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Identity of Common Phosphoprotein Substrates Stimulated by Interleukin 2 and Diacylglycerol Suggests a Role of Protein Kinase C for IL 2 Signal Transduction

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Interleukin 2 (IL 2) is a polypeptide growth factor essential for the proliferation and differentiation of T lymphocytes, large granulocytic lymphocytes, and, potentially, cells of the antibody-producing lineage, B lymphocytes. Many of the biological properties of IL 2 may be mimicked or potentiated by a potent class of tumor promoters, phorbol esters. Phorbol esters have recently been shown to associate with and activate a unique phospholipid/Ca^{2+}-dependent phosphotransferase, protein kinase C (PK-C). Utilizing two-dimensional gel electrophoresis, we have compared the IL 2 and diacylglycerol-induced protein phosphorylation patterns of several IL 2-dependent murine cell lines. Both IL 2 and synthetic diacylglycerol, 1-oleyl-2-acetylglycerol (OAG), stimulated phosphorylation of a number of protein substrates in intact cells compared to unstimulated controls. Three groups of substrates were identified; the first showed increased phosphorylation following stimulation with either IL 2 or OAG, while the second and third groups showed increased phosphorylation following stimulation with IL 2 but not OAG, and with OAG but not IL 2, respectively. Here, we characterize the kinetics of phosphorylation of one cellular substrate, p68, which appears to be phosphorylated in response to direct activators of PK-C or lymphoid or myeloid growth factors in their respective lineage cell lines. The observation that IL 2 also stimulates a unique series of phosphoproteins in addition to those induced by direct PK-C activators suggests that IL 2 may initiate additional protein kinase activities, unrelated to PK-C, which may also be critical for the ligand-receptor signal transduction process regulating growth and gene expression.

Abbreviations used: IFN-γ, gamma interferon; FCS, fetal calf serum; PK-C, protein kinase C; OAG, 1-oleyl-2-acetylglycerol; IL 1, interleukin 1; IL 2, interleukin 2; r IL 2, recombinant interleukin 2; BCGF, B cell growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; PMA, phorbol 12-myristate 13-acetate; PDD, 4 α-phorbol 12,13-didecanoate.

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Interleukin 2 (IL 2), which is the first of the lymphokine family of polypeptide regulatory elements to receive clinical attention, has the properties of both a growth factor and a differentiation signal. Released from T lymphocytes in response to antigen, plant lectins, or phorbol esters, IL 2 acts via a specific high affinity receptor expressed on antigen or mitogen activated T lymphocytes [1]. Although IL 2 was originally suggested to be specific for lymphocytes of the T-cell lineage, recent studies have shown that IL 2 can induce both the activation of large granular lymphocytes to express natural killer cell activity [2,3] and the proliferation of B lymphocytes [4]. Numerous studies have demonstrated that IL 2 receptor interaction contributes to both the transition of cells from G_1 to S phase of cell cycle progression (proliferation), the secretion of gamma interferon (IFN-γ), and the development of the capacity of allogeneic recognition and cytolysis (differentiation) [5–9]. Dissection and identification of the molecular components of IL 2 regulation of T cell growth have followed acquisition of the reagents needed to probe the supramolecular interactions of IL 2 and its receptor. The availability of these reagents has allowed the rapid molecular cloning and nucleotide structure analysis of both IL 2 [10] and its putative receptor which was identified by the anti-Tac monoclonal antibody [11,12]. This progress makes it possible to investigate the basis of the IL 2 receptor signal transduction mechanism.

Protein phosphorylation has been extensively implicated in the regulation of many cellular processes. The state of phosphorylation of certain substrates is modified by protein kinases, and phosphatases may result in conformational changes which determine the chemical stability and physiochemical activity of the substrate [13]. Tyrosine protein kinase activity appears to be intrinsic to several retroviral transforming proteins [14], as well as cellular growth factor receptors such as epidermal growth factor and platelet-derived growth factor receptors [15]. Recently, an additional protein phosphotransferase system, protein kinase C (PK-C), has been suggested to mediate the effects of a potent class of tumor promoters, phorbol esters, as well as potentially mediating the intracellular signaling process of oncogenes that have reported tyrosine kinase activities [16–19]. Given the fact that protein phosphorylation has long been recognized as a rapid and reversible means of regulating protein function, it is clearly important to investigate the coordinate network of phosphorylation events stimulated by IL 2 and to identify the chemical substrates of specific kinase activities.

It has previously been shown that IL 2 induces the subcellular redistribution from cytosol to plasma membrane of protein kinase C in murine IL 2-dependent CT6 T lymphocytes [20]. The membrane association of PK-C induced by phorbol esters is apparently required for the cell activation of PK-C in intact cells and the physiological consequences of phorbol esters. This observation was originally detected in EL4 thymoma cells, which respond to phorbol ester by the synthesis and release of IL 2 [21,22]. The phenomenon has been further observed in unrelated cell types such as PYS yolk sac cells, NIH 3T3 fibroblasts, Dif 5 endoderm cells, and cells of hematopoietic lineage maintained on interleukin 3 [23–25]. The findings that IL 2 induced the subcellular redistribution of a phosphotransferase system shared by phorbol esters and certain oncogenes led us to investigate the role of this kinase in the mechanism of IL 2 regulation of growth and differentiation [20].

The studies presented in this report demonstrate by phosphosubstrate analysis that IL 2 stimulates the phosphorylation of a protein substrate with identical charge to mass ratio as stimulated by diacylglycerol and phorbol myristate acetate, direct activators of PK-C. These studies provide direct biochemical evidence which supports a role for PK-C in the signal transduction process and emphasizes the potential participation of additional coordinate transmembrane events which may be required for the physiological response to IL 2.

MATERIALS AND METHODS

Natural human IL 2 was purchased from Cellular Products, Inc. (Buffalo, NY). Recombinant human IL 2, r IL 2 was purchased from Sandoz (Vienna, Austria). 1-oleoyl-2-acetylglycerol (OAG) was purchased from Avanti Polar Lipids (Birmingham, AL). ^{32}P orthophosphate 10 mCi/ml in 0.02 M HCl was purchased from Dupont/New England Nuclear (Boston, MA). All other chemicals were obtained from standard sources.

Maintenance of Cell Lines

CT6, CTLL-2, and CTB-6 murine T lymphocyte cell lines were maintained in RPMI 1640 (Advanced Biotechnologies, Silver Spring, MD) 2 mM glutamine, 10% fetal calf serum (FCS) (Hyclone, Logan, UT). Two percent CPI-IL 2, 100 U/ml penicillin, and 0.01 mg/ml streptomycin. Culture conditions were 5% CO_2, 91% relative humidity, and 37°C.

Equilibration of Cells in ^{32}P Orthophosphate and Ligand Stimulation

Four hours (CTLL-2, CTB6) or 24 hr (CT6) prior to use, the cells were washed free of serum and IL 2, with two washes in serum-free RPMI made slightly acidic by bubbling CO_2 through the media, and a final wash in neutral RPMI 1640. Before use, the cells were washed twice in 10 mM HEPES-saline and resuspended in phosphate-free RPMI at 1×10^7 cells/ml. Thirty minutes later, 0.5 mCi of ^{32}P orthophosphate neutralized with 20 mM NaOH (New England Nuclear, Boston, MA) was added to each 1 ml. Cells were equilibrated in ^{32}P orthophosphate for 2 hr. Unless otherwise indicated, cells at 1×10^7/ml were stimulated at 10 min at 37°C with either mock material (2 μg/ml final concentration essentially fatty acid-free BSA), r IL 2 (2 μg/ml, 2,000 U/ml), or OAG (1 μg/ml final concentration). Following stimulation, the cells were washed with chilled complete RPMI 1640 (containing phosphate).

Preparation of Particulate Membrane and Cytosol Fractions and Cell-Free Protein Kinase C Phosphorylation

Cells were washed free of serum (two washes in 10 mM Hepes saline) adjusted to 1×10^9 cells/ml in homogenation buffer (20 mM Tris/HCl pH 7.5, 2 mM PMSF, 5 mM EGTA, 2 mM EDTA, 11% w/v sucrose). Cells were then broken in a Dounce homogenizer. Broken cell homogenate was layered over a 41% sucrose cushion and centrifuged at 100,000g for 1 hr. The cytosol fraction was removed from above the interface, while the particulate membrane was removed from the interface. The particulate membrane was washed and pelleted by centrifugation at 100,000g for 1 hr and finally resuspended in homogenation buffer. Membrane and cytosol fractions were frozen on dry ice and stored at −70°C. The basic reaction buffer 20 mM Tris/

HCl pH 7.5, 75 mM Mg^{2+} was used in all reactions. Ca^{2+} (0.5 mM or 5 mM), phospholipid (12 μg/ml phosphatidylserine, 0.4 μg/ml 1,2-Diolein), EGTA, and partially purified protein kinase C were included where indicated (Fig. 5). Reactions carried out at 32°C were started by the addition of ATP adjusted from cold ATP and [$\gamma-^{32}$P]ATP (> 5,000 Ci/mmol) to give a final concentration at 7.5–10 × 10^6 cpm per reaction [25].

Phosphopeptide and Phosphoaminoacid Analysis

Slices of SDS-PAG corresponding to the location of phosphorylated proteins identified by autoradiography were placed in an extraction buffer (20 mM ammonium phosphate 1% SDS, 5% 2-ME) homogenized; and shaken at 37°C for 48 hr. Extracted proteins were precipitated together with 10 μg of bovine serum albumin as carrier protein in 20% TCA. The precipitates were washed twice with ethanol ether and once with ether alone, air dried, and then resuspended in digestion buffer (1.25 mM Tris/HCl pH 6.8, 0.5% SDS, 10% glycerol, and chymotrypsin 10 μg/ml) hydrolyzed with 6 M hydrochloric acid for 1 hr at 110°C. Chymotryptic digests were analyzed by SDS-PAGE using a 30% acrylamide separating gel. To analyze phosphoaminoacids, HCl was removed by heating under vacuum in a rotor evaporator. Single dimension thin layer electrophoresis was carried out at 1,500 V for 40 min, using cellulose thin layer plates and pyridine:glacial acetic acid:water (10:100:1890) buffer, pH 3.5. Phosphoaminoacids were identified using unlabeled standards located with ninhydrin.

Preparation of Samples

To each pellet of 1 × 10^7 cells, 1 ml of lysis buffer was added (1% Triton × 100, 10 mM Tris-HCl, 150 mM NaCl 20 mM NaF, 20 mM EDTA, and 2 mM phenylmethysulfoylfluoride). We had previously determined that this lysis buffer prevented any detectable changes in phosphorylation during the 40 min extraction procedure. In particular, inclusion of the phosphotyrosine phosphatase inhibitor (sodium vanidate) did not affect the observed phosphorylation patterns. After 30 min on ice, the samples were centrifuged at 10,000g for 10 min to remove insoluble particulate material. Part of each sample was then prepared for SDS-PAGE electrophoresis (2:1 with [0.2 m Tris HCl pH 6.8, 30% glycerol, 15% 2-ME, 7% SDS]) or nonequilibrating pH gel electrophoresis (100 μl sample:100 μl [9.5 m urea, 2% NP-40, 2% ampholine, 5% 2 ME]:100 μg urea).

One-Dimensional SDS PAGE and Two-Dimensional (O'Farrell) Electrophoresis

Procedures described in detail elsewhere were followed [26]. Briefly, a 0.75 mm separating gel (7.5–15% acrylamide gradient) with a 3% stacking gel were used. Samples were loaded into preformed slots, and separation was achieved with a constant current, 15 mA/gel and was monitored by migration of bromophenol blue incorporated in sample loading buffer. For the two-dimensional analysis, a 50 μl sample (50 μg protein) was loaded at the anode end of 6% acrylamide, 2% ampholine (pH 3.5–10) tube gels (3 mm × 120 mm). Separation was achieved using a 500 V potential gradient applied for 5 hr. Non equilibrating pH gradient electrophoresis (NEPHGE) gels removed from tubes were equilibrated for 3 hr with 1% 2-ME in stacking gel buffer. Gels were then frozen at −70°C or applied directly onto the top

of the stacking gel of an SDS PAGE (prepared as described above) and fixed in place with 1% agarose in stacking gel buffer.

Treatment of Gels and Autoradiography

Following SDS PAGE, gels were washed in methanol, water, acetic acid (5:5:1), three changes each for 1 hr. Gels were then rehydrated by washing in water, supported on filter paper, and dried (Biorad gel drier). Autoradiography has carried out using Kodak XAR 5 film.

RESULTS

In order to determine and characterize the physiological phosphosubstrates induced by IL 2 interaction with its high affinity receptor, three murine IL 2-dependent cell lines (CT6, CTLL-2, and CTB6) were selected as homogenous target cell populations. Phosphosubstrates of PK-C activity could be determined in cells by the stimulation of intact cells with OAG, a synthetic analog of diacylglycerol, or PMA, both of which directly stimulate PK-C in vitro and in intact cells [27]. A comparison of the total cellular phosphosubstrates stimulated by IL 2 and OAG or PMA would therefore allow us to determine whether IL 2 stimulates the phosphorylation of cellular proteins that are also stimulated by a direct activator of PK-C.

Two-Dimensional Gel Electrophoresis Analysis of IL 2 and OAG Phosphoproteins

In order to resolve the complex changes in phosphorylation following stimulation of cells with IL 2, OAG or PMA, we compared samples using two-dimensional analysis, NEPHGE followed by SDS PAGE. An obvious increase in phosphorylation of several substrates was induced by either IL 2 or OAG. Most of the increase in phosphorylation following IL 2 or OAG (and PMA) treatment represents an increase in the extent of de novo phosphorylation (compared to resting cells) of existing phosphoproteins. The main changes in phosphorylation detected by two-dimensional analysis can be grouped into three categories [28,29]. The first group represents unique proteins phosphorylated following treatment of intact cells with either IL 2 or OAG. The principal members of this group are p68 and p30 (center panel, Fig. 1); these substrates exhibited the greatest net increase in incorporation of ^{32}P. Phosphosubstrates showing increased phosphorylation after treatment with one or other stimulant (IL 2 or OAG) were placed into groups 2 and 3. Examples of group 2 substrates, proteins phosphorylated only after IL 2 stimulation, are p60 and p80, and of group 3 substrates, (proteins phosphorylated only after OAG stimulation) examples are p26 and p20 [28,29]. It is important to note that the two-dimensional phosphorylation patterns obtained for each of the cell lines CT6, CTB6, and CTLL-2 were essentially similar, with the principal changes following stimulation by IL 2 or OAG being well conserved. Since our aim was to establish the role of PK-C in early signal transduction events, we chose to concentrate our subsequent studies on those substrates phosphorylated following stimulation with either IL 2 or OAG, ie, group 1 molecules. The p30 substrate has been identified as 40s ribosomal S6 protein and is the subject of a separate paper [30]. We selected p68 for further investigation because phosphorylation of this molecule was observed in myeloid cells stimulated with interleukin 3 [31] and because it exhibited the greatest incorporation per unit protein of ^{32}P following

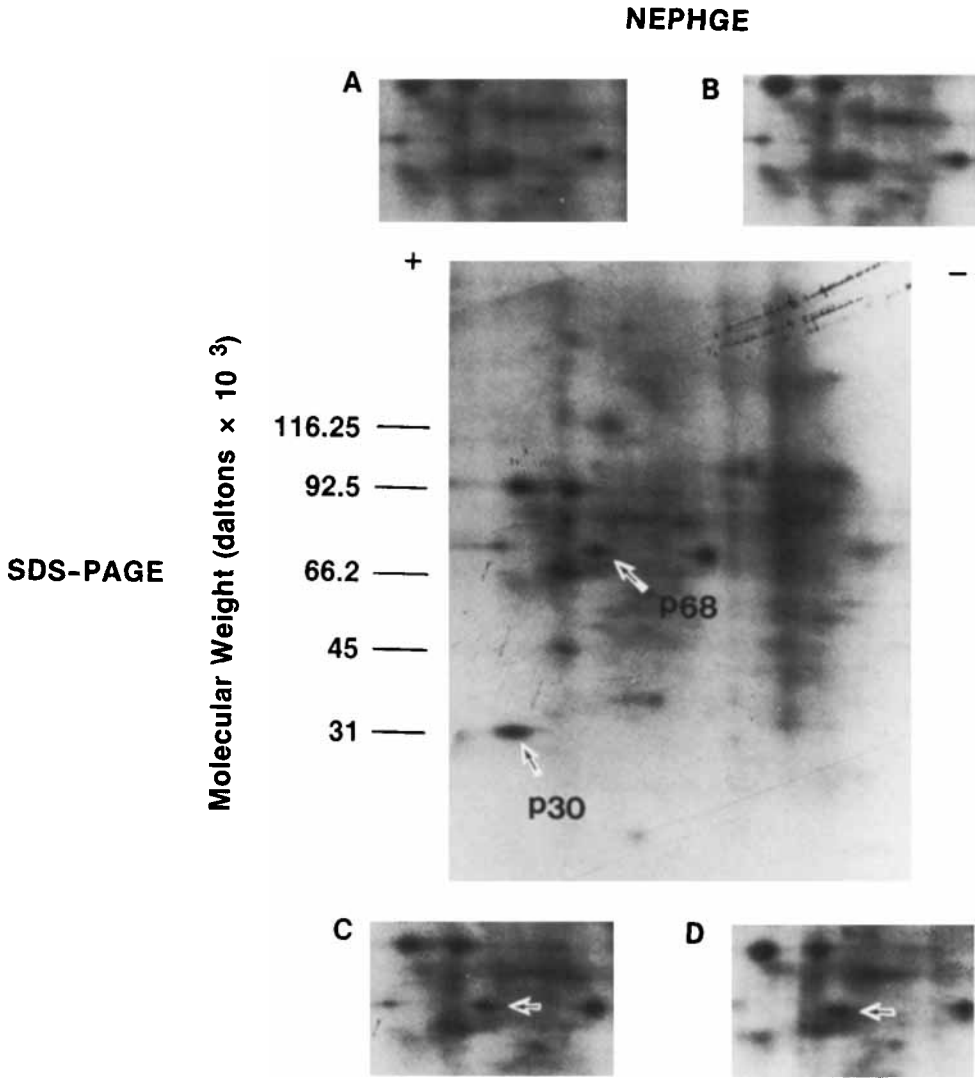

Fig. 1. Two-dimensional NEPHGE/SDS-PAGE (O'Farrell) analysis of phosphosubstrates from intact cells. Orthophosphate-labeled material extracted from intact cells following treatment for 15 min with IL 2 central panel, and with A) mock material, B) PDD, C) OAG, and D) PMA. Phosphosubstrates, p68 and p30, are indicated by arrows. SDS-PAGE molecular weight standards and the anode and cathode for NEPHGE are also indicated on the figure. p68 and p30 are phosphorylated following IL 2, OAG, or PMA, but not PDD stimulation of the cells.

stimulation. In Figure 1, an example of the two-dimensional pattern obtained from CT6 cells is represented. The stimulation of p68 phosphorylation is seen after treating CT6 cells with either IL 2 (center panel, Fig. 1), OAG (Fig. 1C) or PMA (Fig. 1D) but not in mock-treated (Fig. 1A) or PDD- (an inactive analog of phorbol ester) (Fig. 1B) treated cells.

Quantitative and Kinetic Comparison of Protein Phosphorylation Stimulated by IL 2 and OAG.

To quantitate the phosphorylation of p68 following various treatments, spots or bands corresponding to p68 were located via autoradiography were sliced out of the gel, and the extent of Cerenkov radiation measured. The phosphorylation of p68 in a dose response to IL 2, OAG, and PMA was determined (Fig. 2). All these agents stimulated the rapid phosphorylation of p68, the half maximal dose of IL 2 being between 1 and 10 units/10,000 cells (2,000 U/ml), which corresponded well with the ED_{50} growth response of CTB6 cells to IL 2. Occasionally, the IL 2 response at 100 units/10,000 cells was reduced from peak value; while this may not be significant, it could represent a high dose desensitization of IL 2 response (data not shown). OAG and PMA induced half maximal phosphorylation at approximately 1 ng/10,000 cells (2 μg/ml) and 1 pg/10,000 cells (2 ng/ml), respectively.

The next experiment determined the time kinetics of p68 phosphorylation. Following stimulation of CTLL 2 cells with IL 2, OAG, or PMA, a rapid phosphorylation of p68 was induced and was complete approximately 10 min after treatment (Fig. 3A). Further examination of p68 phosphorylation at subsequent times revealed that a dephosphorylation of p68 occurred after 30 min of IL 2 treatment, but that p68 remained phosphorylated for more than 2 hr after PMA treatment (Fig. 3B). In contrast to p68, the observed phosphorylation of p30 was more complex. Phosphorylation of p30 was maximal at around 30 min, as opposed to less than 10 min for p68. The two-dimensional pattern also revealed a difference; p30 was initially detected as a streak which moved towards the cathode and formed a compact spot with maximal incorporation of ^{32}P per unit protein after 30 min treatment with IL 2 [30]. This heterogeneous response suggests possible multiple phosphorylation states of this molecule. In comparison, p68 was rapidly phosphorylated, and only a single compact spot was seen in two-dimensional gels.

Phosphorylation of p68 in a Cell-Free System

To confirm that p68 was a substrate for the calcium/phospholipid-dependent phosphotransferase, cytosolic substrates were phosphorylated in various calcium and

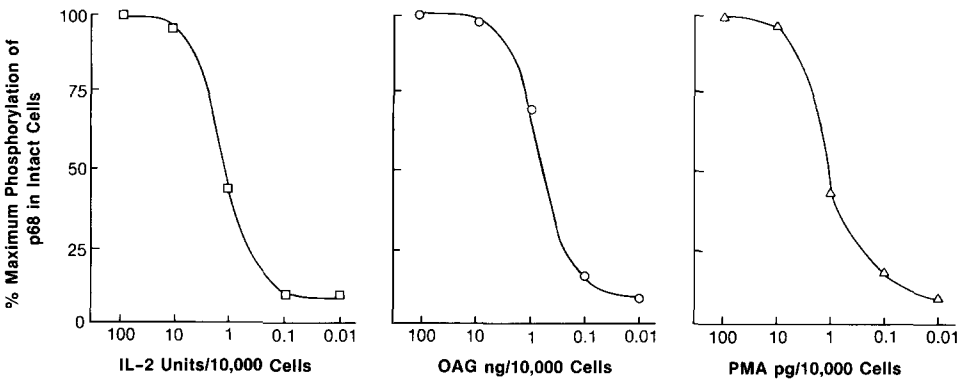

Fig. 2. Phosphorylation of p68 in cells treated with different doses of IL 2, OAG, and PMA. CTB6 cells were pre-equilibrated with orthophosphate and then treated for 15 min with either IL 2, OAG, or PMA. The p68 phosphosubtrate located by autoradiography was excised from gels, and the Cerenkov radiation measured in a scintillation spectrometer.

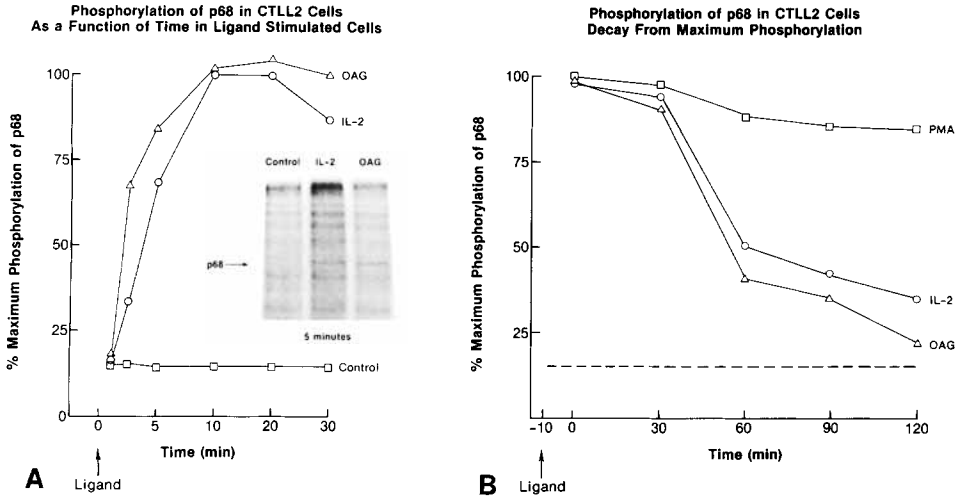

Fig. 3. A) Kinetics of p68 phosphorylation following treatment of CTB6 cells with IL 2 or OAG. The 68,000-dalton molecule was located by autoradiography, excised from the gel, homogenized in 30% hydrogen peroxide for 30 min. The extent of Cerenkov radiation was then determined in a scintillation spectrometer. Single dimension analysis (inset) of phosphosubstrates after treatment of cells for 5 min with mock material, IL 2, or OAG is shown. Position of 68,000-dalton phosphosubstrate is indicated by arrow. B) Dephosphorylation of p68 after stimulation of maximum phosphorylation with IL 2, OAG, and PMA. Cells were stimulated for 5 min with appropriate ligand, and the extent of p68 phosphorylation was determined at subsequent times up to 2 hr after maximum phosphorylation.

phospholipid conditions which facilitate the activation of PK-C. Endogenous PK-C was able to phosphorylate a molecule with identical charge/mass ratio to p68 phosphosubstrate isolated from intact cells. A two-dimensional separation of CT6 cytosolic substrates phosphorylated in a cell-free system is represented in Figure 4. The cell-free phosphorylation of p68 was calcium- and phospholipid-dependent, conditions which characteristically are required for PK-C activation.

Phosphoamino Acid Analysis and Peptide Mapping of p68

To determine the nature of phosphorylated, amino acid residues, p68 phosphosubstrates were extracted from two-dimensional gels subjected to acid hydrolosis and then thin layer electropheresis. Autoradiography of the phosphoamino acids revealed that p68 was phosphorylated only on threonine residues. This result was obtained whether the p68 molecule was either extracted from cells treated with OAG or IL 2 or from the corresponding molecule phosphorylated in a cell-free PK-C reaction (Fig. 5a). Peptide mapping was carried out to confirm the identity as well as the phosphorylation pattern of p68 isolated from cells stimulated with IL-2 or OAG and p68 phosphorylated in a cell-free system (Fig. 5b). Four phosphopeptides could be identified following chymotryptic digestion of p68. The major peptide had a molecular weight of approximately 27 kD, while the three minor peptides had molecular weights of 33, 38, and 43 kD, respectively. p68, therefore, was phosphorylated on the same amino acid species (threonine) with a similar pattern of peptide proteolytic digestion with in vitro PK-C activation, as was seen with IL 2 or OAG in vivo stimulation.

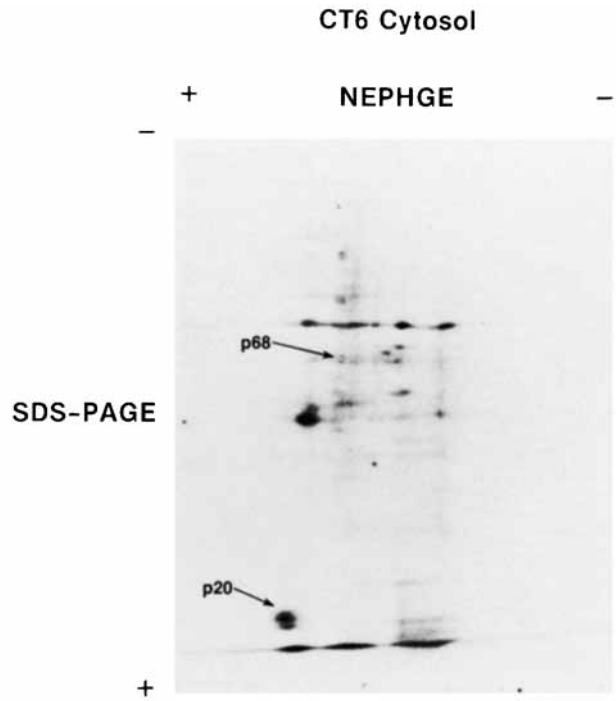

Fig. 4. Two-dimensional NEPHGE/SDS-PAGE analysis of cell-free phosphosubstrates. Cytosolic substrates were incubated in the presence of calcium phospholipid and endogeneous PK-C. The 68,000- and 20,000-dalton substrates are indicated. The 68,000 dalton substrate comigrated with p68 phosphorylated in intact cells following treatment with either IL 2 or OAG. p20 comigrated with myosin light chain and was used as a internal control indicating that PK-C had been activated.

DISCUSSION

In order to understand the mechanism of action of growth factors which induce S phase progression, it is necessary to identify the intracellular signals capable of initiating or modulating the proliferative response. It has been suggested that phorbol esters modulate mitogenesis through pathways that converge with those of hormones [32,33] and that the protein kinase C phosphotransferase system may represent a point of biochemical identity between the mitogenic actions of hormones and phorbol esters [34]. Within the biological framework of the immune system, phorbol esters exhibit many biological effects similar to those of lymphokines. Phorbol esters have been shown to potentiate many of the biological effects of IL 1, IL 2, and BCGF, as well as to induce gene activation of these molecules [35–39]. The activation of PK-C by mitogenic lymphokines is implied by the many biological potentiating effects phorbol esters have in the lymphokine bioassays. Previous studies have demonstrated that phorbol esters such as phorbol myristate acetate (PMA) induce a rapid translocation of PK-C from the cytosol to the plasma membrane, an event associated with activation of the enzyme [21–25]. Using the CT6 murine T cell line, we have shown that IL 2 is able to induce the rapid translocation of PK-C in a manner analogous to the observed PMA effect [20]. Although subcellular redistribution and activation of PK-C by IL 2 was transient compared to the protracted PK-C membrane association induced by PMA, the magnitude of the IL 2-elicited PK-C response correlated with

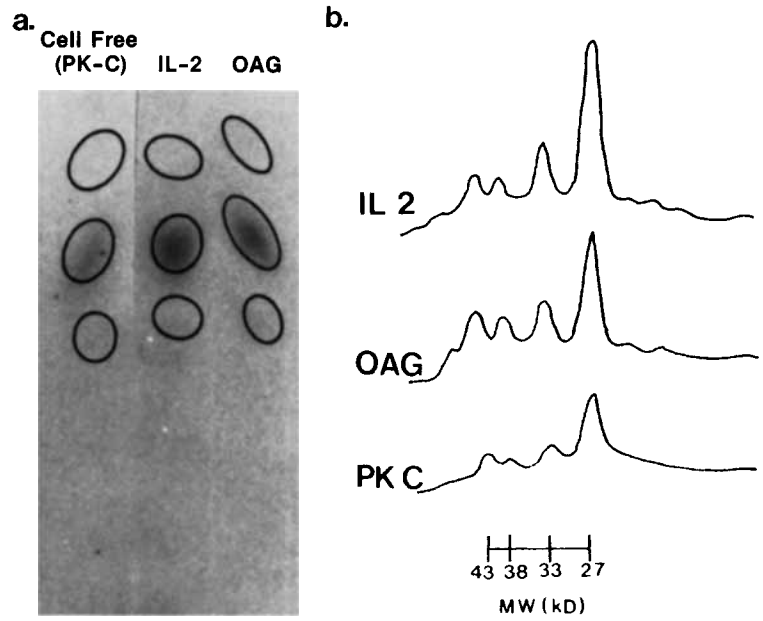

Fig. 5. a) Phosphoamino acid anlaysis of the 68,000-dalton molecule extracted from two-dimensional NEPHGE/SDS-PAGE. Using thin layer electrophoresis, phosphoamino acids released by acid hydrolysis are compared for p68 phosphorylated in intact cells following IL 2 or OAG treatment together with the substrate phosphorylated by protein kinase C in a cell-free system. The positions of cold phosphoamino acid standards for serine threonine and tyrosine are marked as determined by development with ninhydrin. b) Chymotrptic peptide analysis of p68,000-dalton molecule extracted from two-dimensional NEPHGE/SDS-PAGE. p68 extracted from two-dimensional gels was digested with chymotyrpsin (10/ μg/ml), and the resultant peptides separated by SDS-PAGE. Phosphopeptide patterns obtained from p68 phospholyated in intact cells stimulated with IL-2 or OAG and from p68 phophorylated in a cell-free system were compared. Densiometric scans of the phosphopeptides separated by SDS-PAGE are depicted.

the subsequent proliferative response of the cells. This casual quantitative relationship between PK-C activation and the IL 2 proliferative response suggested that IL 2-receptor interaction may result in a signal transduction process mediated in part by PK-C.

Three categories of substrate have been previously identified [28,29]. The first (group 1) is composed of substrates phosphorylated by either IL 2 or OAG treatment (eg, p68 and p30). The second group is composed of substrates phosphorylated following IL 2 treatment but not OAG treatment (eg, p60 and p80). A third group of substrates is phosphorylated following OAG but not IL 2 treatment of cells (eg, p26 and p20).

In this study, we have extensively investigated one of the principal phosphosubstrates associated with IL 2 and OAG- stimulation of T lymphocytes, and, thus, we have extended our original observation that IL 2 stimulates the same charge/mass

ratio in which OAG, a direct activator of PK-C, also induces phosphorylation [28,29]. Investigation of the phosphorylation patterns in three different murine IL 2-dependent cell lines following IL 2, OAG, and PMA stimulation of intact cells revealed similar properties for the phosphorylation of p68. It seems reasonable that the mitogenic stimulation of lymphocytes will invoke coordinate signals in addition to PK-C. The differences between phosphorylation patterns observed following IL 2 stimulation compared to OAG stimulation [28,29] may reflect the absence of one of these second messenger signals. It is possible that the direct stimulation of PK-C with OAG bypasses inositide metabolism and, therefore, does not generate inositol triphosphate, which is believed to be an intracellular ionophore [17]. Whether the phosphoproteins unique to IL 2 stimulation (p80, p60) are the result of a phosphotransferase system that is distinct from PK-C is currently under investigation. It will also be of interest to compare the OAG-induced phophorylation of p26 and p20 with the stress proteins phosphorylated by PMA treatment of fibroblasts [40].

Because of its basic properties, multiple phosphorylation sites and molecular weight of 30,000 daltons, p30 was identified as the S6 phosphoprotein, a component of the 40 s ribosomal subunit. We have investigated the phosphorylation of the S6 protein and control of protein synthesis in CT6 cells. S6 protein is phosphorylated by an S6 kinase distinct from PK-C. The S6 kinase itself appears to be regulated by PK-C, suggesting that complex kinase cascades are induced in IL 2-stimulated cells [30].

The 68,000-dalton molecule which was identified in three different cell lines and was phosphorylated by IL 2, OAG, and PMA was selected for further investigation because it was common to the early response of murine T cells to IL 2. This molecule may also be involved in early signal events induced by colony stimulating factors in murine myeloid cells [31] and human peripheral blood cells (unpublished observation). Phosphorylation of p68 by physiological doses of IL 2 was rapid, maximum phosphorylation occurred within 10 min, and was followed by a gradual dephosphorylation (Fig. 3B), so that by 60 min, p68 phosphorylation returned to the unstimulated level. OAG stimulation gave similar results to IL 2 stimulation; however PMA stimulation resulted in a more extended period of peak p68 phosphorylation. The IL 2 and OAG effects paralleled the kinetics of activation and translocation of PK-C in the CT6 cells [25], as well as the biological proliferation response [25]. The persistant phosphorylation of p68 after PMA stimulation suggested that the decay in phosphorylation observed after IL 2 and OAG stimulation represents dephosphorylation and not protein degradation. Maximum incorporation of ^{32}P into p68 induced by OAG or IL 2 was similar, suggesting that the same number of sites was being phosphorylated. Following phosphorylation, p68 migrated as a single spot in both isoelectric focusing (pI 5.5) and nonequilibrating pH gel systems, suggesting that only one phosphorylated form of p68 exists. Chymotryptic peptide patterns obtained from p68 phosphorylated following IL-2 or OAG stimulation of cells or from p68 phosphorylated in a cell free system were identical, supporting the notion that p68 phosphorylation represents the same event in each system.

A number of growth factor receptors including EGF, PDGF, and insulin have been demonstrated to exhibit tyrosine kinase activities upon interaction with their specific ligand [15]. During the investigation of p68, phosphoamino acid analysis demonstrated that phosphorylation of this substrate occurred at a threonine residue, therefore eliminating a direct role for tyrosine kinase in the phosphorylation of this molecule. However, the data presented in our study cannot exclude the possibility

that a tyrosine kinase system may be integrated among the network of phosphotransferase systems activated by IL 2. Using alkaline hydrolysis techniques, we have been unable to detect any increase in the content of tyrosine residues phosphorylated in cells stimulated with either IL 2 or OAG. Structural studies of the IL 2 receptor identified by the Tac epitope have identified a 13-amino-acid sequence as the probable intracytoplasmic domain of the receptor molecule [11,12], much smaller than those associated with growth factor receptors having intrinsic tyrosine kinase activity [11,12]. In a related study, our laboratory found no evidence for tyrosine kinase activity associated with immunoprecipitated Tac (unpublished findings). Whereas, EGF and PDGF receptors share nucleotide sequence homologies with the catalytic domains of certain oncogenes which also exhibit tyrosine kinase activity, Tac shares no sequence homologies with any defined oncogenes or tyrosine kinases [11,12]. Using a permeabilized cell system, Mine et al [41] were unable to detect any phosphorylation on tyrosine residues induced by IL 2. Collectively, these data suggest that the mechanism(s) of IL 2 receptor signal transduction may differ significantly from descriptions of the EGF and PDGF tyrosine-kinase-mediated activation. It should be noted; however, that a tyrosine kinase activity associated with the particulate fraction of lymphocytes has been described [42,43].

The demonstration that IL 2 and diacylglycerol stimulate identical phosphopeptides [28,29,44,45] provides biochemical evidence that PK-C activation represents a component of the intracellular signaling process initiated by IL 2. Phorbol ester mimicry of IL 2 biological activity is most obvious for the induction of gene products which are also under IL 2 regulatory control. The observations that phorbol ester stimulates gamma interferon production and Tac expression are two notable examples of phorbol ester mimicking IL 2 in the biological expressions of lymphocyte gene products [34–38,46,47]. These observations suggest a functional role of PK-C activation by IL 2 may be related to the regulation of genetic expression of secretory genes (ie, gamma interferon), as well as activation of gene structures involved in the maintenance of cell growth and survival (ie, Tac). Since phorbol esters only potentiate growth of lymphocyte lines in the presence of the physiological ligand, PK-C activation may be necessary (but not sufficient) as a component of an integrated network of phosphotransferases which are required for the initiation of S phase progression. The findings that IL 2 initiates additional phosphotransferase activities unique from those stimulated by diacylglycerol [29] and that S6 protein is phosphorylated by S6 kinase after activation of PK-C [30] reflect the possibility of an integrated network, of which PK-C is only one part.

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