Stimulation of the Antigen and Interleukin-2 Receptors on T Lymphocytes Activates Distinct Tyrosine Protein Kinases*

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The events critical to the mounting of an immune response include the antigenic activation of T lymphocytes and their resultant interleukin 2 (IL-2)-dependent proliferation. Activation signals in response to antigen are generated by the T cell antigen receptor complex (TCR) in conjunction with certain accessory receptors such as CD4 and CD8 (1). The TCR is composed of two functional components. One component is the clonotypic heterodimer, Tc, which recognizes and binds antigen. The other component is the multimeric CD3 receptor which has an invariant structure and appears to be responsible for signal transduction by the antigen receptor. T cell activation results in a series of biochemical events leading to the production and secretion of IL-2 and the expression of high affinity receptors for this growth factor (2, 3). The interaction of IL-2 with its receptor results in the proliferative phase of the T cell response.

The high affinity IL-2 receptor (Kd ~ 10 pm) consists of two distinct subunits with molecular weights of 55,000 (p55) and 75,000 (p75) (4-8). Each subunit can also bind IL-2 independently of the other. The p55 subunit binds IL-2 with a low affinity (Kd ~ 10 nm) while the p75 subunit binds IL-2 with an intermediate affinity (Kd ~ 0.1-1.5 nm). The signal transduction capability of the high affinity receptor resides in the p75 subunit since when expressed by itself this subunit is capable of transducing the mitogenic stimulus of IL-2 (9-12).

Signal transduction by the TCR and IL-2 receptor involves the activation of both serine/threonine and tyrosine protein kinases (13-19). The precise role of these kinases in signal transduction by these two receptors is not clear. However, given the ample precedence for a primary role for tyrosine protein kinases in signal transduction in other cell types it is reasonable to hypothesize that activation of a tyrosine protein kinase is the initial event in signal transduction by both the TCR and the IL-2 receptor. The intracellular domain of several growth factor receptors contain a tyrosine protein kinase that is activated upon stimulation of the receptor (20). While the TCR and IL-2 receptor are coupled to the activation of a tyrosine protein kinase, in contrast to these other growth factor receptors they do not contain a tyrosine protein kinase domain (21-24). Therefore these receptors must activate tyrosine protein kinase(s) by an indirect mechanism.

In addition to the TCR and IL-2 receptor the stimulation of several other T cell surface receptors also results in the activation of a tyrosine protein kinase (19, 24, 25). T cells express several tyrosine protein kinases whose structure requires an indirect mode of regulation (26-28). The potential regulation of these enzymes by multiple receptors raises questions as to what receptor is coupled to which enzyme and whether there is interaction between various pairs of receptors and their kinases. One way to examine the relationships between the tyrosine protein kinases regulated by these different receptors is to compare the tyrosine phosphorylations that occur in response to specific stimulation on a T cell where the receptors are concurrently expressed. This approach reveals whether activation of different receptors results in common or dissimilar sets of proteins being phosphorylated on tyrosine residues, thus providing evidence as to whether a common or distinct tyrosine protein kinase is being

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The abbreviations used are: IL-2, interleukin-2; TCR, T cell antigen receptor complex; SDS, sodium dodecyl sulfate.
activated (24). Using this approach we have recently provided evidence which indicates that stimulation of the CD2 and CD3 receptors on T cells results in the activation of the same tyrosine protein kinase (19). An in vitro comparison of the tyrosine protein kinases activated by the TCR and the IL-2 receptor requires a cloned cell line that co-expresses both receptors. The human leukemic T cell line Hut 78 expresses both the TCR complex (29) and the intermediate affinity (~75 IL-2 receptor (8, 18), similar to that on normal quiescent human T cells (1, 9, 10, 29). Thus, Hut 78 cells appeared to be a suitable model for studying tyrosine protein kinase activation through either of these receptors.

MATERIALS AND METHODS

Cells and Reagents—Hut 78 and CTLL-2 cell lines were purchased from the American Type Culture Collection (ATCC). Jurkat, clone 77 6.8 was obtained from Dr. Kendall Smith, Dartmouth Medical School. The cell lines were maintained on RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (HyClone). Limiting dilutions (~0.1 cell/well) of Hut 78 cells were dispersed into individual wells of a 96 well flat bottomed microtiter plate. Following 2 weeks in culture 8% of the wells contained clones. The cells were transferred into 6-well plates and grown until a 5 x 10^5 cell/ml density was achieved. The cell clones were then placed into culture flasks.

The preparation and use of antibodies against phosphotyrosine conjugated to human immunoglobulin have been described in previous publications (14, 30, 31). The OKT3 hybridoma was obtained from the ATCC and maintained in Iscove’s Modified Dulbecco’s Medium supplemented with 10% NuSerum (Collaborative Research). OKT3 antibodies were purified from the medium of the OKT3 hybridoma using protein A-Sephadex. Recombinant IL-2 was a gift from Cetus Corp. 125I- Protein A was obtained from ICN Radiochemicals. [3H] Thymidine was obtained from Amersham Corp. The molecular weight standards were purchased from Bio-Rad. The phorbol ester 4-phorbol 12-myristate 13-acetate was obtained from Sigma. Cytoscint mixture was supplied by Fisher.

IL-2 Secretion—Hut 78.3 cells were washed three times with RPMI 1640 medium and resuspended in RPMI with 10% fetal calf serum at a density of 5 x 10^4 cells/ml. The cells were either left as a control, stimulated with 2 μg/ml soluble OKT3 antibodies, or co-stimulated with 2 μg/ml soluble OKT3 antibodies in conjunction with 100 ng/ml 4-phorbol 12-myristate 13-acetate. Following a 32-h incubation at 37°C, 5% CO₂ the medium was removed from the cells by centrifugation, passed through 0.22-μm filters, and stored at 4°C. CTLL-2 cells were washed and resuspended in RPMI 1640 medium with 10% fetal calf serum. 100-μl aliquots containing 5 x 10^4 cells were dispersed into wells of a flat-bottomed 96-well plate. The wells, in triplicate, either received serial dilutions of Cetus IL-2 beginning with an initial concentration of 50 pM or serial dilutions of the media from the Hut 78.3 cells beginning with 100-μl additions of medium per well. Final sample volume was 200 μl/well. Following a 20-h incubation at 37°C, 5% CO₂, 20 μl of [3H]thymidine (1 μCi in 20 μl of RPMI 1640 medium) was added to each sample for a 4-h pulse. The cells were harvested onto glass-fiber filter paper in a filter manifold. [3H]Thymidine intake was quantitated by counting the filter paper in Cytoscint mixture in a scintillation counter.

Cellular Stimulation and Monitoring of In Vivo Tyrosine Phosphorylation—Cells were washed three times with 37°C RPMI 1640 medium. The cells were resuspended in RPMI and dispersed into 16 x 100 glass tubes with 1.5 x 10^5 cells/200 μl/sample. The samples were either left as controls or stimulated with saturating concentrations of IL-2 (1-2 nm) or OKT3 antibodies (1-2 μg/ml) as indicated in either the text or the figure legends. All incubations were at 37°C, 5% CO₂ using 10% fetal calf serum using. The reactions were terminated by the addition of 150 μl of SDS sample buffer followed by a 5-min incubation in a boiling water bath. Immunoblots—Samples previously boiled with the reducing SDS sample buffer were analyzed by SDS-polyacrylamide gel electrophoresis. Following electrophoresis on 3-mm 7.5% polyacrylamide gels the gels were stained, blotted onto 0.45 μm nitrocellulose with a 16V current for 16 h. The samples were probed with antibodies and the immune complexes detected with 125I-protein A. The specificity of the phosphotyrosine antibodies has been extensively documented in previous publications (14, 31).

RESULTS AND DISCUSSION

To address whether similar or distinct tyrosine protein kinases were activated following TCR and IL-2 receptor stimulation, it was necessary to utilize a cell line that co-expresses both receptors. This ensures that any differences in tyrosine phosphorylation are due to the kinases and not the cells. To guarantee that we were working with an individual clone expressing both the TCR and IL-2 receptor concurrently, the Hut 78 cell line was cloned and recloned by limiting dilution (<0.1 cell/well). The clone utilized for this investigation, Hut 78.3, was chosen based on its ability to respond to TCR and IL-2 receptor stimulation as demonstrated in the experiments described below. Monoclonal antibodies against the CD3 component of the TCR simulate antigenic stimulation in that under appropriate conditions they can induce both IL-2 production and the expression of the high affinity IL-2 receptor. We utilized OKT3 antibodies, specific for human CD3 (32, 33), and IL-2 to examine the tyrosine protein kinase activity associated with T cell activation and proliferation.

Ability of Hut 78.3 Cells to Produce IL-2—The TCR expressed by Hut 78.3 cells was initially characterized by determining if this receptor was functional and able to induce a physiological response. In an adaptation of a procedure utilized with the extensively characterized human leukemic T cell line Jurkat, Hut 78.3 cells were stimulated with soluble OKT3 antibodies alone or in combination with PMA, a tumor promotor that directly activates protein kinase C, to determine if the cells were capable of synthesizing and secreting IL-2 (34). The cell-free medium from the stimulated Hut 78.3 cells was incubated with the IL-2-dependent cell line CTLL-2 in a 24-h [3H]thymidine assay. The IL-2 secreted by Hut 78.3 cells stimulated with OKT3 antibodies alone was equivalent to basal levels (Fig. 1). However, when the cells were co-stimulated with OKT3 antibodies together with PMA a significant increase in IL-2 secretion was observed. This corresponds to investigations with Jurkat cells and normal T cells where two synergistic signals were required for activation; the first supplied by monoclonal antibodies directed against the TCR and the second by a phorbol ester (34-36). These results indicate that the TCR on Hut 78.3 cells, similar to the parental cell line (37), is capable of generating signals that induce cellular activation resulting in IL-2 production.
Stimulation of Hut 78.3 Cells with OKT3 Antibodies Results in Increased Protein Tyrosine Phosphorylations—To determine if the TCR on Hut 78.3 cells is coupled to the activation of a tyrosine protein kinase the cells were stimulated with OKT3 antibodies and processed for immunoblots with phosphotyrosine antibodies. The basal and OKT3-stimulated phosphotyrosine profile of Hut 78.3 cells are shown in the autoradiogram of a blot with phosphotyrosine antibodies in Fig. 2. OKT3 induced an increase in tyrosine phosphorylation of several proteins with apparent molecular weights ranging from 115,000 to 42,000. In order to show that these tyrosine phosphorylations are representative of T lymphocytes the effects of TCR stimulation on protein tyrosine phosphorylations in another T cell line Jurkat are also shown in Fig. 2. Although the intensities of some of the bands differ between these cell lines the molecular weights of the majority of the proteins with increased tyrosine phosphorylation following stimulation with OKT3 are common to both Hut 78.3 and Jurkat cells. When proteins were separated on 15% acrylamide gels, OKT3 was observed to also stimulate the tyrosine phosphorylation of a 21-kDa protein in both cell lines (data not shown). This protein is likely to be the \( \xi \) subunit of the antigen receptor that Patel et al. (13) reported undergoes rapid tyrosine phosphorylation upon stimulation of the CD3 receptor with anti-CD3 antibodies.

**Different Sets of Substrates Are Phosphorylated by the TCR and IL-2 Receptor Activated Tyrosine Kinases—**Signal transduction by the TCR on Hut 78.3 cells is representative of other T cells based on the ability of this receptor to induce IL-2 secretion and to increase protein tyrosine phosphorylations. We have previously demonstrated IL-2-dependent tyrosine phosphorylations in the parental Hut 78 cell line (18). These phosphorylations are similar to those that occur in several T cell lines and in normal human T cells (14, 18). In order to compare the tyrosine kinase(s) activated by the TCR and IL-2 receptor we examined the proteins phosphorylated following stimulation of Hut 78.3 cells with OKT3 or IL-2.

Equal numbers of Hut 78.3 cells were incubated with either OKT3 or IL-2 at concentrations and times previously determined to elicit maximal responses. The increases in protein tyrosine phosphorylation stimulated by either IL-2 or OKT3 are shown in Fig. 3. As can be seen in the resultant autoradiograms the two stimuli cause completely different patterns of increases in protein tyrosine phosphorylations. IL-2 induced the phosphorylation of proteins on tyrosine residues with molecular weights of 100,000, 94,000, 84,000, and 57,000. In contrast, in the experiment shown in Fig. 3 OKT3 induced increases in the tyrosine phosphorylation of proteins at 115,000, 107,000, a broad and intense band at 78,000, and a protein at 42,000. Thus while stimulation of both the TCR and the IL-2 receptor result in the activation of tyrosine protein kinases, these kinases act on completely different sets of substrates.

**TCR and IL-2 Receptor Stimulations Induce Tyrosine Phosphorylations with Different Time Courses—**Hut 78.3 cells were incubated with either OKT3 or IL-2 for increasing increments of time up to 60 min. The OKT3-induced increases in phospotyrosine in the 78-kDa protein and the IL-2-dependent increases in the 100 kDa protein were quantitated from the autoradiograms. The relative amount of tyrosine phosphorylation in these proteins was graphed as a function of the time of the incubation (Fig. 4). The OKT3-induced phosphorylations achieved maximal levels within 2.5 min, the earliest time point taken. In other experiments maximal phosphorylations were observed within 1 min (data not shown). By the
for the indicated times in minutes with either 2 μg/ml OKT3 or 2 nM IL-2. The samples were processed for an immunoblot with phosphotyrosine antibodies. The OKT3-induced increase of phosphotyrosine in the 78-kDa protein (C) and the IL-2-induced increase of phosphotyrosine in the 100-kDa protein (E) were quantitated by scanning the autoradiograms with a densitometer. The peak heights are graphed as percent of maximum.

5-min time point the response was beginning to decline and after 60 min of stimulation the tyrosine phosphorylation of the 78-kDa protein was near basal conditions. The time course of the OKT3-induced tyrosine phosphorylation of all of the other proteins shown correlated with the 78-kDa protein with the exception of the 21-kDa protein. Although the 21-kDa protein also showed maximal tyrosine phosphorylation within 2.5 min of stimulation with OKT3, the phosphorylation of this protein was still maximal after 30 min when the OKT3-induced tyrosine phosphorylations of the other proteins were significantly decreased (data not shown). The onset and decline of the Hut 78.3 TCR-induced protein tyrosine phosphorylations are similar to that observed following the stimulation of Jurkat cells with OKT3 (19).

In contrast to the TCR response the IL-2-stimulated increases in tyrosine phosphorylation do not approach their maximal levels until 15 min after the initiation of stimulation (Fig. 4). In addition, after 60 min of stimulation the IL-2-dependent phosphorylations remain elevated while the TCR-induced phosphorylations have returned to basal levels. The time course of the IL-2-induced phosphorylation of the other phosphotyrosine bands correlates with that of the 100-kDa protein (data not shown). This time course of the IL-2 response is similar to that which has been observed in other T cell lines and in normal human T cells (14, 17, 18). Thus, based on the initiation and longevity of substrate phosphorylation the tyrosine protein kinases activated following TCR or IL-2 receptor stimulation appear to have different constraints on their regulation.

Tyrosine Phosphorylations Induced by TCR and IL-2 Receptor Stimulation Can Occur Simultaneously in Hut 78.3 Cells—
If the tyrosine protein kinases activated by stimulating the IL-2 and OKT3 receptors are independent and distinct then stimulation of both receptors should result in concurrent increases in tyrosine phosphorylation of both sets of substrates. The ability of OKT3 or IL-2 to induce tyrosine phosphorylation was examined following pretreatment of Hut 78.3 cells with the other agent. The cells were stimulated with IL-2 or OKT3 for times sufficient for inducing maximal levels of tyrosine phosphorylation. These pretreated cells were then either harvested for blotting or stimulated with the other ligand. The results of this experiment are displayed in Fig. 5.

The cells treated only with IL-2 or OKT3 are shown in lanes 2 and 3. In lane 4 the cells were pretreated with OKT3 for 2.5 min prior to a 15-min stimulation with IL-2. The tyrosine phosphorylation that is induced by IL-2 was unaffected by the prior stimulation with OKT3 (compare bands marked with arrows in lanes 2 and 4). Since the pretreatment of cells with OKT3 in lane 4 was followed by a 15-min incubation with IL-2 the protein phosphorylations resulting from TCR stimulation are less intense than that observed in cells treated only with OKT3 for 2.5 min (refer to the time course of OKT3-stimulated phosphorylations; Fig. 4).

In a similar manner IL-2 pretreatment of cells for 15-min did not affect the subsequent tyrosine phosphorylations induced by OKT3 (lane 5) when compared to cells stimulated only with OKT3 (lane 3). In addition, when Hut 78.3 cells were simultaneously co-stimulated with OKT3 and IL-2 together all of the protein tyrosine phosphorylations induced via either the TCR or IL-2 receptor were observed (data not shown). The time course of the tyrosine phosphorylations induced by one agent was not affected by the presence of the other agent. These results not only provide further evidence that the TCR and the IL-2 receptors are coupled to different tyrosine protein kinases but also demonstrate that the tyrosine phosphorylations induced by IL-2 or OKT3 are unaffected by acute stimulation of the other receptor.
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