of both peptides was assayed with PKC, cAMP-dependent protein kinase and calcium/calmodulin-dependent kinase. The ser-195 peptide proved to be a very good substrate for PKC, while the ser-216 peptide was not phosphorylated at all. Neither peptide was phosphorylated to a detectable level with cAMP or the calcium/calmodulin kinase. Kinetic parameters for PKC phosphorylation of ser-195 peptide were calculated using a non-linear least squares algorithm. The K_m for PKC was 38 μM and V_{max} was calculated to be 0.66 μmoles/min/mg. Thus ser-195 of CD8α appears to be an excellent substrate for protein kinase C both in vivo and in vitro.

Phosphorylation of cell surface molecules has been postulated to participate in many phases of transmembrane signalling. In these studies, we demonstrate that the T cell antigen CD8, a molecule which is involved in MHC class restriction in mature T cells, is a substrate of PKC.

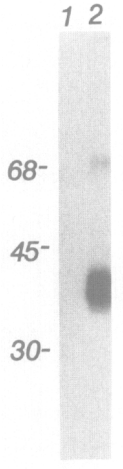

Figure 3. In Vitro Phosphorylation of CD8 by PKC

Membranes were prepared as described in Materials and Methods from L cells (lane 1) and from L cells which had been transfected with the CD8α 38 kDa cDNA (lane 2). Membrane proteins were phosphorylated as described using PKC purified from rat brain. Reactions were stopped with 20 mM EDTA and CD8 immunoprecipitated. Proteins were separated on 10% acrylamide gels and visualized by autoradiography.

Phosphorylation of CD8 has been previously observed in human T cell clones in response to phorbol esters (11, 12). The kinase(s) mediating these effects and the sites of phosphorylation were not rigorously identified.

In these studies we demonstrate that CD8 phosphorylation occurs in vivo in response to activators of PKC but not in response to activators of the cAMP-dependent protein kinase or calcium-dependent kinases or in response to the mitogen concanavalin A. This phosphorylation occurs on a significant percentage of the cell surface molecules and can be abolished by pretreatment of the cells with phorbol ester to deplete cellular PKC.

The in vivo phosphorylation data argues strongly that PKC is required for the phosphorylation of CD8 in response to phorbol esters, but it does not imply that PKC is the direct mediator of these signals. PKC could act by stimulating another protein kinase which would directly phosphorylate CD8. To investigate this possibility we attempted to correlate the in vivo phosphorylation with in vitro activity of this protein kinase and to identify the site of phosphorylation on the CD8α molecule. We have shown that CD8 is a substrate of PKC in vitro and is not phosphorylated by other major protein kinases. In addition, we have identified the site of phosphorylation and shown that a peptide corresponding to this site is kinetically an excellent PKC substrate. Combined with the in vivo data, these in vitro experiments support a direct role for PKC in the phosphorylation of this T cell antigen.

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