The EMBO Journal vol.11 no.12 pp.4549-4556, 1992

p21^{ras} mediates control of *IL-2* gene promoter function in T cell activation

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Communicated by M.J.Crumpton

It has been shown previously in T cells that stimulation of protein kinase C or the T cell antigen receptor leads to a rapid and persistent activation of p21^{ras} as measured by a dramatic increase in the amount of bound GTP. These stimuli are also known to induce the expression of the T lymphocyte growth factor, interleukin-2 (IL-2), an essential growth factor for the immune system. Receptor induced activation of p21^{ras} has been demonstrated in several cell types but involvement of protein kinase C as an upstream activator of p21^{ras} appears to be unique to T cells. In this study we show that p21^{ras} acts as a component of the protein kinase C and T cell antigen receptor downstream signalling pathway controlling *IL-2* gene expression. In the murine T cell line EL4, constitutively active p21^{ras} greatly potentiates the phorbol ester and T cell receptor agonist induced production of IL-2 as measured both by biological assay for the cytokine and by the use of a reporter construct. Active p21^{ras} also partially replaces the requirement for protein kinase C activation in synergizing with a calcium ionophore to induce production of IL-2. Furthermore, using a dominant negative mutant of *ras*, Ha-*ras*N17, we show that endogenous ras function is essential for induction of IL-2 expression in response to protein kinase C or T cell receptor stimulation. Activation of ras proteins is thus a necessary but not sufficient event in the induction of IL-2 synthesis. Ras proteins are therefore pivotal signalling molecules in T cell activation.

Key words: *IL-2* gene/p21^{ras}/promoter function/T cell activation

Introduction

The three members of the *ras* gene family (Ha-, Ki- and N-*ras*) are frequently activated in a wide variety of human tumours (Bos, 1989). Mutationally activated *ras* oncogenes are capable of transforming cells in culture while the normal proto-oncogene products seem to play critical roles in regulating proliferation and differentiation (Haubruck and McCormick, 1991; Downward, 1992b). The *ras* genes encode proteins of 21 000 molecular weight (p21^{ras}) which bind guanine nucleotides, possess an intrinsic weak GTPase activity and require membrane localization for biological activity. Ras proteins are active in their GTP bound state but inactive when bound to GDP: within whole cells, transforming *ras* mutations all give rise to proteins that are more highly GTP bound than the wild type.

The activation state of normal p21^{ras} appears to be controlled by two distinct families of proteins, those that regulate the rate of nucleotide exchange and those that regulate the rate of GTP hydrolysis. Several guanine nucleotide exchange stimulating factors for ras proteins have been identified and partially characterized in a number of organisms but their regulation is as yet poorly understood (Downward, 1992a). At least two mammalian proteins are known that stimulate the rate of hydrolysis of GTP on p21^{ras}: p120^{GAP}, the first GTPase activating protein (GAP) to be discovered for ras superfamily proteins, and neurofibromin, the product of the *NF1* gene which is damaged in the hereditary disease type 1 neurofibromatosis (Downward, 1992b). Transforming point mutations cause the ras proteins to be either insensitive to GAP activity or enhanced in their rate of nucleotide exchange.

Several observations indicate that ras proteins are involved in mediating the delivery of extracellular signals to intracellular targets. Introduction of constitutively activated ras proteins into cells cause many of the same responses as treatment with growth factors, while blocking ras function within the cell prevents normal responses to these stimuli. Recently it has been directly demonstrated that a number of cellular growth stimuli causes activation of ras proteins as measured by the level of GTP bound to them within the whole cell (reviewed in Downward *et al.*, 1992). In peripheral blood T lymphoblasts (PBLs), ras rapidly becomes strongly activated in response to agents that stimulate the T cell antigen receptor (TCR) (Downward *et al.*, 1990). Activation of T cells through the T cell receptor switches on several signalling molecules, including protein-tyrosine kinases and the phosphatidylinositol specific phospholipase Cγ (PLCγ), which catalyses the hydrolysis of phosphatidylinositol bisphosphate to generate the two signalling molecules diacylglycerol (DAG) and inositol trisphosphate (Klausner and Samelson, 1991). Direct activation of protein kinase C with phorbol ester causes a dramatic increase (up to 80%) in GTP bound ras in T cells (Downward *et al.*, 1990). The mechanism by which ras activation appears to be achieved by T cell receptor and protein kinase C activation in T cells is by inhibition of GAP-like activity as measured in cellular lysates. No effect on the rate of guanine nucleotide exchange could be detected. In the T lymphocyte system protein kinase C therefore appears to act as an upstream regulator of ras activity: in other cell types it has not been possible to demonstrate a direct activation of p21^{ras} in response to protein kinase C stimulation. Instead, activation of ras has been found to occur in response to several growth factors thought to be linked to tyrosine kinases in both hematopoietic cells [interleukin 2 (IL-2), interleukin 3 (IL-3), granulocyte macrophage colony stimulating factor (GM-CSF), steel

factor (SLF)], fibroblasts (insulin, EGF, PDGF) and PC12 cells (NGF, EGF) (Downward *et al.*, 1992). The reasons for these differences in ras signalling in different cell types are not yet understood.

Activation of T lymphocytes through the T cell receptor results in progression of cells into the cell cycle (G_0-G_1) and the production of the growth factor interleukin 2 (IL-2) and its receptor (Crabtree, 1989). The interaction of IL-2 with its receptor is essential for cell cycle progression from G_1 to S phase, and is the commitment event in triggering T cell proliferation. The regulation of transcription of the *IL-2* gene is thus a key regulatory point in T cell activation. A recent study has shown that expression of activated ras protein leads to transcription of an *IL-2* reporter construct in a T cell line, particularly in conjunction with a calcium ionophore (Baldari *et al.*, 1992): this raised the question of whether ras proteins were involved in physiological signalling pathways resulting in control of gene transcription. The aim of this study was therefore to investigate the role of ras proteins in signalling pathways from the T cell receptor and protein kinase C which converge on IL-2 enhancer function. The murine T cell line EL4 produces IL-2 in the presence of appropriate signals and provides a model system in which to study pathways involved in mediating induction of IL-2 in T lymphocytes. In this study we show that activated ras provides a signal that dramatically stimulates the T cell receptor and protein kinase C induction of IL-2 expression in these cells. Ras cannot fully substitute for the protein kinase C signal but can partially replace a requirement for protein kinase C in synergizing with a calcium signal in IL-2 induction. However, using a dominant negative *ras* mutant (Ser→Asn17) which blocks the activation of normal ras proteins, we have shown that endogenous ras function is an absolutely required component of both the T cell receptor and protein kinase C signalling pathways involved in the induction of IL-2 expression.

Results

Protein kinase C can activate p21^{ras} in the EL4 T cell line

We have previously reported that stimulation of protein kinase C results in activation of p21^{ras} in normal human peripheral blood lymphoblasts (PBLs); the level of GTP bound to ras as a proportion of total bound guanine nucleotide increases from ~5% in untreated cells to 50–80% after phorbol ester treatment and is also greatly increased upon stimulation of the antigen receptor (Downward *et al.*, 1990). In order to study the role of ras proteins in the regulation of *IL-2* gene expression during T cell activation we chose to use the murine thymoma derived T cell line EL4 since peripheral blood T lymphoblasts were not readily transfectable. To confirm that protein kinase C is indeed able to activate endogenous p21^{ras} in EL4 cells, these cells were labelled with [^{32}P]orthophosphate for 4 h before stimulation of protein kinase C by addition of phorbol ester. The cells were then lysed and p21^{ras} was immunoprecipitated with the monoclonal antibody Y13-259 (Furth *et al.*, 1982). The nucleotides bound to p21^{ras} were separated by thin layer chromatography (TLC). Similarly to our previous findings in PBLs and T cell lines (Downward *et al.*, 1990), phorbol ester increased the amount of GTP bound to p21^{ras} from 5% of total nucleotide in unstimulated cells to 36% (Figure 1), a >7-fold increase in the amount of active ras. As previously observed, the calcium ionophore ionomycin has no effect on p21^{ras} activation (data not shown).

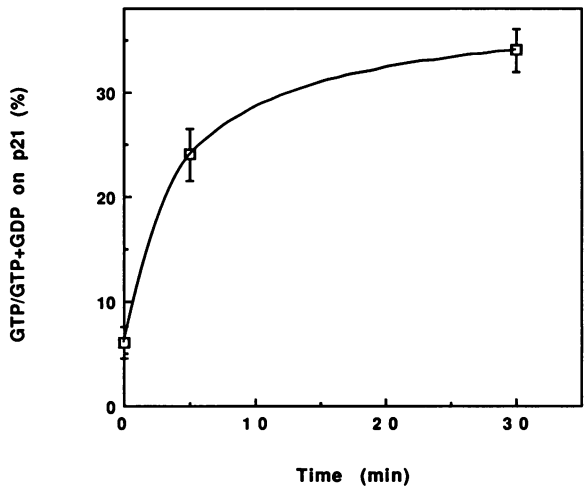

Fig. 1. Effect of phorbol ester treatment on the activation state of p21^{ras} in EL4 cells. Cells were labelled with [^{32}P]orthophosphate for 4 h, 100 ng/ml PDBu was added for either 5 or 30 min, and p21^{ras} was immunoprecipitated from cell lysates using monoclonal antibody Y13-259. The nucleotides bound to p21^{ras} were seperated by TLC and quantitated by direct scanning for β radiation.

EL4 cells produce IL-2 and express IL-2CAT in response to protein kinase C activation

EL4 cells can be used as a model system for studying signalling pathways in normal T lymphocytes since in both these T cell systems stimulation of protein kinase C activates p21^{ras} and regulates interleukin 2 (IL-2) production. Figure 2a illustrates the production of IL-2 by EL4 cells in response to phorbol ester and the synergistic effect of ionomycin on this induction. Ionomycin by itself is unable to stimulate IL-2 production, but increases the response to phorbol ester by up to 5-fold. In this experiment IL-2 levels were measured using a biological assay that utilizes the IL-2 sensitive T cell line CTLL. An experimentally more tractable way of studying the control of *IL-2* gene expression is provided by the use of a construct containing a chloramphenicol acetyltransferase (CAT) reporter gene under the control of the *IL-2* gene enhancer and promoter (Durand *et al.*, 1988). Transfection of this construct (IL-2CAT) into EL4 cells, followed by applying various stimuli 24 h later gave the same induction profile of the IL-2CAT construct as was seen for the endogenous *IL-2* gene (Figure 2b). CAT activity was measured 18 h after applying various stimuli to the transfected EL4 cells; Figure 2b illustrates the fold induction of CAT activity. In the absence of stimuli, expression of IL-2CAT is extremely low. Phorbol ester treatment gave an induction of IL-2CAT expression of ~140-fold which was increased to ~340-fold in the presence of ionomycin. The reporter construct thus mimics the behaviour of the endogenous gene. Stimulation could also be achieved using a monoclonal antibody (2C11) raised against the CD3 component of the murine T cell receptor: this resulted in a ~40-fold induction in the expression of IL-2CAT. As for the endogenous *IL-2* gene, ionomycin by itself has absolutely no effect on CAT expression.

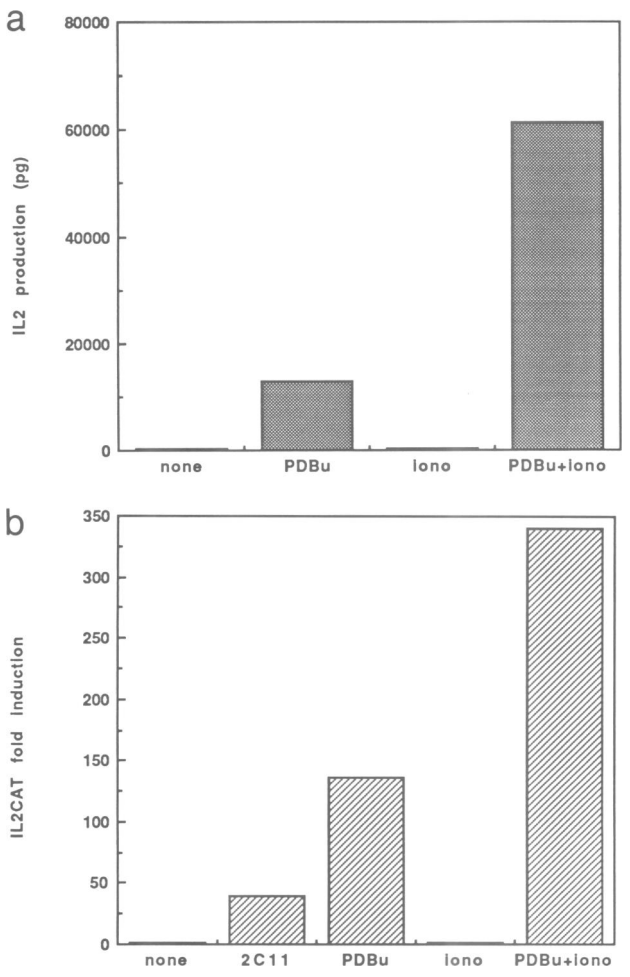

Fig. 2. Activation of TCR and protein kinase C induces expression of IL-2 and IL-2CAT. (**a**) EL4 cells were stimulated with 100 ng/ml PDBu and 0.5 μg/ml ionomycin, as indicated, for 18 h. Cell culture supernatants were harvested and assayed for IL-2 production by measuring [^{3}H]thymidine incorporation into CTLL cells, as described in Materials and methods. (**b**) EL4 cells (2.5×10^7 per point) were transfected with 10 μg IL-2CAT DNA. 24 h post-transfection the cells were stimulated for 18 h with either 100 ng/ml PDBu, 0.5 μg/ml ionomycin or 0.1 mg/ml 2C11 monoclonal antibody coated on to plates, as indicated. Cells were harvested and CAT activity in cell lysates was measured in an 18 h assay, as described in Materials and methods. Results are shown as fold induction of CAT activity compared with unstimulated cells.

p21^{ras} potentiates protein kinase C stimulation of IL-2CAT expression and synergizes with a calcium signal

To determine whether p21^{ras} is involved in signalling pathways linking activation of the T cell receptor to regulation of *IL-2* gene expression, we constructed an expression vector containing a gene encoding a mutationally activated ras protein under the control of the constitutively expressed cytomegalovirus (CMV) promoter. This expression system was used because the CMV promoter allows high level expression in a variety of cell types including T lymphocytes. The v-Ha-*ras* gene was used: this contains mutations at codons 12 (G→R) and 59 (A→T). The encoded protein is insensitive to GTPase activating proteins and has a dominant positive effect on cell proliferation in fibroblast cell lines (Haubruck and McCormick, 1991). The active *ras* sequence was inserted into the *Bam*HI site in the CMV plasmid and clones containing *ras* both in the positive (CMVras+) and negative (CMVras−) orientation were obtained. The expression of activated ras proteins was confirmed by showing that calcium phosphate mediated transfection of Rat-1 fibroblasts by the positive, but not the negative, orientation clone resulted in the appearance of large transformed foci 2 weeks following transfection (data not shown).

EL4 cells were cotransfected with IL-2CAT and either CMVras+, CMVras− or the empty CMV vector, using a 2.5-fold excess of the CMV plasmid. This DNA ratio was found to give optimal stimulation of IL-2CAT expression (data not shown). In Figure 3a a TLC of the CAT assay is shown: the acetylated chloramphenicol products migrate as two species ahead of the non-acetylated chloramphenicol. Cotransfection of CMVras+ increases the phorbol ester and phorbol ester plus ionomycin induction of IL-2CAT expression when compared with cotransfection with CMVras−. Neither activated ras alone nor calcium ionophore alone are able to stimulate IL-2CAT expression in these cells, but together they synergize to give a clear induction of reporter enzyme activity. The empty CMV vector gave similar results to CMVras− (data not shown). Quantitation of this assay, together with a second identical experiment, gave the average values shown in Figure 3b. The presence of activated ras enhances the phorbol ester induction of IL-2CAT by 2.6-fold and the phorbol ester plus ionomycin induction by nearly 2-fold. Ionomycin plus activated ras give an 8-fold induction in expression of IL-2CAT while each alone cause no induction.

In addition to the above pharmacological stimuli, we also determined the effect of activated ras on the more physiological stimulation of EL4 cells through the T cell antigen receptor. The receptor was activated by the specific monoclonal antibody 2C11, which also synergizes with ionomycin. Figure 3c shows that activated ras causes a 4-fold stimulation in IL-2CAT induction by 2C11 and at least a 2-fold increase in the induction of IL-2CAT by the T cell receptor antibodies plus ionomycin.

The stimulatory effect of CMVras+ on the phorbol ester induction of IL-2CAT responded in a dose dependent manner to the amount of *ras* DNA used in the cotransfection experiments (Figure 3d). In these experiments the amount of IL-2CAT used was constant at 10 μg. The dose response to phorbol ester for induction of IL-2CAT shows that concentrations as low as 5 ng/ml begin to induce gene expression (Figure 3e). This is similar to the lowest concentrations of PDBu needed to activate protein kinase C. At all phorbol ester concentrations used, up to 100 ng/ml, the presence of CMVras+ stimulated expression of IL-2CAT by at least 2-fold.

p21^{ras} selectively stimulates AP-1CAT activity in EL4 cells

To show that the inability of CMVras+ to induce IL-2CAT in EL4 cells was not due to insufficient expression of p21^{ras} protein we used a phorbol ester responsive element (TRE) reporter construct, which binds an AP-1 complex and is known to be activated by ras in other cell types. The AP-1CAT construct consists of sequences from −73 to −42 of the 5′ region of the human fibroblast collagenase gene, which contains a single copy of a TRE element, linked to the heterologous herpes simplex virus thymidine kinase

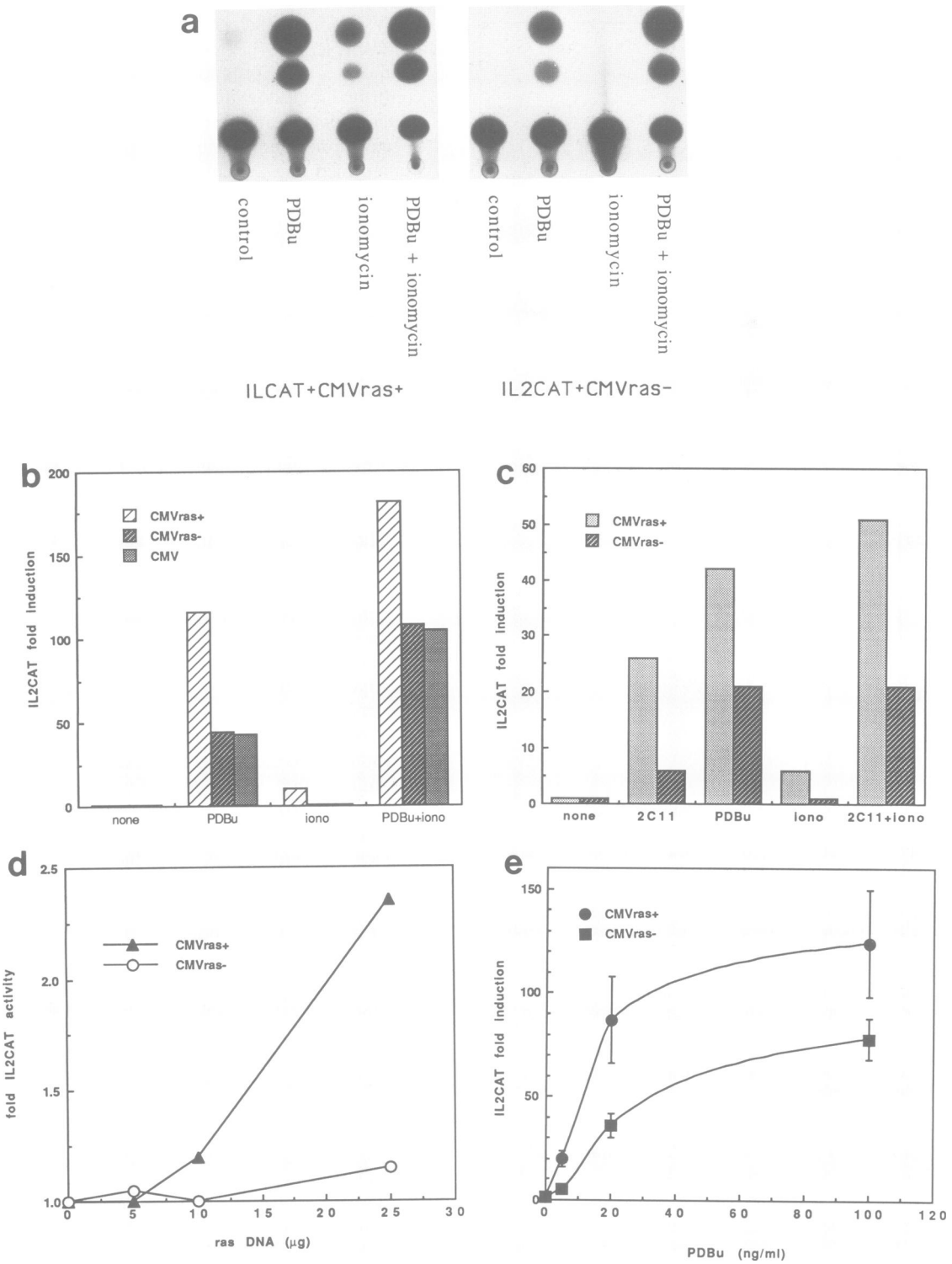

Fig. 3. p21^{ras} potentiates protein kinase C induction of IL-2CAT and synergizes with a calcium signal. EL4 cells were transfected with 10 μg IL-2CAT and 25 μg of the appropriate CMV DNA as indicated. 24 h post-transfection the cells were stimulated for 18 h with either 100 ng/ml PDBu, 0.5 μg/ml ionomycin or 0.1 mg/ml 2C11 monoclonal antibody coated on to plates, as indicated. Cells were harvested and CAT activity in cell lysates was measured in an 18 h assay. Results are shown as (**a**) thin layer chromatogram and (**b** and **c**) quantitated as fold induction of CAT activity compared with unstimulated cells. (**d**) Cells were transfected with 10 μg IL-2CAT DNA and varying amounts of CMV*ras* DNA with CMV empty vector to make up a total of 25 μg cotransfected DNA. 24 h post-transfection the cells were stimulated for 18 h with 100 ng/ml PDBu. (**e**) Cells were transfected with 10 μg IL-2CAT and 25 μg CMV*ras* DNA. 24 h post-transfection the cells were stimulated for 18 h with varying amounts of PDBu as indicated. Results shown are the average of four experiments.

promoter driving the CAT gene (Angel *et al.*, 1987a). Figure 4a shows that transient expression of CMVras+ in EL4 cells is sufficient to stimulate AP1 activity and increase expression from the TRE. Cotransfection of AP-1CAT with CMVras+ results in a maximum 5-fold increase in CAT activity compared with cotransfection with CMVras−. This stimulation responds in a dose dependent manner to the amount of *ras* DNA used in the cotransfection experiments

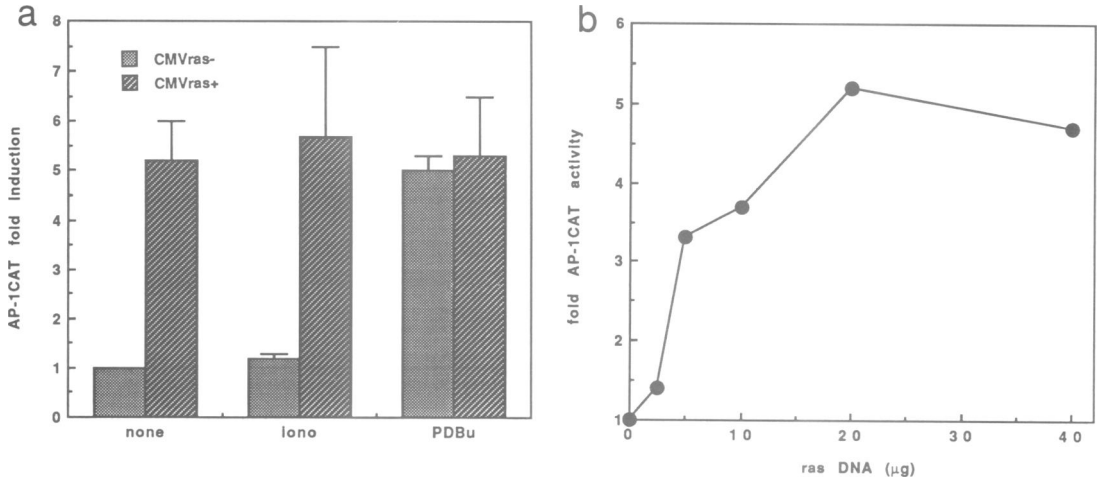

Fig. 4. p21^{ras} can replace phorbol ester in stimulation of AP-1CAT. (**a**) EL4 cells were transfected with 20 μg AP-1CAT and 40 μg CMV*ras* DNA. 24 h post-transfection the cells were stimulated for 18 h with 0.5 μg/ml ionomycin or 100 ng/ml PDBu, as indicated. Cells were harvested and CAT activity in cell lysates was measured in an 18 h assay. Results are shown as fold induction of CAT activity compared with unstimulated cells containing CMVras−. (**b**) EL4 cells were transfected with 20 μg AP-1CAT and varying amounts of CMV*ras* DNA with CMV empty vector to make up a total of 40 μg cotransfected DNA. Cells were harvested 42 h post-transfection and CAT activity in cell lysates was measured in an 18 h assay. Results are shown as fold activity of cells containing CMVras+ compared with CMVras−.

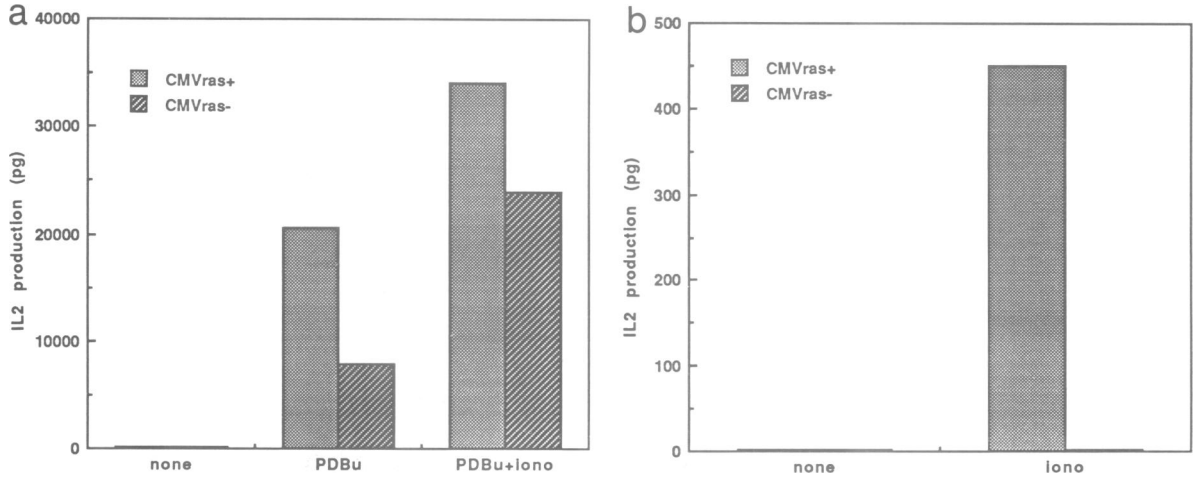

Fig. 5. p21^{ras} potentiates stimulation of IL-2 production. EL4 cells were transfected with 10 μg IL-2CAT and 25 μg CMV*ras* DNA. 24 h post-transfection the cells were stimulated for 18 h with (**a**) 100 ng/ml PDBu or 100 ng/ml PDBu plus 0.5 μg/ml ionomycin, or (**b**) 0.5 μg/ml ionomycin. Cells were harvested and the supernatants assayed for IL-2 production by stimulation of [^{3}H]thymidine incorporation in CTLL cells as described in Materials and methods. Results are shown as the total amount of IL-2 produced in pg from 2.5×10^7 cells.

(Figure 4b). As expected, treatment with phorbol ester also results in the same 5-fold stimulation as seen with ras alone (Figure 4a). The magnitude of the response of the AP-1CAT in EL4 cells is in reasonable agreement with that seen for phorbol ester stimulation of this construct in HeLa tk$^-$ cells (7.2-fold) (Angel *et al.*, 1987b).

To show that there was selectivity in which regulatory elements could be effected by expression of an activated ras protein, we used the constitutively expressed CMVCAT and MoMLV-LTRCAT constructs in cotransfection experiments with CMVras+ and CMVras− DNA. The effects of phorbol ester or ionomycin treatment on the level of CAT activity were measured: expression of activated ras had no effect on these promoters (data not shown).

p21^{ras} potentiates stimulation of IL-2 production

In order to confirm that the increases in CAT activity that were observed in response to activated ras were indeed a consequence of enhancement of signals converging on the *IL-2* gene enhancer, we measured the amount of IL-2 protein produced by *ras* transfected cells. Culture supernatants from the total population of transiently transfected cells were assayed for IL-2 levels. Even against a background of untransfected cells, the presence of CMVras+ in a proportion of these cells was sufficient to enhance the phorbol ester induction of biologically assayable IL-2 by ~2-fold (Figure 5a). A smaller enhancement of the phorbol ester plus ionomycin effect on IL-2 production was seen in response to CMVras+ (~40%). Activated ras was also able to synergize with ionomycin to produce IL-2, which otherwise is undetectable after either ionomycin treatment or transfection of CMVras+ alone (Figure 5b): the absolute levels of IL-2 produced are, however, low compared with induction by phorbol ester. These data confirm the behaviour of the IL-2CAT as an accurate reporter for the expression of the endogenous *IL-2* gene.

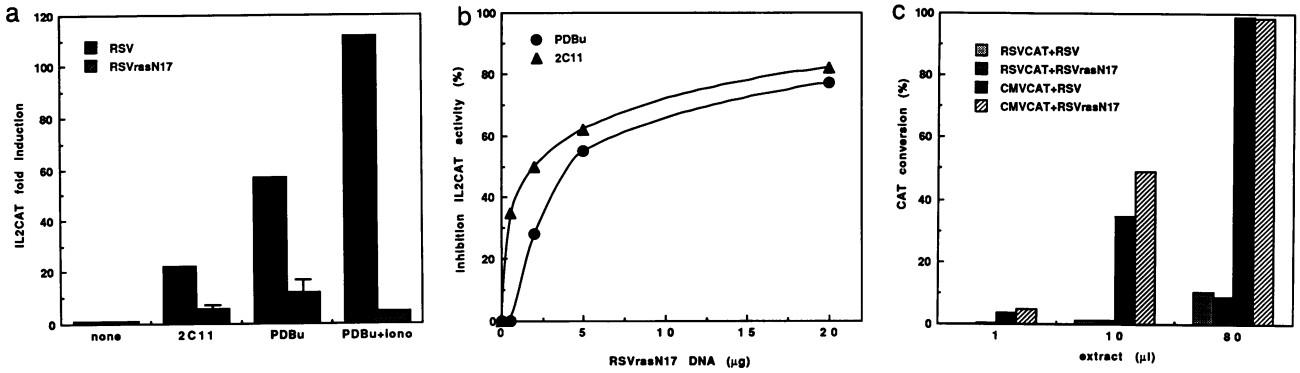

Fig. 6. Dominant negative RSV*ras*N17 mutant blocks TCR and protein kinase C induction of IL-2CAT. (**a**) EL4 cells were transfected with 10 μg IL-2CAT and 20 μg RSV*ras*N17 or 20 μg RSV empty vector. 24 h post-transfection cells were stimulated for 18 h with 0.1 mg/ml 2C11 monoclonal antibody coated on to plates, 100 ng/ml PDBu or 100 ng/ml PDBu plus 0.5 μg/ml ionomycin, as indicated. Cells were harvested and CAT activity in cell lysates was measured in an 18 h assay. Results are shown as fold induction of CAT activity compared with unstimulated cells. (**b**) EL4 cells were transfected with 10 μg IL-2CAT and varying amounts of RSV*ras*N17 and RSV empty vector to give a total of 20 μg cotransfected DNA. 24 h post-transfection cells were stimulated for 18 h with 100 ng/ml PDBu or 0.1 mg/ml 2C11 monoclonal antibody coated on to plates. Cells were harvested and CAT activity in cell lysates was measured in an 18 h assay. Results are shown as % inhibition of CAT activity of cells transfected with RSV*ras*N17 compared with the RSV empty vector. (**c**) EL4 cells were transfected with 2 μg RSVCAT or 2 μg CMVCAT and 20 μg RSV *ras*N17 or 20 μg RSV empty vector. 48 h post-transfection cells were harvested and CAT activity in cell lysates was measured in a 1 h assay, using varying amounts of the lysates as indicated in order to be within the linear range of enzyme activity. Results are shown as percentage conversion of chloramphenicol into the acetylated product.

p21^{ras} is essential for protein kinase C and T cell receptor mediated induction of IL-2 expression

The above data show that protein kinase C activates endogenous p21^{ras} in EL4 lymphocytes and that active, GTP bound ras can stimulate a signalling pathway which both potentiates a protein kinase C mediated pathway and synergizes with a calcium activated pathway in the induction of *IL-2* expression. To determine whether the activation of p21^{ras} is indispensable for induction of *IL-2* gene expression, we used a dominant negative *ras* mutant which appears to block the activation of the endogenous p21^{ras} protein (Feig and Cooper, 1988). In Ha-*ras*N17, serine is replaced by asparagine at codon 17, probably blocking the function of normal p21^{ras} by competing with the proteins that mediate guanine nucleotide exchange. Figure 6a shows that cotransfection of RSV*ras*N17 with IL-2CAT, using a DNA ratio of 2 to 1, blocks the T cell receptor antibody (72% inhibition, $n = 3$, $\sigma = 6\%$), phorbol ester (78% inhibition, $n = 6$, $\sigma = 9\%$) and phorbol ester plus ionomycin (95% inhibition) mediated induction of IL-2CAT activity. The inhibition of the T cell receptor and protein kinase C mediated induction of IL-2CAT by the dominant negative RSV*ras*N17 mutant occurs in a dose dependent manner (Figure 6b). The T cell receptor driven signal is slightly more easily inhibited than the phorbol ester response, possibly reflecting the more potent activation of endogenous p21^{ras} by phorbol ester than by T cell receptor antibodies (Downward *et al.*, 1990). p21^{ras} therefore appears to be an essential component necessary for induction of both the T cell receptor and protein kinase C mediated induction of *IL-2* gene expression in T lymphocytes. However, the dominant negative *ras* mutant was unable to block the IL-2 induction completely; the reason for this incomplete inhibition may be that some cells received IL-2CAT DNA but not RSV*ras*N17 DNA. It is also possible that some very minor signalling pathway from the T cell receptor and protein kinase C does not involve ras and is capable of inducing residual expression of *IL-2*. These effects were not due to a non-specific action of the RSV*ras*N17 since this plasmid does not influence the constitutive expression of RSVCAT or CMVCAT even when cotransfected with DNA ratios of 10-fold that of the CAT reporter plasmid (Figure 6c). The specificity of this dominant negative *ras* mutant was also evident from its inability to inhibit calcium ionophore induction of expression of an NF-AT CAT construct.

Discussion

Our previous studies have demonstrated that the activation state of p21^{ras} in T cells is under the control of protein kinase C and the T cell receptor (Downward *et al.*, 1990). Stimulation of either of these molecules leads to rapid activation of p21^{ras}, apparently through suppression of GAP and/or neurofibromin activity against the background of a constitutively highly active guanine nucleotide exchange factor. Recently we have found evidence that the T cell receptor may use two different signalling pathways to regulate p21^{ras}, one involving protein kinase C and the other protein tyrosine kinases (Izquierdo *et al.*, 1992).

The downstream consequences of activation of p21^{ras} have not previously been determined in T cells: this is in contrast to other cell types where *ras* oncogenes have been shown to cause transformation, differentiation or growth arrest (Haubruck and McCormick, 1991). The effects of the known activators of ras in T cells are, however, well characterized; as shown in Figure 2 and reported previously (Crabtree, 1989), both protein kinase C stimulatory phorbol esters and activating antibodies directed against the T cell receptor cause synthesis of the T cell growth factor IL-2. Production of IL-2 is a critical event in T cell activation and clearly makes a good candidate for control by ras proteins. This response can be mimicked by use of the synthetic IL-2CAT construct in which the chloramphenicol transferase gene is placed under the control of the *IL-2* gene promoter and enhancer (Durand *et al.*, 1988); the enhancer region of the *IL-2* gene binds several transcription complexes which have previously been shown to be activated, at least indirectly, by active p21^{ras} in other cell types, such as the phorbol ester inducible AP-1 complex (Angel *et al.*, 1987b; Wasylyk *et al.*, 1987) (consisting of jun and fos family

members) and the phorbol ester activated NF-κB activity, composed of several members of the rel-related family (Gutman and Wasylyk, 1991). In addition, in T cells phorbol esters are known to synergize with ionomycin in increasing the activity of the NF-AT complex which binds to the *IL-2* enhancer (Mattila *et al.*, 1990; Hivroz-Burgaud *et al.*, 1991).

The data presented here show that expression of a mutationally activated ras protein in the murine T cell line EL4 leads to an enhanced production of biologically assayable IL-2 protein in response to phorbol esters and to T cell receptor agonists. Activated ras also synergizes with the calcium ionophore ionomycin to cause IL-2 production, whereas neither activated ras nor ionomycin alone give any response. Similar effects can be seen when the expression of the cotransfected IL-2CAT construct is measured. Activated ras alone can, however, induce expression of AP-1CAT as strongly as can phorbol ester. This implies that the failure of ras by itself to cause IL-2CAT induction is not due to inadequate levels of ras protein expression. A recent report has shown that control of such an IL-2CAT reporter construct is also boosted by cotransfected *ras* in the EL4 T cell line (Baldari *et al.*, 1992); in this case ras alone was capable of causing a very small stimulation of IL-2CAT expression without the need for a synergizing signal. It is not clear why ras absolutely requires a synergizing signal such as ionomycin in our system: the most likely explanation is clonal variation within the EL4 cell line such that in the work of Baldari *et al.* the cells used might be constitutively receiving a weak activating calcium signal.

The positive effects of activated ras on IL-2 expression indicate that a signalling pathway connects ras proteins to regulation of transcription factors controlling the *IL-2* gene. Since ras alone cannot activate IL-2 expression it would appear that this pathway by itself is not sufficient to transmit to the IL-2 enhancer the stimuli originating from the T cell receptor or from protein kinase C. The ras pathway must synergize with at least a calcium signal in order to stimulate IL-2 expression. This is compatible with the notion that ras activation is one of a number of downstream signalling events stimulated by protein kinase C and by the T cell receptor. However, is the ras signalling pathway essential for the activation of IL-2 expression in response to T cell receptor or protein kinase C stimulation in T cells? Our data using the dominant negative mutant of *ras*, H-*ras*N17, would indicate that this is the case. Removal of endogenous ras function from these cells renders them almost completely incapable of giving IL-2CAT expression in response to protein kinase C or T cell receptor stimulation.

Several examples for $p21^{ras}$ being essential for either receptor tyrosine kinase or phorbol ester induced signalling have recently been reported. For example, signalling through the nerve growth factor (NGF) receptor tyrosine kinase, $p145^{trk}$, was completely blocked and that through protein kinase C was partially blocked by dexamethasone induced expression of c-Ha-*ras*N17 (under the control of the MMTV LTR) in PC12 cells (Szeberenyi *et al.*, 1990) (Thomas *et al.*, 1992; Wood *et al.*, 1992). In an NIH3T3 cell line overexpressing the insulin receptor, transfection of c-Ha-*ras*N17 abolished the insulin induced expression of both collagenase promoter and *fos* promoter–reporter constructs (Medema *et al.*, 1991). For NIH3T3 cells containing MMTVc-Ha-*ras*N17, treatment with dexamethasone completely blocked epidermal growth factor (EGF) and phorbol ester stimulated DNA synthesis, and partially inhibited the stimulation by serum, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) (Cai *et al.*, 1990). While it appears likely from work presented here and previously that protein kinase C lies upstream of *ras* in T cells, in other cell types studied most data point to protein kinase C functioning principally downstream of *ras* (Marshall, 1991). We are currently addressing whether in T lymphocytes protein kinase C acts as a downstream mediator of $p21^{ras}$, as well as an upstream activator.

The data presented suggest that either a calcium signalling pathway or a ras independent protein kinase C mediated pathway must also be activated in addition to the ras mediated pathway in order to stimulate IL-2 expression. As yet the identities of proteins acting as effectors of ras and of the other signalling molecules in this system are unknown, although several candidates exist. From work in other cell types it is possible that NF-κB and AP-1 act as the end points for the ras independent protein kinase C pathway (Gutman and Wasylyk, 1991). It has been shown that the transcription factor complex NF-AT mediates the effects of calcium signals on IL-2 expression (Mattila *et al.*, 1990; Hivroz-Burgaud *et al.*, 1991). Our own recent work implicates NF-AT as one, but probably not the only, target for the ras dependent pathway (M.Woodrow, S.Rayter, J.Downward and D.Cantrell, submitted).

Materials and methods

Reagents

Ionomycin, acetyl CoA and T6520 silica gel on polyester TLC plates were from Sigma. Phorbol-12,13-di-butyrate was from Calbiochem (UK). [^{14}C]chloramphenicol (50–60 mCi/mmol), [^{32}P]orthophosphate (10 mCi/ml) and [^{3}H]thymidine (20–30 mCi/mmol) were from Amersham (UK). MAb 2C11 (reactive against the murine CD3 component of the TCR) was coated on to tissue culture plates overnight before the addition of cells. 2C11 and OKT11 (Becton Dickinson) reactive against human CD2 monoclonal antibodies were protein A–Sepharose purified from cell culture supernatant. Y13-259 rat monoclonal antibodies were purified from cell culture supernatant using rabbit anti-rat protein A–agarose. Rabbit anti-rat polyclonal antibody was from Serotec, fluorescein isothiocyanate conjugated goat anti-mouse polyclonal antibody was from Sera-lab, and protein A–agarose was from Bio-Rad. Recombinant IL-2 was from Eurocetus.

Cells

The murine thymoma EL4 was maintained in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum and 10 μM β-mercaptoethanol (Farrar *et al.*, 1980). The IL-2 dependent cell line, CTLL, was maintained in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 15 μM β-mercaptoethanol and 20 ng/ml recombinant IL-2. Growth was at 37°C and humidified in 5% CO_2/95% air.

Labelling cells and immunoprecipitations

Activation of $p21^{ras}$ in response to phorbol ester was determined from ^{32}P-labelled cells. For each treatment, 5×10^7cells were labelled with 1 mCi [^{32}P]orthophosphate in 10 ml of phosphate-free DMEM medium, 20 mM HEPES buffer pH 7.5 and 1 mg/ml BSA. Cells were then treated with 100 ng/ml PDBu for the desired period and the endogenous $p21^{ras}$ was immunoprecipitated with the monoclonal antibody Y13-259 as previously described (Basu *et al.*, 1992). The nucleotides bound to the immunoprecipitated $p21^{ras}$ were resolved by TLC.

IL-2 assays

CTLL cells which had been fed with IL-2 36 h previously were used for the IL-2 assay. The cells were washed in RPMI medium then incubated in complete medium without IL-2 for at least 1 h. 96 well plates were prepared with 100 μl of serial doubling dilutions of assay supernatants and 100 μl of 5×10^4 CTLL cells were added. IL-2 standards were incorporated

in the assay, starting at a concentration of 20 ng/ml. Cells were incubated for 48 h, 1 μCi [^{3}H]thymidine was added to each well and cells were incubated for a further 4 h. Cells were harvested on to glass filter paper and counted in scintillation fluid.

Plasmids and DNA preparations

IL-2CAT was a gift from G.Crabtree (Stanford, USA) and has been previously described (Durand *et al.*, 1987). Briefly, the IL-2CAT construct contains 275 bp of 5′ sequence from the human IL-2 gene, which contains the IL-2 minimal promoter and enhancer, driving the reporter gene CAT. The construct used for the expression of activated ras was created by inserting the 0.7 kb cDNA fragment of v-Ha-*ras* into the *Bam*HI site of pcDNA-1 (Invitrogen, San Diego, CA, USA) under the control of the CMV promoter. Orientation was determined by restriction analysis, and activity assessed by focus formation in Rat-1 fibroblasts. The dominant negative *ras* construct has been previously described, and contains a 4.8 kb genomic fragment of c-Ha-*ras* which contains a mutation at codon 17, replacing serine with asparagine, expressed under the control of the RSV promoter. CMVCAT was a gift from P.Parker (ICRF), RSVCAT and LTRCAT were gifts from R.Treisman (ICRF), and AP-1CAT was a gift from N.Jones (ICRF).

Plasmids were prepared by alkali lysis, as described in Sambrook *et al.* (1989), and purified by two rounds of caesium chloride–ethidium bromide equilibrium centrifugation.

Transfections

Cells were transfected by electroporation (Gene Pulser, Bio-Rad) as follows; 2.5×10^7 cells in 0.25 ml RPMI medium were mixed with DNA in pre-chilled 0.4 cm cuvettes (Bio-Rad), pulsed at 250 V (960 μF), then left on ice for 10 min prior to addition of complete medium and transferring the mixture to plates. Time constants were typically 75 ± 8. 24 h after transfection various stimuli were added and CAT assays were performed after a further 18 h incubation. The efficiency and reproducibility of the transfection procedure was characterized in preliminary experiments using a vector encoding the human CD2 cell surface expressed protein under the control of a CMV promoter. Eight separate transfections with the CMVCD2 construct were analysed for expression of the CD2 protein on the cell surface, with and without phorbol ester treatment. 48 h post-transfection cells were coated with a mouse monoclonal antibody (OKT11) specific for the extracellular component of the CD2 protein and visualized using a goat anti-mouse polyclonal antibody conjugated to fluorescein isothiocyanate. Live cells positive for CD2 expression were visualized with a FACS scanner at 488 nm wavelength. A mean of 26% (σ = 6) of live cells were found to be positive for expression of CD2 48 h after transfection, whether treated with phorbol ester or not.

CAT assays

Assays were performed according to the method of Gorman *et al.* (1982). Briefly, aliquots of 2.5×10^7 cells were lysed by three cycles of freeze–thaw in 100 μl of 0.25 M Tris–HCl buffer, pH 8, followed by a 10 min incubation at 65°C to heat-kill the lysate. Cellular debris was pelleted and the supernatants were assayed for CAT activity by addition of 0.4 mM acetyl CoA and 0.3 μM [^{14}C]chloramphenicol, then incubated at 37°C for 18 h (IL-2CAT and AP-1CAT) or 1 h (CMVCAT, RSVCAT and LTRCAT). Chloramphenicols were extracted in ethyl acetate and acetylated products separated from non-acetylated chloramphenicol by TLC in 95%/5% chloroform/methanol running buffer. TLC plates were quantitated by an AMBIS β scanner and percentage conversions of acetylated chloramphenicol were calculated. CAT assays were internally controlled for transfection efficiency by expressing activity as fold conversion relative to unstimulated cells.

Acknowledgements

We thank Hans Bos for supplying the H-*ras*N17 expression constructs and Gerry Crabtree for the IL-2CAT construct. Thanks also to Peter Parker and Mike Owen for critical review of this manuscript.

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Received on June 25, 1992; revised on August 24, 1992