The growth factor IL-2 activates p21ras proteins in normal human T lymphocytes.

J D Graves, J Downward, M Izquierdo-Pastor, S Rayter, P H Warne and D A Cantrell

*J Immunol* 1992; 148:2417-2422; ;
http://www.jimmunol.org/content/148/8/2417

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
THE GROWTH FACTOR IL-2 ACTIVATES p21ras PROTEINS IN NORMAL HUMAN T LYMPHOCYTES

JONATHAN D. GRAVES, JULIAN DOWNWARD, MANUEL IZQUIERDO-PASTOR, SYDONIA RAYTER, PATRICIA H. WARNE, and DOREEN A. CANTRELL.

From the *Lymphocyte Activation Laboratory and tSignal Transduction Laboratory, Imperial Cancer Research Fund, P.O. Box 123, Lincoln’s Inn Fields, London WC1A 3PX, U.K.

The T cell growth factor IL-2 induces T cell progression through the cell cycle and ultimately controls T cell mitosis. Here we show that the guanine nucleotide-binding proteins p21ras may be involved in IL-2 signal transduction pathways. IL-2 causes a rapid and prolonged activation of p21ras in both murine and human T cells. The concentration-dependence of IL-2-mediated stimulation of p21ras correlated with IL-2 stimulation of T cell proliferation, which indicates that p21ras activity can be controlled by signals generated via the interaction between IL-2 and its high affinity cellular receptor. These results suggest that p21ras may play a role in the regulation of T cell growth by IL-2.

The products of ras proto-oncogenes (H-, N-, and K-ras) are thought to play a role in signaling pathways that regulate cellular growth and differentiation (1). The ras genes encode highly related 21 kDa proteins (p21ras), located at the inner surface of the plasma membrane, that bind GTP and catalyze its hydrolysis to GDP. Originally identified in certain acutely transforming retroviruses, activated ras oncogenes are capable of transforming mammalian cells in culture and have been implicated in the formation of a high proportion of human tumors. Oncogenic p21ras proteins result from point mutations of cellular ras genes that either inhibit the GTPase activity of p21ras or stimulate guanine nucleotide exchange and allow p21ras to accumulate in the GTP-bound state (2, 3). Ras proteins are able to stimulate cell growth and transformation when GTP bound ("active") but not when GDP bound ("inactive") (4, 5).

Despite accumulated evidence for p21ras involvement in the transduction of cellular growth signals, the precise nature of both upstream regulatory stimuli and downstream effector function of p21ras has remained obscure. However, in lymphocytes, triggering of the TCR/CD3 Ag complex or stimulation of PKC induces the accumulation of p21ras in the active GTP-bound form by a mechanism that involves the inhibition of cellular factors that stimulate p21ras GTPase activity (6, 7). Increases in the proportion of GTP-bound p21ras have also been reported in murine NIH3T3 fibroblasts on stimulation by a variety of oncosgenic or growth factor receptor tyrosine kinases (8) and in lymphocytes and myeloid cells p21ras is thought to act as a transducer of signal from cytokines such as IL-2 and IL-3 (9).

Activation of the TCR/CD3 complex promotes movement of T lymphocytes from G0 to the G1 phase of the cell cycle, induction of IL-2 production, and expression of high affinity IL-2R (10). Progression of T cells through G1 on to the S phase of the cell cycle is subsequently regulated by the interaction between IL-2 and the high affinity IL-2R (10, 11). Thus, although the TCR/CD3 complex determines the clonal specificity of the T cell response to Ag, IL-2 is the key regulator of T cell growth and controls the magnitude of the proliferative response. Although it is clear that IL-2R occupancy results in the transmission of a proliferative stimulus, the mechanism by which the IL-2R transduces its growth-regulatory signal is poorly understood. In the present study, we explored the effect of IL-2 on p21ras activation and examined the intracellular signaling pathway that couples the IL-2R to p21ras proteins in T cells.

MATERIALS AND METHODS

The CD3 antibody UCHT1 was purified from hybridoma supernatants by protein A affinity chromatography. [32P]P50 phosphoprotein and [32P]GTP were obtained from Amersham International (Amersham, Buckinghamshire, UK). Phorbol dibutyrate was obtained from Calbiochem (Lewes, Sussex, UK) and other biochemicals were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Phycocyanin was obtained from Sigma Chemical Co (Poole, Dorset, UK). Phosphomannitin was obtained from Wellcome Diagnostics (Dartford, Kent, UK).

Cells. Human T lymphoblasts were prepared as described (10, 11), by stimulating PBMC (10⁶/ml) in RPMI 1640/10% FCS with 5 μg of PHA/ml for 72 h. After washing, cells were maintained in exponential growth in RPMI 1640/10% FCS supplemented with 0.1 nM rIL-2. Before use, cells were deprived of IL-2 and allowed to quiesce in the G0/G1 stage of the cell cycle. Jurkat cells and the IL-2R-positive T cell clone CTLL-2 were maintained in culture as described (12).

p21ras Activation. p21ras proteins were immunoprecipitated with antibody Y13-259 from activated or quiescent T cells in which guanine nucleotides were [32P]P50-labeled by labeling cells biologically with [32P]P50 phosphoprotein as described (6). For p21ras immunoprecipitations, cell lysis was performed as described (6). In 50 nM HEPES, pH 7.4, 1% Triton X-100, 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 10 nM benzamidine, 10 mM leupeptin, 10 μg/ml aprotinin, and 10 μg/ml soybean trypsin inhibitor. Nuclei were removed by centrifugation at 15,000 g for 2 min and 0.5 M NaCl, 0.5% deoxycholate, and 0.05% SDS were added to the lysate. Immunoprecipitation was for 40 min using antibody Y13-259 pre-coupled to protein A-agarose via rabbit anti-rat Ig. Immunoprecipitates were washed with 8 x 1 ml of 50 mM HEPES, pH 7.4, 500 mM NaCl, 5 mM MgCl2, 0.1% Triton X-100, 0.005% SDS, and nucleotide eluted with 2 mM EDTA, 2 mM dithiothreitol, 0.2% SDS, 0.5 mM GTP, and
Activity of cellular p21H ras GTPase-activating protein. Lysates were made from 5 × 10⁶ T cells that were either quiescent or activated as indicated and assayed for GAP activity at a range of dilutions. Okadaic acid (100 nM) was included in all activated and control incubations. Lysates made by disrupting cells in 0.5 ml of 10 mM Pipes buffer, pH 7.2, containing 120 mM NaCl, 5 nM free Mg²⁺, 100 nM free Ca²⁺, 1% Triton X-100, 10% glycerol, and 100 nM okadaic acid. The lysate was centrifuged at 300,000 × g for 10 min and the supernatants assayed for GAP activity. Fifty nanograms of pure bacterially expressed wild-type human p21H ras were allowed to bind 25 μCi of [γ-³²P]GTP in the presence of 5 mM EDTA at 37°C for 5 min. MgCl₂ 10 mM, 1 mM GTP, and 1 mM GDP were then added and 0.75-mCi aliquots added to varying amounts of GTP extracts made up to the same volume with homogenization buffer. The mixtures were incubated for 10 min at 37°C. The reactions were stopped by addition of 10 volumes of cold lysis buffer containing 0.5 M NaCl, and p21H ras was immunoprecipitated using antibody Y13-259. The proportion of [γ-³²P]GTP to total labeled nucleotide on p21H ras was calculated after separation of GTP from GDP by TLC and quantitation by direct scanning for β-radiation.

RESULTS

To investigate the possibility that p21H ras functions in the intracellular signaling pathways initiated by triggering the IL-2R, the effect of IL-2 treatment on the activation state of endogenous p21H ras was measured in intact T cells. For these studies, IL-2R-positive human T lymphoblasts, the murine IL-2-dependent T cell clone CTLL-2, and a population of IL-2R negative cells, the human T leukemia cell line Jurkat, were used. T cells were metabolically labeled with [³²P]orthophosphate, and p21H ras was immunoprecipitated from cell lysates and the bound guanine nucleotides were analyzed by TLC.

In the absence of stimulation, p21H ras was almost entirely in the GDP-bound state (~95%) in all three cell types (Fig. 1). However, stimulation of T lymphoblasts with IL-2 induced the accumulation of GTP on p21H ras, increasing the amount of GTP bound from 5% to ~35% of total labeled nucleotide. Treatment of T lymphoblasts with the phorbol ester PDBU induced a greater stimulation of p21H ras than IL-2 (~80% accumulation of p21H ras-GTP complexes). Both PDBU and IL-2 treatment were also observed to stimulate an increase in the level of GTP on p21H ras in murine CTLL-2 cells such that the proportion of GTP-bound cellular p21H ras rose to ~40% and 60%, respectively (Fig. 1). In contrast, IL-2 did not influence GTP levels on p21H ras in the IL-2R-negative human T leukemia line Jurkat. In these cells, PDBU was able to increase the amount of GTP bound to p21H ras from ~5% to ~90% of labeled p21H ras-associated nucleotide (Fig. 1). T cells express p21H ras and p21N ras and the Y13-259 mAb used for these experiments recognizes both of these Ras proteins. In parallel experiments, we observed that p21H ras-GTP complexes could be immunoprecipitated from IL-2, TCR, and PDBU-activated T cells with the Y13-238 mAb, which recognizes p21H ras but not p21N ras. However, approximately twofold more p21H ras-GTP complexes were immunoprecipitated with Y13-259 than Y13-238, which indicates that IL-2 and the TCR, as previously described for PKC, activate both p21H ras and p21N ras.

Kinetics of p21H ras stimulation by IL-2. IL-2 binds rapidly, within minutes, to its physiologically active high affinity receptor and yet a minimum 4 to 6 h duration of IL-2R occupancy is necessary to commit T cells to progression into the S phase of the cell cycle and hence mitosis (12). Accordingly, we explored the kinetics and duration of IL-2-mediated p21H ras activation to determine whether IL-2-induced activation of p21H ras was an immediate response to the occupation of IL-2R, or whether a prolonged period of IL-2R occupancy was necessary for p21H ras stimulation.

Accumulation of p21H ras-GTP complexes in response to IL-2 treatment of T lymphoblasts was detectable within 1 min, reached a maximum of 40% by 3 min, and was maintained over a period of 20 min (Fig. 2A). Over a more prolonged time course, maximal IL-2-induced accumulation of p21H ras-GTP complexes was maintained over a period of 30 to 60 min (Fig. 2B). Moreover, in IL-2-stimulated T lymphoblasts, ~15% of cellular p21H ras was in an active GTP-bound state 4 h after exposure to IL-2 (Fig. 2B). These data suggest that p21H ras activation in response to IL-2R occupancy is rapid and persistent.

IL-2 concentration dependence for stimulation of p21H ras and DNA synthesis. T cells express at least two IL-2-binding molecules, the IL-2Rα or p55 subunit, which has a relatively low affinity (Kd ~ 10⁻⁸ M) and the IL-2Rβ or p75 subunit which has a Kd of ~10⁻⁹ M. These two

Figure 1. Effect of IL-2 on the nucleotide bound to p21H ras. TLC of the nucleotides eluted from immunoprecipitates of p21H ras from [³²P]orthophosphate-labeled human peripheral blood-derived T lymphoblasts, murine T-leukemic CTLL cells, and human T-leukemic J6-Jurkat cells. Cells were unstimulated or stimulated for 5 min with 20 ng/ml IL-2 or 50 ng/ml PDBU before lysis and immunoprecipitation of p21H ras with mAb Y13-259 as described. The position at which GTP and GDP standards ran is indicated. The data given are representative of the results obtained from several experiments.
subunits physically associate in the cell membrane to yield the high affinity ($K_d \sim 10^{-11}$ M) IL-2R complex that mediates the proliferative response to IL-2 (10). To determine whether the binding of IL-2 to its high affinity receptor was responsible for inducing the intracellular signals that activate p21ras, the concentration of IL-2 necessary to induce proliferation in T cells was compared with the concentration of IL-2 required to reduce p21ras activation. Stimulatory effects of IL-2 on p21ras were detected at a concentration of ~0.7 ng human rIL-2/ml (Fig. 3A); and the half-maximal dose of IL-2 for inducing the accumulation of p21ras-GTP was 2.5 ng/ml. This dose response of IL-2 for activation of p21ras coincided with the IL-2 dose response for the initiation of T cell proliferation, as judged by examination of the IL-2 dose response for incorporation of $[^3H]$Tdr into cellular DNA (Fig. 3B). These data indicate that levels of IL-2 that occupy the high affinity of IL-2R activate p21ras and suggest that there is a signaling pathway in T cells that couples the high affinity IL-2R to p21ras proteins.

**Mechanism of IL-2-induced p21ras activation.** The ratio of GTP-GPD on p21ras can, by analogy with the effects of activating mutations, be regulated by altering the rate of guanine nucleotide exchange and/or the GTPase activity of p21ras. Kinetic experiments in streptolysin O-permeabilized T cells have demonstrated that the stimulatory effect of TCR/CD3 agonists or PKC activation on the proportion of cellular p21ras-GTP in the GTP-bound "active" state is mediated by inhibition of the GTPase activity of p21ras and that these stimuli do not influence the rate of guanine nucleotide exchange on p21ras (6). In contrast, IL-2 proved unable to activate p21ras in permeabilized T lymphoblasts (data not shown). Because the IL-2R appeared to be uncoupled from regulation of p21ras in permeabilized cells, it was not possible to analyze the mechanism of IL-2-mediated p21ras activation by this method.

In T lymphocytes, regulation of the activation state of p21ras by phorbol esters or TCR/CD3 triggering appears to be mediated via the ability of PKC to control the function of a specific GTPase-activating protein. This conclusion is based on the observation that lysates prepared from T lymphoblasts stimulated by these agonists are impaired for their ability to stimulate the GTPase activity of p21ras relative to control preparations (6). Because cells contain multiple proteins with p21ras GTPase-stimulating activity, this effect may be mediated by the GAP (13, 14), the product of the NF-1 gene that is known to possess GAP-like activity (15–17), or possibly another as yet uncharacterized GTPase-activating protein. To determine whether interaction of IL-2 with its cellular receptor induces an inhibition of GAP-like activities toward p21ras, we compared cell extracts from T cells that were either quiescent, stimulated with IL-2, or antibodies against the TCR for their ability to promote the GTPase activity of p21ras. Figure 4 shows that whereas GAP-like activity was reduced by threefold upon TCR/CD3 triggering, treatment of responsive cells with IL-2 did not alter the GAP-like activity measurable in lysates. To measure TCR regulation of GAP activity it is essential to prepare cell lysates in the presence of phosphate inhibitors. Repeated experiments preparing cell lysates in the presence of serine/threonine phosphatase inhibitors such as okadaic acid or tyrosine phosphatase inhibitor such as sodium orthovanadate failed to reveal any differences in GAP activity in lysates from IL-2-stimulated T cells compared with controls.

To further investigate the mechanism by which IL-2R occupancy stimulates accumulation of GTP on p21ras, the effect of TCR/CD3 triggering or PHA stimulation in combination with IL-2 treatment was investigated. Stimulation of T lymphoblasts with IL-2, the mitogenic lectin PHA, or via the TCR/CD3 complex induced the accumulation of GTP on p21ras, increasing the amount of GTP bound from 5% to between 30 and 40% of total labeled nucleotide (Fig. 5). PDBU treatment increased the amount of GTP bound to p21ras from ~5% to ~70% of p21ras-associated nucleotide (Fig. 5). In combination, the effects of PHA treatment or TCR/CD3 triggering and IL-2 on p21ras were neither synergistic or additive (Fig. 5) and did not combine together to allow an accumulation of p21ras-GTP to the maximum levels observed in response to PDBU (Fig. 5).

**DISCUSSION**

This study demonstrates that, in T lymphocytes, p21ras can be regulated by occupancy of the high affinity receptor for IL-2 and that p21ras may function in the signal transduction pathways initiated by this receptor. IL-2 treatment of human peripheral blood-derived T lymphoblasts or the murine IL-2-dependent T cell line induced a 6- to 10-fold increase in the level of GTP bound to endogenous p21ras. In T lymphoblasts, IL-2-induced activation of p21ras was rapid and persistent; accumulation of p21ras-GTP was detectable within 1 min and, in the continuous presence of IL-2, remained elevated for at least 4 h. Because exposure to IL-2 for a period of 4 to 6 h is required for commitment of T cells to mitosis (11), these results suggest that p21ras may play a role in the regulation of cell growth by IL-2.

The ability of receptors like the TCR/CD3 complex and the CD2 Ag to regulate p21ras is mediated via control of ras GTPase activity rather than by control of the rate of guanine nucleotide exchange on p21ras. We could not measure directly IL-2 effects on the dynamics of GTP binding and hydrolysis on p21ras and thus cannot propose a mechanism of IL-2-induced p21ras activation. However, if IL-2 regulated guanine nucleotide exchange on p21ras proteins it would be predicted that IL-2 would synergize...
to stimulate p21\textsuperscript{ras} with agonists that controlled p21\textsuperscript{ras} GTPase activity. Accordingly, the lack of synergy between TCR agonists and IL-2 with regard to p21\textsuperscript{ras} activation suggests that these agonists exert their regulatory effect on p21\textsuperscript{ras} GTPase activity.

Our previous studies have suggested that the GTPase activity of p21\textsuperscript{ras} is controlled in T cells by a mechanism involving PKC regulation of the function of a p21\textsuperscript{ras} GTPase-activating protein. However, whereas receptors previously shown to activate p21\textsuperscript{ras} in T cells (TCR/CD3 complexes and CD2 Ag) can stimulate PKC (6, 7), current knowledge regarding IL-2R signaling does not indicate a role for PKC. IL-2R occupancy is known to induce serine/threonine (18-20) and tyrosine (19-24) phosphorylation of cellular substrates but the kinases thought to be regulated by IL-2 include c-raf and p56\textsuperscript{cbl} but not PKC (25, 26). Most notably, PKC activation is not required for IL-2 to regulate T cell growth (27-30) although it is necessary for T cell activation via the TCR/CD3 complex. The simplest hypothesis is therefore that IL-2 is not regulating p21\textsuperscript{ras} via PKC but via alternative mechanisms. Accordingly, it would appear that in T cells at least two signaling pathways, one involving PKC and one as yet uncharacterized, couple cell-surface receptors to p21\textsuperscript{ras}. It has been described that the IL-2R associates in the cell membrane with the tyrosine kinase 56\textsuperscript{Itk} and one obvious possibility is that the pathway that couples the IL-2R to p21\textsuperscript{ras} involves 56\textsuperscript{Itk} and some of its cellular substrates.

Support for the idea that the IL-2R stimulates different signal transduction pathways than the TCR/CD3 complex is provided by the observation that IL-2 treatment of T cells does not alter the p21\textsuperscript{ras} GAP-like activity measurable in cell extracts whereas TCR/CD3 or PKC stimulation can do this. We would not, however, use these data to eliminate a p21\textsuperscript{ras}-GAP protein from the intracellular signaling pathway that couples the IL-2R to p21\textsuperscript{ras}. Cells may contain multiple proteins with p21\textsuperscript{ras} GTPase-stimulating activity including p120\textsuperscript{CBG} (13, 14) and the product of the NF-1 gene (15-17). We do not know whether PKC and the TCR/CD3 complex regulate
achieve this through a different, possibly less stable modification that is not maintained in the cell extracts during the assay period.

These results demonstrate that, in normal human T cells, an intracellular signaling pathway couples the high affinity IL-2R to p21<sup>ras</sup> proteins. Thus, in addition to receptors that promote movement from G<sub>0</sub> to G<sub>1</sub>, such as the TCR/CD3 complex, p21<sup>ras</sup> is responsive to signals generated by the IL-2R that regulates T cell progression from G<sub>1</sub> to S phase of the cell cycle. Further analysis of the mechanisms by which these receptors regulate the activity of cellular p21<sup>ras</sup> and of the cellular consequences of p21<sup>ras</sup> activation should provide valuable insight into ras function in this cell system.

References

2422
IL-2R COUPLING TO p21<sup>ms</sup>


