Induction of Expression and Phosphorylation of the Human Interleukin 2 Receptor by a Phorbol Diester*

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The receptor for interleukin 2 (IL 2) is induced on normal mature T cells activated by antigen or mitogen. Receptor-negative human T leukemic cell lines were induced to express the IL 2 receptor after incubation with 12-O-tetradecanoyl phorbol 13-acetate. The Mr of the IL 2 receptor expressed on the surface of four different T cell lines and normal peripheral blood lymphocytes varied. The source of the heterogeneity appeared to be due to differences in post-translational processing of the receptor. The precursor of the IL 2 receptor isolated from tunicamycin-treated HUT102B2 or 12-O-tetradecanoyl phorbol 13-acetate-induced CCRF-CEM cells had the same Mr, and isoelectric point. Incubation of cells with 12-O-tetradecanoyl phorbol 13-acetate also induced the rapid phosphorylation of serine and threonine residues on the IL 2 receptor from HUT102B2 cells and activated peripheral blood lymphocytes. This phosphorylation may be mediated by the Ca++ and phospholipid-dependent protein kinase C.

Induction of an immune response to foreign antigen requires the activation of subsets of T lymphocytes by receptors for the specific antigen. Activation of T lymphocytes by antigen or mitogen induces the expression of membrane receptors for the polypeptide hormone IL 2, and is produced by a subpopulation of activated T cells (5, for review see Ref. 6). The complete sequence of the protein has been deduced from the nucleotide sequence of cDNA clones (7).

A plasma membrane glycoprotein of Mr = 50,000 to 55,000, termed T cell activation antigen or Tac, has been identified as the receptor for IL 2 on human T cells (8, 9). A monoclonal antibody (anti-Tac) to this molecule inhibits the proliferation of IL 2-dependent continuous T cell lines and blocks the binding of radiolabeled IL 2 to a cell line (HUT102B2) which expresses a large number of IL 2 receptors. Conversely, a large excess of IL 2 can inhibit the binding of anti-Tac antibody to these cells. After cross-linking IL 2 to HUT102B2 cells, anti-Tac or anti-IL 2 antibodies immunoprecipitate an antigen which is 14,000 daltons larger than the IL 2 receptor recognized by anti-Tac from untreated cells (10). These results indicate that the glycoprotein recognized by anti-Tac directly binds IL 2.

Most long-term human T and B cell lines of neoplastic or normal origins do not express receptors for IL 2 as assessed by their ability to bind radiolabeled IL 2 (1). The cell line HUT102B2, established from a patient with a cutaneous T cell lymphoma, is unusual in that it both expresses a high level of IL 2 receptors and produces IL 2 (11). The IL 2 receptor on HUT102B2 cells as defined by the anti-Tac antibody migrates as a diffuse band in SDS-polyacrylamide gel electrophoresis with Mr = 47,000 to 53,000 (p50) (8). The IL 2 receptor found on normal activated peripheral blood lymphocytes differs from the receptor on HUT102B2 cells having Mr = 52,000 to 57,000 (p55) (10, 12, 13).

In the present report, IL 2 receptor-negative T cell lines were induced to express the receptor after incubation with the tumor-promoting phorbol ester, TPA. Phorbol esters induce a variety of changes in different cell types affecting morphology, growth potential, and apparent differentiation (for review see Ref. 14). In particular, human T leukemia cell lines undergo changes in the expression of cell surface antigens, including an increase in the receptor for sheep red blood cells, and a decrease in the level of terminal deoxynucleotidyltransferase activity in response to TPA (15, 16). These changes are associated with the maturation of normal T cells. The IL 2 receptor expressed by four different cell lines varied in size. The structural basis for these differences was investigated.

Incubation with TPA also induced phosphorylation of the IL 2 receptor on HUT102B2 cells. Recently protein kinase C has been identified as a receptor for phorbol esters (for review see Ref. 17). Incubation of cells with phorbol esters causes a rapid activation of kinase C which phosphorylates a wide variety of substrates including some membrane proteins.

**EXPERIMENTAL PROCEDURES**

**Cells**—The cells used, HUT102B2, CCRF-CEM, JURKAT, MOLT 4D, HPB-MLT, HPB-ALL, Tall-1, JM, HS-2, and RPMI 8402, were human T leukemic cell lines (18–20). The cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS. Ten percent horse serum was substituted for FBS for the CCRF-CEM cell line. To analyze the effects of phorbol esters, cells were resuspended at 5 × 10⁶ cells/ml and incubated with 50 ng/ml of TPA (Consolidated Midland Corp.) for 2 days unless otherwise stated.

**Radiolabeling Procedures**—Cell surface iodination was performed using lactoperoxidase and glucose oxidase as described (21). To metabolically label cells with [35S]methionine, cells were preincubated
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RESULTS

Induction of the IL 2 Receptor—Most human T cell lines do not express the receptor for IL 2 as assayed by their ability to adsorb out IL 2 activity or bind radiolabeled IL 2. However, it was found that the IL 2-binding assay underestimated by 10-fold the number of receptors on the HUT102B2 cell line detected by the anti-Tac antibody (1, 8). This was interpreted to mean that the IL 2-binding assay detected only the high-affinity receptors whereas the anti-Tac antibody recognizes both high- and low-affinity receptors (12). Therefore, to re-analyze this question, a panel of T cell lines was analyzed for the expression of receptors for IL 2 by measuring the binding of anti-Tac antibody in an indirect 32P-labeled antibody assay. The cell lines were also incubated with the phorbol ester, TPA, for 18 h prior to being assayed since TPA has been shown to cause cellular differentiation and changes in cell surface antigen expression in T cells and other cell lineages. All the cell lines analyzed except HUT102B2 were essentially negative for anti-Tac binding. However, among nine cell lines tested, CCRF-CEM showed a significant increase in anti-Tac binding after incubation with TPA. The plant lectin, phytohemagglutinin, which activates normal T cells and induces the expression of the IL 2 receptor had no effect on anti-Tac binding to CEM cells. The human T cell lines JURKAT, MOLT 4D, and RPMI 8402 displayed a moderate increase in anti-Tac antibody binding after incubation with TPA, whereas HPB-ALL, Tall-1, JM, HPB-MLT, and HSB-2 showed no significant change. The amount of IL 2 receptor on CEM cells detected by trace antibody binding increased with longer exposure to TPA and concomitantly the expression of the transferrin receptor decreased.

The induction of IL 2 receptors on CEM cells following exposure to TPA was analyzed quantitatively by flow cytometry. As shown in Fig. 1b, CEM cells initially did not express the IL 2 receptor. However, after 2 to 3 days incubation with 50 ng/ml of TPA, greater than 70% of the population was anti-Tac-positive. The expression of the antigen T3, which is found on all T cells and a small percentage of thymocytes, also increased upon exposure of CEM cells to TPA as reported previously (36) (Fig. 1c). Not all membrane proteins were affected by this treatment. The high level of expression of the T200 glycoprotein on CEM cells was not modulated by TPA. The major histocompatibility HLA-DR antigen is induced on activated T cells subsequent to the appearance of the IL 2 receptor. However, CEM cells were negative for HLA-DR before and after incubation with TPA (data not shown).
were stained as described (35) with (a) anti-Tac, that continued to grow were found to be IL-2 receptor negative. The IL-2 receptor expressed on HUT102B2 and TPA-induced cells.

The IL-2 receptors were immunoprecipitated from cell surface-iodinated cells, purified by SDS-PAGE, and digested with trypsin. The tryptic peptides were analyzed on thin-layer plates with high-voltage electrophoresis in the first dimension and chromatography in the second dimension. Ten to eleven labeled peptides were resolved but differences in the polypeptide moieties of the IL-2 receptors from HUT102B2 and TPA-induced CCRF-CEM cells could be detected.

Neuraminidase, Endo F, and Tunicamycin Treatment—The IL-2 receptor is a glycoprotein with N-linked and probably O-linked carbohydrates (10, 13). To determine if the size heterogeneity of the IL-2 receptor on different cell lines was due...
to glycosylation differences, the proteins were treated with Endo F to remove N-linked glycans or neuraminidase to remove sialic acid residues. As shown in Fig. 4, digestion with either Endo F or neuraminidase reduced the Mr of the IL 2 receptor on TPA-induced CEM or HUT102B2 cells by approximately 8,000 to 10,000. Incubation of the lysate with neuraminidase followed by immunoprecipitation with anti-Tac and digestion of the immunoprecipitate with Endo F further reduced the Mr of the IL 2 receptor but did not abolish the Mr heterogeneity among cell lines. It was not determined why neuraminidase and Endo F treatment of the JURKAT IL 2 receptor generated minor species of lower Mr than the major digestion product.

Leonard and co-workers (10) demonstrated that in the presence of tunicamycin, a 33,000-dalton precursor of the IL 2 receptor was detected in HUT102B2 cells. This protein is resistant to digestion by Endo F and neuraminidase suggesting that it is not glycosylated. The addition of asparagine-linked oligosaccharides gives rise to 35,000- and 37,000-dalton forms of the protein. Further processing of these glycans and other post-translational modifications including the presumptive addition of O-linked carbohydrates account for Mr = 47,000 to 53,000 observed for the mature receptor found on the cell surface. HUT102B2 and TPA-induced CEM cells were labeled with [35S]methionine in the presence of Tm to compare the precursor forms of the IL 2 receptors. In Fig. 5A, the glycosylated and nonglycosylated IL 2 receptors from the two cell lines are compared by SDS-PAGE. The metabolically labeled receptor from the TPA-induced CEM cells (lane 3) was approximately 8,000-daltons larger than the receptor on the HUT102B2 cells (lane 4) as observed for the surface-iodinated receptors. However, in the presence of Tm, a protein of Mr = 33,000 was isolated from both cell lines (lanes 5 and 6). Incompletely processed IL 2 receptor of Mr = 44,000 was also observed with the HUT102B2 cells (10). Thus, most of the apparent difference in size was due to differences in glycosylation or other post-translational processing. The nonglycosylated IL 2 receptors from HUT102B2 and TPA-induced CEM cells were also compared by two-dimensional gel electrophoresis with SDS-PAGE in the first dimension and isoelectric focusing in the second dimension. The glycosylated receptor is an acidic protein with an isoelectric point of 5.5 to 6.0 (10). The nonglycosylated protein was more basic with an isoelectric point of 6.5 to 7.0 (Fig. 5B). The IL 2 receptors from the two tunicamycin-treated cell lines focused as a single band at the same isoelectric point suggesting the conclusion that the polypeptide moieties of the two proteins are identical (Fig. 5B, lanes 1 and 2).

In Vivo Phosphorylation of the IL 2 Receptor—Several growth factor receptors, including the EGF and insulin receptors, are phosphorylated on tyrosine residues in response to ligand binding and have tyrosine kinase activity (37–39). The role of this phosphorylation in growth control is unknown. To determine if the IL 2 receptor has similar properties to other growth factor receptors, HUT102B2 cells and TPA-induced CEM or JURKAT cells were incubated with 32P04. No phosphorylation of the IL 2 receptor was observed (Fig. 6, lanes 1 and 2).
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FIG. 6. Phosphorylation of the IL 2 receptor in vivo. Cells were labeled with H$_3$32P04, lysed, and immunoprecipitated with anti-Tac as described under “Experimental Procedures.” The IL 2 receptors were isolated from: lane 1, surface iodinated HUT102B2 cells; lane 2, 32P-labeled HUT102B2 cells pulsed with 50 ng/ml of TPA for 15 min; lane 3, 32P-labeled untreated HUT102B2 cells; lanes 4–6, 32P-labeled HUT102B2, CCRF-CEM, and JURKAT cells, respectively, which were incubated with 50 ng/ml of TPA for 2 days prior to labeling. The immunoprecipitates were from 2 × 10$^6$ cell equivalents of each sample. The arrow indicates the M$_r$ of the IL 2 receptor from HUT102B2.

FIG. 7. Analysis of phosphoamino acids of IL 2 receptor from 32P-labeled HUT102B2 cells pulsed with TPA for 15 min. The acid hydrolysate of the labeled IL 2 receptor was resolved in two dimensions by electrophoresis toward the anode (left side) at pH 1.9 followed by electrophoresis toward the anode (top) at pH 3.5. The origin is designated by the arrow. Phosphoserine, (S), phosphothreonine (T), and phosphotyrosine (Y) standards were mixed with the labeled sample and co-analyzed.

lanes 3, 5, and 6). However, if HUT102B2 cells were incubated with TPA for 2 days, the receptor was phosphorylated (Fig. 6, lane 4). In addition to causing phenotypic changes and differentiation of cells, phorbol esters cause an increase in phosphorylation. This appears to be due to the rapid activation of protein kinase C which phosphorylates a wide variety of substrates (17). Since changes in phosphorylation caused by kinase C are rapid, HUT102B2 cells were preincubated with 32P0, for 4 h to label the cellular pools and then pulsed with TPA for 15 min. This also caused a dramatic increase in phosphorylation of the receptor (Fig. 6, lane 2). A similar TPA pulse of 32P-labeled TPA-induced CEM cells did not result in phosphorylation of the receptor (data not shown).

The phosphorylated IL 2 receptor was eluted from an SDS gel and acid hydrolyzed to determine the phosphoamino acid composition. The hydrolysate was analyzed on thin-layer plates by two-dimensional electrophoresis. The major phosphorylated residue was serine with a minor amount of phosphothreonine (Fig. 7). No phosphotyrosine was observed.

DISCUSSION

The present study demonstrated that the IL 2 receptor can be induced by TPA on the receptor-negative human T leu-

kemic cell lines, CCRF-CEM, MOLT 4D, and JURKAT. Leukemic cell lines are thought to represent clonal expansions of cells blocked at specific stages of normal hematopoietic cell differentiation. The cell lines, CCRF-CEM, MOLT 4D, and JURKAT were established from patients with acute lymphoblastic leukemias (ALL) and have phenotypes representative of immature T cells. The HUT102B2 cell line, derived from a patient with a cutaneous T lymphoma, has characteristics of a mature T cell. Within the normal T cell lineage, the IL 2 receptor is expressed on mature T cells. Induction of the receptor with TPA correlates with other previously reported changes in the expression of T cell antigens and activities characteristic of normal T cell differentiation (15, 16, 36, 40). Activation of normal T cells by antigen or mitogen induces the expression of the IL 2 and transferrin receptors at the cell surface within the first 4 to 6 h (3, 4). Other membrane antigens such as HLA-DR appear later and by 24 h DNA synthesis is initiated (2–4). The level of expression of the IL 2 receptor and the rate of growth are regulated by the availability of IL 2 (41). In contrast to normal T cell activation, in this report, the expression of the transferrin receptor decreased on the leukemic cell lines in response to TPA and paralleled the inhibition of proliferation by TPA. Thus the expression of IL 2 receptors and transferrin receptors is not obligatorily linked. The HL-60 promyelocytic leukemic cell line is induced by TPA to differentiate along the monocytic/macrophage pathway as assessed by changes in morphology and membrane antigen expression (42, 43). As observed for the T leukemic cell lines, the expression of the transferrin receptor on HL-60 cells decreases and cell growth is arrested as the cells terminally differentiate in response to TPA (44).

The IL 2 receptor expressed by the four cell lines analyzed migrated on SDS gels as a diffuse band varying in M$_r$ between 50,000 and 70,000. The M$_r$ heterogeneity appeared to be due to differences in post-translational modifications. No difference between the IL 2 receptors isolated from the surface of HUT102B2 and TPA-induced CEM cells was detected by two-dimensional peptide map analysis. Furthermore, no differences in the M$_r$ or isoelectric point of the receptors from the two cell lines metabolically labeled in the presence of tunicamycin were observed when they were compared by one- and two-dimensional gel electrophoresis. The primary action of tunicamycin is to inhibit N-linked glycosylation (45). However, the M$_r$ variability of the IL 2 receptor was not due exclusively to differences in the N-linked glycans. Endoglyco-

sidase F, which cleaves N-linked oligosaccharides, reduced the M$_r$ of the glycosylated IL 2 receptors on both HUT102B2 and induced CEM cells by approximately 8,000 to 10,000 to 41,000 and 51,000, respectively, indicating that the N-linked carbohydrates on the two molecules were similar in M$_r$. The Endo F-treated receptors from both cell lines still differed in M$_r$, and each had additional, possibly O-linked, sialic acid-containing carbohydrates which could be the source of the M$_r$
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Phosphorylation of the IL 2 receptor may be catalyzed by protein kinase C which is widely distributed in the tissues of many species and is found in particularly high levels in lymphocytes (for review see Ref. 17). The enzyme, which phosphorylates serine and threonine residues, is found in both the cytosol and membrane fractions of cells but upon activation, the fraction of kinase C associated with the membrane increases (48). Protein kinase C is normally activated in the presence of Ca\textsuperscript{2+} and phospholipid by diacylglycerol formed during the turnover of phosphatidylinositol but tumor-promoting phorbol esters can replace the requirement for diacylglycerol (49, 50).

An increase in the phosphorylation of the receptors for insulin (51), somatomedin C (51), EGF (52, 53), and transferrin\textsuperscript{a} can also be induced by phorbol esters. The EGF receptor has been shown directly to be a major substrate for kinase C in vitro (52). Phosphorylation of growth factor receptors by kinase C may play a role in regulating their activity. Phosphorylation of the EGF receptor by kinase C decreases the EGF-stimulated tyrosine kinase activity of this receptor (52). Incubation of some cell lines with phorbol esters results in a decrease in binding of insulin or EGF due to either a decrease in the number or affinity of the receptors depending on the cell line (14, 54, 55). Consequently the demonstration that the IL 2 receptor may also be a substrate for kinase C raises the possibility that the regulation of T cell growth may share features in common with other cell types.

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heterogeneity. In contrast, the IL 2 receptor precursors isolated from both cell lines after tunicamycin treatment were smaller having an identical $M_r = 33,000$. Inhibition of glycosylation by tunicamycin prevents the migration of some proteins to the plasma membrane (46). In the case of the IL 2 receptor, tunicamycin may indirectly prevent translocation of the receptor molecules from the endoplasmic reticulum to other membrane organelles where additional post-translation modifications occur.

Previously, it was reported that the IL 2 receptor found on activated normal human T cells was 5,000 to 8,000 daltons larger than the receptor on HUT102B2 cells (10, 12, 13). Since HUT102B2 cells are infected with human T cell leukemia virus, it was possible that these cells expressed an aberrant receptor due to the virus. The current observation of extensive $M_r$ heterogeneity indicates that variations in processing of the receptor are cell line specific and not due to viral infection.

It was not determined if the differences in the IL 2 receptors expressed by the four cell lines affected the binding of IL 2. However, the high-affinity receptors on HUT102B2 and activated normal peripheral blood blood cells displayed the same dissociation constant suggesting that the post-translational differences do not affect ligand binding (1).

The anti-Tac antibody was reported to precipitate additional proteins of $M_r$, 113,000 and 180,000 from $[^{35}S]$methionine-labeled HUT102B2 cells (8, 10). They were not observed when the cells were surface iodinated. In this study no metabolically labeled higher $M_r$ bands were observed in the anti-Tac immunoprecipitates that were not precipitated by S. aureus alone. However, proteins of $M_r$, 75,000 and 27,000 (p27) were consistently observed in the anti-Tac immunoprecipitates from iodinated HUT102B2 cells. They were not co-precipitated with the IL 2 receptor from the other three cell lines analyzed. The p27 polypeptide was insensitive to Endo F but sensitive to neuraminidase suggesting that it did not have N-linked oligosaccharides but might have O-linked sugars. The identity of this protein is unknown.

The mechanism by which a growth factor binding to its receptor transmits a signal across the membrane is unknown. Phosphorylation of growth factor receptors may be one of the initial signals in a cascade of events which affect cell growth. For example, one of the earliest detectable cellular changes induced by epidermal growth factor binding to its receptor is the phosphorylation of several proteins including the receptor itself (47). The IL 2 receptor on untreated HUT102B2 cells, which constitutively produce IL 2, was not phosphorylated in vivo but exposure to TPA caused a rapid phosphorylation of the protein on serine and threonine residues. TPA-induced phosphorylation of the IL 2 receptor on PHA-stimulated peripheral blood lymphocytes was also observed. Phosphorylation of the IL 2 receptor induced on CEM or JURKAT cells was not detected even using five times more cells than HUT102B2. The difference between the cell lines may be because: 1) the level of phosphorylation was below detection in CEM and JURKAT cells, 2) the rate of turnover of the receptor varied between cell lines, or 3) the amount of phosphatase activity in the cell lines varied. The CEM and JURKAT cells were incubated with TPA for 2 days before labeling with $[^{32}P]O_4$, but similar treatment of the HUT102B2 cells did not prevent phosphorylation of the IL 2 receptor. Phosphorylation of the IL 2 receptor presumably occurs on the cytoplasmic side of the plasma membrane indicating that the IL 2 receptor is a transmembrane protein.

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* D. A. Shackelford and I. S. Trowbridge, unpublished data.
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