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p21^{WAF1} is dynamically associated with JNK in human T-lymphocytes during cell cycle progression

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SUMMARY

We have examined the regulation of the c-Jun NH_2-terminal kinase (JNK) subfamily of mitogen-activated protein kinases (MAPKs) in response to inhibition of DNA replication during the cell cycle of human T-lymphocytes. In this study, we demonstrate that JNK is rapidly activated following release of T-lymphocytes from G_1/S-phase arrest and that this activation precedes resumption of DNA synthesis upon S-phase progression. We also show that activation of JNK correlates with dissociation of the cyclin-dependent protein kinase (CDK) inhibitor, p21^{WAF1}, from JNK1. Since JNK1 isolated from T-lymphocytes by immunoprecipitation can be inhibited by recombinant p21^{WAF1} in vitro, these data suggest that JNK activation may be regulated in part by its dissociation from p21^{WAF1}. The observation of a dynamic, physical association of native JNK1 and p21^{WAF1} in vivo has not previously been described and suggests a novel mechanism for JNK-mediated regulation of the cell cycle of human T-lymphocytes.

Key words: Cell cycle, T-lymphocyte, JNK

INTRODUCTION

Mammalian cells ensure that DNA replication and repair are completed before entering M-phase (Murray, 1992). The presence of unreplicated or damaged DNA activates the G_2/M-phase checkpoint, thereby blocking entry into mitosis (Hartwell and Weinert, 1989). Cell cycle arrest in response to DNA damage is mediated by the cyclin-dependent protein kinase (CDK) inhibitor p21^{WAF1} (also termed Cip1 or Sdi1) and the tumour suppressor p53. DNA damage in the G_1-phase activates p53, a transcriptional activator of p21^{WAF1}. Increased levels of the p21^{WAF1} protein then block entry into S-phase by inhibiting the activity of cyclin D/CDK4, cyclin E/CDK2 and proliferating cell nuclear antigen (PCNA) (for review, see Hunter and Pines, 1994). The cytoplasmic signals that communicate the presence of damaged or unreplicated DNA to the DNA synthetic and mitotic machinery remain largely unknown. However, recent work suggests involvement of the c-Jun NH_2-terminal kinases/stress-activated protein kinases (JNKs/SAPKs) in regulating the cell cycle. Activation of the JNKs has been linked both to cell cycle arrest and to cell cycle progression. For example, inflammatory stimuli such as IL-1 and TNF-α, and environmental stresses such as ionizing radiation, heat shock and hyperosmolarity activate the JNK pathway and lead to growth arrest (Woodgett et al., 1996; Kyriakis and Avruch, 1996) while the activation of the JNKs by the GTPases Rho and Cdc42 may lead to G_1 progression and DNA synthesis in Swiss 3T3 fibroblasts (Yamamoto et al., 1993; Olson et al., 1995). In accord with these observations, we demonstrate that JNK is activated in human T-lymphocytes in response to the inhibition of DNA replication and also following release of these cells from G_1/S-phase arrest. We also show for the first time that p21^{WAF1} is dynamically associated with JNK1 in vivo and that JNK activation correlates with dissociation of the p21^{WAF1}/JNK1 complex and entry into S-phase in human T-lymphocytes.

MATERIALS AND METHODS

Cell culture

Human T-lymphocytes were isolated from peripheral blood (donated by healthy volunteers) using a Ficoll gradient (Pharmacia) according to the manufacturer's instructions. The T-lymphocytes were suspended in RPMI 1640 containing 10% (v/v) foetal calf serum (FCS), penicillin (50 i.u./ml) and streptomycin (50 μg/ml), and stimulated with phytohaemagglutinin (PHA, 2 μg/ml) for 72 hours. PHA was then removed by washing the cells three times with Dulbecco's phosphate buffered saline (PBS). The cells were then expanded in the above medium containing 20 ng/ml recombinant interleukin-2 (IL-2, Eurocetus) at 37°C in a humidified atmosphere of

95% air, 5% CO_2 for 2 weeks prior to experimentation. For some experiments T-lymphocytes were synchronized in G_1 (Cantrell and Smith, 1984). Stimulation of G_1-arrested T-lymphocytes with IL-2 results in cell cycle entry and proliferation (Cantrell and Smith, 1984). HeLa cells were cultured in Dulbecco's minimum essential medium (MEM; Gibco-BRL) containing 10% (v/v) FCS, penicillin (50 i.u./ml) and streptomycin (50 μg/ml).

Aphidicolin treatment and preparation of cell extracts

T-lymphocytes were arrested in early S-phase by treating exponentially growing cells with 1 μg/ml aphidicolin (5 mg/ml stock in dimethylsulfoxide (DMSO; Sigma)) for 24-36 hours. Inhibition of DNA polymerase α by aphidicolin causes a block to the replication fork (Sheaff et al., 1991), thereby arresting the cells in early S-phase. The aphidicolin block was removed to allow resumption of DNA replication by washing the cells three times with PBS. Strictly speaking, the release from aphidicolin allows for S-phase progression from a block to the replication fork in early S-phase. However, to simplify terminology, aphidicolin-release by this method is described throughout the text as S-phase entry from a G_1/S-phase arrest. Control cells were washed in PBS containing aphidicolin (1 μg/ml) and then returned to aphidicolin-containing medium. T-lymphocytes (5×10^7 cells) were washed once in PBS and lysed at intervals after release from aphidicolin in 200 μl of buffer A (10 mM Tris-HCl, pH 7.4, 1% v/v Nonidet P-40, 0.1% w/v bovine serum albumin (BSA), 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, 5 μg/ml pepstatin and 5 μg/ml chymostatin) for preparation of total cell extracts and immunoprecipitation of either JNK1, p21*WAF1*, p38, extracellular-regulated kinase-1 (ERK1) or ERK2, or in 200 μl of buffer B (20 mM Tris-HCl, pH 7.6, containing 0.5% v/v Nonidet P-40, 250 mM NaCl, 3 mM ethylenediaminetetra-acetic acid (EDTA), 3 mM ethylene glycol-bis(β-aminoethylether)N,N,N′,N′-tetraacetic acid (EGTA), 1 mM PMSF, 2 mM Na_3VO_4, 20 μg/ml aprotinin, 5 μg/ml leupeptin and 1 mM dithiothreitol (DTT)) for assay of JNK activity. In some experiments 2.5 mM hydroxyurea (2.5 M stock in water; Sigma) was used instead of aphidicolin to arrest T-lymphocytes at the G_1/S boundary.

HeLa cells were blocked in early S-phase with aphidicolin (1 μg/ml) for 16 hours and either released or maintained in the block as described for T-lymphocytes. HeLa cell lysates were prepared by scraping the cells into ice-cold PBS containing 0.4 mM EDTA. The cells were collected by centrifugation at 1,000 rpm for 5 minutes and then lysed in either buffer A for JNK immunoprecipitation or in buffer B for assay of JNK activity.

Immunoprecipitation and western blotting

T-lymphocytes (5×10^7 cells) were washed once in PBS and lysed in 200 μl of buffer A at intervals after release from aphidicolin. Insoluble material was removed from cell lysates by centrifugation at 14,000 ***g*** for 10 minutes at 4°C. JNK1 was isolated by immunoprecipitation using a polyclonal antiserum sc-517 (Santa Cruz). After incubation for 16 hours at 4°C with 0.2 μg of antibody, immune complexes were collected by adding 100 μl of Protein A-Sepharose beads (0.03 g/ml) and incubating for a further 2 hours at 4°C with constant rotation. The immunoprecipitates were washed three times in PBS containing 2 mM Na_3VO_4, 2 mM NaF, 5 μg/ml leupeptin, 5 μg/ml pepstatin and 5 μg/ml chymostatin and then boiled in sample buffer. Both immunoprecipitated proteins and total cell extracts were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 10% or 12.5% acrylamide) and electroblotted onto Immobilon membranes (Millipore) as described previously (Edgecombe et al., 1991). The membranes were blocked for 1 hour with 5% (w/v) BSA and 1% (w/v) ovalbumin in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) and then incubated with either a polyclonal phospho-specific JNK (Thr183/Tyr185) antibody (New England Biolabs, 1:1,000 dilution), a polyclonal phospho-specific ERK (Thr202/Tyr204) antibody (Promega, 1:20,000 dilution), a monoclonal anti-p21*WAF1* antibody (sc-817, Santa Cruz; 1:500 dilution) or with the anti-JNK1 antibody (1:500 dilution) for 12 hours at 4°C. After washing with TBST, the membranes were incubated with either a horseradish peroxidase-conjugated goat anti-mouse antibody or goat anti-rabbit antibody (Sigma, 1:10,000 dilution) for 2 hours at room temperature. Immunoreactive proteins were visualised using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham).

Preparation of recombinant p21*WAF1*

The cDNA encoding human p21*WAF1* with an N-terminal glutathione-S-transferase (GST) tag and C-terminal hexahistidine (His6) tag in pET21d (Novagen) was transformed into BL21 (DE3) PlysS for protein expression. The double tag was introduced into the recombinant protein as this increased its solubility and yield compared with either single-tagged protein. Bacterial cultures were grown in LB medium containing 50 μg/ml ampicillin to an OD_{595} of 0.6 and then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at room temperature for 4 hours. The bacterial pellet was resuspended in buffer C (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.25 mM PMSF) containing 5 mM imidazole and lysed by freeze-thawing. The lysate was centrifuged at 12,000 ***g*** and the supernatant filtered through a 0.45 μm filter (Millipore). Proteins were allowed to bind to Ni-NTA agarose (Qiagen) for 1 hour at 4°C. The beads were collected in a chromatography column and washed with 5 bed volumes of buffer D (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.25 mM PMSF, 0.5% v/v Triton X-100, 0.5% v/v Tween-20) containing 50 mM imidazole. The column was then eluted stepwise with 2 volumes each of buffer D sequentially containing 65 mM, 85 mM, 105 mM, 125 mM and 150 mM imidazole. Purified GST-p21*WAF1*-His6 from the 105 mM, 125 mM and 150 mM imidazole fractions was pooled and dialysed at 4°C against 20 mM Tris-HCl, pH 8.0, containing 0.15 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 0.25 mM PMSF and 1 mM DTT, and then concentrated using a Centricon-10 device (Amicon). GST-p21*WAF1*-His6 was prepared at 0.6 mg/ml as determined by the Bradford assay (Pierce) and its purity was greater than 90% as estimated by Coomassie blue staining of an SDS-PAGE gel.

Protein kinase assays

T-lymphocytes (5×10^7 cells) were washed once with PBS and lysed in buffer B. Insoluble material was removed from cell lysates by centrifugation at 14,000 ***g*** for 10 minutes at 4°C. Lysates were either incubated for 1 hour at 4°C with 5 μg of GST-c-Jun coupled to glutathione-Sepharose (Pharmacia-LKB) or for 2 hours at 4°C with 0.5 μg of JNK1 antibody. The JNK1 antibody was recovered by addition of 100 μl of Protein A-Sepharose beads (0.03 g/ml) and incubation for a further 2 hours at 4°C. Beads were collected by centrifugation and washed twice in 200 μl of buffer B and twice in 200 μl of buffer E (20 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethane sulfonic acid] (Hepes), pH 7.5, containing 20 mM β-glycerophosphate, 10 mM $MgCl_2$, 1 mM DTT and 50 μM Na_3VO_4). Kinase assays were initiated by addition of 40 μl of buffer E containing 20 μM ATP and 2 μCi [γ-^{32}P]ATP. After incubation for 20 minutes at 30°C, reactions were terminated by the addition of an equal volume of Laemmli sample buffer and boiling. Phosphorylated proteins were resolved by SDS-PAGE through 12% acrylamide and visualised by autoradiography. Incorporated radioactivity was quantified by liquid scintillation counting of the excised bands.

To assay the effect of recombinant p21*WAF1* on JNK activity in vitro, varying concentrations of the GST-p21*WAF1*-His6 protein were added to JNK1 immunoprecipitates immediately before addition of buffer E containing 20 μM ATP and 2 μCi [γ-^{32}P]ATP to a final volume of 40 μl. For these experiments, activated JNK1 was isolated by immunoprecipitation from T-lymphocytes at thirty minutes after release from an aphidicolin block as described above.

For the parallel assay of JNK and p38 activities, lysates of T-lymphocytes (15×10^7 cells) were prepared in buffer B at intervals after release from aphidicolin and divided into 3 aliquots. One aliquot was assessed for JNK activity using GST-c-Jun as affinity ligand and substrate. JNK1 and p38 were separately isolated from the remaining 2 aliquots by immunoprecipitation following incubation for 90 minutes at 4°C with 0.4 μg of polyclonal antisera sc-517 and sc-535 (Santa Cruz), respectively. JNK and p38 assays were performed as described above, except that reactions were initiated by addition of either 5 μg GST-c-Jun or 5 μg GST-activating transcription factor 2 (ATF2) (Gupta et al., 1995), respectively, and 40 μl of buffer E containing 20 μM ATP and 2 μCi [γ-^{32}P]ATP.

To assay ERK1 and ERK2 activities, T-lymphocyte (10×10^7 cells) extracts in buffer B were divided into 2 aliquots and incubated with 0.2 μg of polyclonal antibody to either ERK1 (sc-093, Santa Cruz) or ERK2 (sc-154, Santa Cruz) for 12 hours at 4°C. Immune complexes of ERK1 and ERK2 were collected as described above and assayed using myelin basic protein (MBP; Gibco-BRL) as substrate (Tamemoto et al., 1992).

To measure cyclin-dependent kinase activity, capped mRNAs of *Xenopus* cyclin E and cyclin A were translated in rabbit reticulocyte lysate for 1 hour at 30°C in the presence of L-[^{35}S]methionine as described by Jackson and Hunt (1993). To ensure correct folding and activation of the translated proteins, *Xenopus* egg extracts and bacterially expressed GST-cdk2 were added to the translation mix. The active cyclin/GST-cdk2 complexes were recovered by incubation with glutathione-Sepharose for 1 hour at 4°C. The beads were collected and then washed 5 times with buffer F (80 mM β-glycerophosphate, 20 mM EGTA, 50 mM NaF, 0.25 M NaCl and 0.25 mM PMSF) and twice with buffer G (50 mM β-glycerophosphate, 5 mM NaF, 1 mM DTT, 15 mM magnesium acetate, 0.3 mM EDTA and 0.25 mM PMSF). Excess buffer was removed and the beads were incubated with varying concentrations of GST-p21^{WAF1}-His6 protein for 10 minutes at room temperature. The reactions were started by addition of 10 μl of a kinase buffer containing 50 mM β-glycerophosphate, 5 mM NaF, 1 mM DTT, 15 mM magnesium acetate, 0.3 mM EDTA, 0.25 mM PMSF, 100 μM ATP, 1 μCi [γ-^{32}P]ATP and 2 μg of histone H1 as substrate. Incubations were for 45 minutes at 23°C. Phosphorylated proteins were resolved by SDS-PAGE through 15% acrylamide and visualised by autoradiography. Phosphorylation of histone H1 was quantitated by scanning densitometry.

Flow cytometry

HeLa cells or T-lymphocytes (1×10^6 cells) were fixed in cold (–20°C) methanol and stained with propidium iodide. Analysis of the DNA content was performed by flow cytometry (FACSCAN, Becton Dickinson). The detection of apoptotic cells by flow cytometry was performed as described (Martin et al., 1994), using fluorescein isothiocyanate (FITC)-labelled annexin V (Bender MedSystems) binding to phosphatidylserine.

Measurement of DNA synthesis

T-lymphocytes were analysed for DNA synthesis by incorporation of [^{3}H]thymidine (Amersham) as described (Churcher and Moss, 1993).

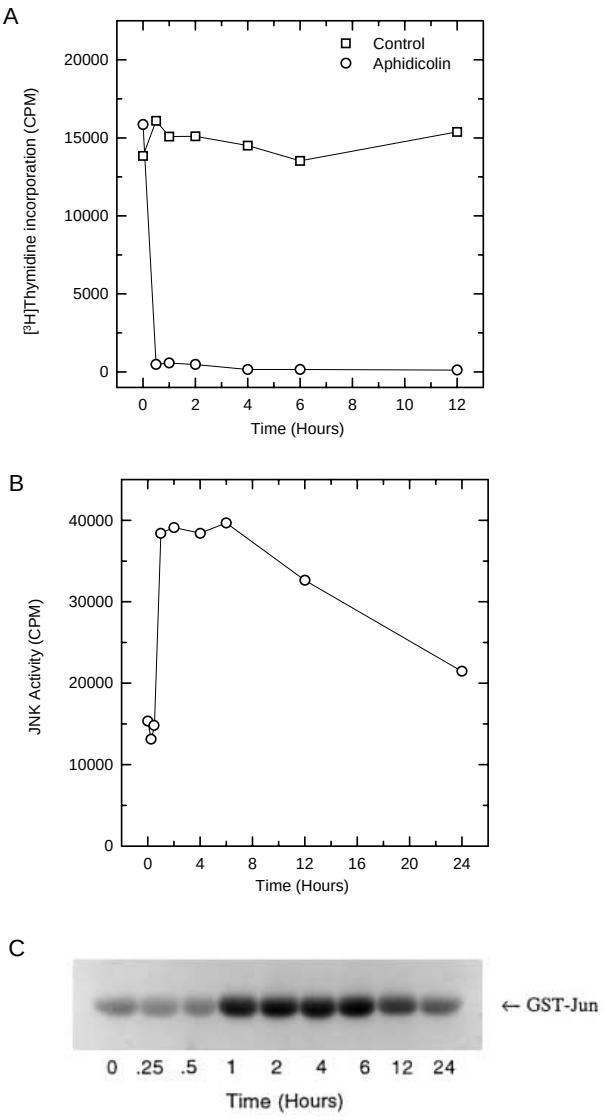

Fig. 1. Aphidicolin inhibits DNA replication and stimulates JNK in T-lymphocytes. (A) The effect of aphidicolin (1 μg/ml) on [^{3}H]thymidine uptake was measured following its addition to an asynchronous population of IL-2-dependent T-lymphocytes. Data shown are representative of two experiments. Each data point is the mean of quadruplicate samples. (B) The effect of aphidicolin on JNK activity was measured in the same experiment shown in A using GST-Jun as affinity ligand and substrate. Data are means of duplicate values obtained from 2 separate experiments. (C) The phosphorylated proteins were visualised after SDS-PAGE by autoradiography. A representative autoradiogram of the quantified data presented in B is shown.

RESULTS

Inhibition of DNA synthesis causes JNK activation

We first examined whether inhibition of DNA replication, like DNA damage (Derijard et al., 1994), causes activation of the c-Jun NH_2-terminal kinase (JNK) signalling pathway in IL-2-stimulated human peripheral blood T-lymphocytes. Treatment of these cells with aphidicolin, which inhibits DNA polymerase α (Sheaff et al., 1991) without inducing DNA damage (Canman et al., 1992; Kizaki et al., 1992; Lavin et al., 1994), resulted in the rapid inhibition of DNA synthesis as determined by [^{3}H]thymidine uptake (Fig. 1A). Furthermore, flow cytometry confirmed that treatment of T-lymphocytes with aphidicolin (1 μg/ml) for 24-36 hours caused G_1/S-phase arrest (90.2±2% of cells arrested in G_1/S-phase, mean ± s.e.m., n=4). However, this treatment did not appear to cause apoptosis since the proportion of apoptotic cells (as a percentage of the total number of viable cells) in the aphidicolin-treated population

was 5.6%, compared with 1.6% in the control cell population. In addition, cell viability in both populations was greater than 95% at 36 hours, as determined by Trypan Blue exclusion. Indeed, western blot analysis of T-lymphocyte lysates with an anti-p21^{WAF1} antibody indicated that the level of p21^{WAF1} protein did not vary over the time course of the aphidicolin block (0-24 hours, data not shown), supporting the absence of an apoptotic response.

Recent work has established that all known isoforms of JNK derived from the JNK1, JNK2 and JNK3 genes are capable of binding to, and phosphorylating to a similar extent, the substrate c-Jun (Gupta et al., 1996). We therefore measured JNK activity in T-lymphocytes at intervals after aphidicolin addition by using a recombinant protein fragment of c-Jun as affinity ligand and substrate (glutathione-S-transferase[GST]-c-Jun [1-79]; Derijard et al., 1994). Addition of aphidicolin to the asynchronous population of T-lymphocytes caused a sustained activation of JNK (Fig. 1B and C) which was delayed in onset relative to the rapid inhibition of [^{3}H]thymidine incorporation (Fig. 1A). Thus, the inhibition of DNA replication, like DNA damage, induces JNK activation.

JNK activation is associated with entry of T-lymphocytes into S-phase

We next determined the level of JNK activity in T-lymphocytes following their release from G_1/S-phase arrest. Cells were blocked in G_1/S-phase with aphidicolin for 24-36 hours and then washed free of aphidicolin. A rapid increase in DNA synthesis was observed under these conditions (half-maximal [^{3}H]thymidine incorporation at 40±7 minutes after release, mean ± s.e.m., n=3) and was maximal by 2 hours (Fig. 2A). As shown in Fig. 2B and C, a transient 4.9-fold activation of JNK was observed at 30 minutes after release from G_1/S-phase arrest (mean JNK activation = 13±5.1-fold (± s.e.m.), n=8, range = 2.3-39.7-fold). This activation was not due to a change in the amount of the 46 kDa form of JNK associated with GST-c-Jun as this was found to be constant compared to control by immunoblot analysis using an antiserum to JNK1 (not shown). In control experiments, JNK activity remained unchanged in G_1/S-phase-arrested cells that had been washed and returned to aphidicolin-supplemented medium (Fig. 2B). In some experiments hydroxyurea, an inhibitor of ribonucleotide reductase, was used in place of aphidicolin to inhibit DNA synthesis in T-lymphocytes. Release of these cells from the hydroxyurea block also caused a transient JNK activation that was maximal at 30 minutes (data not shown).

The activation of the JNKs is regulated by dual phosphorylation on threonine and tyrosine within the motif Thr-Pro-Tyr (Kyriakis and Avruch, 1990; Kyriakis et al., 1991; Mukhopadhyay et al., 1992). Western blot analysis of T-lymphocyte extracts using an antiserum raised to the phosphorylated threonine and tyrosine residues within this sequence indicated that activation of the kinase at 30 minutes (Fig. 2B and C) correlated with phosphorylation of the 46 kDa and 55 kDa forms of JNK (Fig. 2D). Reprobing of these blots with an antibody to JNK1 showed that this antiserum only cross-reacted with the 46 kDa form of JNK in T-lymphocytes and that the level of this protein did not vary over the time course of these experiments (not shown). Therefore, these observations suggest that JNK activation, which precedes the onset of DNA synthesis, may serve as a positive signal for cell

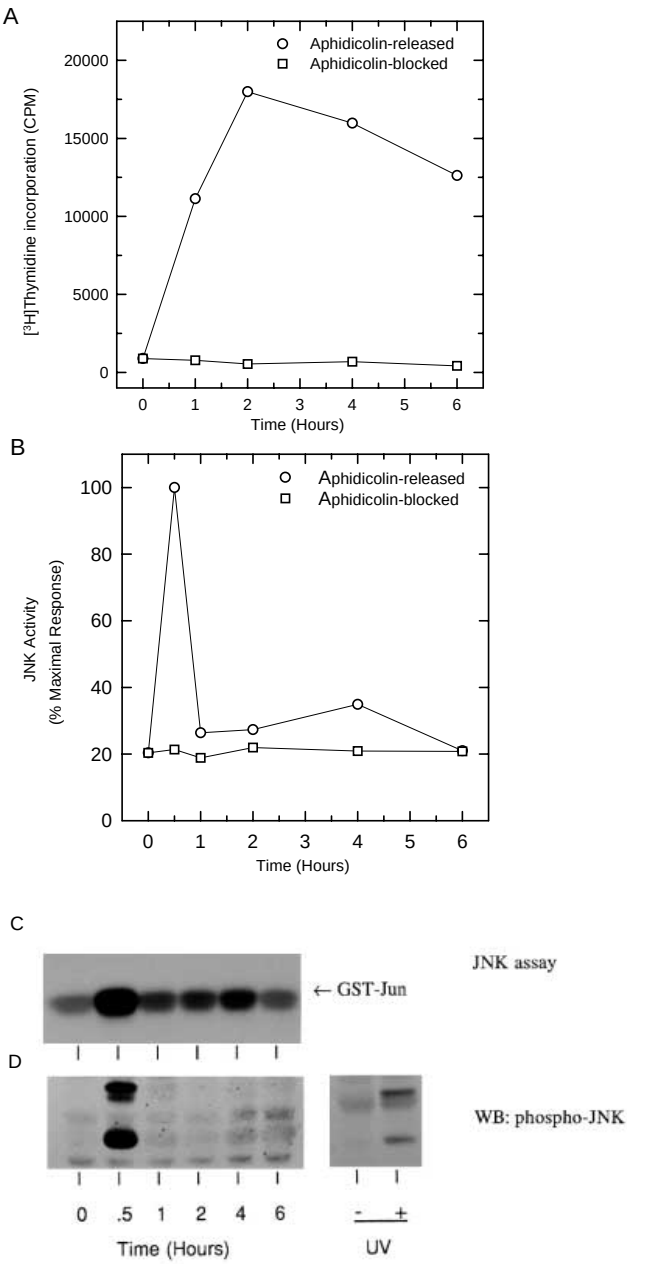

Fig. 2. Transient JNK activation precedes DNA synthesis in T-lymphocytes following release from an aphidicolin block. (A) [^{3}H]Thymidine uptake was measured in cells at intervals following their release from an aphidicolin block. Control cells were washed and returned to aphidicolin-containing medium to retain the block. (B) JNK activity was measured in the same experiment shown in A, using GST-Jun as affinity ligand and substrate. (C) The phosphorylated proteins were visualised after SDS-PAGE by autoradiography. A representative autoradiogram of the quantified data presented in B is shown. (D) Total cell lysates were prepared from T-lymphocytes at the same time points as shown in A-C and blotted with an antiserum that recognises the dual phosphorylated form of activated JNK (WB:Phospho-JNK). The fast and slow migrating proteins visualized at 0.5 hours post-release correspond to the 46 and 55 kDa forms of JNK, respectively. Extracts from control and UV-stimulated 293 cells were obtained from New England BioLabs and used as markers for activated JNK isoforms. WB, western blot.

cycle progression and entry into S-phase following release from G_1/S-phase arrest.

JNK1 but not ERK1 or ERK2 is activated upon S-phase entry

To determine whether stimulation of the c-Jun NH_2-terminal kinase activity was specific for the JNK subfamily of MAPKs, T-lymphocytes were released from the G_1/S-phase block and the protein kinase activities of the immunoprecipitated JNK1, p38, ERK1 and ERK2 were determined (Fig. 3). An immune complex kinase assay of JNK1 activity indicated that it was maximally activated 30 minutes after release from the aphidicolin block (Fig. 3A) and followed that observed using GST-c-Jun as an affinity ligand to isolate c-Jun NH_2-terminal kinase activities (Figs 3A and 2C). p38 kinase is another class of mammalian MAP kinases that are activated by cellular stress (Han et al., 1994; Rouse et al., 1994; Raingeaud et al., 1995). Immune complex analysis of p38 kinase activity using GST-ATF2 as substrate indicated that p38 was activated weakly (~2-fold) at 30 minutes post-release (Fig. 3A). In other experiments, activation of the p38 kinase was either similarly low or not detectable.

The activity of ERK1 and ERK2 was measured by using an immune complex kinase assay and by immunoblot analysis of total cell extracts using an antiserum that recognizes the dual phosphorylated Thr-Glu-Tyr sequence present in activated ERK1 and ERK2. Data presented in Fig. 3B show that neither ERK1 nor ERK2 was activated following release of T-lymphocytes from the aphidicolin block. Activation of both ERK1 and ERK2 was, however, observed in T-lymphocytes treated with phorbol myristate acetate (PMA) (Fig. 3B). Therefore, these data indicate that there is a selective activation of the JNK subgroup of MAP kinases in T-lymphocytes upon S-phase entry.

p21^{WAF1} dissociates from JNK upon S-phase entry in T-lymphocytes and HeLa cells

The p21^{WAF1} protein has recently been shown to bind to and inhibit the activation of the JNKs (Shim et al., 1996), although whether native JNKs and p21^{WAF1} associate in vivo has not been established. Since p21^{WAF1} is known to be present in proliferating human peripheral T-lymphocytes (Nourse et al., 1994), we investigated whether JNK was associated with p21^{WAF1} protein in T-lymphocytes arrested in G_1/S-phase by aphidicolin and following release from this block. JNK1 was immunoprecipitated at intervals after release and then immunoblotted with a monoclonal antibody to p21^{WAF1}. JNK activity was also determined in the same experiment using GST-c-Jun as affinity ligand and substrate. Immunoblot analysis of the JNK1 immunoprecipitates indicated that p21^{WAF1} was associated with JNK1 in the aphidicolin-arrested T-lymphocytes (Fig. 4A). However, 30 minutes post-release, p21^{WAF1} association with JNK1 was no longer detectable (Fig. 4A) and this correlated with maximal JNK activity (Figs 3A and 4A). Reassociation of p21^{WAF1} and JNK1 was detectable at 4 hours post-release and increased at 6 hours, whereas JNK activity had returned to basal by 2 hours. Thus, the temporal loss of p21^{WAF1} association with JNK1 correlated with JNK activation but not with its inactivation (Fig. 4A). Importantly, in control cells, washed and returned to an aphidicolin-containing medium to retain the block, the level of p21^{WAF1} protein associated with JNK1 was constant ('Aphidicolin-blocked', Fig. 4A). Immunoblot analysis of JNK1 and p21^{WAF1} immunoprecipitates indicated that the level of each protein did not vary over this time course (Fig. 4A and Dulic et al., 1998),

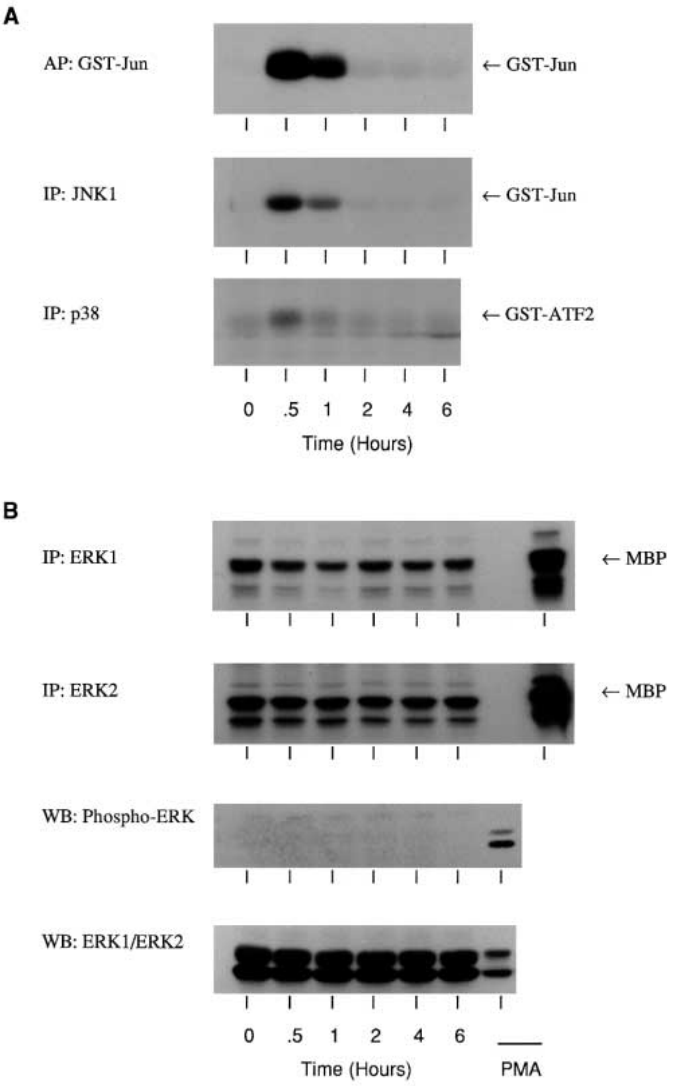

Fig. 3. Release of T-lymphocytes from an aphidicolin block is associated with activation of JNK but not ERK1 or ERK2. The activities of the JNK, p38, ERK1 and ERK2 MAP kinases were assayed after the release of T-lymphocytes from an aphidicolin block. (A) JNK activity was assayed using either GST-Jun as affinity ligand and substrate (AP: GST-Jun) or following immunoprecipitation and assay using GST-Jun as substrate (IP: JNK1). p38 immunoprecipitates were assayed using GST-ATF2 as substrate. (B) ERK1 and ERK2 were immunoprecipitated with the relevant antisera and their kinase activities assessed using myelin basic protein (MBP) as substrate (IP: ERK1; ERK2). As a positive control for ERK activation, T-lymphocytes were stimulated for 30 minutes with phorbol myristate acetate (PMA, 250 nM) in the presence of the Ca^{2+} ionophore A23187 (10 μM). Cell extracts were also immunoblotted with a phospho-specific ERK antiserum that recognises activated ERK1 and ERK2 (WB: Phospho-ERK). The same blot was reprobed with a mixture of two antisera to ERK1 and ERK2 to show the presence of these 44 kDa and 42 kDa proteins, respectively (WB: ERK1/ERK2). AP, affinity purification; IP, immunoprecipitation. WB, western blot.

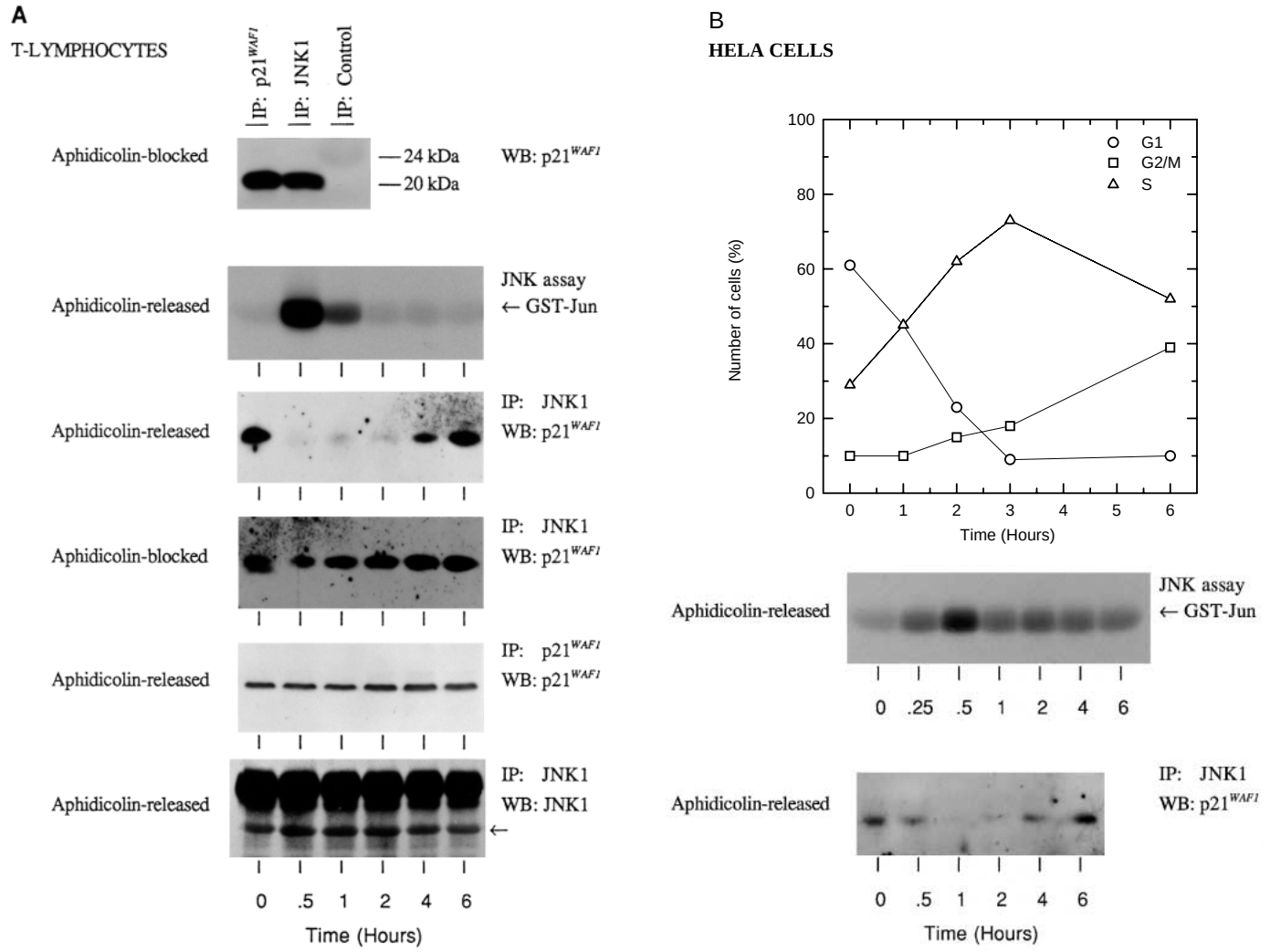

Fig. 4. Activation of JNK following release from an aphidicolin block correlates with dissociation of p21^{WAF1} from JNK1 in T-lymphocytes (A) and HeLa cells (B). (A) p21^{WAF1} is bound to JNK1 in aphidicolin-blocked T-lymphocytes. JNK1 was immunoprecipitated from aphidicolin-blocked T-lymphocytes and western blotted with an anti-p21^{WAF1} antibody. Positive and negative controls involved immunoprecipitation with either anti-p21^{WAF1} antibody or with Protein A-Sepharose beads only, respectively. Molecular mass standards are indicated in kDa. JNK activity and the level of p21^{WAF1} protein associated with JNK1 were measured in parallel following the release of T-lymphocytes from an aphidicolin block. JNK activity was assayed using GST-Jun as affinity ligand and substrate. The amount of p21^{WAF1} protein associated with JNK1 was determined by western blotting of JNK1 immunoprecipitates with an anti-p21^{WAF1} antibody in both aphidicolin-released and aphidicolin-blocked T-lymphocytes. The levels of p21^{WAF1} and JNK1 protein do not vary in T-lymphocytes following their release from an aphidicolin block. p21^{WAF1} was immunoprecipitated and western blotted with an anti-p21^{WAF1} antibody. Similarly, JNK1 was immunoprecipitated and blotted with an anti-JNK1 antiserum. The small arrow indicates the position of the 46 kDa JNK species. (B) Release of HeLa cells from an aphidicolin block results in the stimulation of DNA replication, JNK activation, and dissociation of p21^{WAF1} from JNK1. HeLa cells were synchronised at the G_1-S-phase boundary by an aphidicolin block. Samples were taken at the indicated times following release from the aphidicolin block for cell cycle analysis, for the assay of JNK activity and for JNK1 immunoprecipitation. DNA replication in the cell samples was monitored by flow cytometry after staining the DNA with propidium iodide. JNK activity was assayed using GST-Jun as affinity ligand and substrate. The JNK1 immunoprecipitates were western blotted with an anti-p21^{WAF1} antibody to determine the level of p21^{WAF1} protein associated with JNK1.

confirming that the JNK activity measurements were not complicated by such factors. These data therefore support a functional role for p21^{WAF1} and JNK1 association in vivo and are consistent with the notion that this interaction may contribute to the regulation of JNK1 during progression of T-lymphocytes through the cell cycle.

To determine whether JNK activation may constitute a general signal for cell cycle progression, we studied JNK activation in HeLa cells following the release of cells from G_1/S-phase arrest. Fig. 4B shows that JNK was also maximally activated 30 minutes after release from the aphidicolin block, although the magnitude of the response was considerably less than that observed in T-lymphocytes (2.0±0.15-fold, mean ± s.e.m., n=6). JNK activation was not seen in control cells washed and maintained in the presence of aphidicolin (not shown). Flow cytometry indicated that 45% of the cells were

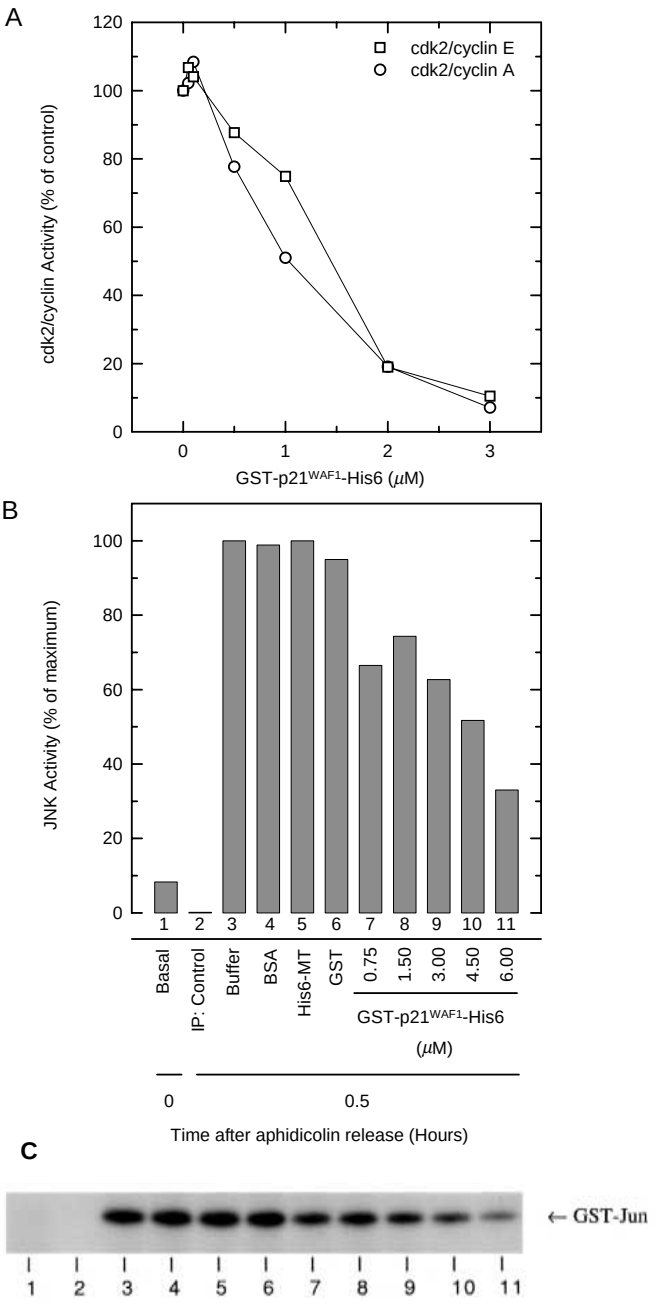

Fig. 5. Recombinant p21^{WAF1} inhibits cdk2/cyclins and JNK in vitro. (A) The effect of His6-p21^{WAF1}-GST on cdk2/cyclin A and cdk2/cyclin E activity was assayed in vitro as described in Materials and Methods. Data shown represent one of three independent experiments. (B) The effect of His6-p21^{WAF1}-GST on JNK1 activity. JNK1 was immunoprecipitated either from aphidicolin-blocked T-lymphocytes (Basal, lane 1) or 0.5 h after release from the aphidicolin block (lanes 3-11). IP:Control (lane 2) represents a control assay in which Protein A-Sepharose was used under standard immunoprecipitation conditions but in the absence of JNK1 antiserum. Activated JNK1 was assayed in the presence of either buffer alone (lane 3), BSA (9 μM, lane 4), His6-O^6-methylguanine-DNA methyltransferase (His6-MT, 30 μM, lane 5), free glutathione-S-transferase (GST, 22 μM, lane 6) or increasing concentrations of His6-p21^{WAF1}-GST (0.75-6 μM, lanes 7-11). Each bar represents the mean of two independent determinations. (C) A representative autoradiogram showing the JNK activity data quantified in (B).

in S-phase 60 minutes after release from the aphidicolin block (Fig. 4B). Immunoblot analysis revealed that p21^{WAF1} was associated with JNK1 in the aphidicolin-blocked HeLa cells (Fig. 4B) and that the partial loss of this association (30 minutes post-release) correlated with JNK activation. Therefore, these data are consistent with JNK activation providing a positive signal for entry into S-phase.

p21^{WAF1} inhibits JNK activity in vitro

We next analysed whether p21^{WAF1} was able to inhibit the activity of JNK1, as has been reported in recombinant systems (Shim et al., 1996). p21^{WAF1} was purified from bacteria as a recombinant epitope-tagged protein (GST-p21^{WAF1}-His6) and found to be functionally active as a cyclin-dependent kinase (CDK) inhibitor by immune complex kinase analysis (Fig. 5A). p21^{WAF1} inhibited the activity of cdk2/cyclin A and cdk2/cyclin E protein kinases in vitro with a half maximal effect observed at approximately 1 μM and 1.5 μM p21^{WAF1}, respectively (Fig. 5A). Having confirmed its effectiveness as a CDK inhibitor, this preparation of p21^{WAF1} was used to determine its effect on JNK1 activity in vitro. Activated JNK1 was isolated by immunoprecipitation from lysates of T-lymphocytes prepared at 30 minutes following release from the aphidicolin block. Recombinant p21^{WAF1} inhibited JNK1 activity in vitro in a dose-dependent manner with the highest concentration of p21^{WAF1} tested (6 μM) giving approximately 70% inhibition of the maximal JNK1 activity (Fig. 5B and C). This inhibition was specific for the p21^{WAF1} protein since BSA (6 μM), free GST (22 μM) and a control protein containing a hexahistidine tag (His6-O^6-methylguanine-DNA methyltransferase (His6-MT), 30 μM; Moore et al., 1994) were all without effect (Fig. 5B and C). Thus activated JNK1 from human T-lymphocytes can be inhibited by recombinant p21^{WAF1} in vitro, supporting the findings of Shim et al. (1996).

DISCUSSION

The current study provides evidence for the involvement of JNK1 in both cell cycle arrest and cell cycle progression (Woodget et al., 1996; Kyriakis and Avruch, 1996; Yamamoto et al., 1993; Olson et al., 1995). In the case of induction of G_1/S-phase arrest, inhibition of DNA synthesis preceded JNK activation, indicating that inhibition of DNA replication may signal JNK activation. Previous reports have shown that, in T-lymphocytes, JNK is activated by γ-irradiation, UV light, Fas ligand and DNA-damaging drugs (Yu et al., 1996; Chen et al., 1996). It is known that cells can respond to DNA damage induced by UV- or γ-irradiation by arresting in either G_1- or G_2-phase of the cell cycle (Elledge, 1996) or by undergoing apoptosis (Chen et al., 1996; Verheij 1996). As our data indicate that inhibition of DNA replication does not result in apoptosis, JNK activation in response to inhibition of DNA replication may act primarily to signal cell cycle arrest. Although the mechanism by which JNK mediates cell cycle arrest is unknown, it may involve transcriptional regulation of one or more factors such as c-Jun (Xia et al., 1995).

JNK is also activated in response to mitogenic stimuli and may be required for cell cycle progression through G_1/S (Yamamoto et al., 1993; Olson et al., 1995; Mitsui et al., 1997). JNK is implicated in mediating the positive effects of the

monomeric G-proteins Rac and Cdc42 on cell cycle progression and DNA synthesis in Swiss 3T3 cells (Yamamoto et al., 1993; Olson et al., 1995), and on v-Src- and α_{12}-induced transformation of NIH3T3 cells (Minden et al., 1995; Collins et al., 1996). Similarly, transformation induced by the human leukemia oncogene Bcr-Abl appears to be mediated by activation of JNK (Raitano et al., 1995; Dickens et al., 1997). Furthermore, Ras- and Rac-dependent activation of JNK has recently been shown to be a necessary pathway for α_{12}-induced G_1/S-phase gene expression and entry into S-phase (Mitsui et al., 1997). In accord with these findings, the present study shows that JNK activation preceded the onset of DNA replication following the release of T-lymphocytes from G_1/S-phase arrest. Although correlative, this observation is consistent with the notion that JNK activation provides a positive signal for the onset of DNA synthesis. Taken together, therefore, these observations suggest that JNK may be involved in regulating S-phase progression. The apparent paradox of how JNK activation can be involved in both cell cycle arrest and cell cycle progression remains to be resolved. Our data suggest the possibility that the temporal regulation of JNK activation during the cell cycle is a factor in determining either cell cycle arrest or progression. An alternative explanation would involve differential translocation of JNK and/or p21^{WAF1} to distinct subcellular locations in response to differing stimuli, thereby allowing their interaction with selective targets.

The recent identification of cytoplasmic inhibitors of JNK that appear to act selectively on this subfamily of MAPKs provides an additional level of complexity to JNK regulation. Recent data from in vitro and co-transfection experiments suggest that p21^{WAF1}, a DNA damage-inducible inhibitor of the cell cycle, is capable of acting as an inhibitor of the JNK subgroup of MAPKs (Shim et al., 1996). In the present study we have confirmed that recombinant p21^{WAF1} can inhibit the activity of JNK1 in vitro using T-lymphocytes as a source of activated JNK1, although p21^{WAF1} appears to act as a more potent inhibitor of cyclin-dependent kinase-2 (CDK2). Shim et al. (1996) report a similar potency of effect of p21^{WAF1} for inhibition of CDK2 and recombinant JNK3. Another cytoplasmic inhibitor of the JNK subfamily of MAP kinases has been identified using a yeast two-hybrid screen to isolate binding partners for JNK1. This protein, designated JNK-interacting protein-1 (JIP-1), is reported to be a specific inhibitor of JNK, and may act by retaining JNK in the cytoplasm (Dickens et al., 1997). In addition, heat shock protein 72 (hsp72), a member of the hsp70 family, has been reported to inhibit activation of both the JNK and p38 signal transduction pathways, although its mechanism of action remains unclear (Gabai et al., 1997).

The present study shows that JNK1 exists as a complex with p21^{WAF1} in human T-lymphocytes and that this interaction is cell cycle-dependent. Activation of JNK at S-phase entry is associated with increased phosphorylation on threonine and tyrosine within the motif Thr-Pro-Tyr of the 46 kDa and 55 kDa forms of JNK but also correlates with dissociation of p21^{WAF1} from JNK1. Our data show the existence of a dynamic association between p21^{WAF1} and JNK1 in vivo, an observation that has not previously been reported, and suggest that relief of an inhibitory constraint mediated by p21^{WAF1} may contribute to JNK activation at S-phase entry. The data do not, however, support a role for p21^{WAF1} in JNK inhibition at later stages of S-phase where dephosphorylation rather than p21^{WAF1} reassociation appears to be the principal mechanism involved in JNK inactivation. It is well established that p21^{WAF1} can inhibit both CDK activation and proliferating cell nuclear antigen (PCNA)-mediated DNA replication (Hunter and Pines, 1994). The association of p21^{WAF1} with JNK1 in cells arrested in G_1/S-phase will likely contribute to the observed low basal activity of the kinase prior to aphidicolin release. If JNK is causally involved in cell cycle progression as our own and previous data suggest (Yamamoto et al., 1993; Minden et al., 1995; Olson et al., 1995; Collins et al., 1996; Mitsui et al., 1997), then these observations are consistent with the idea that inhibition of JNK1 by p21^{WAF1} association forms part of the checkpoint mechanism by which p21^{WAF1} prevents entry into mitosis in the presence of unreplicated or damaged DNA. This is supported by the observation that tumour cells that are deficient in p21^{WAF1} fail to arrest entry into mitosis in response to DNA-damaging agents (Waldman et al., 1996; Jacks and Weinberg, 1996).

Although the immediate upstream regulators of the JNK signalling cascade have recently been characterized in detail (Kyriakis and Avruch, 1996; Woodget et al., 1996), the downstream target(s) through which the JNKs signal either cell cycle arrest or cell cycle progression remain to be determined. JNKs are believed to mediate their effects by regulating gene expression (Xia et al., 1995; Raitano et al., 1995); JNKs are known to phosphorylate and regulate the transcriptional activity of c-Jun, ATF-2, Elk-1 (Gupta et al., 1995; Derijard et al., 1994; Whitmarsh et al., 1995), although the tumour suppressor p53 may also be a substrate for JNK1 in vivo (Milne et al., 1995). The activities of the cyclin-dependent protein kinases (CDKs), cdk2/cyclin A and cdk2/cyclin E, are known to be regulated by phosphorylation and their activation is essential for initiating DNA replication (Heichman and Roberts, 1994). Based on the present findings it appears likely that the JNKs may be involved in a signalling pathway whose ultimate targets include these CDKs.

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REFERENCES

Canman, C. E., Tang, H. Y., Normolle, D. P., Lawrence, T. S. and Maybaum, J. (1992). Variations in patterns of DNA damage induced in human colorectal tumour cells by 5-fluorodeoxyuridine: Implications for mechanisms of resistance and cytotoxicity. *Proc. Nat. Acad. Sci. USA* **89**, 10474-10478.

Cantrell, D. A. and Smith, K. A. (1984). The interleukin-2 cell system: a new cell growth model. *Science* **224**, 1312-1316.

Chen, Y.-R., Wang, X., Templeton, D., Davis, R. J. and Tan, T-H. (1996). The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and radiation. *J. Biol. Chem.* **271**, 31929-31936.

Churcher, Y. and Moss, S. E. (1993). Modulation of protein tyrosine phosphorylation during G_1/S transition in activated human T-lymphoblasts. *J. Biol. Chem.* **268**, 26144-26149.

Collins, L. R., Minden, A., Karin, M. and Brown, J. H. (1996). Gα_{12} stimulates c-Jun NH_2-terminal kinase through the small G proteins Ras and Rac. *J. Biol. Chem.* **271**, 17349-17353.

Derijard, B., Hibi, M., Wu, I.-H., Barett, T., Su, B., Deng, T., Karin, M. and Davis, R. J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76**, 1025-1037.

Dickens, M., Rogers, J. S., Cavanagh, J., Raitano, A., Xia, Z., Halpern, J. R., Greenberg, M. E., Sawyers, C. L. and Davis, R. J. (1997). A cytoplasmic inhibitor of the JNK signal transduction pathway. *Science* **277**, 693-696.

Dulic, V., Stein, G. H., Far, D. F. and Reed, S. I. (1998). Nuclear accummulation of p21^{cip1} at the onset of mitosis: a role at the G_2/M-phase transition. *Mol. Cell. Biol.* **18**, 546-557.

Edgecombe, M., Patel, R. and Whitaker, M. (1991). A cyclin-abundance cycle-independent p34^{cdc2} tyrosine phosphorylation cycle in early sea urchin embryos. *EMBO J.* **10**, 3769-3775.

Elledge, S. J. (1996). Cell cycle checkpoints: preventing an identity crisis. *Science* **274**, 1664-1672.

Gabai, V. L., Meriin, A. B., Mosser, D. D., Caron, A. W., Rits, S., Shifrin, V. I. and Sherman, M. Y. (1997). Hsp70 prevents activation of stress kinases. *J. Biol. Chem.* **272**, 18033-18037.

Gupta, S., Campbell, D., Derijard, B. and Davis, R. J. (1995). Transcription factor ATF2 regulation by the Jnk signal transduction pathway. *Science* **267**, 389-393.

Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B. and Davis, R. J. (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. *EMBO J.* **15**, 2760-2770.

Han, J., Lee, J. D., Bibbs, L. and Ulevitch, R. J. (1994). A MAP kinase targeted by endotoxins and hyperosmolarity in mammalian cells. *Science* **265**, 808-811.

Hartwell, L. and Weinert, T. (1989). Checkpoints: controls that ensure the order of cell cycle events. *Science* **246**, 629-634.

Heichman, K. A. and Roberts, J. M. (1994). Rules to replicate by. *Cell* 79, 557-562.

Hunter, T. and Pines, J. (1994). Cyclins and cancer II: cyclin D and CDK inhibitors come of age. *Cell* **79**, 573-582.

Jacks, T. and Weinberg, R. A. (1996). Cell-cycle control and its watchman. *Nature* **381**, 643-644.

Jackson, R. J. and Hunt, T. (1983). Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. *Meth. Enzymol.* **96**, 50-74.

Kizaki, H., Ohnishi, Y., Azuma, Y., Mizuno, Y. and Ohsaka, F. (1992). 1-β-D-arabinosylcytosine and 5-azacytidine induce intranucleosomal DNA fragmentation and cell death in thymocytes. *Immunopharmacology* **24**, 219-227.

Kyriakis, J. M. and Avruch, J. (1996). Sounding the alarm: Protein kinase cascades activated by stress and inflammation. *J. Biol. Chem.* **271**, 24313-24316.

Kyriakis, J. M. and Avruch, J. (1990). pp54 microtubule-associated protein-2 kinase: A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. *J. Biol. Chem.* **265**, 17355-17363.

Kyriakis, J. M., Brautigan, D. L., Ingebritsen, T. S. and Avruch, J. (1991). pp54 microtubule-associated protein-2 kinase requires both tyrosine and serine/threonine phosphorylation for activity. *J. Biol. Chem.* **266**, 10043-10046.

Lavin, M. F., Khanna, K. K., Beamish, H., Teale, B., Hobson, K. and Watters, D. (1994). Defects in radiation signal transduction in ataxia-telangiectasia. *Int. J. Rad. Biol.* **66**, S151-S156.

Martin, S. J., Reutelingsperger, C. P. M., McGahon, A. J., Rader, J. A., Van Schie, R. C. A. A., LaFace, D. M. and Green, D. R. (1994). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* **182**, 1545-1556.

Milne, D. M., Campbell, L. E., Campbell, D. G. and Meek, D. W. (1995). p53 is phosphorylated *in vitro* and *in vivo* by an ultraviolet radiation-induced protein kinase characteristic of the c-Jun kinase, JNK1. *J. Biol. Chem.* **270**, 5511-5518.

Minden, A., Lin, A., Claret, F-X., Abo, A. and Karin, M. (1995). Selective activation of the JNK signalling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* **81**, 1147-1157.

Mitsui, H., Takuwa, N., Kurokawa, K., Exton, J. H. and Takuwa, Y. (1997). Dependence of activated G_{12}-induced G1 to S phase cell cycle progression on both Ras/mitogen-activated protein kinase and Ras/Rac/Jun N-terminal kinase cascades in NIH3T3 fibroblasts *J. Biol. Chem.* **272**, 4904-4910.

Moore, M. H., Gulbis, J. M., Dodson, E. J., Demple, B. and Moody, P. C. E. (1994). Crystal structure of a suicidal DNA repair protein: the Ada O^6-methylguanine-DNA methyltransferase from *E. coli*. *EMBO J.* **13**, 1495-1501.

Mukhopadhyay, N. K., Price, D. J., Kyriakis, J. M., Pelech, S., Sanghera, J. and Avruch, J. (1992). An array of insulin-activated, proline-directed serine/threonine protein kinases phosphorylate the p70 S6 kinase. *J. Biol. Chem.* **267**, 3325-3335.

Murray, A. W. (1992). Creative blocks: cell-cycle checkpoints and feedback controls. *Nature* **359**, 599-604.

Nourse, J., Firpo, E., Flanagan, W. M., Coats, S., Polyak, K., Lee, M.-H., Massague, J., Crabtree, G. R. and Roberts, J. M. (1994). Interleukin-2-mediated elimination of the p27^{kip1} cyclin-dependent kinase inhibitor by rapamycin. *Nature* **372**, 570-573.

Olson, M. F., Ashworth, A. and Hall, A. (1995). An essential role for Rho, Rac and Cdc42 GTPases in cell cycle progression through G1. *Science* **269**, 1270-1272.

Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J. and Davis, R. J. (1995). Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.* **270**, 7420-7426.

Raitano, A. B., Halpern, J. R., Hambuch, T. M. and Sawyers, C. L. (1995). The *Bcr-Abl* leukemia oncogene activates Jun kinase and requires Jun for transformation. *Proc. Nat. Acad. Sci. USA* **92**, 11746-11750

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T. and Nebrada, A. (1994). A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78**, 1027-1037.

Sheaff, R., Ilsley, D. and Kutcha, R. (1991). Mechanism of DNA polymerase alpha inhibition by aphidicolin. *Biochemistry* **30**, 8590-8597.

Shim, J., Lee, H., Park, J., Kim, H. and Choi, E-J. (1996). A non-enzymatic p21 protein inhibitor of stress-activated protein kinases. *Nature* **381**, 804-807.

Tamemoto, H., Kadowaki, T., Tobe, K., Ueki, K., Izumi, T., Chatani, Y., Kohno, M., Kasuga, M., Yazaki, Y. and Akanuma, Y. (1992). Biphasic activation of two mitogen-activated protein kinases during the cell cycle in mammalian cells. *J. Biol. Chem.* **267**, 20293-20297.

Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J., Szabo, E., Zon, L. I., Kyriakis, J. M., Haimovitz-Friedman, A., Fuks, Z. and Kolesnick, R. N. (1996). Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature* **380**, 75-79.

Waldman, T., Lengauer, C., Kinzler, K. W. and Vogelstein, B. (1996). Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. *Nature* **381**, 713-716.

Whitmarsh, A. J., Shore, P., Sharrocks, A. D. and Davis, R. J. (1995). Integration of MAP kinase signal transduction pathways at the serum response element. *Science* **269**, 403-407.

Woodgett, J. R., Avruch, J. and Kyriakis, J. (1996). The stress activated protein kinase pathway. *Cancer Surveys* **27**, 127-138.

Yamamoto, M., Marui, N., Sakai, T., Morii, N., Kozaki, S., Ikai, K., Imamura, S. and Narumiya, S. (1993). ADP-ribosylation of the *rhoA* gene product by botulinum C3 exoenzyme causes Swiss 3T3 cells to accumulate in the G1 phase of the cell cycle. *J. Biol. Chem.* **268**, 21509-21512.

Yu, R., Shtil, A. A., Tan, T-H., Roninson, I. B. and Kong, A-N, T. (1996). Adriamycin activates c-Jun N-terminal kinase in human leukemia cells: A relevance to apoptosis. *Cancer Lett.* **107**, 73-81.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. and Greenberg, M. E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270**, 1326-1331.