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DIRECT PHOSPHORYLATION OF THE IL-2 RECEPTOR TAC ANTIGEN EPITOPE BY PROTEIN KINASE C

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Summary: Phorbol esters induce a rapid phosphorylation of the antigenic epitope of the human IL-2 receptor identified by anti-Tac monoclonal antibody. The physiological activator of protein kinase C, diacylglycerol also stimulated the phosphorylation of the Tac epitope in intact activated human T lymphocytes. Stable derivatives of cyclic nucleotides had no effect on the stimulation of Tac phosphorylation with cultured lymphocytes. Immunoprecipitated Tac derived from particulate membranes could serve as a direct substrate for purified protein kinase C *in vitro*. The Ca^{2+}/phospholipid dependency of the *in vitro* phosphorylation reaction substantiated that the phosphorylation of Tac observed in intact cells stimulated by phorbol ester or diacylglycerol was the result of the physiological activation of protein kinase C.

Interleukin 2 (IL-2) is a lymphocytotropic peptide synthesized and secreted by T lymphocytes upon stimulation by antigen, lectin, or phorbol esters (1-3). IL-2 promotes the growth and differentiation of antigen-sensitized lymphocytes as well as of large granular lymphocytes which have been closely associated with natural killer cell activity (4, 5). The biological activity of IL-2 is believed to involve the interaction of the peptide with a high-affinity receptor acquired by antigen or lectin activation. The receptor for IL-2 has been antigenically and biochemically characterized with a defined monoclonal antibody designated anti-Tac (6-8). Although the ligand-binding

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Abbreviations: IL-2, interleukin-2; PK-C, protein kinase C; OAG, 1-oleoyl-2-acetylglycerol; DG, diacylglycerol; PHA, phytohemagglutinin; TRF, anti-transferrin receptor; TAC, anti-IL-2 receptor antibody; RPC, control IgG monoclonal antibody.

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characteristics of IL-2 and the high-affinity Tac receptor have been well defined (6, 9) and the cloning of the cDNA for Tac recently accomplished (10, 11) little is known about the biochemical mechanism of IL-2-receptor signal transduction. In a previous report IL-2 was shown to stimulate the rapid membrane-association of a phosphotransferase system, protein kinase C (12). Recent studies have shown that protein kinase C has a crucial role in signal transduction of various biological hormones (13) as well as phorbol esters (14) and has been suggested to mediate some of the biological effects of oncogenes (15-16). Phorbol esters have recently been shown to stimulate the phosphorylation of the antigenic epitope of the human IL-2 receptor, Tac, on retrovirus infected lymphocytes (17). In contrast, HTLV-infected HUT102B2 cells have also been shown to exhibit a constituative state of "autophosphorylation" of the Tac antigen epitope expressed on surface membranes (18). The study presented here provides biochemical data which demonstrate that phorbol esters and the physiological protein kinase C activator, diacylglycerol, stimulate the phosphorylation of the Tac antigen epitope in intact normal human T lymphocytes. Furthermore, purified protein kinase C phosphorylated in vitro immunoprecipitated Tac derived from solubilized lymphocyte membranes.

Materials and Methods

Preparation of Cultured Lymphocytes and Immunoprecipitation Gel Electrophoresis. Human peripheral blood T cells were separated using nylon wool columns and discontinuous Percoll density gradient sedimentation as described (19). T cells were cultured for 2 days in RPMI-1640 supplemented with 5% FCS and 2 µg/ml PHA (Wellcome) and were cultured for an additional 3 days in RPMI-1640 containing 5% FCS and purified IL-2 (100 units/ml, Cellular Products). Ten million cells were washed twice in a balanced salt solution, resuspended in 2 ml of methionine-free (RPMI-1640 Selectamine medium) (Gibco) and preincubated at 37°C for 30 min. 0.5 mCi of NEN translation grade [35S]-methionine was added and the culture was incubated at 37°C for 4 hr. Cells were washed once with cold RPMI-1640 and lysed with 10 mM Tris-HCl-NaCl pH 7.5 buffer containing 1% Triton X-100. After centrifugation at 100,000 g for 1 hr at 4°C, the supernatant was subjected to immunoprecipitation as described previously (8, 18). After extensive washing of the immunoabsorbents, bound antigens were eluted by boiling for 5 min in 2% SDS-sample buffer and resolved on SDS-polyacrylamide gel (7.5%). After staining with Coomasie brilliant blue R250, the gel was treated with ENHANCE (NEN) and dried. Fluorography was performed at -80°C.

^{32}P-Orthophosphate Labeling of Intact T Lymphocytes. Activated T cells were prepared as described in figure 1. Five million million activated T cells were washed twice with Hepes-buffered saline and suspended in 1 ml of

phosphate-free RPMI-1640 and preincubated at 37°C for 1 hr. Cells were labeled with 0.5 mCi of [^{32}P]orthophosphate (Amersham) for 2 hr and then incubated with (+) or without (-) 100 ng/ml PMA for 10 min. The cells labeled with [^{32}P]orthophosphate were also treated with 50 µg/ml OAG, 1 mM 8-bromo cyclic GMP, or 1 mM 7-chloro-cAMP for 10 min. Cells were washed once with cold RPMI-1640 and lysed with the buffer containing 1% Triton X-100 and 20 mM NaF. The same procedure for immunoprecipitation was done except use of the washing buffer containing 20 mM NaF (R = anti-RPC, T = antiTac). Bound antigen was eluted in 2% SDS-sample buffer by boiling for 5 min and resolved on SDS-polyacrylamide gradient gels (7.5-15%). Phosphoproteins were detected by autoradiography using intensifying screens (DuPont, Lightening Plus) and Kodak X-Omat AR films. Exposure was done at -80°C.

In Vitro Phosphorylation of Tac by Protein Kinase C. About 1 billion activated T cells were prepared as mentioned (fig. 1). Membranes were obtained by modification of a previously described procedure (12). Briefly, the cells were washed twice with cold phosphate-buffered saline and resuspended in the buffer; 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 2 mM EDTA, 0.33 M sucrose, and 1 mM phenylmethylsulfonyl fluoride. Cells were disrupted in a Wheaton Dounce homogenizer. Homogenates were layered on 41% sucrose in the same buffer and centrifuged at 100,000 g for 1 hr at 4°C. The membrane bands were removed from the interphase and then diluted 10-fold and collected by centrifugation at 100,000 g for 1 hr at 4°C. Membranes were solubilized with 1% Triton X-100. Immunoprecipitates were prepared as described. The phosphorylation reaction was carried out at 30°C for 15 min in an Eppendorf tube. The reaction mixture (total volume of 250 µl) contained 14 µg of purified rat brain protein kinase C (20), 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 30 µl of immunoprecipitate, 20 mM NaF, 50 µM [γ-^{32}P] ATP (Amersham) with or without 1 mM $CaCl_2$, 5.0 µg of phosphatidylserine, and 1.0 µg of 1,2-dioleoylglycerol. The reaction was terminated by the addition of 50 µl of 100 mM ATP and 100 mM EDTA and the mixture was immediately centrifuged with a Beckman Microfuge for 15 sec.

Results

IL-2 receptors identified by anti-Tac or by [^{3}H]-leu-lys-IL-2 binding analysis, reach maximal levels on the surface of activated T cells at d 5-7 following stimulation with phytohemagglutinin (PHA) and IL-2 (9). Activated T cells were labelled with [^{35}S]methionine and cellular lysate was subjected to analysis by immunoprecipitation and SDS-poly acrylamide electrophoresis (PAGE)-fluorography. Figure 1 shows the fluorographic profile of labelled cell lysates immunoprecipitated with monoclonal antibodies to Tac (IL-2-receptor), TFR (transferrin receptor), or a nonspecific control immunoglobulin (RPC, IgG2a). Tac antigens extracted from normal activated T cells had apparent molecular weights of 60,000 daltons and 40,000 daltons when separated by 7.5% SDS-PAGE. The 40 Kd species has been reported to be a precursor molecule which can be further glycosylated and expressed on the plasma membrane (7). The 60 Kd Tac^{+} molecules were slightly larger than those present in HTLV-transformed T-cells (7) but this molecular weight is consistent with

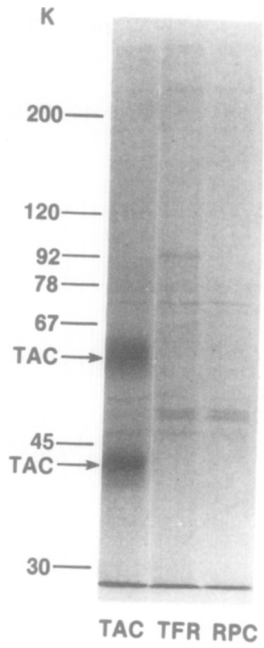

Fig. 1. Immunoprecipitation of ^{35}S-methionine-labeled Tac. Activated T lymphocytes were labeled as described in Materials and Methods. Immunoprecipitates were applied to a 7.5% SDS-PAGE. Fluorography was performed at -80°C for 3 days.

that described for the Tac antigen associated with the IL-2-receptor found on activated T lymphocytes (6-8). The immunoprecipitation of the transferrin receptor (TFR) at the apparent molecular weight of 92 Kd agreed with published values.

Phorbol esters have been shown to stimulate phosphorylation of a number of membrane-associated receptors including high-affinity receptors for epidermal growth factor (EGF), insulin, and somatomedin C (21, 22). In order to ascertain whether IL-2-receptors or transferrin receptors were similarly phosphorylated, activated T cells were prelabeled with [^{32}P]orthophosphate in phosphate-free RPMI-1640 for 2 hr at 37°C. The cells were stimulated with phorbol myristate acetate (PMA) for an additional 10 min. The reaction was terminated with phosphate containing RPMI-1640, and immunoprecipitation of Tac and TRF was analyzed by SDS-PAGE and autoradiography. Figure 2a demonstrates that in the presence ([+] lane 1) of PMA, IL-2 receptors (Tac) were significantly phosphorylated. The transferrin receptor (TRF) complex was not phosphorylated in the presence or absence (±) of PMA. Limited phos-

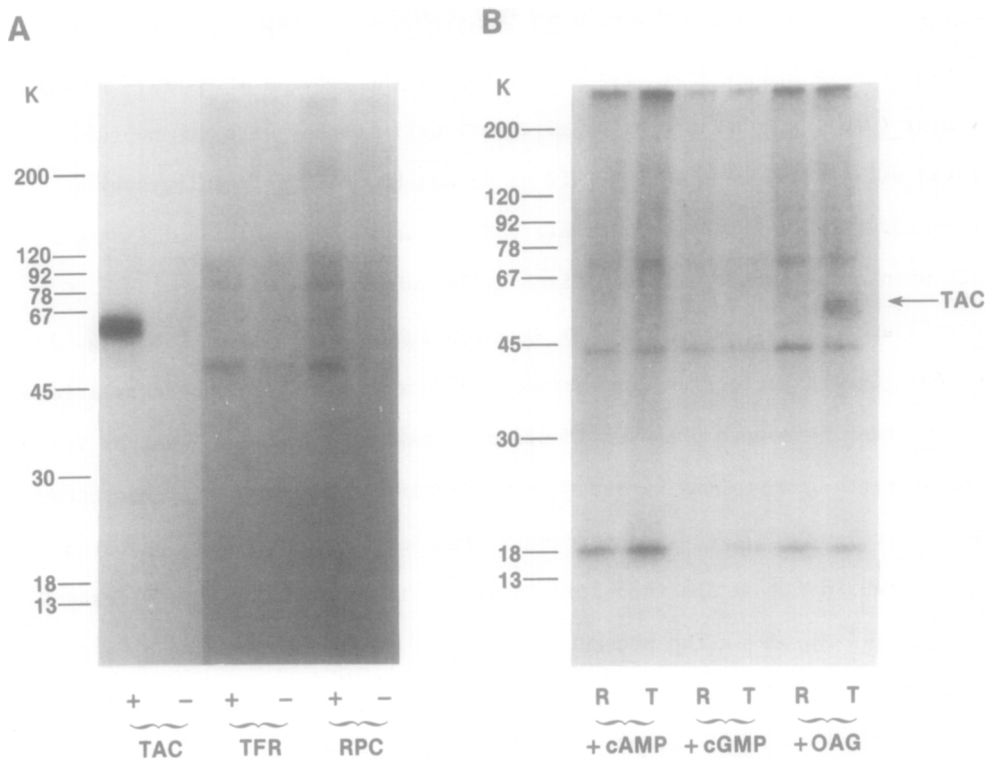

Fig. 2. Immunprecipitation of phosphorylated Tac. ^{32}P-orthophosphate labeled activated lymphocytes were stimulated with PMA (+) or control diluent (-) and cellular lysates precipitated with anti-Tac, anti-TRF, or control antibody anti-RPC. Panel B; labeled cells were stimulated with cyclic nucleotide derivatives or synthetic diacylglycerol, OAG. Immunoprecipitates were prepared with anti-Tac (T) control anti-RPC (R).

phorylation of a few non-Tac-associated peptides was seen after PMA stimulation in the unrelated control immunoprecipitate (RPC) which was attributed to nonspecific binding of peptides to the protein A sepharose matrix. Since phorbol esters have been shown to associate physiochemically with and activate protein kinase C, the demonstration of PMA-stimulated phosphorylation of Tac with intact cells suggests that PK-C may be involved in the phosphorylation of Tac. Whether Tac is a direct substrate of protein kinase C or alternatively a substrate of a protein kinase whose activity is regulated by protein kinase C cannot be inferred from intact cell phorbol ester treatment.

T lymphocytes also have considerable protein kinase A and G activities regulated in part by intracellular levels of cyclic AMP and cyclic GMP,

respectively (23). Activated T cells labeled with [^{32}P]orthophosphate were stimulated with either 7-Cl-cyclic AMP, 8Br-cyclic GMP, or 1-oleoyl-2-acetyl-glycerol (OAG), a synthetic diacylglycerol. Cell lysates were immunoprecipitated with anti-Tac or anti-RPC (T or R) and SDS-PAGE autoradiography performed. Figure 2b shows that only the synthetic diacylglycerol (DG) OAG, which when added exogenously to intact cells substitutes for endogenous DG and activates protein kinase C (24), induced phosphorylation of Tac (Fig. 2b, lane 6). Cyclic nucleotide derivatives had no effect on Tac phosphorylation.

In order to obtain direct evidence concerning the issue of whether Tac is a substrate for protein kinase C enzyme activity, an in vitro reconstitution protocol was designed. Accordingly, immunoprecipitates were prepared as described in fig. 1 and incubated in vitro with purified protein kinase C (20) and [γ-^{32}P]-ATP in the presence or absence of phospholipids and Ca^{2+}. Fig. 3 shows that the Tac immunoprecipitate was directly phosphorylated in

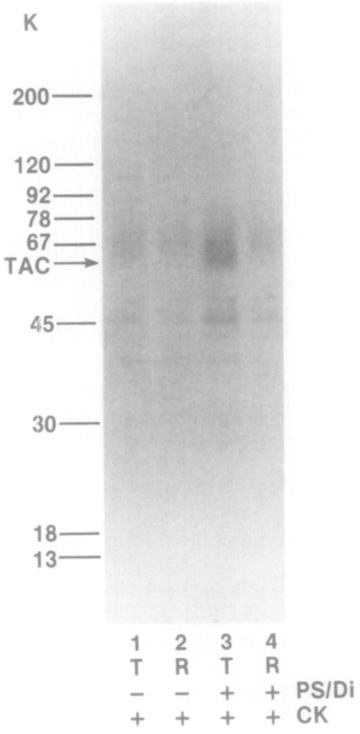

Fig. 3. In vitro phosphorylation of Tac by purified PK-C. Immunoprecipitates of Tac were prepared from isolated particulate membranes and admixed with purified PK-C plus Tac without phospholipid; Lane 2, PK-C with anti-RPC without phospholipid; Lane 3, PK-C plus Tac ppt. and phospholipid cofactors; Lane 4, PK-3, phospholipid plus control RPC ppt.

vitro in the presence of the phospholipid protein kinase C activators phosphatidylserine and diolien (diacylglycerol). The absence of detectable phosphorylation of Tac without the addition of phospholipids substantiated the dependence of protein kinase C for phospholipid co-factors (23). Phosphorylation of the control immunoprecipitate (RPC) did not occur in the presence or absence of protein kinase C and phospholipids in the molecular weight range of Tac antigen (lanes 2 and 4). Acid hydrolysis and thin layer cellulose electrophoresis of the phosphorylated Tac immunoconjugate revealed the major phosphorylated residues were threonine and serine. No phosphotyrosine was observed (data not shown). Taken together, stimulation of Tac phosphorylation by PMA and the synthetic diacylglycerol (OAG) as well as the direct phosphorylation of immunoprecipitated Tac by purified protein kinase C provided in situ (intact cell) and in vitro evidence for a direct role of protein kinase C in the phosphorylation of IL-2 Tac-associated receptors.

Discussion

The data presented in this study demonstrate that the IL-2 receptor identified by the antigenic Tac moiety is phosphorylated in intact cells and *in vitro* by protein kinase C (figs. 2,3). Recently, Shackelford and Towbridge (17) demonstrated that phorbol esters induced the phosphorylation of Tac expressed on HTLV-retrovirus infected cells HUT102B2. Unstimulated HUT102B2 cells did not possess demonstrable Tac phosphorylation. In contrast, Depper et al. (18) have shown that HUT102B2 express constituatively phosphorylated Tac residues. Our data does not reconcile the differences observed between these studies with HTLV-infected T lymphocytes. The data presented here, however, provide conclusive evidence that the protein moiety of the Tac epitope may serve as a direct substrate of protein kinase C activity. The data confirm the supposition for the role of protein kinase C previously implied by the addition of phorbol esters to cultured cells.

Although the physiological consequences of Tac phosphorylation by protein kinase C are unknown, other cellular receptors have been reported to be modified by protein kinase C (21-22). Protein kinase C has been

demonstrated to phosphorylate the epidermal growth factor (EGF) receptor which resulted in diminished activity of the EGF receptor-associated tyrosine kinase (25). Phorbol esters have also been shown to stimulate phosphorylation of receptors for insulin and somatomedin C on B lymphocytes (22). The phorbol ester-treated cells had reduced receptor affinity for their respective ligands (21, 22). These studies have suggested a role of protein kinase C in the regulation of receptor-ligand interactions and signal transduction. In view of the present findings, it is interesting to speculate that protein kinase C phosphorylation of IL-2 receptors may mediate the alterations of affinity of the IL-2 receptor which occur under a variety of physiological or pathological circumstances. Alternatively, the phosphorylation of membrane-associated receptors by protein kinase C may suggest a common crucial transmembrane signaling event shared by growth factors, tumor promoters, and certain oncogenes.

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