

## Interleukin-2 Promoter Activation in T-Cells Expressing Activated Ha-ras\*

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**Antigen triggering of the T-cell receptor results in an accumulation of activated GTP-bound p21<sup>ras</sup> protein. To assess the role of ras protein in T-cell activation we have cotransfected the murine thymoma line EL4 with a construct capable of expressing a constitutively active, oncogenic form of Ha-ras and a reporter construct containing the human interleukin-2 promoter fused upstream of the bacterial gene for chloramphenicol acetyltransferase. We show that the ras oncoprotein contributes to interleukin-2 promoter activation. Its pattern of synergism with a calcium ionophore or the lymphokine interleukin-1 indicates that it replaces a signal mediated by protein kinase C. Interleukin-2 promoter activity in the presence of ras oncoprotein was inhibited by H7, a potent inhibitor of protein kinase C, but not by HA1004, an inhibitor of cyclic nucleotide-dependent kinase, suggesting that protein kinase C mediates the ras effect. In addition, we show that in these cells, expression of activated ras results in activation of a synthetic promoter containing several copies of an NF $\kappa$ B binding site.**

Proliferation and subsequent differentiation of resting T-cells is initiated by antigen receptor (TCR)<sup>1</sup> triggering in combination with accessory signals such as IL-1. Prolonged TCR stimulation is necessary for commitment to proliferation after which activating signals can be removed without affecting the capacity of the cells to complete the differentiation process. Commitment to proliferation coincides with activation of interleukin-2 (IL-2) and IL-2 receptor expression. Signals derived from the TCR induce several known second messenger pathways including limited activation of protein kinase C (PKC), mobilization of calcium and activation of protein tyrosine kinases (reviewed by Clevers et al. (1988) and Crabtree (1989)). *In vitro*, phorbol esters and calcium ionophores can mimic, at least in part, these signals and induce expression of IL-2 or an IL-2 promoter/reporter gene con-

struct in T-cell culture lines (Truneh et al., 1985; Macchia et al., 1990; Baldari et al., 1991).

The proto-oncogene product p21<sup>Ha-ras</sup> is a GTP-binding protein related to the low molecular weight GTPases which are involved in coupling transmembrane receptors to membrane associated enzymes such as adenylate cyclase and phospholipase C (reviewed by Barbacid (1987)). The oncogenicity of ras results from mutations which block GTPase activity or increase the rate of exchange of bound nucleotide such that the GTP-bound active form of the protein accumulates in the cell (Satoh et al., 1987, 1988; Field et al., 1987). Its location at the inner surface of the plasma membrane and its relatedness to G proteins suggests that normal p21<sup>Ha-ras</sup> may mediate extracellular mitogenic stimuli. In support of this, activation of T-cells via the TCR results in an increase in the GTP-bound active form of p21<sup>Ha-ras</sup> (Downward et al., 1990). However, a direct role for ras in T-cell activation has not been shown.

We have addressed the question of the role of p21<sup>Ha-ras</sup> in T-cell activation using a gene coding for an oncogenic form of the protein transfected into T-cell culture lines.

### MATERIALS AND METHODS

**Cell Culture, Transfections, and CAT Assays**—Transfections, activations, protein determinations, and CAT assays were carried out as described (Macchia et al., 1990; Baldari et al., 1991). Transfection efficiency varied between experiments. For this reason, in each experiment, activations, for 12 h beginning 30 h after transfection, were done on equal aliquots of a single pool of transfected cells. When ras-transfected cells were compared with control cells, the transfection mix, including the indicator plasmid, was prepared, then divided into equal aliquots to which were added either the T24 ras plasmid or the vector lacking only T24 sequences. Autoradiograms were scanned using an UltrascanXL enhanced laser densitometer and evaluated using GSXL software. The results of representative experiments are shown.

**Plasmids**—The ras expression plasmid was constructed by insertion of the 1400-bp *ClaI/DraI* restriction fragment containing the Moloney leukemia virus long terminal repeat from pDOL<sup>-</sup> (Korman et al., 1987) into the *HindIII* site into the plasmid pT24C3 (Santos et al., 1982) after filling of the overhanging ends with Klenow polymerase. IL-2/CAT, which contains approximately 2000 bp of the human IL-2 promoter upstream of the bacterial chloramphenicol acetyltransferase (CAT) coding sequence, has been described elsewhere (Macchia et al., 1990). The NF $\kappa$ B-containing plasmid was described as PRDII (Fan and Maniatis, 1989).

**Analysis of RNA**—RNA was prepared from transfected cells as described (Macchia et al., 1990). Polymerase chain reaction amplification was carried out using the Invitrogen cDNA cycle kit for reverse transcription-polymerase chain reaction on 2  $\mu$ g of purified RNA according to the manufacturers' instructions. The oligonucleotide primers were 17-mers designed to be complementary to sequences in the 5'- and 3'-nontranslated regions of the human Ha-ras mRNA.

### RESULTS AND DISCUSSION

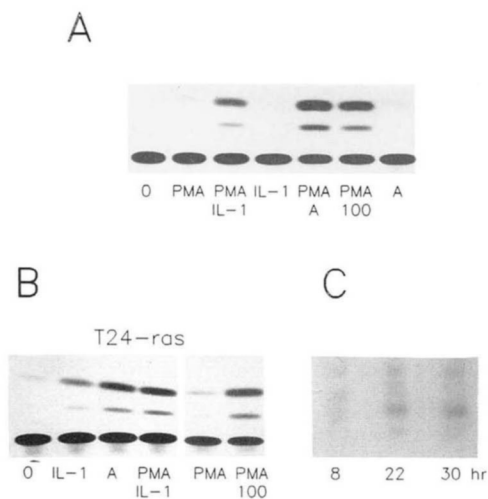
In cells of the murine thymoma line EL4, an IL-2 promoter/CAT fusion gene responds to treatment with high concentrations (>5 ng/ml) of phorbol myristate acetate (PMA) or suboptimal concentrations (0.5–1 ng/ml) of PMA in combination with either IL-1 or the calcium ionophore A23187. In the absence of PMA, IL-1 and A23187 either alone or in combination have no effect (Macchia et al., 1990) (Fig. 1A).

PMA can replace diacyl glycerol (DAG), the natural activator of PKC, and cause activation and translocation of PKC

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<sup>1</sup> The abbreviations used are: TCR, antigen receptor; IL, interleukin; PKC, protein kinase C; CAT, chloramphenicol acetyltransferase; bp, base pair(s); PMA, phorbol myristate acetate; DAG, diacyl glycerol.



**FIG. 1. Activated ras expression contributes to IL-2 promoter activation.** A, thin layer chromatogram of CAT assays of EL4 cells transiently transfected with the IL-2/CAT plasmid and activated with PMA, IL-1, and A23187 alone or in combination. 0, no treatment; PMA, 0.7 ng/ml PMA; PMA100, 100 ng/ml PMA; IL-1, 1 ng/ml recombinant human IL-1 $\beta$ ; A, (1  $\mu$ g/ml A23187). B, CAT assays of EL4 cells cotransfected with the IL-2/CAT construct and a construct capable of expressing the T24 ras oncoprotein. Symbols are as in A. C, blot hybridization of polymerase chain reaction products of RNA from ras-transfected EL4 cells showing accumulation of ras-specific RNA with time. Cells were collected at the times shown after transfection.

to the plasma membrane (Nishizuka, 1984). Considerable evidence implicates PKC as a mediator of activation of IL-2 gene expression by PMA (Berry *et al.*, 1990; Macchia *et al.*, 1990, and references therein). Neither IL-1 nor A23187 activate PKC but instead activate different signal transduction pathways which synergize with PMA at points distal to PKC activation (Abraham, *et al.*, 1987; Macchia, *et al.*, 1990).

We have transfected EL4 cells with a plasmid containing the gene coding for the T24 form of p21<sup>Ha-ras</sup> (Santos *et al.*, 1982). The T24 gene product has no GTPase activity and remains in the GTP-bound active form. Messenger RNA transcribed from this gene can be detected 22 h after transfection (Fig. 1C). In cells cotransfected with the T24 gene and the IL-2/CAT reporter gene, activation of the IL-2 promoter was no longer dependent on PMA. IL-1 or A23187 alone were sufficient to induce CAT activity. Low but detectable CAT activity was found in untreated ras-transfected cells and control cells treated with suboptimal PMA (0.7 ng/ml). A small increase in the 0.7 ng/ml PMA signal seen in ras-transfected cells may indicate additive effects but is of questionable significance since slight variation in the level of suboptimal activation was often seen (Fig. 1B). Thus the ras oncoprotein replaces suboptimal concentrations of PMA in synergism with both IL-1 and A23187. These results indicate that p21<sup>Ha-ras</sup> and PKC are involved in the same pathway of TCR-induced IL-2 expression.

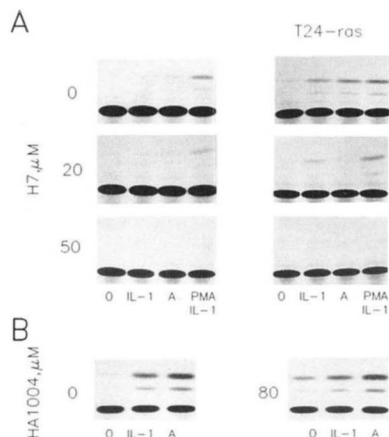
Downward *et al.* (1990) have shown that, in addition to TCR agonists, PMA results in accumulation of GTP-bound p21<sup>ras</sup> protein in the human lymphoma cell line Jurkat. On the basis of this result, the authors suggested that PKC activates p21<sup>ras</sup>. Others, however, report that oncogenic ras causes an increase in DAG, a natural activator of PKC, suggesting that GTP-bound ras activates PKC (Wolfman and Macara, 1987; Haliotis *et al.*, 1990). To investigate this further we have tested the effect of a PKC inhibitor on T24-ras activation of the IL-2 promoter. If PKC mediates ras activation of the IL-2 promoter, then blocking PKC should block

ras activity. On the other hand, if PKC mediates TCR activation of ras, constitutively active ras should be independent of PKC activity. H7 inhibits PKC and to a lesser extent cyclic nucleotide-dependent kinases (Hidaka *et al.*, 1984). Activation of IL-2/CAT expression by IL-1 or A23187 in ras-transfected cells was blocked by H7 in a dose-dependent fashion (Fig. 2). Interestingly, IL-1/ras synergism was more resistant to H7 than was A23187/ras synergism. Similar results were reported for IL-1/PMA synergism compared with A23187/PMA synergism (Macchia *et al.*, 1990) strengthening the similarity between ras effect and PKC activation. The related compound HA1004, which inhibits cyclic nucleotide-dependent protein kinases effectively but PKC only weakly (Hidaka *et al.*, 1984), had no effect on ras-mediated activation even at higher molar concentration. Activated p21<sup>Ha-ras</sup> cannot therefore replace activation of PKC in induction of IL-2 expression.

It may be that PMA causes the accumulation of GTP-bound ras protein by an alternative mechanism not involving PKC. DAG has been shown to activate GTPase inhibitory protein, a cytoplasmic protein which inhibits ras hydrolysis of GTP (Tsai *et al.*, 1990). Since PMA is believed to substitute for DAG in PKC activation (Nishizuka, 1984), it is likely that PMA can also substitute for DAG in GTPase inhibitory protein inhibition of ras GTPase. This would also explain why cytosolic extracts of PMA treated cells inhibited ras GTP hydrolysis *in vitro* (Downward *et al.*, 1990).

Both activation of PKC and expression of ras oncoprotein are known to result in site-specific dephosphorylation and elevated DNA binding of c-Jun protein, a major component of the AP-1 transcription factor (Boyle *et al.*, 1991; Binétruy *et al.*, 1991). In addition PKC phosphorylation of the NF $\kappa$ B inhibitor results in nuclear translocation and activation of the transcription factor NF $\kappa$ B (Ghosh and Baltimore, 1990). Both AP-1 and NF $\kappa$ B are believed to be involved in IL-2 transcription (Ullman *et al.*, 1990). We tested whether activated ras could modulate NF $\kappa$ B activity in EL4 cells.

Fig. 3 shows the results of cotransfection of EL4 cells with the ras construct and a CAT construct under the control of a synthetic promoter containing several copies of an NF $\kappa$ B binding site. In control cells the NF $\kappa$ B promoter was constitutively active. In agreement with a previous report (Espel *et al.*, 1990), treatment with IL-1 alone or suboptimal PMA alone resulted in a 2–3-fold activation of the NF $\kappa$ B promoter. In the presence of activated ras, CAT activity in untreated



**FIG. 2. ras activation of the IL-2 promoter is inhibited by the PKC inhibitor H7.** CAT assays of EL4 cells cotransfected with the IL-2/CAT plasmid and either T24-ras (right) or control plasmids (left). Cells were treated with the activators shown in the presence of A, H7, (0, 20, or 50  $\mu$ M, or B, HA1004 (80  $\mu$ M). Symbols are as in Fig. 1.

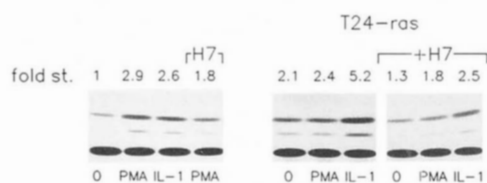


FIG. 3. A synthetic multimeric NF $\kappa$ B binding site is activated by ras. CAT assays of extracts of EL4 cells cotransfected with a plasmid containing eight copies of an NF $\kappa$ B binding site upstream of the CAT gene and either the ras plasmid or the vector without the ras insert. The -fold stimulation compared with untreated cells in the absence of ras is shown above each track. Symbols are as in Fig. 1. H7 (20  $\mu$ M) was included during the activation where indicated.

cells was found to be similar to stimulation of control cells by PMA. Treatment of ras-transfected cells with IL-1 resulted in an approximately 5-fold increase in CAT activity, whereas treatment with 0.7 ng/ml PMA did not augment the CAT activity. ras-induced activation of the NF $\kappa$ B promoter was also inhibited by H7. In the presence of H7 expression of the NF $\kappa$ B/CAT construct was reduced almost to constitutive level, whereas IL-1 activation was reduced to the level obtained by IL-1 activation in the absence of ras (Fig. 3).

We conclude that TCR-induced accumulation of GTP-bound p21<sup>Ha-ras</sup> plays an important role in activation of IL-2 gene expression and consequently T-cell activation. In addition, we suggest that p21<sup>Ha-ras</sup> controls PKC activation of both AP-1 and NF $\kappa$ B like transcription factors. Since in some cells, transformation by oncogenic ras results in release of DAG (Wolfman and Macara, 1987) it is possible that p21<sup>Ha-ras</sup> couples the TCR to a phospholipase. However the mechanism by which the TCR controls accumulation of the GTP-bound form is as yet still unclear.

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