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The co-operation between Ras/Raf and SV40 large T (LT) in the transformation of primary rat Schwann cells is a strikingly synergistic process. We have explored the signalling pathways and the molecular mechanisms involved. Either activated Ras or an inducible Raf onco-protein alone, induce a G1-specific cell cyle arrest. This requires prolonged activation of MAP kinase activity followed by the inhibition of cyclin-dependent kinases (cdk) via induction of the cyclin/cdk inhibitory protein, $p21^{Cip1}$. In contrast, when Raf is activated in the presence of LT or dominant-negative (dn)-p53, the cell cycle arrest and $p21^{Cip1}$ induction are specifically abolished and the ability of Raf to increase the specific activity of cyclin-dependent kinases is revealed. Thus Raf-dependent G1 arrest and $p21^{Cip1}$ induction require p53 function. This indicates that in this scenario p53 plays a key role in signal integration and determines the specificity of the cellular response to the Ras/Raf pathway independent of DNA damage. It appears that this particular feature of p53 together with its involvement in the induction of apoptosis may represent an important element in its role as a tumour suppressor gene in Schwann cells. In addition, we show that super-induction of cyclin A/cdk2 activity is causally involved in the stimulation of Schwann cell proliferation in the absence of mitogens by Raf and LT. This indicates that co-operation between Raf and LT involves involves at least two levels of regulaton: the loss of $p21^{Cip1}$ induction and the co-operative activation of cyclin/cdk complexes.

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Introduction

Neurofibromatosis is a multi-faceted disease involving the hyperproliferation and malignancy of Schwann cells and fibroblastic cells. In our laboratory a model system to study mechanisms that control rat Schwann cell proliferation and transformation has been established (Ridley et al., 1988; 1989). This system has been used to investigate the role of ras-controlled signalling and the cooperation between ras and nuclear oncogenes, such as SV40 large T (LT), for cell division and tumour progression. This approach appears of particular relevance to the understanding of neurofibromatosis for two reasons:

1) Genetic lesions responsible for the autosomal dominant disorder 'von Recklinghausen neurofibromatosis', have been shown to lead to the decreased expression of a gene named NF1 (Viskochil et al., 1990; Cawthon et al., 1990; Wallace et al., 1990; Declue et al., 1992; Basu et al., 1992). NF1 codes for a protein, neurofibromin, which has a catalytic domain with homology to GAP, a protein which activates the intrinsic GTPase of ras proteins. The GAP-related domain (GRD) of neurofibromin has also been shown to stimulate the $p21^{ras}$ GTPase activity (Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990). Inhibition of the Ras pathway in malignant schwannomas from patients with neurofibromatosis type 1, using either neutralising antibodies to Ras or by increasing Ras-GAP activity, results in a reversion of the tumour cells, confirming the role of Ras in tumour formation in this disease (Basu et al., 1992; DeClue et al., 1992).

2) Neurofibromatosis patients frequently develop multiple benign neurofibromas, which are composed mainly of Schwann cells. They also have an increased risk of developing malignant tumours derived from Schwann cells or other neural crest derived cell-types. The development of these lesions is likely to involve the accumulation of multiple genetic events. Indeed, in addition to the loss of NF1, malignant neurofibrosarcomas were found to be associated with deletions and mutations of the tumour suppressor gene p53 (Menon et al., 1990). The loss of this tumour suppressor is mimicked by LT, as one of its functions appears to be the inhibition of p53 activity via stable direct interaction (Gannon et al., 1990). In addition, transgenic animals in which LT expression is directed to Schwann cells develop neurocristopathies which resemble Neurofibromatosis type 1, demonstrating that LT expression can also contribute to the development of tumours in this cell type *in vivo* (Mazarakis et al., 1996).

The accumulation of multiple genetic lesions including losses of tumour suppressor gene function and oncogene activation can be mimicked in our in vitro Schwann cell transformation system. Moreover, our studies indicate that the elucidation of links between ras signalling and the mechanisms controlling cell cycle progression appears of central importance to the understanding of Schwann cell transformation. The co-operation of Ras and LT in the transformation of primary Schwann cells is a synergistic process in which the cellular response to Ras is dependent on the presence of LT (Ridley et al., 1988). Introduction of activated Ras alone, results in morphologically transformed cells that are growth arrested and only when co-expressed with LT does Ras induce the formation of highly proliferative, anchorage-

independent cultures. In contrast, LT alone lowers the growth factor requirement of cells which otherwise exhibit normal behaviour.

Schwann cells from NF-/- embryos have elevated levels of Ras-GTP, resemble Ras-infected Schwann cells and grow much more slowly than the wild-type cells (Kim, et al., 1995), demonstrating that the observable NF1-/- phenotype is mimicked by activation of the Ras/Raf pathway. The poor growth properties of primary Schwann cells in which the Ras pathway is activated, suggest that further genetic events are required for tumour formation in this cell-type.

Taken together, the convergence and integration of oncogene and/or suppressor gene-controlled signalling pathways in particular at the level of cell cycle regulators (see below) appears essential to the process of tumour progression or oncogene cooperation in Schwann cells. Detailed analysis of these mechanisms should lead to a greater understanding of how Ras-signalling and nuclear oncogenes cooperate to abrogate the growth control of Schwann cells. In the long term this work should provide a basis for the development of stratagies and reagents to specifically interfere with the genetic events and the transforming activities that cause Neurofibromatosis and related diseases.

Initially proposed research goals

1) Identification and characterisation of the signalling pathways activated by ras.

2) Testing whether the expression of proteins involved in the control of the cell cycle can alleviate the ras induced G1 block of the cell cycle.

3) Genetically and biochemically characterising the cdc 2 and cyclin A promoter response elements and their corresponding DNA binding proteins which mediate the effects of ras and nuclear oncogenes.

4) Development an *in vivo* model to study the tumour forming capabilities of the Schwann cell lines.

Goals 1 and 2 have been intensively pursued. We have made significant progress in these areas during the granting period. The results obtained are detailed and discussed below.

The funding for goal 3 was cut by the site visiting comitte in May 1995. Progress in this area up until then was reported previously and is not included in this final report.

Work towards goal 4 has been fraught with technical difficulties such that it became impossible to design informative animal experiments. It was therefore necessary to abandon this part of the work. The reasons for this are discussed below.

Results

Ras can be replaced by an inducible Raf protein

In order to explore the molecular basis for the co-operation between Ras and LT we have made use of an inducible Raf protein in which an activated Raf kinase has been fused to the oestrogen receptor hormone binding domain (ΔRaf-1:ER) (Samuels et al., 1993). Raf has been shown to act directly downstream of Ras (Moodie et al., 1993; Van Aelst et al., 1993; Vojtek et al., 1993; Warne et al., 1993) and elicits a similar phenotype in some cell types; for example in PC12 cells both activated Ras and Raf proteins are capable of inducing neurite outgrowth (Wood et al., 1993). In primary Schwann cells we

show that the effects of Raf are indistinguishable from those of Ras (see below).

Raf alters Schwann cell morphology and causes G1 arrest

The Raf/oestrogen receptor fusion protein (ΔRafER-1:ER) exhibits kinase and transforming activity in a strictly hormone-dependent fashion (Samuels, et al., 1993). Low passage normal rat Schwann cells were infected with the retroviral vector LXSN (Miller and Rosman, 1989) or its derivative LXSN-ΔRaf-1:ER. Several hundred G418 resistant colonies infected with the empty vector (NSE) or the vector encoding the inducible Raf protein (NSΔRafER) were pooled and expanded. The growth properties of these cells were maintained over several months, in that the cells did not appear to enter crisis or show any significant changes in their ability to quiesce or respond to mitogenic signals. Early passage NSΔRafER cells were then infected with a second retroviral vector, Babe-Puro (Morgenstern and Land, 1990) or with the Babe-Puro vector expressing SV40 LT. Puromycin resistant colonies expressing both ΔRaf-1:ER and LT (NSΔRafERLT) or ΔRaf-1:ER and the empty vector (NSΔRafERBp) were pooled and expanded.

The addition of 4-hydroxy-tamoxifen (TMX) to the NSΔRafER cells resulted in a dramatic change in cell morphology (Fig 1A). When viewed by time-lapse video microscopy, elongation of the normally flat cells could be seen as early as six hours. In addition to the cells exhibiting a highly refractile phenotype, they also extended processes and became more motile. This motility was not inhibited by cell-cell contact as the cells would move across each other. These morphological changes were indistinguishable from those seen when Schwann cells are injected with Ras protein (Ridley, et al., 1988) or those reported in

Schwann cells isolated from late-stage embryos with a homozygous deletion of the NF1 gene (Kim et al., 1995). The activation of Raf in the cells co-expressing LT (NSΔRafERLT) led to similar morphological changes and increased cell motility while the addition of TMX to Schwann cells expressing the empty LXSN vector (NSE) had no detectable effects on the morphology of the cells (data not shown).

In order to address the effects of the activation of ΔRaf-1:ER on the proliferation of normal Schwann cells, the cells were analysed for BrdU incorporation and DNA content by flow cytometry. The addition of TMX to the NSΔRafER cells resulted in a cell-cycle arrest in the G1 phase of the cell-cycle, whereas TMX had no effect on the cell-cycle profile of the control NSE cells (Fig1B). This inhibition of cell growth was confirmed by ^{3}H-thymidine uptake assays, which showed an approximately 80% decrease in DNA synthesis (Figure 1C, right hand panel). When the cells were followed by time lapse microscopy a complete cessation of cell division was observed within 30 hours. This was not associated with any observable cell-death during the 72 hours of the experiment (data not shown). In contrast, the cells co-expressing LT (NSΔRafERLT) were not inhibited in response to the activation of ΔRaf-1:ER; indeed ΔRaf-1:ER increased the rate of DNA synthesis (Fig. 1C, right hand panel). The mitogenic effect of Raf in the NSΔRafERLT cells was seen more clearly when the cells were incubated in DMEM containing 2% FCS without GGF or forskolin, conditions in which the NSΔRafER cells quiesce and the LT cells grow more slowly (Fig. 1C, left hand panel). Under these conditions, addition of TMX to the NSΔRafERLT cells led to a greater than 3-fold increase in the rate of DNA synthesis as measured by ^{3}H-thymidine uptake. The activation of ΔRaf-

1:ER had no significant effect on DNA synthesis in the quiescent NSΔRafER cells (Fig 1C, left hand panel).

Thus Raf behaves similarly to Ras in Schwann cells by inducing distinct morphological changes and a cell-cycle arrest. As with Ras, the cell-cycle block induced by Raf is overcome by co-expressing LT and in this scenario the Raf signal is re-interpreted as a mitogenic stimulus. This would suggest that, in Schwann cells it is the Raf signalling pathway which is responsible for both Ras-induced morphological transformation and the effects of Ras on the cell-cycle.

Constitutive activation of MAP kinase by ΔRaf-1:ER in Schwann cells

As an indicator of the activation of signalling pathways downstream from Raf, we compared the kinetics of MAP kinase activation following mitogen stimulation in the presence and absence of activated ΔRaf-1:ER. Treatment of quiescent NSΔRafER cells with mitogens resulted in a transient activation of p42-ERK-2, as measured by an in-vitro kinase assay and a phosphorylation-dependent mobility shift (Fig. 1D) which has been shown to be characteristic for MAP kinase activity. In contrast, the addition of TMX to the NSΔRafER cells in the presence (Fig.1D) or absence (not shown) of exogenous mitogens resulted in the prolonged activation of p42-ERK-2. It therefore appears that the constitutive activity of ΔRaf-1:ER, which results in a cell-cycle arrest, is overriding the growth-promoting signals by the mitogens. The activation of ΔRaf-1:ER in cells co-expressing LT (NSΔRafERLT), also led to the prolonged activation of p42-ERK-2, with indistinguishable kinetics to those seen in the NSΔRafER cells (not shown). Thus depending on the presence of LT, a constitutive Raf signal has opposing effects on the cell-cycle.

Raf arrests Schwann cells prior to induction of cyclin A

To investigate the mechanism by which Raf causes a cell cycle arrest and how this is overcome in the NSΔRafERLT cells, we decided to study the effects of Raf on cyclin/cdk activity. In an initial experiment, we addressed whether the activation of Raf was capable of blocking NSΔRafER cells from entering the first S phase following growth factor stimulation of quiescent cells, as this would enable us to analyse the effects of Raf in a synchronised cell population. Subconfluent NSΔRafER cells were rendered quiescent by incubating them in medium containing 2% FCS for 2 days. Pulse-labelling with ^{3}H-thymidine showed that entry into S phase occurred 16 to 20 hours following mitogen addition with a peak of DNA synthesis between 20 and 28 hours (Fig. 2A). The co-addition of TMX resulted in an approximately 80% decrease in the proportion of cells reaching the first S phase. This inhibition was not due to a delay in the cells entering S phase since further incubation did not lead to a significant increase in the incorporation of ^{3}H-thymidine in cells treated with TMX, whereas the untreated cells continued to cycle (not shown).

To determine whether this Raf-induced arrest was linked to changes in cyclin or cdk expression, cell lysates were prepared at various time points and analysed by immunoblotting. Cyclin D1 levels were very low in the quiescent cells (Fig. 2B) and were induced as the cells entered G1. In the presence of TMX, the levels of cyclin D1 were super-induced. Other studies have reported that Ras expression and transformation leads to a marked elevation of cyclin D1 levels (Liu et al., 1995; Winston et al., 1996). Our results demonstrate that the activation of the Raf pathway is sufficient to induce cyclin D1. Cyclin E levels, appeared to be

invariant as the cells progressed from the quiescent state through G1 and were unaffected by the activation of ΔRaf-1:ER, suggesting that modulation of cyclin E levels, is not the principal mechanism controlling cell cycle progression in primary Schwann cells. Cyclin A protein, although present in significant amounts in the quiescent cells, was induced as the cells progressed towards and entered S phase. However, in the cells treated with TMX, this induction of cyclin A was not seen (Fig. 2B). Cdk4 expression increased slightly during G0/G1 progression, while cdk2 levels remained relatively unchanged. However neither cdk4 nor cdk2 levels appeared to be affected by ΔRaf-1:ER activation. These results suggest, that activation of ΔRaf-1:ER leads to a cell cycle arrest in G1 prior to the induction of cyclin A.

Raf inhibits cyclin E- and cyclin A -dependent kinase activity

The finding that the block in G1 appeared to occur prior to the induction of cyclin A was also reflected by the low levels of cyclin A protein observed when growing cells were arrested by activation of ΔRaf-1:ER (Fig 3A, top left hand panel). Immunoprecipitation of cyclin A complexes from these cells showed that there was a corresponding decrease in cyclin A/cdk2 complexes (Fig. 3A top right hand panel) and cyclin A-dependent kinase activity (Fig. 3A, bottom panel). It appears that in Schwann cells, virtually all the cdk2 associated with cyclin A is the faster migrating isoform of cdk2, which has been shown to correspond to the active form phosphorylated on residue Thr160 (Gu et al., 1992). This may reflect high CAK activity in Schwann cells or possibly a preferential association of cyclin A with the activated kinase.

Unlike cyclin A, cyclin E levels were unaffected by activation of ΔRaf-1:ER (Fig. 3B, left hand panel). Moreover, immunoprecipitation of cyclin E complexes from

both growing and arrested NSΔRafER cells showed that there were similar levels of cyclin E/cdk2 complexes in the growing and arrested cells (Fig 3B, upper right hand panel). The cdk2 complexed to cyclin E, as for the cyclin A complexes, was mostly the CAK- phosphorylated form. Interestingly however, the cyclin E-dependent kinase activity associated with these complexes was reduced almost to background levels in the arrested cells (Fig 3B, bottom panel). Thus activation of Raf leads to a dramatic decrease in the specific activity of cyclin E/cdk complexes. The loss of both cyclin E and cyclin A-dependent kinase activities in the arrested cells was reflected by a similar inhibition of cdk2 precipitable H1 kinase activity (data not shown). Cyclin E, cyclin A and cdk2 activity have each been shown to be required for entry into S phase (Girard et al., 1991; Pagano et al., 1992; Zindy et al., 1992; Tsai et al., 1993; van den Heuvel and Harlow, 1993; Ohtsubo et al., 1995). Therefore it is likely that the inhibition of these cyclin/cdk complexes is sufficient to arrest the cells in G1.

Raf induces $p21^{Cip1}$ expression

Since cyclin E/cdk2 activity was reduced without a concomitant decrease in the expression of the proteins, we investigated whether Raf activation affected the expression of cdk inhibitors, such as $p21^{Cip1}$ (el-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993; Noda et al., 1994) and $p27^{Kip1}$ (Polyak et al., 1994; Toyoshima and Hunter, 1994). Lysates were prepared from NSΔRafER cells grown in the presence or absence of TMX and the inhibitor levels were analysed by western blotting. Activation of Raf had no effect on $p27^{Kip1}$ levels (Fig. 4A). However, Raf activation induced a large increase in the levels of $p21^{Cip1}$ (Fig. 4B). The addition of TMX to the control NSE cells had no effect on the levels of $p21^{Cip1}$ (data not shown). Samples of each lysate were also

immunoprecipitated with cyclin E antibodies and the precipitated proteins were immunoblotted with $p21^{Cip1}$ antibodies. Figure 4C shows that the induction of Raf in the NSΔRafER cells resulted in a corresponding increase in the amount of $p21^{Cip1}$ associated with cyclin E complexes. Moreover, the induction of $p21^{Cip1}$ as well as the activation of p42-ERK-2 require MEK activity, as both events are sensitive to the MEK inhibitor PD98059 (Fig. 4 D). This clearly indicates the involvement of the MAP kinase pathway in Raf-dependent $p21^{Cip1}$ induction.

To further address whether the induction of $p21^{Cip1}$ is responsible for the suppression of cyclin E-dependent kinase activity and the inhibition of DNA synthesis in Raf arrested cells, we determined the kinetics of these three events. The rate of the ΔRaf-1:ER-induced inhibition of DNA synthesis was analysed by measuring the incorporation of ^{3}H-thymidine in NSΔRafER cells pulse-labelled at various time points. These experiments showed that the growth inhibition induced by ΔRaf-1:ER commenced after a 12 hour lag period with significant decreases in ^{3}H-thymidine uptake apparent after 14 hours and complete inhibition after 24 hours (Fig. 5A). The late onset of the growth arrest induced by ΔRaf-1:ER was paralleled by a similar delay in the induction of $p21^{Cip1}$, with a small increase seen at 10 hours and maximal induction at approximately 22 hours (Fig. 5B). This delayed appearance of $p21^{Cip1}$ was in contrast to the rapid induction of cyclin D1, which could be seen as early as 3 hours following TMX addition to growing(not shown) or quiescent cells (Fig. 2B). The decrease in cyclin E-dependent kinase activity coincided with the induction of $p21^{Cip1}$ and preceded the inhibition of DNA synthesis (Fig. 5A), arguing that $p21^{Cip1}$ is responsible for the inhibition of the kinase activity which results in the growth arrest of the cells.

Raf induction of $p21^{Cip1}$ and cell cycle arrest are p53 dependent

Schwann cells co-expressing ΔRaf-1:ER and LT (NSΔRafERLT), no longer arrest in response to ΔRaf-1:ER activation. We were therefore interested to explore whether ΔRaf-1:ER would induce $p21^{Cip1}$ in these cells. Pools of NSΔRafERLT cells and control NSΔRafERBp cells, were grown for 30 hours in the presence or absence of TMX. Lysates prepared from these cells were then subjected to western blot analysis to detect $p21^{Cip1}$. We found that although ΔRaf-1:ER induced $p21^{Cip1}$ in the control cells, LT co-expression resulted in both a decrease in the basal level of $p21^{Cip1}$ expression and an apparent loss of $p21^{Cip1}$ induction (Fig. 6A).

One of the properties of LT is that it can bind to and sequester p53 (Lane and Crawford, 1980; Maltzman et al., 1981). p53 is known to control the regulation of $p21^{Cip1}$ expression (el-Deiry, et al., 1993; Li et al., 1994; Liu, et al., 1995; Macleod et al., 1995) and in recent experiments it has been shown that embryonic fibroblasts isolated from p21-/- mice and a colon cancer cell line with a homozygous deletion of the p21 gene are significantly or completely deficient in their ability to arrest in G1 in response to p53-dependent growth inhibitory signals (Brugarolas et al., 1995; Deng et al., 1995; Waldman et al., 1995). We were therefore interested as to whether the induction of $p21^{Cip1}$ in response to Raf was p53 dependent and whether inactivation of p53 was capable of abolishing the ΔRaf-1:ER-induced growth arrest. NSΔRafER cells were infected with a BabePuro retroviral vector constructed to encode a dominant negative mutant (dn) of p53 ($p53^{175}$), which has been shown to inhibit the activity of endogenous p53 (Kern et al., 1992). Puromycin-resistant colonies were pooled and expanded. Proliferating NSΔRafER$p53^{175}$ were incubated in the presence or absence of TMX. Lysates were prepared and FACS

analysis was carried out on the cells 30 hours after the addition of hormone. Immunoblot analysis of the lysates showed that as with the LT expressing cells, the basal levels of $p21^{Cip1}$ expression were reduced in the cells expressing dn-p53 and ΔRaf-1:ER activation no longer stimulated a significant induction of $p21^{Cip1}$ (Fig. 6A). Consistent with this finding, equivalent levels of $p21^{Cip1}$ were precipitated by cyclin E antibodies from the same lysates (Fig. 6B). Similar results were obtained with NSΔRafER cells infected with a retrovirus expressing the carboxy-terminal oligomerisation domain of p53 (data not shown). Thus ΔRaf-1:ER induction of $p21^{Cip1}$ in Schwann cells is p53-dependent.

Cyclin D1 induction by ΔRaf-1:ER, unlike $p21^{Cip1}$, does not appear to be p53 dependent. Immunoblot analysis of lysates of the NSΔRafER$p53^{175}$ cells showed that Raf stimulated a large induction of cyclin D1, similar to that seen in the parental cells (Fig. 6A). This observation demonstrates that p53 is only required for a specific subset of Raf signals. Cyclin D1 levels are lower in NSΔRafERLT cells, a finding consistent with other reports which have shown that RB-binding proteins such as LT, down regulate cyclin D1 expression and these cells no longer require cyclin D1 function to cycle (Lukas et al., 1994). Raf activation is still able to stimulate cyclin D1 expression in these cells but only to levels found in the uninduced NSΔRafER cells (Fig.6A) indicating that the pathway required for cyclin D1 induction remains active in the LT expressing cells.

Cyclin E-dependent kinase assays performed on aliquots of lysates from NSΔRafER$p53^{175}$showed that in the presence of dn-p53, ΔRaf no longer inhibited the kinase activity but instead stimulated a 2-fold increase in kinase activity (Fig. 6C). Likewise in the NSΔRafERLT cells Raf also stimulated an

increase in cyclin E-dependent kinase activity. Thus, in the absence of $p21^{Cip1}$ induction, signals from Raf increase cyclin E-dependent kinase activity.

FACS analysis of the NSΔRafERp53^{175} cells showed that their cycling was unaffected by ΔRaf-1:ER activation (Fig. 6C) demonstrating that the Raf induced growth arrest is dependent on normal p53 function.

Raf and LT co-operate to increase cyclin A/cdk2 activity and induce DNA synthesis

Although Schwann cells expressing dn-p53 do not arrest in response to ΔRaf-1:ER , it is only in LT expressing Schwann cells that ΔRaf-1:ER causes an increase in cell cycling. This indicates that in addition to inhibiting p53 activity, LT has additional effects on the cell-cycle (Fig. 7A). It is unlikely that this involves the ΔRaf-1:ER-induced increase in cyclin E-dependent kinase activity, as similar levels of kinase activity were found in cells expressing dn-p53 or LT (Fig.6D). In contrast, cyclin A-dependent kinase activity was found to be consistently higher in the LT cells (Fig 7B). Western blot analysis showed that NSΔRafERLT cells had elevated levels of cyclin A and cdk2 when compared with NSΔRafER or NSΔRafERp53^{175} cells (Fig.7B),whereas cyclin E levels were unaffected by LT expression (not shown). Cyclin A and cdk2 levels were unaffected by the removal of mitogens (not shown) or by the activation of Raf (Fig. 7B). These findings are consistent with those reported by Oshima et al., 1993, showing that the expression of LT in primary rat lung epithelial cells resulted in a dramatic increase in the expression of cyclin A and cdc2 proteins.

In order to address whether the elevated levels of cyclin A complexes induced by LT may contribute to the ability of Raf to stimulate DNA synthesis in the

NSΔRafER cells we infected NSΔRafERp53^{175} cells with a Babe hygro retroviral vector constructed to encode cyclin A. Consistent with a role of cyclin A in the increased proliferative response to ΔRaf-1:ER, polyclonal populations of these cells showed an increased proliferative response to ΔRaf-1:ER, whereas Babe hygro infected control cells behaved as the parental cells (Fig. 7A, right-hand panel).

When we measured cyclin A-dependent kinase activity in NSΔRafERLT cells we found that in the absence of exogenous mitogens, the complexes were mostly inactive. Activation of ΔRaf-1:ER in these conditions stimulated the cells to enter S phase, as measured by FACS analysis or ^{3}H-thymidine uptake (not shown) and resulted in a 8-20 fold increase in kinase activity (Fig. 7C upper panel). This activation was not associated with a detectable change in the levels of cyclin A/cdk2 complexes (Fig. 7C lower panel) and thus represents an increase in the specific activity of the complexes. In the presence of mitogens the cyclin A-dependent kinase activity was elevated but the activation of Raf resulted in a further 2-5 fold further stimulation of kinase activity (Fig. 7C). Activation of cdk2-dependent kinase activity was stimulated to a similar extent (not shown), indicating the increase in kinase activity was at the level of the cyclin A/cdk2 complexes. Thus Raf signalling and LT function converge not only to regulate cyclin E/cdk2 activity but also co-operate to increase cyclin A/cdk2 activity with LT inducing cyclin A/cdk2 levels and Raf increasing the specific activity of these complexes. Thus co-operation between Raf and LT involves both the loss of a cell cycle inhibitor protein and the synergistic activation of cyclin A complexes.

In vivo model

A prerequisite to carry out meaningful experiments with our cell system in vivo is the regulatable expression of Ras or Raf proteins in a living animal. However insurmountable technical difficulties have prevented us from achieving this goal. Although we have constructed a RafER fusion protein with a mutant ER motif which can only bind and be activated by 4-OH-tamoxifen but not by any other steroid hormone (Littlewood et al., 1995), so far regulation of similar fusion proteins in transgenic mice has not been observed (Gerard Evan and Martin Eilers, personal communication). Moreover, attempts to achieve a regulation of Ras proteins in Schwann cells based on the tetracyclin repressor system, which has been shown to be useful for in vivo studies, have also failed. Although we have observed regulation of Ras expression in NIH 3T3 cells after inserting a ras cDNA into a retrovirus carrying a tetracyclin-regulatable cassette (Paulus et al., 1996), Ras expression was constitutive when this virus was introduced into Schwann cells. Therefore the proposed in vivo studies have not been carried out. Not in the least this decision was made in order unnecessary use of experimental animals.

Discussion

Co-operating oncogenes target cyclin/cdk inhibitors

In Schwann cells co-operating oncogenes regulate cyclin/cdk complexes by distinct inhibitory and activating mechanisms. Such regulation plays a key role in connecting cellular signalling pathways to cell-cycle control and determines the specificity of the cellular response to the Ras/Raf pathway. Activation of an inducible Raf protein alone, results in a G1-specific cell cyle arrest associated with a MEK-dependent induction of $p21^{Cip1}$ and the concomitant inhibition of

cyclin/cdk activity. In the presence of LT or dominant-negative mutants of p53 however, the $p21^{Cip1}$ induction is suppressed, the ability of Raf to increase the specific activity of cyclin-dependent kinases is revealed and the growth arrest abolished. This effect is most evident in cells containing LT, as they express higher levels of cyclin A/cdk2 complexes and in these cells Raf activation is sufficient to stimulate proliferation in the absence of exogenous growth factors.

The suppression of Cdk inhibitor proteins may be a common property of immortalizing oncogenes. E1A rescues TGFb cell-cycle arrest by binding to and blocking the inhibitory effects of $p27^{Kip1}$ (Mai et al., 1996). Similarly Myc can overcome cell cycle inhibiton by $p27^{Kip1}$ and $p16^{INK4a}$ in fibroblasts via a yet unknown mechanism (B. Amati, personal communication and S. Shellard & H.L., unpublished observations). In addition, fibroblasts isolated from mice carrying a targeted deletion of the INK4a tumour suppressor locus, which encodes the cdk-4/ cdk-6 inhibitory protein $p16^{INK4a}$ can be transformed by Ras or Raf alone (Serrano et al., 1996). This suggests that similar to Raf in Schwann cells, a reduction in the level of cyclin/cdk inhibitory proteins reveals the transforming potential of Ras in fibroblasts.

DNA damage-independent role of p53

The activity of p53 is pivotal to the switch in Raf from a growth inhibitory to a stimulatory signal. Previous work has demonstrated a critical role of p53 in G1 arrest and apoptosis induced by DNA damage after UV irradiation (Kastan et al., 1992; Kuerbitz et al., 1992). Here we show that the activation of the Raf pathway arrests Schwann cells in a p53-dependent fashion in the absence of DNA damage. Thus, it is tempting to speculate that the ability of p53 to modulate the proliferative response to signals such as Ras/Raf activation may

play an important role in its function as a tumour suppressor gene. It is possible that the constitutive activation of the Raf pathway may mimic a damage signal and therefore be sensed by p53 as inappropriate. However, the expression of dominant-negative p53 mutants reduces the growth factor requirement of Schwann cells in the absence of activated Raf (Fig. 7A and data not shown) which suggests a function for p53 in the regulation of normal proliferative signals.

Raf induces $p21^{Cip1}$ by a p53-dependent mechanism

The Raf-induced G1 arrest is preceded by a loss of cyclin E- and cyclin A-dependent kinase activity. The inhibition of cyclin A-dependent kinase activity appears to result from a corresponding decrease in the levels of cyclin A/cdk2 complexes, reflecting a block prior to the induction of cyclin A expression. In contrast, the suppression of cyclin E-dependent kinase is due to an inhibition of the specific activity of cyclin E/cdk2 complexes and correlates with increased expression and binding of the inhibitor protein $p21^{Cip1}$ to the complex. The arrested state induced by Raf is very different from the quiescent state resulting from the removal of growth factors in terms of the balance of cyclin/cdk complexes and inhibitor levels. In addition to elevated $p21^{Cip1}$ expression, Raf-arrested cells also overexpress cyclin D1, whereas $p21^{Cip1}$ and cyclin D1 are barely detectable in quiescent cells. Moreover, in quiescent cells the amount of cdk2 bound to cyclin E is very low whereas the levels of cyclin E/cdk2 complexes in Raf-arrested cells are similar to those in proliferating cells (A. L. & H. L., unpublished observations).

It has previously been shown that overexpression of $p21^{Cip1}$ leads to a cell cycle arrest in G1 (Harper et al., 1995). Other reports have looked at kinase activity

following stimuli which lead to a G1 arrest associated with the induction of cdk inhibitory proteins. p53-dependent G1 arrest in response to DNA damage (Dulic et al., 1994), which is known to be significantly dependent on $p21^{Cip1}$ or the induction of the G1 inhibitory proteins $p21^{Cip1}$ and $p27^{Kip1}$ following the detachment of cells from the substratum (Fang et al., 1996) each results in a G1 arrest associated with a decrease in both cyclin E and cyclin A -dependent kinase activity. Interestingly in both cases, this involves an inhibition of the specific activity of cyclin E kinases, resulting in a block in G1 prior to the induction of cyclin A. Thus, cyclin E/cdk2 complexes appear to be a target for $p21^{Cip1}$ and $p27^{Kip1}$ when induced by various mechanisms. This is consistent with cyclin E/cdk2 activity being required for the induction of cyclin A expression. It is possible that $p21^{Cip1}$ is also inhibiting cyclin D-dependent kinase activity, although due to the technical difficulties in measuring cyclin D-dependent kinase activity, this was not further investigated.

The regulation of $p21^{Cip1}$ expression appears complex. DNA damage signals are known to result in the induction of $p21^{Cip1}$ in a p53-dependent fashion via two conserved p53 binding sites in the promoter (Dulic, et al., 1994; Michieli et al., 1994; Macleod, et al., 1995). The G1 arrest associated with these signals has been shown to be partly or fully dependent upon $p21^{Cip1}$ (Brugarolas, et al., 1995; Deng, et al., 1995; Waldman, et al., 1995). However, $p21^{Cip1}$ expression is induced as an immediate early gene, by various mitogens and differentiation agents and by the growth inhibitory peptide TGFb, in a p53-independent fashion (Jiang et al., 1994; Li, et al., 1994; Michieli, et al., 1994; Steinman et al., 1994) and the response to mitogens can be blocked or mimicked by inhibitors or activators of the MAP kinase pathway (Liu et al., 1996). The role of the induction of $p21^{Cip1}$ as cells enter the cycle is unclear, although it has been

proposed that $p21^{Cip1}$ may act as an assembly factor of cyclin/cdk complexes (Zhang et al., 1994). Elevated levels of $p21^{Cip1}$ expression are also associated with the differentiated state of specific tissues and have been postulated to be involved in the maintenance of the quiescent differentiated state (Halevy et al., 1995; Macleod et al., 1995). The latter has also been shown to be a p53-independent mechanism. In Schwann cells, we show that the Raf-dependent induction of $p21^{Cip1}$ is a p53-dependent process and thus differs from the immediate early response seen following mitogen stimulation. It will be of interest to dissect the mechanisms involved in this p53-dependent induction.

In contrast to the rapid induction of cyclin D1 in response to Raf activation, the induction of $p21^{Cip1}$ is delayed. This suggests that the mechanism by which Raf stimulates $p21^{Cip1}$ expression may be indirect. The kinetics of the induction however, may explain how activation of the same pathway i.e. the MAP-kinase pathway, can result in opposing effects on the cell cycle. If constitutive activation of the MAP kinase pathway for several hours is required to induce $p21^{Cip1}$, this may partly explain why transient activation of the same pathway by mitogens, stimulates rather than inhibits the cell cycle.

Raf activates cyclin/cdk activity in presence of a co-operating partner

In the absence of normal p53 function, Raf activation results in an increase in cyclin/cdk activity, presumably by a mechanism which can be suppressed by $p21^{Cip1}$. This demonstrates that the effects of an oncogene cannot be gauged solely by the introduction of a single oncogene into a primary cell, as the specificity of the cellular response can depend on the expression of other co-operating genes. In addition to inhibiting p53, LT leads to the constitutive over-

expression of cyclin A/cdk2 complexes with Raf activation causing a super-induction of the kinase activity. In this context it is noteworthy that although loss of functional p53 abolishes the Raf-induced cell cycle arrest, only cells expressing LT respond to Raf activation with increased proliferation. The elevation of the cyclin A complexes appears to be at least partly responsible for the ability of Raf to stimulate proliferation in these cells, as p53-defective cells infected with a cyclin A carrying retrovirus are similarly induced to cycle by Raf. Preliminary experiments indicate that the mechanism of this activation does not involve further alterations in the levels of $p21^{Cip1}$ or $p27^{Kip1}$ or cdc25-dependent dephosphorylation of cdk2 (AL & HL unpublished observations). As the activation of cyclin-dependent kinase activity appears to be induced in a similar fashion by growth factors (Fig. 7c) it will be important to identify the mechanisms involved in this process.

Implications for Neurofibromatosis

The model system which we have developed demonstrates how progressive genetic changes contribute to the transformed phenotype and describe some of the molecular mechanisms involved. Raf alone, leads to a change in the morphology of the cells, coincident with an increase in cell motility and the induction of growth factor secretion, all of which can be imagined to disrupt the micro-environment of the cell. However, these cells are growth arrested due to the induction of $p21^{Cip1}$. Inactivation of p53, abolishes the growth arrest, whilst retaining the other Raf-dependent properties. The co-expression of LT, in addition, results in the co-operative activation of cyclin A- dependent kinases and these cells acquire the ability to proliferate in response to the Raf signal in the absence of exogenous mitogens.

In Neurofibromatosis Type I, the Ras pathway is activated via inactivation of neurofibromin, a Ras-GAP (Ballester, et al., 1990; Martin, et al., 1990; Xu, et al., 1990; Xu, et al., 1990). Inhibition of the Ras pathway in tumour cells derived from these patients, results in a reversion of the tumour cells, confirming the role of Ras in tumour formation in this disease (Basu, et al., 1992; DeClue, et al., 1992). We have shown that activation of Raf in primary Schwann cells, as previously reported for Ras (Ridley, et al., 1988), results in a growth arrest of the cells. In addition, a recent report described the isolation of Schwann cells from NF-/- embryos. These Schwann cells were shown to have elevated levels of Ras-GTP, resembled Ras- infected Schwann cells and grew much more slowly than the wild-type cells (Kim, et al., 1995), demonstrating that the observable NF-/- phenotype is mimicked by activation of the Ras/Raf pathway. The poor growth properties of primary Schwann cells in which the Ras pathway is activated, suggest that further genetic events are required for tumour formation in this cell-type. It will be of great interest to investigate whether the benign and malignant tumours found in patients suffering from Neurofibromatosis type I, have further genetic defects which result in the suppression of the effects of $p21^{Cip1}$. These may involve the loss of $p21^{Cip1}$ or p53 expression, mutations which affect cyclin/$p21^{Cip1}$ interaction or increases in the levels of cyclin/ cdk complexes. Interestingly, reports of p53 mutations in neurofibrosarcomas from NF1 patients have previously been reported (Menon et al., 1990).

Methods

Cell culture

Schwann cells were purified from 2-3 day old Wistar rats, as previously described (Brockes, et al., 1979; Ridley, et al., 1988). The Schwann cells were

routinely cultured at 37° C (10% CO_2) in Dulbecco's modified Eagles medium (DMEM) with 1.5 mg/ml glucose, supplemented with 3% FCS, 1μM forskolin (Calbiochem) and Glial Growth Factor (GGF) (a kind gift from Mark Noble), on dishes pre-coated with poly-L-lysine (Sigma). Throughout all the experiments phenol red -minus medium and charcoal-stripped serum were used. To render the cells quiescent, the cells were washed twice in DMEM and incubated in either DMEM supplemented with 2%FCS or SATO mix, a mitogen-free supplement (Raff et al., 1983), for 48 hours.

Retroviral vectors

The Xho1/Cla1(blunted) fragment encoding ΔRaf-1:ER (Samuels, et al., 1993) was subcloned into the Xho1/BamH1(blunted) site of the retroviral vector (Miller and Rosman, 1989). The BamH1 fragment encoding SV40LT (Jat et al., 1986) and a BamH1 fragment encoding human $p53^{175}$ (provided by David Lane)(Vojtesek et al., 1992) were subcloned into the BamH1 site of the Babe-Puro retroviral (Morgenstern and Land, 1990). A sequenced PCR fragment encoding amino acids 302-390 of murine p53 ($p53^{CT}$) was subcloned into the BamH1/EcoR1 site of Babe-puro (a kind gift of Trevor Littlewood). A BamH1/Sal1 fragment encoding the human cyclin A gene (a kind gift from Jonathan Pines) was subcloned into the BamH1/Sal1 sites of the Babe Hygro retroviral vector (Morgenstern and Land, 1990). Each of the constructs were transfected, using the standard calcium phosphate method, into the packaging cell line GP+E (Markowitz et al., 1990) and G418, hygromycin or puromycin colonies were pooled and expanded.

Infection of Schwann cells

Schwann cells were infected by co-cultivation, at a 1:2 ratio, with the producer cell lines, which had been pretreated for 2 hours with 20μg/ml mitomycin C

(Sigma). 2-3 days after plating the cultures were transferred into selective medium containing 400μg/ml G418 (Gibco) or 0.4μg/ml puromycin (Sigma) as appropriate. Drug resistant colonies were pooled and expanded.

Facs analysis and DNA synthesis assays

1-2 x 10^6 cells were preincubated for 4 hours with 10μM BrdU(Sigma), trypsinised and then fixed in 80% ethanol. The fixed cells were then incubated with fluorescein isothiocyanate-cojugated anti BrdU antibodies (Becton-Dickinson) and stained with propidium iodide containing RNase (20 μg/ml). Replicative DNA synthesis and DNA content were analysed using bivariate flow cytometry. For DNA synthesis assays, 5 x10^4 cells were seeded in triplicate into 6 well dishes in conditions as described in the figure legends. ^{3}H-thymidine was used at a concentration of 0.5μCi/ml. At the indicated times the cells were lysed in 1% SDS and the TCA precipitable material was filtered and counted.

Western Blot Analysis

The cells were lysed in buffer A (1% NP40, 50mM Tris pH. 8, 150mM Nacl, 10μg/ml aprotinin, leupeptin and pepstatin, 20mM NaF, 1mM Na_3VO_4, 100μg/ml PMSF). Protein concentration was determined using the Biorad protein assay. 30 μg of lysate were resolved by SDS-PAGE and electroblotted onto Immobilon P membranes (Millipore). The following antibodies were used :anti-p42 ERK-2(122), provided by Chris Marshall (Leevers and Marshall, 1992); anti-cyclin.D1, provided by Gordon Peters(Bates et al., 1994); anti-cyclin E (Santa-Cruz, sc-481); anti-cyclin A-E-23, provided by Julian Gannon and Tim Hunt; anti cdk-4 (Santa-Cruz, sc-749); anti cdk-2 (Santa Cruz, sc-163); anti-p27 (Santa Cruz, sc-528); anti-p21(CP36), provided by Wade Harper:

Imunoreactive bands were visualised using enhanced chemiluminescence detection (Amersham International PLC).

Immunoprecipitations and kinase assays

Cells were lysed in buffer A. 300μg of lysate were incubated with 10μg of antibody (cyclin E-Santa-Cruz 481, cyclin A-E72, provided by Julian Gannon and Tim Hunt (Slingerland et al., 1994), cdk-2-Santa-Cruz 163) for 1 hour at 37°C, followed by incubation for a further hour with protein A or protein G sepharose, as appropriate. The beads were washed five times in buffer A and either subjected to Western blot analysis or kinase assays. For kinase assays the beads were washed a further two times in kinase buffer (50mM Tris pH7.5, 10mM $MgCl_2$, 1mM DTT) and then resuspended in 50μl of kinase buffer supplemented with 50μM ATP, 5μCi[^{g32}P]ATP and 10μg of histone H1(Boehringer Mannheim) for 30 minutes at 37°C. The samples were resolved by SDS-PAGE and exposed to Kodak X-OMAT AR. The kinase assays were quantified using ImageQuant by Molecular Dynamics.

Conclusions

We show that in rat Schwann cells Raf and LT co-operate to regulate cyclin/cdk activity and have investigated the molecular mechanisms involved. Activated Ras and Raf cause a G1- specific cell cycle arrest, while together with SV40 large T (LT) or dominant-negative p53 Ras/Raf co-operate to induce rapid proliferation. The G1 arrest induced by Raf alone requires MEK activity and involves prolonged activation of MAP kinase activity followed by the inhibition of cyclin-dependent kinases (cdk) via induction of the cyclin/cdk inhibitory protein, $p21^{Cip1}$. In parallel, Raf also induces an altered cell morphology and increased cell motility. When Raf is activated in the presence of LT or dominant-negative (dn)-p53, cell cycle arrest and $p21^{Cip1}$ induction are specifically abolished while the other Raf-induced parameters remain unchanged. Thus Raf-dependent G1 arrest and $p21^{Cip1}$ induction require normal p53 function. This indicates that in this scenario p53 plays an important role in the integration of cellular signals as well as in determining the specificity of cellular responses. LT is more potent in co-operation with Raf than dn-p53. In contrast to dn-p53, LT permits Raf to induce Schwann cell proliferation in the absence of exogenous growth factors. A LT-induced large increase in the levels of cyclin A/cdk2 complexes is at least partly responsible for this effect. However, in the absence of exogenous mitogens these complexes are mostly inactive. Activation of Raf in the presence of LT stimulates cyclin A/cdk2 activity which is followed by G1/S transition. This indicates that co-operation between Raf and LT involves both the loss of $p21^{Cip1}$ induction and the co-operative activation of a cyclin/cdk complex (see Fig. 8). Our findings suggest that it will be of great interest to investigate whether the benign and malignant tumours found in patients suffering from Neurofibromatosis type I, have genetic defects which result in the suppression of the effects of $p21^{Cip1}$. These may not only be p53 mutations (Menon et al.,

1990) but also involve the loss of $p21^{Cip1}$ expression, mutations which affect cyclin/$p21^{Cip1}$ interaction or increases in the levels of cyclin/ cdk complexes.

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Appendices

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Frank Obermüller, graduate student

Susan Staddon B.A. technician

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Lloyd, A.C., Obermüller, F., Staddon, S., Barth, C.F., McMahon, M. and Land, H. 1996. Co-operating oncogenes converge to regulate cyclin/cdk complexes. Genes Dev. in revision.

Meeting abstracts

Signalling by co-operating oncogenes converges on cyclin/cdk regulation.

Land, H, Lloyd, A.C. and Obermüller, F.

EMBL Conference on 'Oncogenes and Growth Control', Heidelberg, April, 1996

Co-operating oncogenes target cyclin/cdk activity

Lloyd, A.C., Obermüller, F. and Land, H.

EMBL Conference on 'Oncogenes and Growth Control', Heidelberg, April, 1996

Oncogene co-operation in Schwann cells targets cyclin/cdk activity

Lloyd, A.C., Obermüller, F. and Land, H.

FASEB Conference on Neurofibromatosis, Snowmass Co., July, 1996

Figure legends

Figure 1. Activation of Raf in primary Schwann cells leads to the constitutive activation of MAP kinase, morphological alterations and a G1 specific cell-cycle arrest.

(A) Phase-contrast micrographs of pools of primary Schwann cells infected with a retrovirus encoding ΔRaf-1:ER, grown in the presence or absence of 200nM 4-OH-tamoxifen (TMX) for 30 hours.

(B) Pools of asynchronously growing NSΔRafER cells and control NSE cells were cultured in the presence or absence of 200nM TMX for 30 hours. Cells were trypsinised and examined for DNA content by propidium iodide staining and flow cytometry and also analysed for BrdU uptake in the four hours prior to lysis (see insets).

(C) NSE, NSΔRafER and NSΔRafERLT were seeded into DMEM supplemented with 2% FCS. 48 hours later the cells were treated with TMX or ethanol (- TMX) in the absence (left hand panel) or presence (right hand panel) of fresh medium supplemented with 3% FCS, forskolin and GGF. ^{3}H-thymidine was added 12 hours later and the cells were lysed after a further 16 hours. TCA precipitable material was filtered and counted.

(D) NSΔRafER cells were quiesced for 48 hours in DMEM supplemented with 2% FCS. The cells were lysed at the indicated time points following the addition of fresh medium supplemented with GGF and forskolin and 200nM TMX. The lysates were equalised for protein content and then western blotted using the anti-ERK-2 polyclonal antiserum 122.

Figure 2. Raf arrests Schwann cells in G1 prior to the induction of cyclin A.

(A) NSΔRafER cell were quiesced for 48 hours in DMEM supplemented with 2% FCS. The cells were stimulated with fresh medium supplemented with forskolin and GGF in the presence or absence of 200nM TMX The cells were pulse-labelled with ^{3}H-thymidine at four hourly intervals and TCA precipitable material was filtered and counted in a scintillation counter.

(B) Protein lysates were prepared at the time points indicated and were analysed by western blotting with antibodies specific for the indicated proteins.

Figure 3. Raf activation inhibits cyclin A- and cyclin E-dependent kinase activity. Asynchronously growing NSΔRafER cells were cultured in the presence (+) or absence (-) of 200nM TMX for 30 hours. Protein lysates were prepared and standardised for protein content. The left hand upper panels show western analysis for cyclin A (A) and cyclin E (B).

300μg of the lysates were subjected to immunoprecipitation with a cyclin A MAb (A) or a cyclin E antibody (B). The controls were protein G-sepharose beads alone, for the cyclin A antibody or a peptide block for the cyclin E antibody. The immunoprecipitates were either western blotted with a cdk2 antibody (upper right hand panels) or assayed for histone H1 kinase activity (lower panels). * Alternative spliced form of cdk2 seen in rodent cells (Matsushime et al., 1994).

Figure 4. Activation of Raf leads to an increase in $p21^{Cip1}$.

NSΔRafER cells were grown for 30 hours in the presence of TMX(+) or the solvent control (-). 30μg of protein lysates were subjected to immunoblot analysis with an anti-p27 antibody (A) or an anti-p21 antibody (B).

(C) 300μg of the lysates were immunoprecipitated with the cyclin E antibody and western blotted with an anti-p21 antibody. (D) NSΔRafER cells were

quiesced for 48 hours in DMEM supplemented with 2% FCS. The cells were then exposed to fresh medium supplemented with GGF, forskolin and 200nM TMX in the presence and absence of PD98059. The cells were lysed at the indicated times. The lysates were equalised for protein content and then western blotted using the anti-ERK-2 polyclonal antiserum 122 (top panel) or an anti-p21 antibody (bottom panel).

Figure 5. The kinetics of $p21^{Cip1}$ induction, cyclin E-dependent kinase activity and DNA synthesis following Raf activation.

Asynchronously growing NSΔRafER cells were incubated in the presence of TMX (+) or control solvent (-).

(A)(o) Protein lysates were prepared at the times indicated. 300μg of the lysates were immunoprecipitated with an anti-cyclin E antibody and assayed for histone H1 kinase activity. (+) DNA synthesis was measured by the uptake of ^{3}H-thymidine added between the times indicated. The results are expressed as the amount of activity relative to the corresponding control activity

(B) Protein lysates were prepared at the indicated time points or from quiescent NSΔRafER cells (Q) and were subjected to immunoblotting analysis with an anti-p21 antibody.

Figure 6. The induction of $p21^{Cip1}$ in Raf-arrested cells is p53-dependent.

NSΔRafER cells were infected with retroviral vectors expressing a dominant-negative form of p53 ($p53^{175}$). Pools of NSΔRafERBp, NSΔRafER$p53^{175}$ and NSΔRafERLT cells were grown in the presence of TMX (+) or the control solvent (-) for 30 hours. (A)Protein lysates were prepared and analysed by western blotting with an anti-p21 antibody or an anti-cyclin D1 antibody as indicated.

(B) 300μg of lysates were immunoprecipitated with an anti-cyclin E antibody and then analysed by western blotting with an anti-$p21^{Cip1}$ antibody.
(C) 300μg of lysates were immunoprecipitated with an anti-cyclin E antibody and assayed for histone H1 kinase activity
(D) The NSΔRafER$p53^{175}$ cells were trypsinised and analysed for DNA content by propidium iodide staining and flow cytometry and for BrdU incorporation in the four hours prior to trypsinisation (see insets).

Figure 7. Raf and LT co-operate to induce high levels of cyclin A-dependent kinase activity and DNA synthesis.
(A) Left hand panel - Equal numbers of NSΔRafERBp, NSΔRafER$p53^{175}$, NSΔRafERLT cells were seeded into DMEM supplemented with 2% FCS. 48 hours later the cells were stimulated with fresh medium supplemented with 3% FCS, forskolin and GGF in the presence of TMX(+) or the control solvent(-). ^{3}H-thymidine was added 12 hours later and the cells were harvested after a further 18 hours. TCA precipitable material was filtered and counted.
Right hand panel - NSΔRafER$p53^{175}$ cells were infected with the Babe-hygro retroviral vector constructed to express the cyclin A gene or the empty vector control to generate NSΔRafER$p53^{175}$cycA and NSΔRafER$p53^{175}$BH cells respectively. Hygromycin resistant colonies were pooled and expanded. DNA synthesis assays were performed as described above.
(B) Protein lysates were prepared from growing NSΔRafER, NSΔRafER$p53^{175}$ and NSΔRafERLT cells. 30μg of protein was analysed by western blotting with either an anti-cyclin A or anti-cdk2 antibody as indicated (upper two panels). 300μg of the lysates were immunoprecipitated with an anti-cyclin A antibody and assayed for histone H1 kinase activity (lower panel).

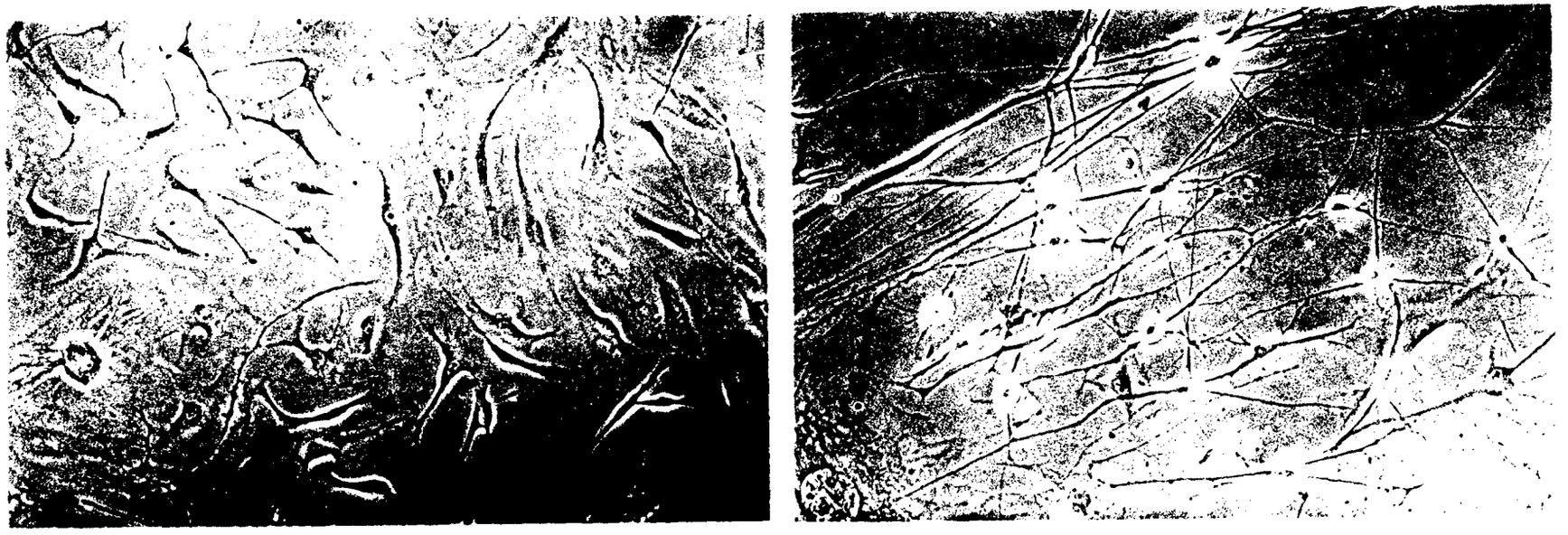
(C) NSΔRafERLT cells were seeded into DMEM supplemented with SATO mix (- mitogens) or DMEM supplemented with 3% FCS, GGF and forskolin (+ mitogens) for 48 hours prior to the experiment The cells were then treated with TMX (+) or control solvent (-) for 24 hours and protein lysates were prepared. 300μg of protein was immunoprecipitated with an anti-cyclin A antibody and kinase assays were performed with the precipitates. The kinase reactions were subjected to SDS-PAGE and then transferred onto a PVDF membrane The filter was exposed to film (upper panel) and then western blotted with an anti-cdk2 antibody (lower panel).

Figure 8. Mechanisms of oncogene cooperation in Schwann cells (see also Conclusions).

Fig 1

A

NSΔRafER

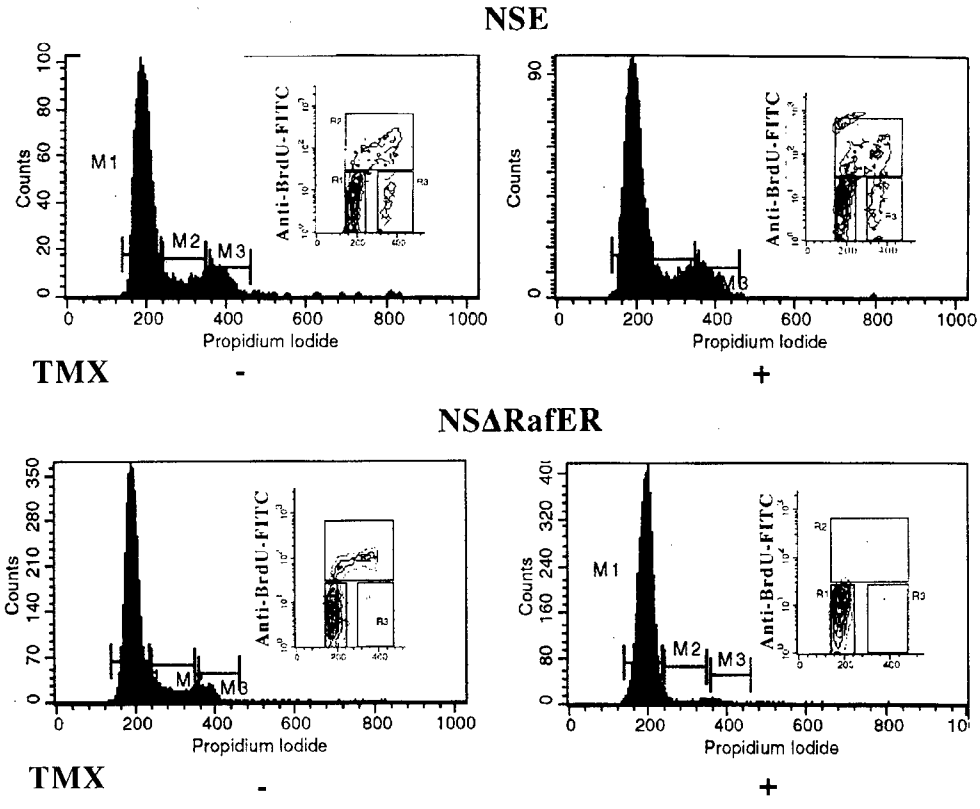


- TMX

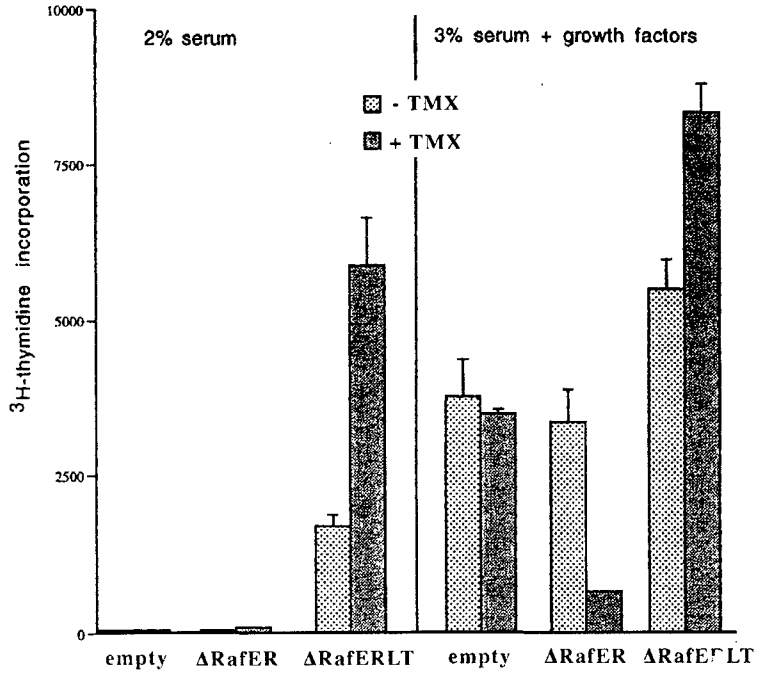
+ TMX

Fig 1

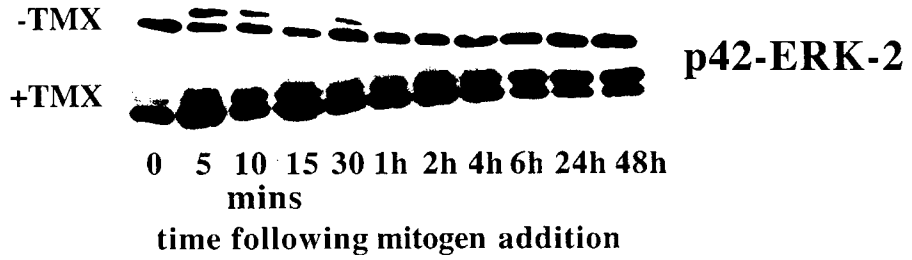
B


NSE
Counts
Propidium Iodide
M1
M2
M3
Anti-BrdU-FITC
TMX
-
+
NSΔRafER
Counts
Propidium Iodide
M1
M2
M3
Anti-BrdU-FITC
TMX
-
+

C


2% serum
3% serum + growth factors
- TMX
+ TMX
3H-thymidine incorporation
empty
ΔRafER
ΔRafERLT
empty
ΔRafER
ΔRafERLT

D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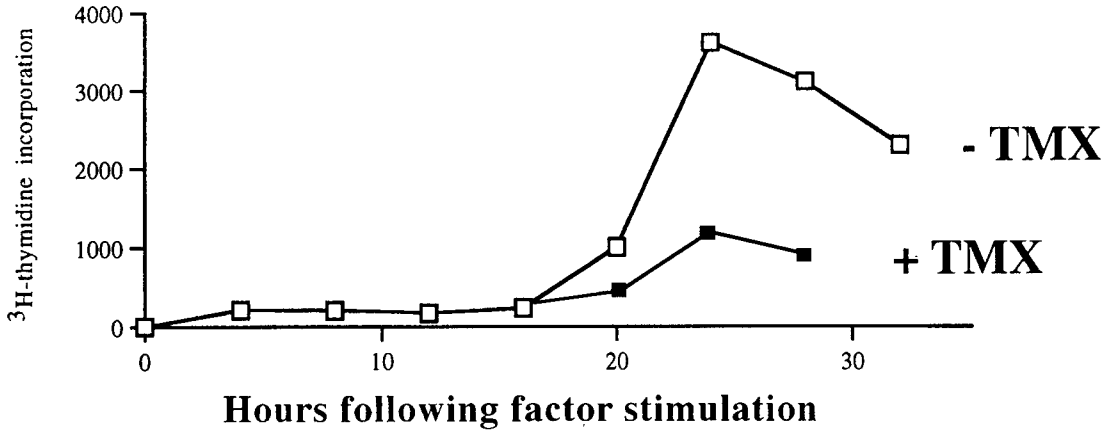
+TMX
p42-ERK-2
0 5 10 15 30 1h 2h 4h 6h 24h 48h
mins
time following mitogen addition

A


4000
3000
2000
1000
0
^{3}H-thymidine incorporation
0
10
20
30
- TMX
+ TMX
Hours following factor stimulation

B

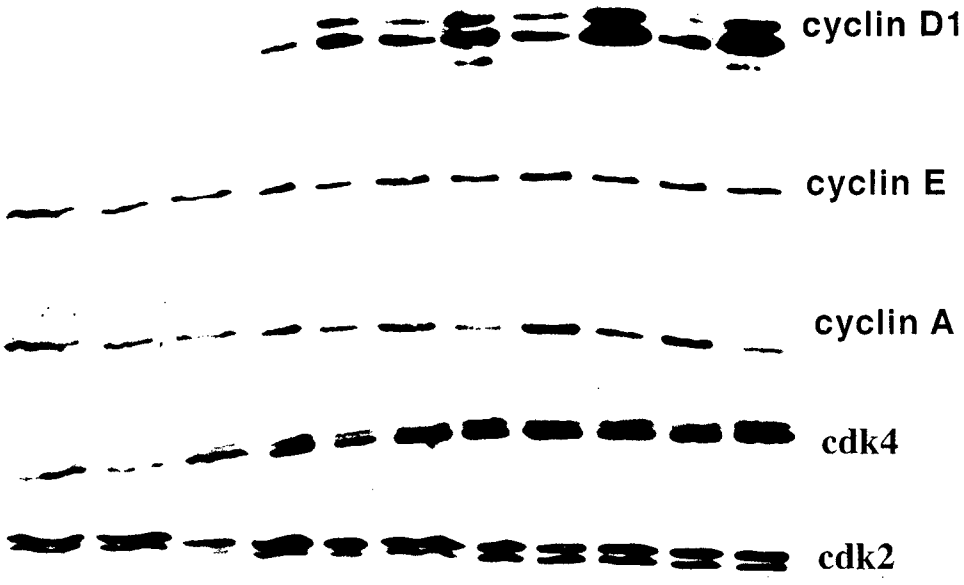
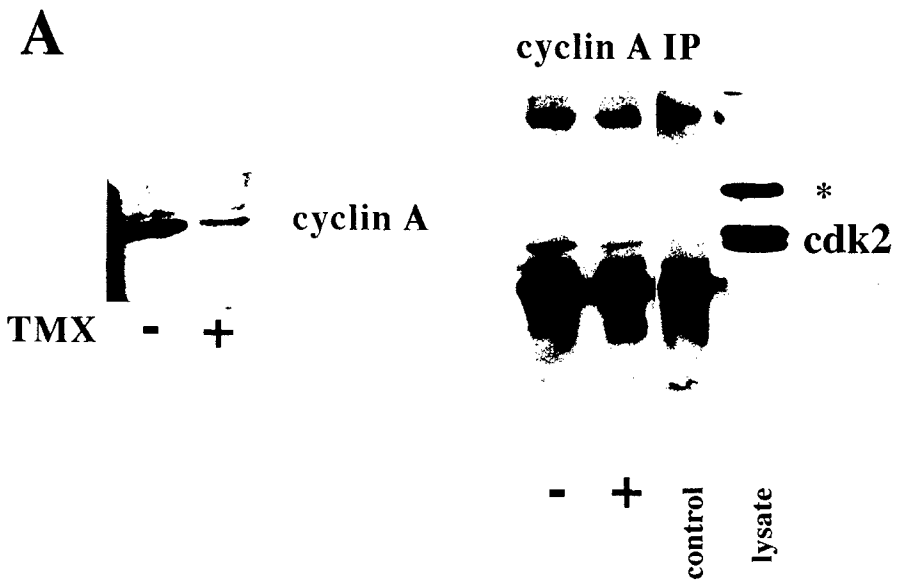

cyclin D1
cyclin E
cyclin A
cdk4
cdk2
0 3- 3+ 10- 10+ 18- 18+ 26- 26+ 30- 30+
Hours following factor stimulation (-/+ TMX)

Fig 3


A
cyclin A IP
cyclin A
TMX - +
*
cdk2
- +
control
lysate

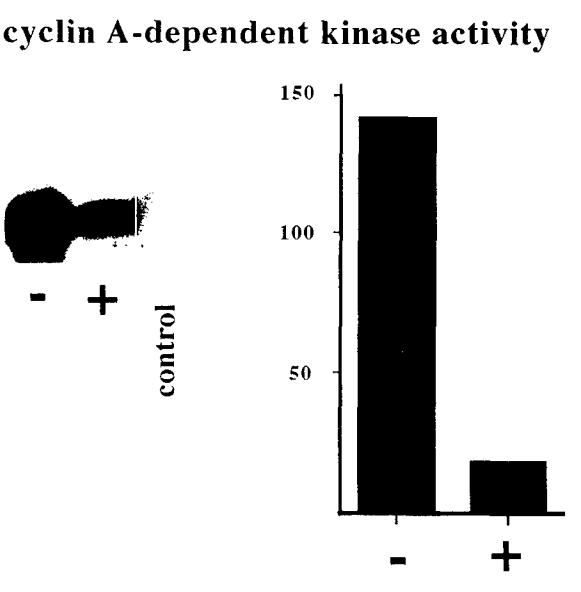

cyclin A-dependent kinase activity
150
100
50
- +
control
- +

Fig 3

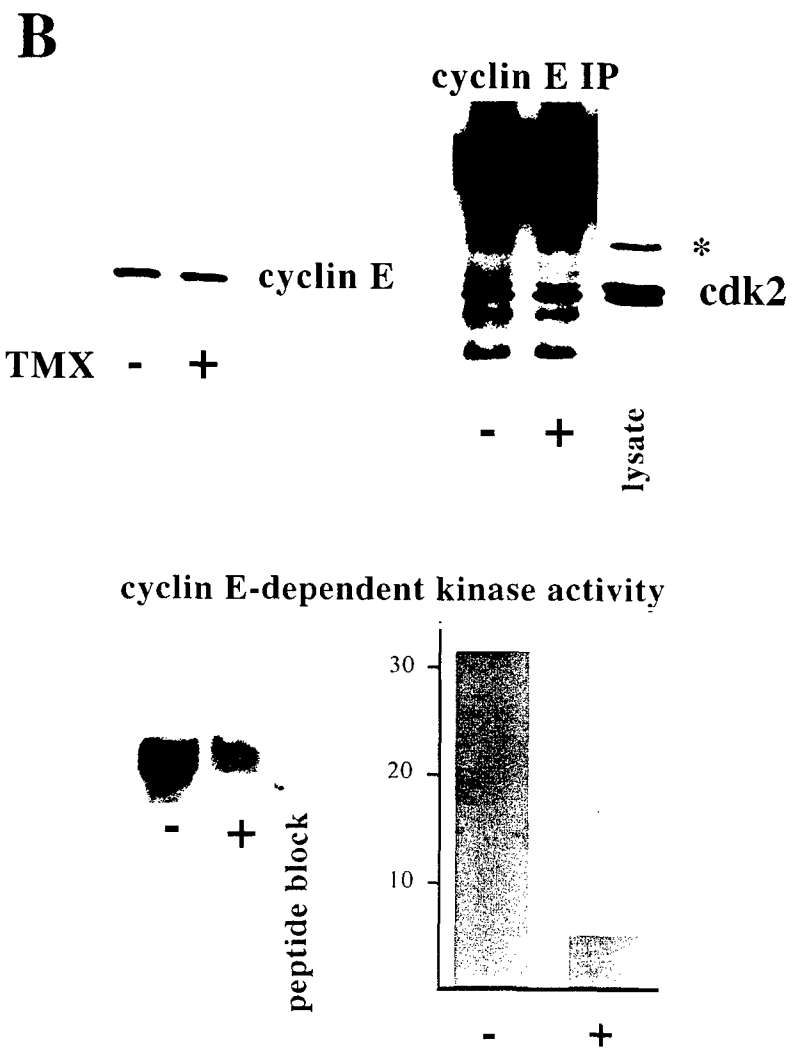

B
cyclin E IP
cyclin E
cdk2
*
TMX - +
- +
lysate
cyclin E-dependent kinase activity
30
20
10
- +
peptide block

Fig 4

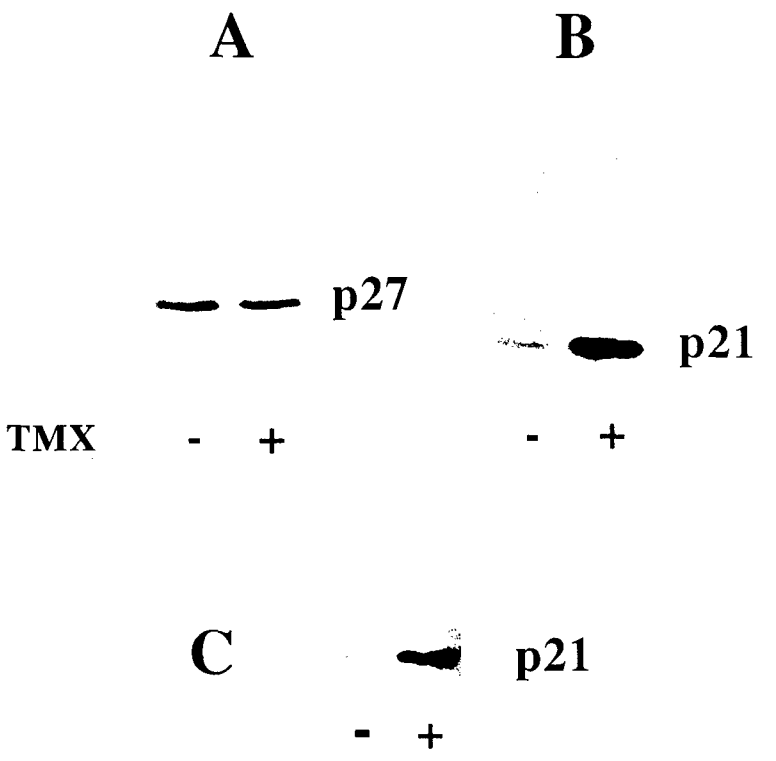


Fig 4D

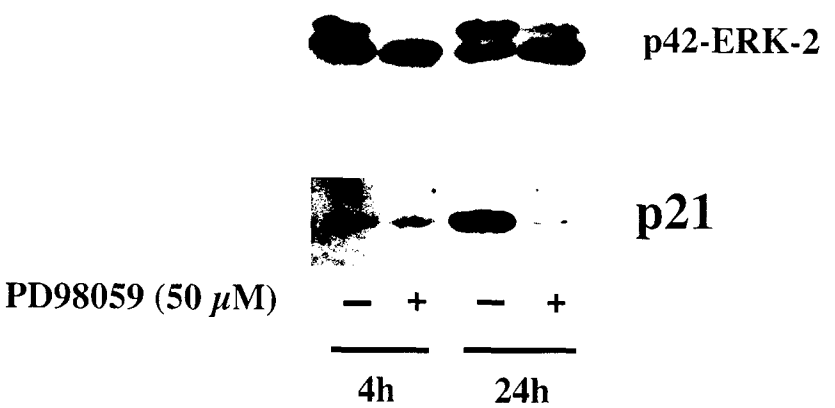
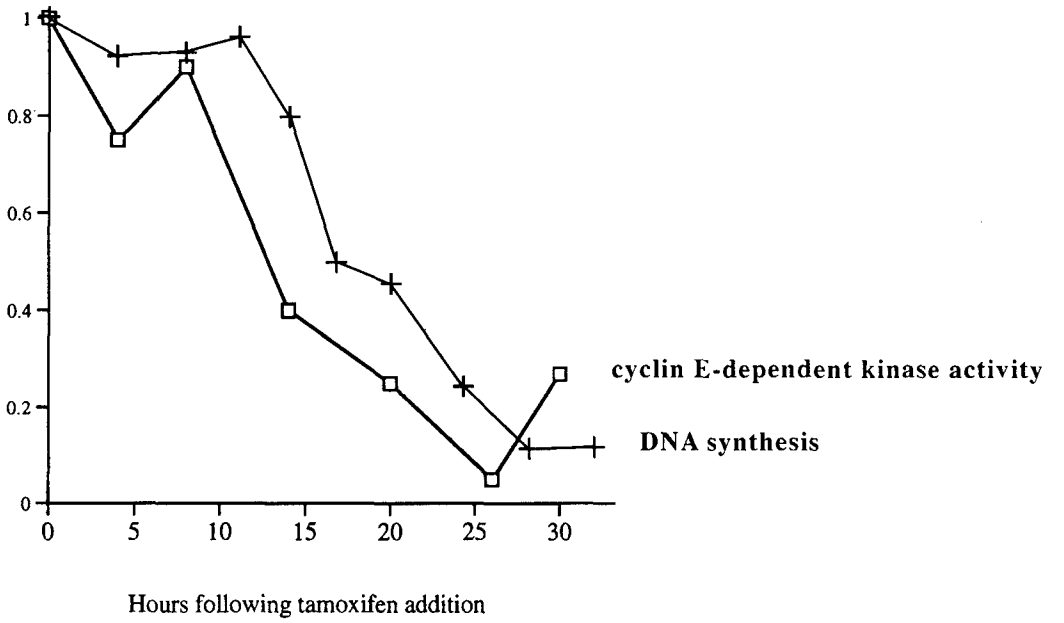


Fig 5

A



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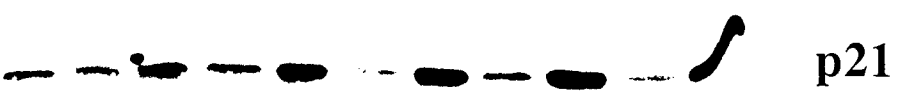


Q 0 4- 4+ 10- 10+ 14- 14+ 18- 18+ 22- 22+ 30- 30+

Hours following tamoxifen addition

Fig 6

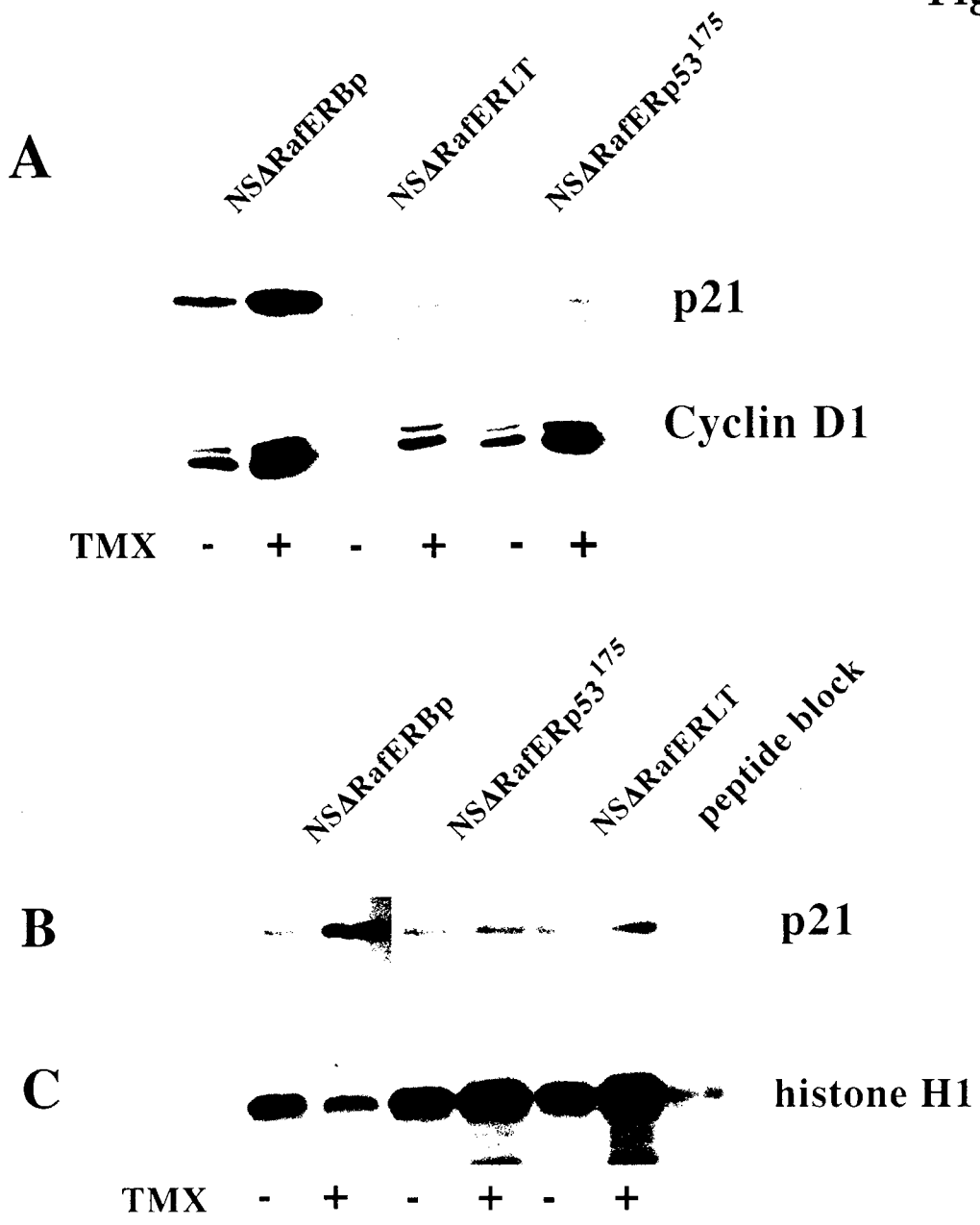
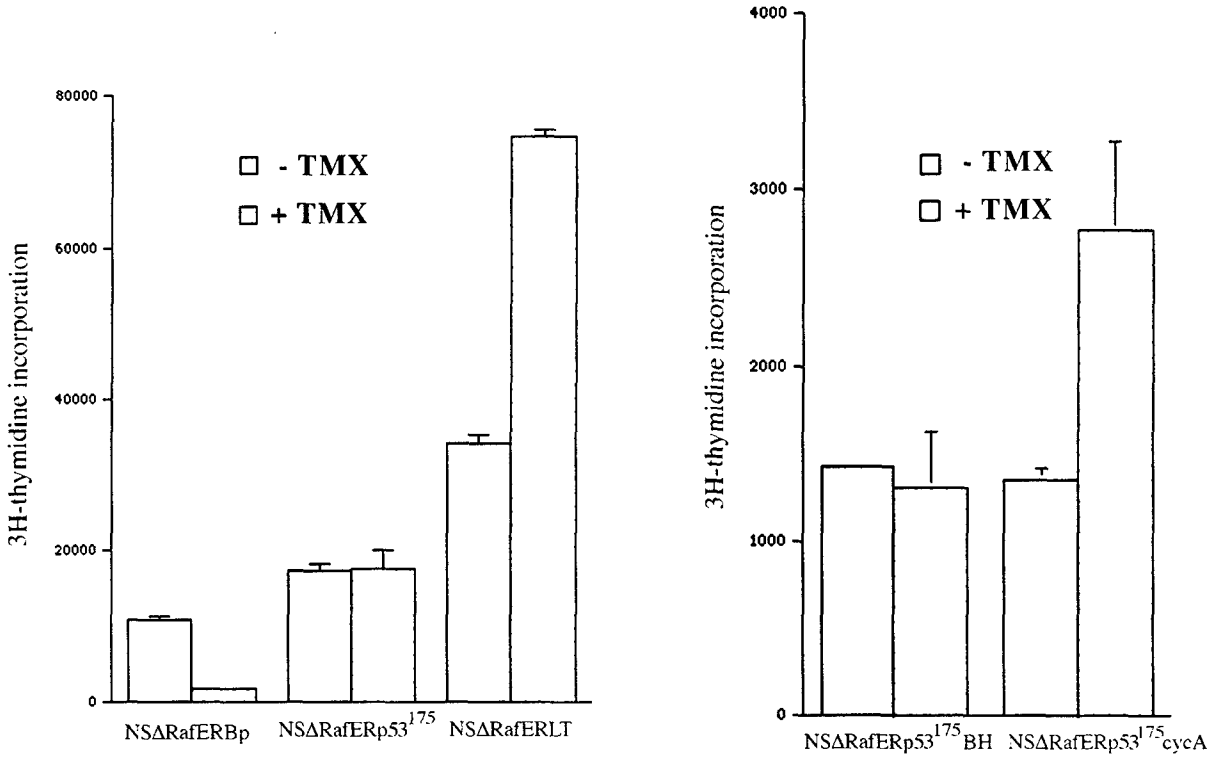
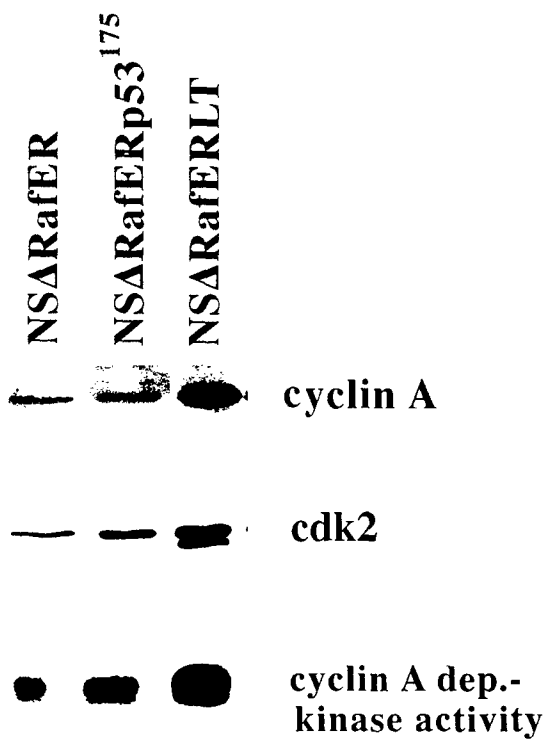

A
NSΔRafERBp
NSΔRafERLT
NSΔRafERp53175
p21
Cyclin D1
TMX - + - + - +
B
NSΔRafERBp
NSΔRafERp53175
NSΔRafERLT
peptide block
p21
C
histone H1
TMX - + - + - +
D
counts
M1
M2
M3
Anti-BrdU FITC
Propidium Iodide
TMX - +
NSΔRafERp53175

Fig 7

A


3H-thymidine incorporation
80000
60000
40000
20000
0
- TMX
+ TMX
NSΔRafERBp
NSΔRafERp53175
NSΔRafERLT
4000
3000
2000
1000
0
- TMX
+ TMX
NSΔRafERp53175BH
NSΔRafERp53175cycA

B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NSΔRafERp53175
NSΔRafERLT
cyclin A
cdk2
cyclin A dep.-
kinase activity

C

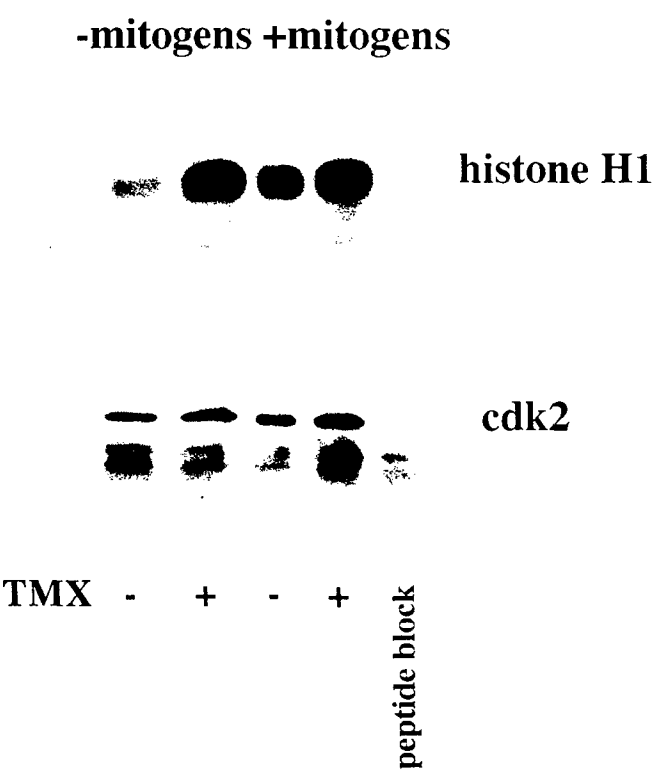
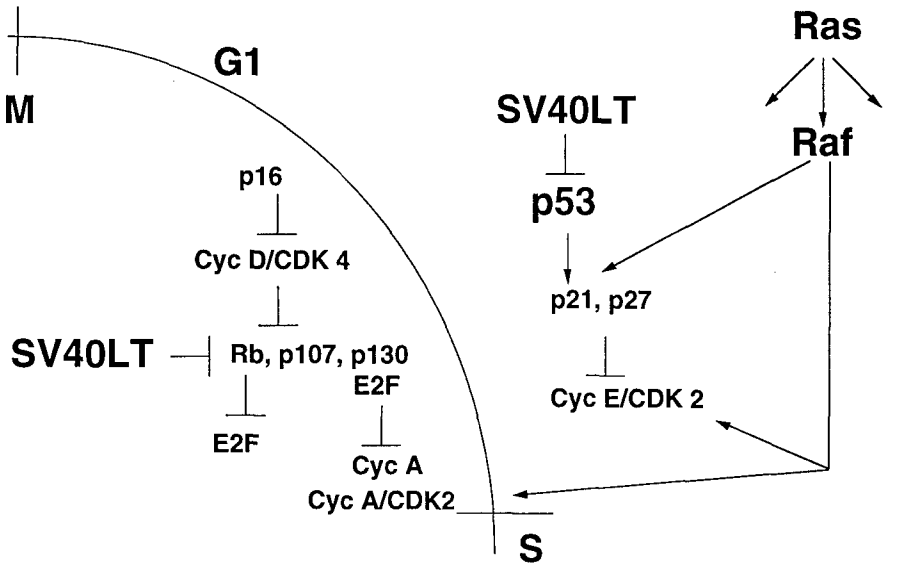

-mitogens +mitogens
histone H1
cdk2
TMX - + - +
peptide block

Fig.8


Ras
G1
M
SV40LT
Raf
p16
p53
Cyc D/CDK 4
p21, p27
SV40LT
Rb, p107, p130
E2F
Cyc E/CDK 2
E2F
Cyc A
Cyc A/CDK2
S