Oncogene (1997) 15, 1635–1642

Rapamycin and p53 act on different pathways to induce G1 arrest in mammalian cells

SM Metcalfe, CE Canman[2], J Milner[3], RE Morris[4], S Goldman[2] and MB Kastan[2]

[1]Department of Surgery, Addenbrooke's Hospital, University of Cambridge, Cambridge, CB2 2QQ UK: [2]Oncology Centre, Johns Hopkins Hospital, 600 N Wolfe Street, Baltimore, Maryland, 21287 USA; [3]YCRC p53 Research Group, University of York, York, YO1 5DD UK; [4]Transplantation Immunology Laboratory, Department of Cardiothoracic Surgery, Stanford University School of Medicine, Stanford, California, USA

Certain growth regulatory kinases contain a common domain related to the phospho-inositol 3 (PI-3) kinase catalytic site. These include the ATM gene product, DNA-PKcs, and the target of rapamycin (TOR in yeast; and FRAP in mammalian cells). Rapamycin inhibits growth factor signalling and induces G1 arrest in many cell types. Some growth regulatory PI-3 kinases appear functionally linked to p53 and we have explored potential links between cellular effects induced by rapamycin and p53. In p53 null cells rapamycin inhibited cell cycling but did not induce G1 arrest. In cells which showed selective G1 arrest in response to rapamycin, rapamycin had no effect on basal levels of p53 protein. Similarly p21(WAF1) protein was not induced by rapamycin. The kinetics of the cellular p53/p21(WAF1) response to ionising radiation was unaffected by rapamycin; and the ability of growth factor to protect against p53-mediated apoptosis in response to DNA damage was also unaffected by rapamycin. The ATM gene is mutated in the cancer susceptibility syndrome ataxia telangiectasia (AT) but such mutant cells showed a similar sensitivity to rapamycin compared to their normal counterparts. RKO cell lines of common genetic background, but with different levels of functional p53 protein, also responded similarly to rapamycin. Thus, although rapamycin and p53 are each able to induce G1 arrest, they appear to act through independent growth regulatory pathways.

Keywords: rapamycin; p53; G1 arrest

Introduction

Rapamycin is an immunosuppressive, anti-proliferative macrolide which inhibits growth factor-mediated signal transduction required for cell cycle progression (Morris, 1992); it also may augment apoptotic signalling pathways (Shi *et al*., 1995). Rapamycin becomes active when bound to specific binding proteins known as FKBPs. The rapamycin-FKBP complex inhibits targets of rapamycin (TOR1 and TOR2) in yeast (Kunz *et al*., 1993; Cafferkey *et al*., 1993) and FRAP in mammalian cells (Brown *et al*., 1994), also known as RAFT1 and mTOR (Sabatini *et al*., 1995; Sabers *et al*., 1995). Both TOR and FRAP belong to a family of growth regulatory kinases which show homology with the catalytic domain of IP-3 / IP-4 kinases (recently reveiwed by Jackson, 1995; Abraham, 1996). Mutation analyses of FRAP has located the kinase domain towards the carboxy terminus whilst the binding domain for rapamycin-FKBP mapped to 90 amino acids lying amino terminal relative to the kinase domain (Brown *et al*., 1995; Chen *et al*., 1995). FRAP behaves like both protein and phosphoinositol kinases in that it autophosphorylates (serine) *in vitro*: this is necessary for FRAP activity and autophosphorylation is blocked by rapamycin but not by wortmannin (Brown *et al*., 1995). Rapamycin-FKBP does not directly inhibit PI-3 kinase activity (Hollis and Hutchinson, 1996) nor the PI-4 kinase activity of TOR or FRAP (Carendas and Heitman, 1995; Sabatini *et al*., 1995) but may dislocate the enzyme from its substrate (Carendas and Heitman, 1995).

The present study was prompted by the established homology between FRAP and (i) the catalytic subunit of DNA-dependent protein kinase (dsDNA PK) (Hartley *et al*., 1995), and (ii) the ataxia telangiectasia (AT) gene product, ATM (Savitsky *et al*., 1995). AT is a human autosomal recessive disorder in which ATM homozygous patients show a range of pathologies including neurodegeneration, immunosuppression and a predisposition to malignancy. The PI-3 kinase-like domain common to the carboxytermini of DNA-PK and ATM suggests that ATM may be a protein kinase. Moreover, several lines of evidence indicate that signal transduction pathways involving DNA-PK and ATM may lead to p53 activation and this could be important during cellular responses to DNA damage (Lees-Miller *et al*., 1990; Kastan *et al*., 1992; reviewed by Jackson, 1995). In particular the p53 response to ionising radiation is significantly delayed in AT cells (Khanna and Lavin, 1993; Canman *et al*., 1994), possibly contributing to hypersensitivity, or enhanced chromosome breakage, in AT cells after exposure to radiation or other DNA damaging agents. We have asked if rapamycin affects p53 and p53 functions in response to ionising radiation. We also ask if cells from ataxia telangiectasia patients show an altered sensitivity to rapamycin. Our results indicate that rapamycin can induce cell cycle arrest by p53-independent pathway(s) and that sensitivity of cells to rapamycin is unaffected by mutation of the AT gene.

Results

We looked for commonalities between p53 and rapamycin using BAF-3 cells which is a non-malignant murine haematopoietic cell line dependent upon IL3

Correspondence: SM Metcalfe
Received 22 November 1996; revised 11 June 1997; accepted 11 June 1997

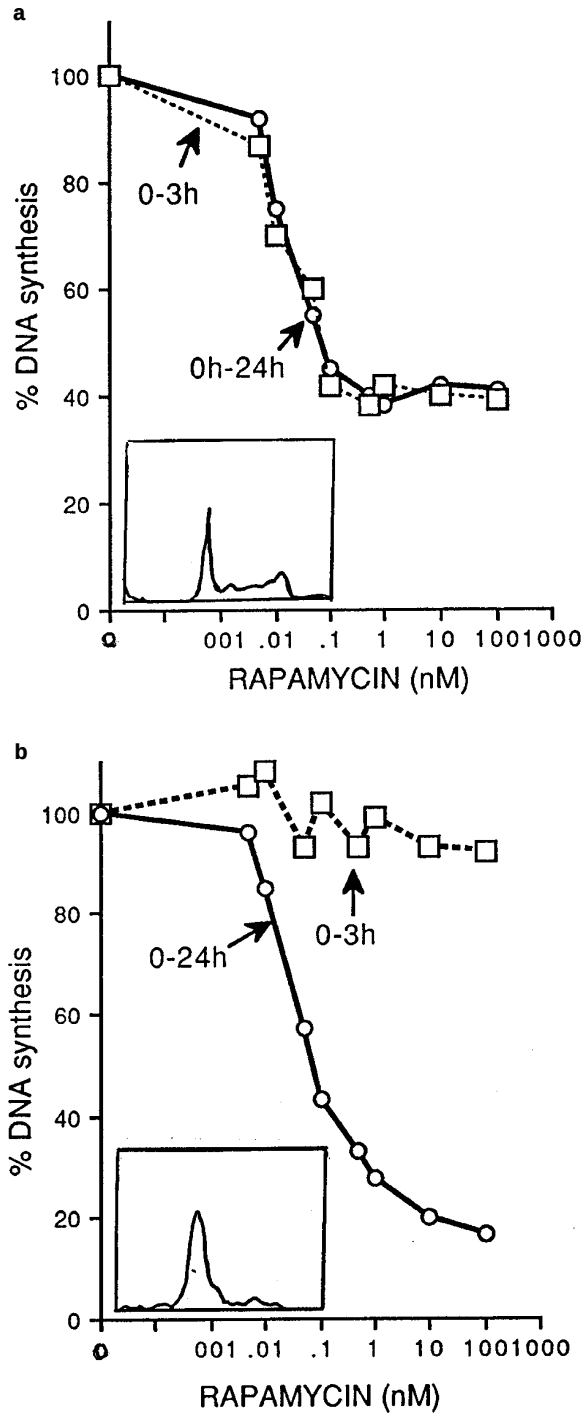

Figure 1 Cells were seeded into 96 well microtitre plates at a defined number per experiment (ranging from 10 000 to 50 000 per well) in 200 μl of growth medium. Rapamycin was added to each well to give the final concentration shown and the cultures were incubated at 37°C. Repeated experiments showed that the cell number did not alter the dose response curve to rapamycin. Replicate plates were labelled with tritiated thymidine to assay DNA synthesis at 0 h to 3 h, or 21 h to 24 h, following drug treatment to provide data following 3 h exposure, or 24 h exposure, to rapamycin respectively. The mean c.p.m. of radiolabelled thymidine incorporated in triplicate cultures was 10 302 c.p.m. at 3 h and 29 380 c.p.m. at 24 h for the 0536 cells. Similarly, for BAF-3 cells at 3 h, 16 368 c.p.m. and at 24 h, 58 400 c.p.m. The standard error between triplicates was less than 10%. In some experiments cell samples were taken for FACS analysis: these showed that control cultures were distributed thoughout the cell cycle. This profile was maintained in BAF-3 cells after 24 h culture in 10 nM rapamycin (**a**) but 0536 cells in 10 nM rapamycin became arrested in G1 as shown in the 24 h FACS analysis (**b**)

for survival and proliferation (Collins *et al*., 1992; Palacios and Steinherz, 1985). Importantly, BAF-3 cells express wild type p53 which is upregulated in response to irradiation to induce G1 arrest in the presence of IL3, or apoptosis in the absence of IL3 (Canman *et al*., 1995). By treating irradiated BAF-3 cells with rapamycin, in the presence or absence of IL3, we were able to ask if the drug has any detectable effect on signalling to p53.

Does rapamycin block the protective effect of growth factor against apoptosis?

In order to ask if rapamycin alters p53-mediated growth factor responses it was necessary to first demonstrate that rapamycin has a physiological effect on BAF-3 cells. DNA synthesis in the BAF-3 cultures was inhibited by some 50% (ID50%) at 0.1 nM rapamycin, and the dose response curve was constant at both 3 h and 24 h rapamycin treatment, as shown in Figure 1a. FACS analysis of BAF-3 cultures treated with rapamycin for 24 h showed that the cells remain in various phases of the cell cycle (see insert to Figure 1a, and note the contrast with Figure 1b where the lymphoblastoid cell line, 0536, became arrested in G1 in response to rapamycin: this is discussed in detail later).

When the BAF3 cells were starved of IL3 for 13 h they became arrested in G1 (Figure 2a) and proceded to apoptosis by 37 h of IL3 starvation (Figure 2d); this progression to apoptosis also occured in the presence of rapamycin resulting in a similar flow cytometry profile to that shown in Figure 2d. Those cultures which received IL3 after the 13 h starvation period recovered from G1 arrest and re-entered the cell cycle: this recovery was independent of the presence of rapamycin (compare Figure 2b with c). Thus rapamycin did not affect growth factor derived signals which support constitutive cell cycling and protection against apoptosis.

Does rapamycin mimic ATM dysfunction by inhibition of an ATM-like PI-3 kinase?

The induction of p53 in response to ionising radiation in ATM cells is quantitatively defective. Since the ATM protein belongs to a family of proteins with a PI-3 kinase-like domain and thus a potential target for rapamycin, we asked if rapamycin treatment mimics the effect of ATM dysfunction. Basal and radiation-induced levels of p53 in BAF-3 cells were unaffected by the presence of rapamycin, as shown in a time course study (Figure 3a; lanes 1, 3, and 5 for basal levels; and lanes 2, 4 and 6 for induced levels, each measured at 1.5 h; 3 h; and 6 h post irradiation). As a functional assessment of p53 we also evaluated p21 protein levels, since upregulated p53 normally transactivates this cyclin kinase inhibitor. Levels of p21 increased following p53 induction, consistent with p21 transactivation by p53, and this transactivation was not altered by rapamycin (compare upper and lower blots in Figure 3a). In this experimental series aliquots of cells were taken at 8 h and at 22 h for flow cytometrical analysis. The results showed that rapamycin had no effect on IL3-mediated protection against apoptosis following radiation of BAF-3 cells; nor was progres-

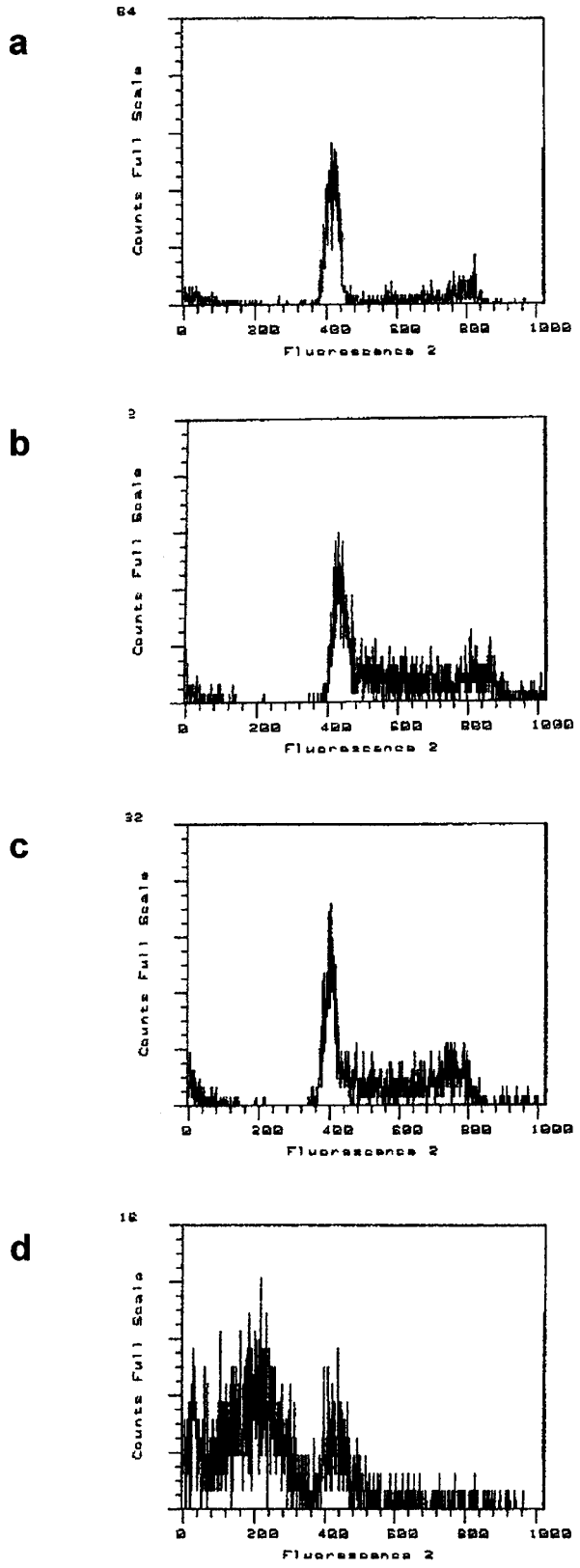

Figure 2 Rapamycin does not prevent IL3-mediated rescue of IL3-starved BAF-3 cells from apoptosis. Flow cytometry of BAF-3 cells cultured for 13 h in the absence of IL3 (**a**) and then after their further culture for 24 h in IL3 (**b**), or 24 h in IL3 plus 1 μM rapamycin (**c**); or for 24 h without IL3, totalling 37 h IL3 starvation (**d**). Cells treated with rapamycin coincident with 37 h IL3 starvation also became apoptotic and gave a similar flow cytometric profile to (**d**)

sion to apoptosis following radiation in the absence of IL3 altered by rapamycin (as illustrated in Figure 3b). Thus rapamycin had no effects on radiation-induced p53 or p21 and did not affect the BAF-3 cell p53 response to ionising radiation in the presence or absence of growth factor.

Rapamycin causes primary cultures of mouse spleen cells to arrest at a specific cell cycle stage, about halfway through G1 (Metcalfe and Milner, 1991). Certain cell lines also arrest in G1 when treated with rapamycin (Albers *et al*., 1993), whilst others arrest at the G1 to S interface (Morice *et al*., 1993a). Given the possibility of variation in response to rapamycin, we extended these experiments to other cell lines known to express wild type p53. MCF-7, an adherent cell line derived from human breast cancer tissue, shows a characteristic p53 response to ionising radiation. When MCF-7 cells were irradiated and then cultured for 3 h with or without high dose rapamycin (1 μM) there was a marked upregulation of p53 and corresponding induction of p21 compared to un-irradiated controls: this upregulation of p53 and p21 was not altered by rapamycin. These results are shown in Figure 3c and are in accord with our findings in the BAF-3 cells.

The possibility that rapamycin may cause a progressive effect on basal p53 levels was also explored. Here we looked at both MCF 7 and 0536 cells, which are a human B cell line immortalised by Epstein–Barr viral infection and which express wild type p53. Rapamycin doses known to partially, or fully, inhibit G1 progression had no chronic effect on the basal level of p53 expression in either 0536 cells or MCF7 cells (Figure 3d). We also noted that rapamycin did not alter the levels of $p21^{Waf\text{-}1/Cip1}$ in any of these cell cultures (data not shown).

To ask directly if the AT mutation alters responsiveness to rapamycin, we compared two human lymphoblastoid which cell lines express wild type p53, one from a normal individual (0536), and one from an individual with ataxia telangiectasia (0719b). Both cell lines arrested in G1 in response to rapamycin with an ID50% of 0.05 nM, as measured by inhibition of DNA synthesis (Figure 4). Other stages of the cell cycle were unaffected by rapamycin even at 10 nM or 100 nM. Thus, at 3 h, there was little drug effect since only a very small proportion of the total cell population would be at the rapamycin-sensitive stage. Later, as the cells accumulate behind the rapamycin-sensitive stage, the effect of G1 arrest became more marked as revealed at 27 h and 52 h. Flow cytometric analysis confirmed that the cells were in G1 arrest (see Figure 1b).

Does rapamycin require p53 to inhibit cell cycle progression?

All the results given above suggest that rapamycin may inhibit mammalian cell cycle progression by a mechanism which is independent of p53. We addressed this directly by asking if p53 null cells are sensitive to inhibition by rapamycin. The SAOS cell line was derived from a human osteosarcoma and lacks expression of the p53 protein. SAOS cells grown in rapamycin showed a dose related inhibition of cell replication (Figure 5). There was no visible toxicity even at 100 nM rapamycin, and all cultures proceeded

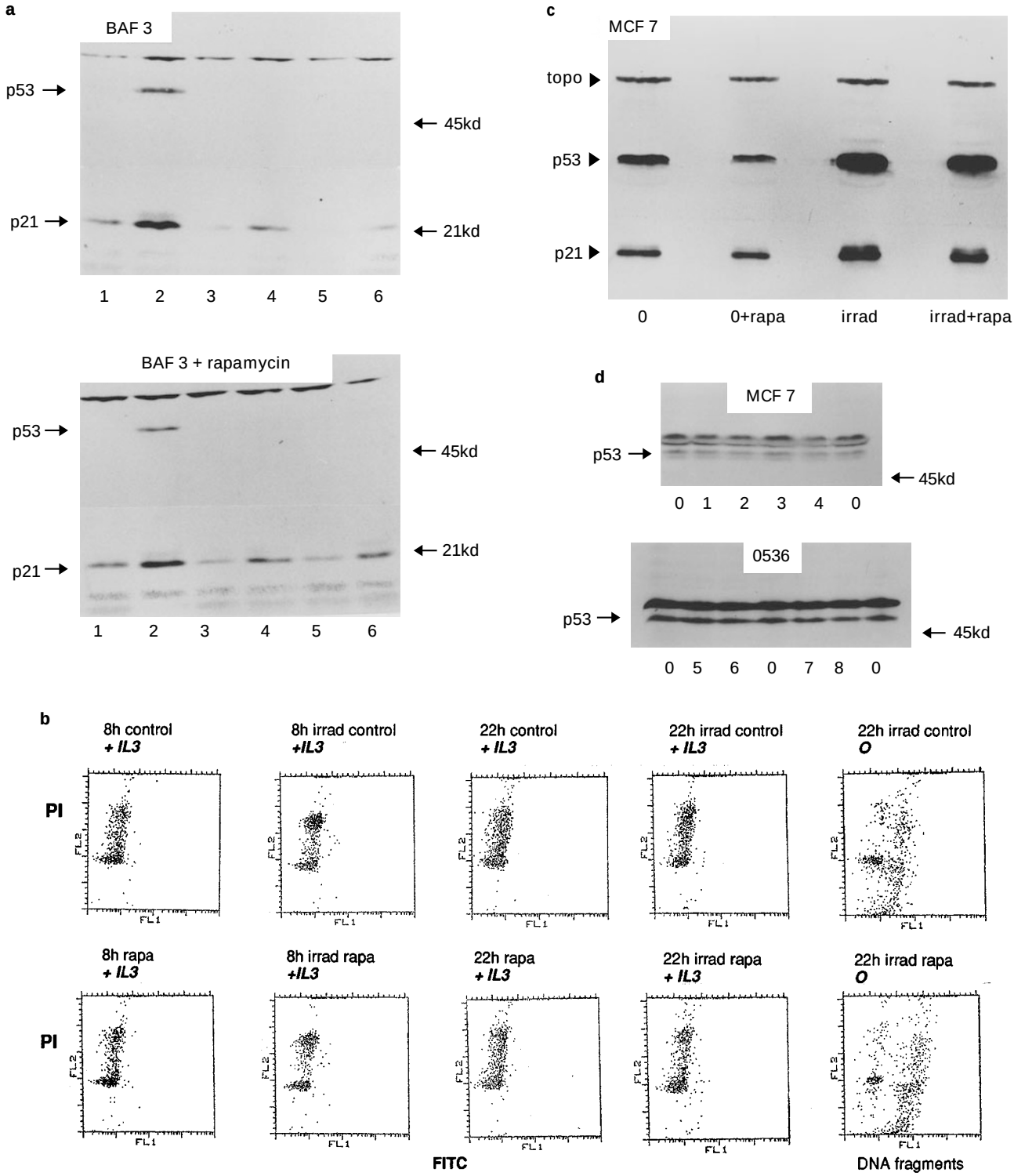

Figure 3 Rapamycin does not alter p53 and p21 induction following radiation damage, nor basal levels of p53 expression. (**a**) Equivalent numbers of BAF-3 cells were cultured in two groups of four flasks each, one group being maintained in IL3 and the second without IL3. Two flasks in each group were treated with 10 nM rapamycin. After 0.5 h incubation one control from each group and one rapamycin flask from each group was subjected to 4 Gy gamma irradiation. The cells were then cultured at 37°C, and samples were removed at 1.5 h; 3 h; 6 h; and 24 h. These samples were processed to measure p53 levels and p21 levels by Western blotting after SDS–PAGE. The sample lanes were loaded with equal concentrations of protein from the following cultures: 1: untreated control at 1.5 h; 2: irradiated control at 1.5 h showing induced p53 and induced p21; 3: untreated control at 3 h; 4: irradiated control at 3 h showing return of p53 to basal levels and prolonged induction of p21; 5: untreated control at 6 h; 6: irradiated control at 6 h showing prolonged induction of p21. The gel portrayed is of the group without IL3: the gel (not shown) of the IL3 treated cultures gave a similar pattern of p53 and p21 levels according to treatment. (**b**) Aliquots of cells from the experiment 3**a** was reserved for flow cytometric analysis of the cell cycle and apoptosis at 8 h and 22 h. At 8 h after radiation there was increased number of cells arrested in G1, both in the presence and absence of rapamycin, when IL3 was present. At 22 h irradiated cell populations cultured in the presence of IL3, with or without rapamycin, showed no significant apoptosis: in contrast the cell populations irradiated and without IL3 showed extensive apoptosis, independent of the presence or absence of rapamycin. The flow cytometric plots show propidium iodide staining (y axis) versus FITC labelled apoptotic DNA fragments (abscissa). (**c**) Equivalent cultures of MCF 7 cells were incubated for 3 h alone (0), or with 1 μM rapamycin (rapa), or after 4 Gy gamma irradiation (irrad), or with 1 μM rapamycin during and following 4 Gy gamma irradiation (irrad + rapa). Nuclear proteins were then extracted and equal aliquots run on SDS–PAGE for Western blotting to measure levels of p53 and p21. Topoisomerase was measured as an internal standard of equivalent

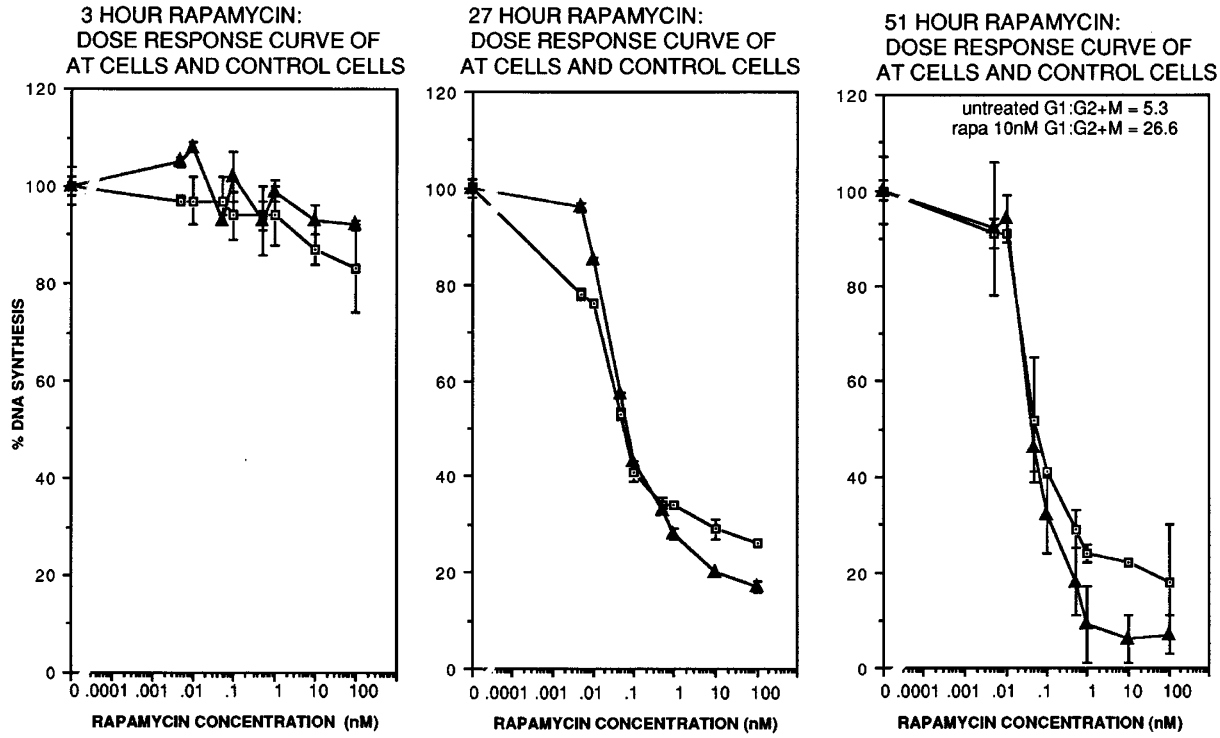

Figure 4 Kinetics of cell cycle arrest in B cell lines derived from normal and AT homozygous donors. Dose response of human lymphoblastoid cell lines 536 (control) ▲ and 719 (homozygous ataxia telangiectasia mutant) □. Both cell lines are immortalised by EBV transformation and both cell lines arrested in G1 in response to rapamycin. Cells were seeded in triplicate into 96 well microtitre plates at 50 000 per well in 200 μl of RPMI plus 10% FCS growth medium. Ten microlitres of ethanol containing rapamycin were added to each well to give the final rapamycin concentration shown; the cultures were then incubated at 37°C. Repeated experiments showed that the initial seed cell number did not alter the dose response curve to rapamycin (range 10 000–50 000 per well). Replicate plates were labelled for 3 h with tritiated thymidine to assay DNA synthesis between 0 h to 3 h, or 24 h to 27 h, or 48 h to 51 h, following drug treatment to provide data following 3 h, 27 h and 51 h exposure to rapamycin. In the experiment shown the mean c.p.m. of radiolabelled thymidine incorporated into the untreated (100%) 0536 cell DNA was 10301 c.p.m.; 29379 c.p.m.; and 49595 c.p.m. at 3 h; 27 h; and 51 h respectively. Similarly incorporation into untreated 0719 (AT) cell DNA was: 5180 c.p.m.; 16368 c.p.m.; and 34075 c.p.m. at 3 h; 27 h; and 51 h respectively. Percent standard error bars are shown. Flow cytometry showed that the cells arrest in G1 (see insert to Figure 1b and G1:G2+M ratio characteristics shown above for 536 cells. The corresponding AT cell G1:G2+M ratio was 5.3 in untreated controls and 9.3 in rapamycin-treated cells)

to replicate after washing in 20% FCS and re-seeding in drug-free growth medium showing that the rapamycin had had no long term effects on the ability of the cells to proceed through the cell cycle.

To confirm the lack of dependence of the action of rapamycin on p53 we compared the responses of RKO cell lines which have a common genetic background but express different levels of functional p53 (Kessis *et al*., 1993). In all cases rapamycin caused an increase in the G1:G2+M ratio which was independent of p53 expression. For example, 16 h treatment with 10 nM rapamycin resulted in the G1:G2+M ratio to increase from 3.7 to 5.1 in the parental RKO cells; from 2.35 to 3.5 in the RKO-neo cells which contain a neo-resistant plasmid; and from 2.6 to 5.2 in RKO-neo-E6 cells which are HPV E6 transfectants with undetectable baseline levels of p53 protein and loss of G1 arrest in response to DNA damage.

Discussion

Growth factors induce phosphorylation of the S6 protein of the small (40S) ribosomal subunit via p70S6 kinase, resulting in increased protein synthesis (recently reviewed by Proud, 1996). The p70S6 kinase itself may be activated by the serine/threonine kinase Akt/PKB (Bos, 1995). Although Akt/PKB is insensitive to direct inhibition by rapamycin, rapamycin prevents phosphorylation and activation of p70S6 kinase (Chung *et al*., 1992), indicating that FRAP is involved at some point in the control of this signalling pathway. A second pathway controlled by FRAP is required for cap-dependent mRNA translation, wherein 4EBP-1 (an inhibitor of the initiation factor eIF-4E) is phosphorylated by a PI3 kinase to release an active eIF-4E: rapamycin blocks phosphorylation of 4EBP-1 and cap-dependent translation (Beretta *et al*., 1996). Translation

protein loading per lane. (**d**) Basal levels of p53 were assessed after cell culture in rapamycin for different time periods. MCF 7 cells and 0536 cells were incubated in flasks, with flask '0' being untreated control cells in each cell line. Flasks of MCF 7 cells were treated with rapamycin @ 2×10^{-8} M for 3 h (flask 1); 2×10^{-6} M for 24 h (flask 2); 2×10^{-8} M for 24 h (flask 3) and 2×10^{-6} M for 3 h (flask 4). The 536 cells were treated with rapamycin @ 2×10^{-8} M for 3 h (flask 5); 2×10^{-8} M for 24 h (flask 6); 2×10^{-9} M for 24 h (flask 7) and 2×10^{-10} M rapamycin for 48 h (flask 8). The cells from each flask were harvested, lysed, and proteins separated by SDS–PAGE followed by Western blotting and staining for p53 levels. Each lane number corresponds to the flask number as detailed above

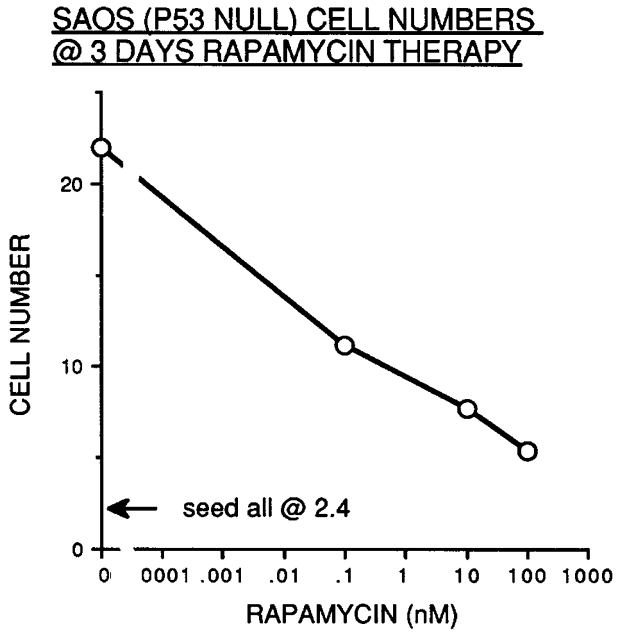

Figure 5 SAOS cells were seeded into flasks at 2.4×10^5 cells/flask and cultured in the presence of rapamycin at 0.1 nM, 10 nM or 100 nM concentrations for 3 days. The cells were then harvested by trypsinization and counted. The abscissa shows the numbers of cells recovered ($\times 10^5$) from each flask. Microscopic observations showed that the rapamycin had no apparent toxic effect at any dose, and after washing and counting the cells, each culture was seeded separately in control growth medium: all cultures proceeded to replicate

in both yeast (Barber *et al*., 1996) and mammalian cells is potently inhibited by rapamycin although, in yeast, translation is independent of S6 (Proud, 1996). This suggests that TOR/FRAP controls at least two discreet signalling pathways which are sensitive to inhibition by rapamycin. Interestingly, inhibition of eIF-4E expression in mammalian cells slows mammalian cell proliferation (De Benedetti *et al*., 1991) but results in early G1 arrest in yeast (Brenner *et al*., 1988). In this paper and in previous studies it has been shown that rapamycin slows the division rate of some mammalian cells whilst inducing G1 arrest in others (Brown *et al*., 1994). Thus rapamycin appears to effect more than one signalling pathway in mammalian cellular regulation.

We have asked if rapamycin-induced inhibition of G1 and cell cycle progression is functionally linked to p53. Our results show that rapamycin acts through a p53 independent pathway to inhibit the cell cycle or cause G1 arrest. Our finding that cells with a common genetic background, but with specific differences in p53 protein functional ability, were similarly sensitive to rapamycin supports the concept of discreet signalling pathways able to influence G1 arrest. The ability of IL3 to protect BAF-3 cells from p53-mediated apoptosis was not altered by rapamycin, showing that certain cell/cytokine-responsive pathways are resistant to rapamycin whilst others (e.g. IL2; platelet-derived growth factor; and beta-fibroblastic growth factor; Morris, 1992; Cao *et al*., 1995) are potently inhibited by rapamycin. Rapamycin only binds and inhibits TOR/FRAP when it is complexed to specific cytoplasmic receptors, known as FKBPs (Heitman *et al*., 1991 and recently reviewed by Galat and Metcalfe, 1995). Different FKBPs exist at different cellular sites (Perrat-Applanat *et al*., 1995; Galat *et al*., 1992) and it is likely that the effects of rapamycin in a given cell type, or for a given signalling pathway, are qualified by the nature and localisation of that cell's FKBP profile. Of note is the observation that p53 may directly down regulate transcription of FKBP25 (Dr Maureen Murphey and Dr Arnold Levine, personal communication). Since FKBP25 has a high affinity for rapamycin and also encodes a nuclear localisation sequence which distinguishes it from cytoplasmic FKBP12 (Galat *et al*., 1992), this implies that rapamycin and p53 may be functionally linked at a subtle level which does not impinge on the cellular responses measured in our experiments. Further elucidation of the relationship between the various rapamycin-FKBP complexes with FRAP(s) together with p53-mediated regulation of FKBP25 synthesis will allow a more accurate understanding of rapamycin's action on cell cycle regulation.

Down stream, rapamycin influences the activity of cyclin kinases such $p34^{cdc2}$ and its relatives (Morice *et al*., 1993a), but the details linking rapamycin to these downstream effects are unknown. Transforming growth factor beta may induce p21 expression independent of the p53 pathway in certain ovarian cancer cells to contribute to cell cycle arrest (Elbendary *et al*., 1994), supporting the concept of discreet, rather than convergent, regulatory mechanisms. However, it is relevant to note that we found no evidence of rapamycin causing induction of p21 protein in the absence of p53 induction. The recent finding that p27(Kip1) null cells remain sensitive to cell cycle arrest mediated by rapamycin (Nakayama *et al*., 1996) suggests that p27(Kip1) is not required for the rapamycin inhibition pathway. We have looked for convergence at the level of p53 within the growth-regulatory PI-3 kinases/PI-4 kinases which bind to rapamycin-FKBP, but found that rapamycin targets p53-independent regulatory pathway(s). This is in accord with our finding that normal cells and cells homozygously defective for ATM showed no difference in sensitivity to rapamycin: both became arrested in G1 with an ID50% of around 0.05 nM (Figure 4). The recent report that functional loss of the ATM protein leads to rapamycin resistance at 1 nM (Beamish *et al*., 1996) appears to differ from our results. However, since a cell line may respond to rapamycin either by G1 arrest, or by reduced rate of progression through the cell cycle (Brown *et al*., 1994; Morice *et al*., 1993a,b), it is possible that the results reported by Beamish *et al*. were influenced by such differences, since the AT cell lines they used did not show rapamycin-induced G1 arrest, in contrast to their control cells which did. Since we found that rapamycin therapy did not alter p53 induction or affect irradiation-induced apoptosis, it appears that the ability of the wild type AT gene product to phosphorylate its putative substrate(s) is not inhibitable by rapamycin. Biochemical evidence indicates that DNA-PK phosphorylates proteins but has no detectable affinity towards lipids (Hartley *et al*., 1995) and a similar substrate specificity may also be true of the ATM protein. If rapamycin, via FRAP, effects lipid phosphorylation but not protein phosphorylation, then the drug might be expected to target a signalling pathway which is discreet from that affected by any ATM activity. In conclusion, although both

rapamycin and p53 each induce G1 arrest, they appear to act through independent growth regulatory pathways.

Materials and methods

Cell culture

The BAF-3 cell line was maintained in RPMI 1640 supplemented with 10% foetal calf serum and 10% Wehi 3B conditioned medium as a source of IL3. Other cell lines were maintained in RPMI 1640 supplemented with 10% foetal calf serum: these were the human MCF-7 breast cancer cell line; the human lymphoblast cell lines 0536 (control) and 0526 or 0719 (homozygous ataxia telangiectiasia mutants) obtained from the human genetic mutant cell repository, Camden, New Jersey, USA; the p53 null human SAOS osteosarcoma cell line; and the human colorectal carcinoma cell line, RKO. There was no microscopically visible toxicity due to rapamycin at doses up to 1 μM in any of these cell lines, and cultures recovered normal growth when drug was removed.

Reagents

Rapamycin was kindly donated to SMM by Suren Sehgal of Wyeth Ayerst Pharmaceuticals. The drug is highly hydrophobic and adsorbs to plastic: it is unstable in aqueous solution and therefore drug stocks of 1mg/ml in ethanol were stored at -20°C and used at 1/1000 dilution as described by Cao *et al*., (1995). Control cultures were treated with an equivalent dose of ethanol alone. Primary monoclonal antibodies (mabs) used in Western blotting were as follows: mouse anti-mouse p53 mabs from Oncogene (a cocktail of PAb 421 and PAb 240); rabbit polyclonal anti-p21 (Santa Cruz); mouse anti-human p53 (a cocktail of Oncogene PAb 421, 1801 and DO-1); mouse anti-human p21 mab (Pharminogen); and human anti-human topoisomerase (Topogen). Secondary antibodies were horse radish peroxidase (HRP) conjugated goat anti-mouse serum (Pierce), goat anti-human HRP; goat anti-rabbit HRP (Pierce).

Flow cytometry

Cells for flow cytometric analysis were fixed in 1% formaldehyde PBS followed by 70% methanol at -20°C, with thorough mixing throughout. These were treated with RNA-ase and stained with propidium iodide for FACS assay of DNA content. Some samples were also labelled with FITC using the terminal transferase assay for DNA fragments in apoptotic cells as described in Canman *et al*. (1995) and adapted from Goczyca *et al*. (1993).

Measurement of p53 and p21 levels

Western blots of experimental cell cultures were prepared from cells lysed in Laemmli sample buffer using standard techniques (Canman *et al*., 1995). SDS-polyacrylamide gel electrophoresis (SDS–PAGE) gels (12% acrylamide) were loaded with equivalent protein concentrations based either on equal cell numbers, or protein measurements using a BioRad kit for the Lowry assay. After SDS–PAGE, separated proteins were electroblotted onto nitrocellulose and stained with 1% fast green to confirm equivalence in loading per lane. P53 and p21 were detected by their respective mabs and autoradiographed by enhanced chemiluminescence (Amersham ECL). In the MCF-7 cells, topoisomerase was stained as an internal reference standard for comparison with induced levels of p53.

DNA synthesis

Cell dose response curves to rapamycin were measured in 200 μl aliquots containing equal numbers of cells using 96 well microtitre plates. At 0 h, or after 24 h or 48 h incubation, the rate of DNA synthesis was measured by labelling with 1 μCi tritiated thymidine per well for 3 h (Amersham International, specific activity 70 Ci/mM). Cultures were then harvested onto glass fibre paper and radioactivity counted using standard, automated techniques. The results are presented as percentage DNA synthesis for ease of comparison: actual radioactivity counts are presented in the Figure legends.

Cell counts

In the SAOS (p53 null) cell line measured levels of DNA synthesis were relatively low. Therefore cell counts were used to record any effect of rapamycin. SAOS cells were seeded into flasks at 2.4×10^5 cells/flask and cultured in the presence of rapamycin at 0.1 nM, 10 nM or 100 nM for 3 days. The cells were then harvested by trypsinization and counted.

Acknowledgements

The authors are grateful to Maureen Murphey and Arnold Levine for allowing us to cite their unpublished observations; to Raj Nair at Stanford University School of Medicine, and Beverely Plunkett and Chris Leonard at Johns Hopkins Hospital, for their helpful assistance during parts of this work. Financial support to SMM was provided by a European Society of Organ Transplantation Travel Scholarship, by Wyeth-Ayerst Pharmaceuticals, and by the European Community Health Resources Grant Number CT 93-0180. MBK, CEC and SG are supported by grants from the NIH (CA61949, ES05777, T32CA60441). MBK is the Steven Birnbaum Scholar of the Leukaemia Society of America.

JM is the Yorkshire Cancer Research Campaign Professor of Cell Biology, UK.

References

Abraham RT. (1996). *Current Opinion in Immunology*, **8,** 412–418.

Albers MW, Williams RT, Brown EJ, Tanaka A, Hall FL and Schreiber SL. (1993). *J. Biol. Chem*., **268,** 22825–22829.

Barber NC *et al*., (1996). *Mol. Biol. Cell*, (in press).

Beamish H, Williams R, Chen P, Khanna KK, Hobson K, Watters D, Shiloh Y and Lavin M. (1996). *Oncogene*, **13,** 963–970.

Beretta L, Gingras A-C, Svitkin YV, Hall MN and Sononberg N. (1996). *EMBO J*., **15,** 658–664.

Bos JL. (1995). *TIBS*, **20,** 441–442.

Brenner C, Nakayama N, Goebl M, Tanaka K, Toh-e A and Matsumoto K. (1988). *Mol. Cell. Biol*., **8,** 3556–3559.

Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WA and Schreiber SL. (1994). *Nature*, **369,** 756–758.

Brown EJ, Beal PA, Keith CT, Chen J, Shin TB and Schreiber SL. (1995). *Nature*, **377,** 441 – 446.

Cafferkey R, Young PR, McLaughllin MM, Bergsma DJ, Koltin Y, Sathe GM, Faucette L, Eng W, Johnson RK and Livi GP. (1993). *Mol. Cell. Biol.*, **13,** 6012 – 6023.

Canman CE, Wolff AC, Chen C-Y, Fornace AJ and Kastan MB. (1994). *Cancer Res.*, **54,** 5054 – 5058.

Canman CE, Gilmer TM, Coutts SB and Kastan MB. (1995). *Genes and Dev.*, **9,** 600 – 611.

Cao W, Mohasci P, Shorthouse R, Pratt R. and Morris RE. (1995). *Transplantation*, **59,** 3.

Cardenas ME and Heitman J. (1995). *EMBO J.*, **14,** 5892 – 5907.

Chen J, Zheng XF, Brown EJ and Schreiber SL. (1995). *Proc. Natl. Acad. Sci.*, **92,** 4947 – 4951.

Chung J, Kuo CJ, Crabtree GR and Blenis J. (1992). *Cell*, **69,** 1227 – 1236.

Collins MKL, Marvel I, Malde P and Lopez-Rivas A. (1992). *J. Exp. Med.*, **176,** 1043 – 1051.

DeBenedetti A, Joshi-Barve S, Rinker-Schaeffer C and Rhoads RE. (1991). *Mol. Cell. Biol.*, **11,** 5435 – 5445.

Elbendary A, Berchuck A, Davis P, Havrllesky L, Bast RC, Ingehart JD and Marks JR. (1994). *Cell Growth Differ.*, **5,** 1301 – 1307.

Galat A, Lane WS, Standaert RF and Schreiber SL. (1992). *Biochem.*, **31,** 2427 – 2434.

Galat A and Metcalfe SM. (1995). *Prog. Biophys. Mol. Biol.*, **63,** 69 – 119.

Gorczyca W, Gong J and Darzynkiewicz Z. (1993). *Cancer Res.*, **53,** 1945 – 1951.

Hartley KO, Gell D, Smith GCM, Zhang H, Divecha N, Connelly MA, Admon A, Lees-Miller SP, Anderson CW and Jackson SP. (1995). *Cell*, **82,** 849 – 856.

Heitman J, Morra NR and Hall MN. (1991). *Science*, **253,** 905 – 909.

Hollis CM and Hutchinson R. (1996). *Biochem. Soc. Trans.*, **24,** 66S.

Jackson SP. (1995). *Curr. Biol.*, **5,** 1210 – 1212.

Kastan MB, Zhan Q, El-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B and Fornace Jr, AJ. (1992). Cell, **71,** 587 – 597.

Khanna KK and Lavin MF. (1993). *Oncogene*, **8,** 3307 – 3312.

Kessis TD, Slebos RJ, Nelson WG, Kastan MB, Plunkett BS, Han SM, Lorincz AT, Hedrick L and Cho KR. (1993). *Proc. Natl. Acad. Sc.*, **90,** 3988 – 3992.

Kunz J, Henriquez R, Schneider U, Deuter-Reinhard M, Movva RN and Hall MN. (1993). *Cell*, **73,** 585 – 596.

Lees-Miller SP, Chen YR and Anderson CW. (1990). *Mol. Cell. Biol.*, **10,** 6472 – 6481.

Metcalfe SM and Milner AJ. (1991). *Transplantation*, **51,** 1318 – 1320.

Morice WG, Brunn GJ, Wiederrecht G, Siekierka JJ and Abraham RT. (1993a). *J. Biol. Chem.*, **268,** 3734 – 3738.

Morice WG, Wiederrecht G, Brunn GJ, Siekierka JJ and Abraham RT. (1993b). *J. Biol. Chem.*, **268,** 22737 – 22745.

Morris RE. (1992). *Transplant. Rev.*, **6,** 39 – 87.

Nakayama K, Ishida N, Shirane M, Inomata A, Inoue T, Shishido N, Horii I, Loh DY and Nakayama K. (1996). *Cell*, **84,** 707 – 720.

Palacios R and Steinherz M. (1985). *Cell*, **41,** 727 – 734.

Perrot-Applanat M, Cibert C, Geraud G, Renoir JM and Ballieu EE. (1995). *J. Cell Sci.*, **108,** 2037 – 2051.

Proud CG. (1996). *TIBS*, **21,** 181 – 185.

Sabatini DM, Pierchala, Barrow RK, Schnell MJ and Snyder S. (1995). *J. Biol. Chem.*, **270,** 20875 – 20878.

Sabers CJ, Martin MM, Brunn GJ, Williams JM, Dumont FJ, Wiederrecht G, Abraham RT. (1995). *J. Biol. Chem.*, **270,** 815 – 822.

Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S, Ashkenazl M, Pecker I, Frydman M, Harnik R, Patanjali SR, Simmons A, Cllnes G, Sartlel A, Gattl RA, Chessa L, Sanal O, Lavin MF, Jaspers NGJ, Taylor MR, Arlett MF, Miki, T, Weissman SM, Lovett M, Collins FS and Shiloh Y. (1995). *Science*, **268,** 1749 – 1753.

Shi Y, Frankel A, Radvanyl LG, Penn LZ, Miller RG and Mills GB. (1995). *Cancer Res.*, **55,** 1982 – 1988.