Expression and phosphorylation of fibroblast-growth-factor-inducible kinase (Fnk) during cell-cycle progression

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Fnk is a member of the polo family of cell-cycle-regulated serine/threonine kinases. We report here that it is present in serum-starved quiescent cells and that mitogenic stimulation of quiescent cells with calf serum results in the modification of a significant fraction of the Fnk pool. This modification results in a slower migrating form when analysed by SDS/PAGE. The modification is transient and by 9 h after stimulation all of the Fnk is again present as the faster migrating form. We also show that the Fnk protein increases in abundance as cells progress from G_1 to mitosis and is post-translationally modified as cells enter and exit mitosis. The Fnk modification is again manifested as a slower migrating species by SDS/PAGE and is due to phosphorylation of the protein. The mitotic-specific phosphorylation of Fnk correlates with an increase in its kinase activity, and this activity is dramatically reduced by phosphatase treatment of mitotic Fnk immunoprecipitates. During the later stages of mitosis, Fnk is dephosphorylated such that, by the time the cells enter G_1, it is all present as the dephosphorylated form. These results suggest that Fnk has two functions, one during the entry of cells into the cell cycle and a second during mitosis of cycling cells.

INTRODUCTION

The molecular events that constitute cell-cycle progression including passage through start, DNA synthesis and repair and the equal partitioning of chromosomes to newly formed daughter cells are tightly controlled. Recently, a new family of cell-cycle-regulated, cyclin-independent kinases has been discovered, and initial evidence suggests that they play a vital role in the successful completion of the cell cycle. *polo*, the founding member of this new family, was initially identified as a gene required for successful mitosis in *Drosophila melanogaster*. Mutations within this gene were shown to result in aberrant spindle formation leading to abnormal mitosis in larval neuroblasts [1]. Polo has been shown to phosphorylate several proteins in *Drosophila* extracts, including β-tubulin, and binds to at least one microtubule-associated protein of 85 kDa [2]. Since the initial characterization of *polo*, homologues of this gene have been identified in a number of organisms including yeasts, *Xenopus* and mammals [3–8]. The *Saccharomyces cerevisiae* homologue, CDC5, was identified as a temperature-sensitive mutation ($cdc5^{ts}$) which resulted in cell-cycle arrest in mitosis at the restrictive temperature resulting in large budded cells with undivided nuclei [9]. Cdc5p also appears to be required for the process of adaptation after cell-cycle arrest caused by DNA damage [10]. The *Schizosaccharomyces pombe* homologue, PLO1, has been shown to be an essential gene required for both spindle formation and septation [5]. The polo homologue from *Xenopus laevis* was purified from mitotic egg extracts by virtue of its ability to associate with Xcdc25 *in vitro*, suggesting a critical function for initiation of mitosis in this organism [3]. This protein, called Plx1 for Polo-like kinase from *Xenopus*, was further shown to phosphorylate Xcdc25 *in vitro* on some of the residues that are phosphorylated *in vivo*. Each of the *polo* family members from yeast to man contains two highly conserved domains. Residing in the N-terminal half of the proteins is a serine/threonine-specific kinase domain. This region contains all 11 subdomains described by Hanks et al. [11] for serine/threonine kinases. The C-terminal half of the proteins contains what is now referred to as the polo box and defines the polo family of kinases. The polo box consists of 28 amino acids which are strictly conserved in all family members. Of the 28 conserved residues, 14 are identical in all polo kinases. To date, this motif has not been found in any other proteins and its function has yet to be determined.

Workers from our laboratory and others have described the cloning of a mammalian homologue of the *polo* gene known as PLK for Polo-like kinase [6,7,12–14]. The murine Plk mRNA is expressed predominantly in tissues with a high mitotic index [7,11,14]. In NIH-3T3 fibroblasts, the protein is differentially expressed as cells pass through the cell cycle with protein accumulating during S phase and peaking in abundance in late G_2 [15]. The kinase activity of Plk increases dramatically as cells cross the G_2/M border [15,16]. Immunofluorescence studies localize Plk to centrosomes during both interphase and mitosis. As mitosis proceeds, Plk moves from the centrosomes to the mitotic spindles and by anaphase is localized to the developing midzone. During the final stages of mitosis, Plk becomes concentrated at the post-mitotic bridge [17,18]. Microinjection of anti-Plk antibodies into asynchronous cultures of HeLa cells has been shown to impair the ability of these cells to complete mitosis. Fluorescent microscopy of these injected cells which had been arrested in mitosis revealed that duplicated centrosomes failed to separate, leading to monoastral spindle arrays [19]. Finally, Plk has been shown to interact with MKLP-1, a kinesin-like protein required for mitotic progression [15].

Three additional polo homologues have been isolated from mammalian cell cDNA libraries: SNK for *s*erum-*i*nducible *k*inase [6], FNK for *f*ibroblast growth factor-*i*nducible *k*inase [8], and PRK for *p*roliferation-*r*elated *k*inase [20]. Both SNK and FNK

Abbreviations used: PAP, potato acid phosphatase; PP2A, protein phosphatase 2A; DTT, dithiothreitol; DMEM, Dulbecco's modified Eagle's medium.

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have been shown to be immediate-early response genes, the mRNA expression of which is induced by the addition of growth factors to serum-deprived cells [6,8].

In this paper we show that the Fnk protein is present in serum starved cells and is transiently modified when these cells are stimulated to re-enter the cell cycle by the addition of serum. We also show that Fnk is expressed throughout the cell cycle and becomes phosphorylated as cells enter mitosis and then dephosphorylated as cells exit mitosis. This mitotic phosphorylation of Fnk correlates with an increase in kinase activity. Thus we provide evidence that Fnk may have a signal-transducing function in the release of cells from quiescence and may also function during mitosis.

MATERIALS AND METHODS

Materials

Anti-(cyclin B) antibody was purchased from Santa Cruz Biotechnology Inc., and anti-Flag antibody as well as its cognate peptide were purchased from Eastman Kodak. Peroxidase-labelled goat anti-rabbit IgG and peroxidase-labelled goat anti-mouse IgG were purchased from Kirkegaard and Perry Laboratories. NIH-3T3 and 293T cells were purchased from the American Type Culture Collection, and FT210 cells were a gift from M. Yamada. Protein A–Sepharose was from Pharmacia Biotech. Immobilon-P membrane was from Millipore. Aphidocolin, mimosine, nocodazole, and potato acid phosphatase (PAP) came from Sigma. Expand PCR kit was from Boehringer Mannheim. Protein phosphatase 2A (PP2A) was obtained from Upstate Biotechnology Inc. The pcDNA3 vector was purchased from Invitrogen. [γ-^{32}P]ATP came from NEN Research Products. The BCA* protein assay kit was from Pierce. The Renaissance Chemiluminescence kit used for enhanced chemiluminescence was from Dupont–NEN. All other reagents were purchased from Life Technologies.

Generation of anti-Fnk serum

The polyclonal antibody against murine Fnk used in these experiments (8045) was raised against the peptide sequence Pro-Ser-Ala-Pro-Pro-Ala-Gly-Pro-Gly-Pro-Pro-Ala-Asn-Ala-Ser located in the N-terminal region of the murine Fnk protein. This peptide was synthesized with an N-terminal cysteine, conjugated to keyhole limpet haemocyanin and injected into rabbits (Macromolecular Resources, Boulder, CO, U.S.A.). The crude serum from these rabbits was used in the Western-blot analyses at a dilution of 1:500.

DNA constructs

The plasmid DNA3-Flag was constructed by insertion of annealed primers corresponding to the Flag epitope into the *Hin*dIII site of pcDNA3. This destroyed the original *Hin*dIII site but recreated a *Hin*dIII site at the 3′ end of the Flag epitope followed by the multi-cloning site of pcDNA3. The primers were: (1) 5′-AGCTGCACCATGGACTACAAGGACGACGACGACA-3′ and (2) 5′-AGCTTGTCGTCGTCCTTGTAGTCCATGGTGC-3′. This resulted in a Kozak consensus sequence followed by the Flag epitope: Asp-Lys-Asp-Asp-Asp-Asp-Lys. The Flag–Fnk fusion used in these studies was constructed by amplification of the Fnk coding region by PCR using the primers: (1) 5′-GGAATTCCCATGGAGCCCGCCGCCGGC-3′ and (2) 5′-CCCGCGGCCGCTAAGCAGGGCTTTGGTCCCT-3′. The amplified product was digested with *Eco*RI and *Not*I and inserted into the *Eco*RI–*Not*I sites in DNA3-Flag to create the expression vector Flag–Fnk.

Cell culture, synchronization and transfection

NIH-3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. FT210 cells were grown in RPMI supplemented with 20% fetal bovine serum, and 293T cells were grown in DMEM supplemented with 10% fetal bovine serum. All media were supplemented with 2.0 mM L-glutamine and the antibiotics penicillin (50 units/ml) and streptomycin (50 μg/ml). The NIH-3T3 and 293T cells were cultured in a 37 °C incubator adjusted to 7% CO_2. The FT210 cells were cultured in a 7% CO_2 incubator adjusted to 32 °C.

FT210 cells were synchronized in late G_2 by overnight incubation at 39 °C. NIH-3T3 cells were arrested in late G_1 by overnight incubation in the presence of mimosine at a final concentration of 1.0 μM. They were blocked at the G_1/S boundary and in S phase by overnight incubation in aphidocolin at a final concentration of 0.25 ng/ml, and in prometaphase by overnight incubation in 100 ng/ml nocodazole. Mitotic NIH-3T3 cells were harvested by shake-off. In the nocodazole-release experiment the cells were treated with nocodazole for 8 h and then harvested by shake-off, washed three times in prewarmed DMEM and released into warm DMEM. The 293T cells were transfected with 10 μg of the Flag–Fnk construct using the calcium phosphate method, washed 14 h later and then re-fed. At 36 h after transfection, the medium was exchanged with either normal growth medium for asynchronous cultures or with normal medium supplemented with 20 ng/ml nocodazole for mitotic arrest. All transfectants were harvested 8 h later. Because 293T cells are loosely adherent, the mitotic extract represents a mixture of about 70% mitotic and 30% interphase cells.

For the serum-starvation experiments, we plated NIH-3T3 cells to 40% confluence on 10 cm dishes the night before starvation. Once the cells became adherent they were washed with prewarmed DMEM and then cultured for 48 h in DMEM containing 0.1% calf serum along with L-glutamine and antibiotics. After 48 h the cells were washed once in DMEM and then allowed to re-enter the cell cycle by the addition of DMEM containing 10% calf serum.

Cell lysis and Western-blot analysis

All adherent cultures were washed three times with cold PBS and lysed directly on the plates on ice with TNEN lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2.0 mM EDTA, 1.0% Nonidet P40, 1.0 μg/ml each aprotinin and leupeptin, 1.0 mM PMSF, 10.0 mM NaF and 1.0 mM Na_3VO_4). Non-adherent FT210 cells were collected by centrifugation at 500 ***g*** for 2 min at 4 °C and washed three times in cold PBS before lysis in TNEN buffer. Mitotic NIH-3T3 and 293T cells were collected by shake-off and washed three times in cold PBS followed by lysis in TNEN buffer. Lysis was performed for 10 min at 4 °C. Protein extracts were centrifuged at 18000 ***g*** at 4 °C for 15 min to clear debris. Protein concentration was determined using the BCA* protein assay kit, and 30 μg of protein was subjected to electrophoresis on SDS/7.0% polyacrylamide gels (100 μg of protein was used for FT210 cells). Proteins were transferred to an Immobilon-P membrane and membranes were blocked in TBST buffer (20 mM Tris/HCl, pH 8.2, 150 mM NaCl, 0.05% Tween 20) containing 5% (w/v) dried milk. Anti-Fnk antibody was added at a dilution of 1:500. Anti-(cyclin B) antibody was used at a 1:500 dilution. Anti-Flag antibody was used at a 1:1000 dilution. Peroxidase-labelled goat anti-rabbit IgG was used as the secondary antibody for anti-Fnk and was added at a dilution of 1:8000. Peroxidase-labelled goat anti-mouse IgG was used at 1:8000 dilution as the secondary antibody for the anti-Flag and

anti-(cyclin B) antibodies. All secondary antibodies were detected by enhanced chemiluminescence.

Competition for Western-blot analysis using the anti-Fnk antibody was performed in 5 ml with 10 μg of cognate peptide. Competition of the Flag immunoprecipitations was performed in 600 μl with 10 μg of Flag peptide (Eastman Kodak). Each was added in conjunction with the respective antibody. For immunoprecipitations we used 3 μl of anti-Flag antibody, 60 μl of a 50 % slurry of Protein A–Sepharose in TNEN buffer and 200 μg of protein extract and incubated overnight in a final volume of 600 μl at 4 °C.

Kinase assays

Kinase assays were performed on 293T transfectants which were lysed in TNEN buffer as described previously. The Flag–Fnk fusion protein was immunoprecipitated from the lysates as described above. After three washes in TNEN buffer the immunoprecipitates were washed twice in kinase buffer [30 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 7.0 mM $MnCl_2$, 1.0 mM dithiothreitol (DTT)] and resuspended in 37.5 μl of kinase buffer supplemented with 25 μCi of [γ-^{32}P]ATP and 1.0 μg of dephosphorylated casein. The reaction mixtures were incubated at room temperature for 10 min and stopped by the addition of sample buffer and then boiled for 5 min. The samples were then loaded on to 9 % gels, transferred to Immobilon-P membranes and exposed to film.

Phosphatase assay

For protein phosphatase 2A (PP2A) reactions of cell lysates, cells were lysed in a modified PP2A buffer (40 mM Hepes, pH 7.4, 40 mM NaCl, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 1.0 mM DTT, 0.1 mg/ml BSA, 1 % Nonidet P40, 1.0 μg/ml aprotinin, 1.0 mM PMSF) for 10 min and centrifuged at 18000 ***g*** at 4 °C for 12 min. The resulting supernatant was removed, assayed for protein concentration, and 30 μg of protein was used for phosphatase reactions. PP2A (0.5 unit) was added and incubated at room temperature for 45 min. For inhibition of PP2A activity, okadaic acid was added to a final concentration of 10 nM. PAP treatment of immunoprecipitated Flag–Fnk was performed with 0.5 unit of PAP in 100 mM Mes, pH 6.0, for 60 min at 37 °C. These cells were lysed in TNEN buffer. The immunoprecipitations were performed overnight at 4 °C and the immunoprecipitates washed twice with TNEN buffer followed by three washes in 100 mM Mes buffer. All samples were separated by electrophoresis on either 7.5 % or 9.0 % acrylamide gels, transferred to Immobilon-P, and probed with either anti-Fnk or anti-Flag antibody.

RESULTS

Characterization of anti-Fnk antibody

To begin biochemical analyses of the Fnk protein, we synthesized a 21-amino acid peptide corresponding to a region near the N-terminus of the predicted protein sequence for murine Fnk (amino acids 19–33). This peptide was conjugated to keyhole limpet haemocyanin and injected into rabbits. To test the specificity of the resulting antiserum, we performed Western-blot analysis using lysates from asynchronous cultures of NIH-3T3 cells. As shown in Figure 1(A), the anti-Fnk serum was capable of recognizing a protein with the expected molecular mass for Fnk of approx. 75 kDa. This protein was specifically competed for when the antiserum was exposed to excess cognate peptide during Western-blot analysis. To confirm further that this serum specifically recognized the Fnk protein, we created a Flag-epitope-tagged version of the protein and expressed this fusion protein transiently in transfected 293T cells. The entire Fnk coding region was subcloned into the pcDNA3 vector which had been modified to include the Flag epitope 5′ to the multicloning site (see the Materials and methods section). The fusion protein was immunoprecipitated from the lysates of these transfectants with a monoclonal antibody to the Flag epitope in the presence or absence of excess Flag peptide (Figure 1B). The Fnk antiserum recognized the fusion protein immunoprecipitated by the Flag antibody. A protein of identical size was recognized when the lysates were probed with anti-Flag antibody, and this signal was not present when excess Flag peptide was included in the immunoprecipitation. This protein was not present in untransfected cell lysates, which confirmed the identity of the reactive protein as Fnk.

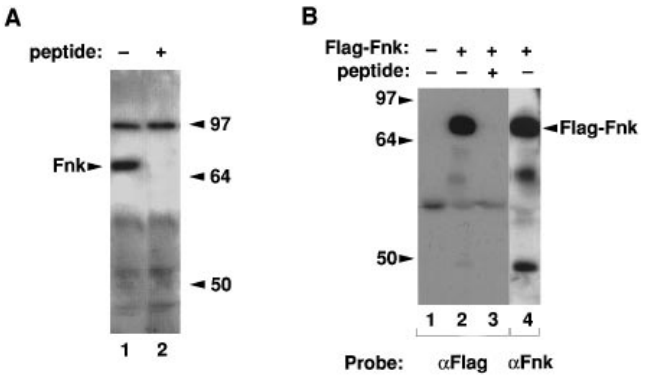

Figure 1 Characterization of the anti-Fnk antibody

(**A**) An NIH-3T3 cell lysate was prepared and 30 μg of protein was separated by SDS/PAGE, transferred to Immobilon-P and probed with anti-Fnk antibody in the absence (lane 1) or presence (lane 2) of excess cognate peptide. (**B**) 293T cells transfected with the Flag–Fnk expression vector were lysed and 200 μg of protein was used for immunoprecipitation with the Flag antibody in the absence (lanes 1, 2 and 4) or presence (lane 3) of excess Flag peptide. Lane 1 is an immunoprecipitate from 293T cells that were not transfected. Lanes 1–3 were immunoblotted with the anti-Flag antibody, and lane 4 was probed with anti-Fnk antibody. Molecular-mass standards are indicated (in kDa) on the right of (**A**) and the left of (**B**).

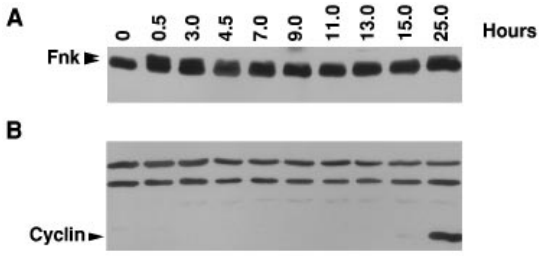

Figure 2 Modification of Fnk as cells exit G_0

Lysates were prepared from NIH-3T3 cells that had been arrested in G_0 by starvation and then stimulated to re-enter the cell cycle by the addition of 10 % calf serum for the indicated times. A 30 μg portion of protein extract was separated by SDS/PAGE. (**A**) Immunoblot with anti-Fnk serum; (**B**) same membrane as in (**A**) reprobed with anti-(cyclin B) antibody.

Modification of Fnk on serum stimulation of quiescent cells

We examined Fnk protein expression in NIH-3T3 cells that were first arrested in G_0 by starvation in 0.1 % calf serum and then stimulated to re-enter the cell cycle by the addition of 10 % calf serum. Fnk protein was detectable in serum-starved and serum-stimulated cells (Figure 2A). After 30 min of serum stimulation, a form of Fnk with reduced mobility was detected, and this

modified form could be detected for 9 h after stimulation (Figure 2A). The stimulated cells were allowed to continue through the first cycle of division, and no further modification of Fnk was seen until 25 h after stimulation when the slower migrating form of Fnk reappeared. By this time synchrony of the population had been lost, but this later modification was almost certainly due to cells that had entered mitosis (where we have found the protein to be modified; see below). To confirm that a significant proportion of the cells had entered M phase by 25 h after stimulation, we reprobed the membrane with an antibody to cyclin B. Previous studies have shown that cyclin B expression is cell-cycle-regulated and peaks during mitosis [21]. No expression of cyclin B was detected during the time course of the experiment until 25 h after stimulation which correlated with the later modification of Fnk (Figure 2B).

Expression of Fnk throughout the cell cycle

To examine Fnk protein expression as cells progressed through the cell cycle, we blocked NIH-3T3 cells in various stages of the cell cycle as described in the Materials and methods section. Protein extracts from these cells as well as from asynchronous cultures were separated by SDS/PAGE and subjected to Western-blot analysis using the anti-Fnk serum. The Fnk protein was expressed at every stage of the cell cycle (Figure 3). The abundance of the protein increased as cells entered S phase and reached peak levels during mitosis. Further, the mobility of Fnk in mitotic extracts was reduced relative to G_1 and S phase extracts, suggesting that Fnk was modified during mitosis.

Modification of Fnk as cells enter and exit mitosis

To establish more precisely when in the cell cycle Fnk modification occurs, we employed FT210 cells, a mouse mammary tumour cell line carrying a temperature-sensitive mutation in the CDC2 gene ($cdc2^{ts}$). When these cells are grown at the restrictive temperature of 39 °C the Cdc2 protein is inactivated and at least partially degraded and the cells arrest at the G_2/M border [22]. Incubating these cells at the permissive temperature of 32 °C allows the cells to enter mitosis once Cdc2 protein has accumulated to sufficient levels [22]. As shown in Figure 4(A), the Fnk protein in FT210 cells grown at 39 °C migrated as a single band and co-migrated with the Fnk detected in asynchronous cultures. When these cells were shifted to the permissive temperature in medium containing nocodazole, we began to see the appearance of the modified form of Fnk. Nocodazole was added to these cells when they were shifted to the permissive temperature to ensure that we were studying only a G_2 to M phase event. During the time course of the experiment more of the Fnk protein became modified, suggesting that release from the G_2 block was slow, probably a result of the gradual accumulation of the Cdc2 protein. When this membrane was reprobed with antibody to the Cdc2 protein we observed that the increase in Fnk modification correlated with an increase in the expression of Cdc2 (results not shown).

This slower migrating form of Fnk is not seen in G_1-arrested cells and we therefore wanted to determine what happened to the modified form of Fnk that we saw in mitotic cells. NIH-3T3 cells arrested in mitosis with nocodazole were collected by mitotic shake-off, washed to remove the nocodazole and then released into fresh medium. Lysates were prepared at various times after release from the mitotic block and analysed by Western blot using the anti-Fnk antibody (Figure 4B). Within 1 h of release from mitotic arrest the faster migrating form of Fnk can be seen below the slower migrating band, and within 3 h of release the slower migrating form of Fnk was barely detectable as the faster migrating form grew in abundance. FACS analysis of these cells showed that nearly all the cells had completed mitosis and entered G_1 3 h after release (results not shown). Thus the decrease in abundance of the slower migrating form of Fnk and the concomitant increase in the faster unmodified form correlate with the exit of the cells from mitosis.

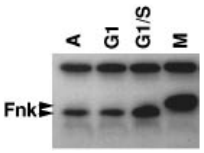

Figure 3 Expression of Fnk throughout the cell cycle

NIH-3T3 cells were arrested at various stages of the cell cycle as described in the Materials and methods section. Protein extract (30 μg) was separated by SDS/PAGE and immunoblotted with the anti-Fnk antibody. A, Asynchronous cells; G_1, cells blocked with mimosine; G_1/S, cells blocked with aphidocolin; M, mitotic cells blocked with nocodazole.

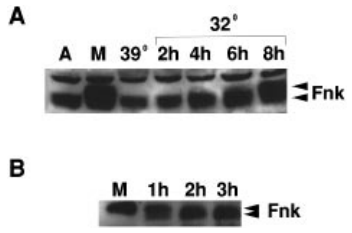

Figure 4 Modification of Fnk on entry into and exit from mitosis

(**A**) FT210 cells carrying a temperature-sensitive CDC2 mutation ($cdc2^{ts}$) were blocked in late G_2 by incubation at the restrictive temperature of 39 °C and then released into nocodazole-containing medium which was prewarmed to the permissive temperature of 32 °C. Cells were harvested at the G_2 block and at various times after release, and 100 μg of protein extract was separated by SDS/PAGE followed by immunoblotting with the anti-Fnk antibody. A, Asynchronous FT210 cultures grown at 32 °C. M, FT210 cells grown at 32 °C and blocked overnight with nocodazole to arrest the cells in prometaphase. (**B**) NIH-3T3 cells were blocked for 8 h in growth medium containing nocodazole to arrest the cells in prometaphase. The cells were collected by shake-off and washed three times in warm DMEM before release into drug-free DMEM. The released cells were collected at various times and 30 μg of each protein sample was separated by SDS/PAGE and immunoblotted with the anti-Fnk antibody. M, nocodazole-arrested mitotic cell lysates.

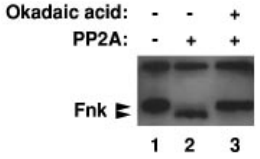

Figure 5 Phosphorylation of Fnk during mitosis

Mitotic NIH-3T3 cells were lysed in modified PP2A lysis buffer. Protein (30 μg) was either left untreated (lane 1) or incubated with PP2A (lane 2) or PP2A and 10 nM okadaic acid (lane 3) and then separated by SDS/PAGE followed by immunoblotting with anti-Fnk antibody.

Phosphorylation of Fnk during mitosis

To determine the cause of the modification observed in mitotic extracts, lysates from nocodazole-arrested cells were treated with the serine/threonine-specific PP2A. This resulted in the complete conversion of the slower migrating form of Fnk into the faster migrating species (Figure 5). This dephosphorylation of Fnk was

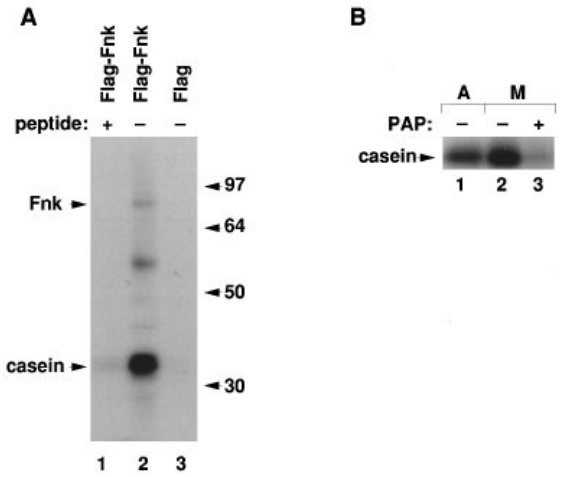

Figure 6 Demonstration that Fnk is active as a kinase and its activity is regulated by phosphorylation

(**A**) Flag–Fnk was immunoprecipitated from transfected 293T cells with the Flag antibody and utilized in an *in vitro* kinase reaction with casein as exogenous substrate. The immunoprecipitation was carried out in the presence (lane 1) or absence (lanes 2 and 3) of excess Flag peptide. Lane 3 represents the immunoprecipitation and *in vitro* kinase reaction from cells transfected with the empty Flag vector. Molecular-mass standards are indicated (in kDa) on the right of the gel. (**B**) 293T transfectants expressing the Flag–Fnk protein were immunoprecipitated with the Flag antibody and were left untreated (lanes 1 and 2) or were treated with PAP (lane 3) and then washed to remove the phosphatase. After the PAP treatment the precipitate was used in an *in vitro* kinase reaction with casein as the exogenous substrate. A, Lysates from asynchronous cultures; M, lysates from nocodazole-arrested mitotic cultures.

also seen in mitotic lysates treated with PAP (results not shown). There was no such conversion when the PP2A-specific inhibitor okadaic acid was included in the phosphatase reaction, which demonstrated that the modification of Fnk in mitotic lysates was due to phosphorylation of the protein.

Correlation of Fnk phosphorylation during mitosis with an increase in kinase activity

Although the anti-Fnk antibody specifically recognized the Fnk protein in a Western-blot analysis, it was not capable of immunoprecipitation. Therefore, to study the kinase activity of Fnk, we used the Flag–Fnk construct and expressed this fusion protein in transiently transfected 293T cells. Transfection of this construct into 293 cells resulted in the overexpression of a Flag–Fnk fusion protein as shown earlier in Figure 1(B).

Immunoprecipitation of this fusion protein with anti-Flag antibodies from transfected cells followed by an *in vitro* kinase assay revealed that Fnk was indeed a kinase capable of phosphorylating casein (Figure 6A). When this immunoprecipitation was carried out in the presence of excess Flag peptide, or from cells that had been transfected with the Flag vector alone, we saw a complete loss of *in vitro* kinase activity, which demonstrated that the kinase activity seen was due to the Flag–Fnk fusion protein.

To determine whether the phosphorylation of Fnk in mitosis correlated with an increase in kinase activity, we transfected the Flag–Fnk fusion construct into 293T cells and blocked the cells with nocodazole 36 h after transfection. A comparison of Fnk activity in these cells with that in transfectants that had not been arrested showed that mitotic Fnk was significantly more active than that found in asynchronous cultures (Figure 6B). Treatment of the immunoprecipitates from mitotic transfectants with PAP drastically reduced the kinase activity of the fusion protein, which demonstrated that phosphorylation of Fnk is required for the increased kinase activity.

DISCUSSION

Fnk belongs to the polo family of proteins which have been shown to be essential for cell survival and the successful completion of the cell cycle. Recent reports also suggest that at least one family member, Plk, may be implicated in the origin or progression of some human tumours [23,24]. Lower eukaryotes appear to contain only a single polo family member and evidence suggests that it may function in several cell-cycle processes including the initiation of DNA replication [25], centrosome maturation [1] and septation [5]. In contrast, mammals contain at least three polo family members (Plk, Fnk and Snk) which probably have distinct, although possibly overlapping, functions. Lee et al. [26] have shown that Plk can complement the mitotic defects associated with a temperature-sensitive mutation in *S. cerevisiae* CDC5, suggesting that Plk may be the *bona fide* mammalian *polo*/CDC5 homologue; however, more recent work has indicated that human Prk/Fnk can also complement the $cdc5^{ts}$ mutation [27]. Thus it may be that any of the related mammalian family members will prove capable of complementing mutants in lower eukaryotes, thereby complicating assessment of the physiological functions of the mammalian genes. Although Li et al. [20] have proposed that the kinase Prk may be a fourth family member, comparison of the amino acid sequence of human Prk with that of murine Fnk shows that the two proteins are 99.5% identical except for a 17-amino acid insertion in Prk between the kinase domain and the polo box. Careful alignment of the 5′ ends of the human Prk and murine Fnk nucleotide sequences demonstrates that the similarity of the two clones extends beyond what Li et al. [20] propose to be the initiating methionine. Of the 37 nucleotides 5′ to the putative translation start site of the Prk cDNA, 24 are identical with the murine Fnk 5′ coding sequence. Consideration of the deduced amino acid sequence of this putative non-coding region indicates that five of the 12 potential amino acids would be identical with those found in the murine protein sequence. Since there is no in-frame stop codon in the 5′ region of the PRK clone, nor is there a Kozak consensus sequence surrounding the putative initiation codon, there would appear to be no particular reason for the assumption that the entire open reading frame was present in their cDNA clone. Indeed, we have obtained a human polo-like cDNA clone that is distinct from PLK and SNK and is essentially identical with the reported PRK sequence except that our clone extends 41 nucleotides further into the 5′ region and continues to show extensive identity with the murine FNK sequence at both the nucleotide and amino acid levels (results not shown). Therefore we suggest that PRK represents a truncated clone of the human homologue of FNK and is not a unique polo kinase family member.

We have shown that Fnk is modified as quiescent cells are stimulated to re-enter the cell cycle. The appearance of this modified form of Fnk is coincident with the induction of FNK transcription on growth factor stimulation, and suggests a function for Fnk in cell-cycle entry. Because FNK is an immediate-early gene [8] and the protein becomes modified on mitogenic stimulation, we suspect that Fnk plays an early role in cell-cycle re-entry. Injection of Plk mRNA into quiescent NIH-3T3 cells has been shown to drive quiescent cells into S phase [14]. Since Plk is not normally expressed in quiescent cells and its kinase activity in G_1 is negligible [14,18], we suspect that its

dysregulated expression in these injection experiments may mimic the normal activity of Fnk on mitogenic stimulation. If the polo relative of Fnk, Plx1, does in fact interact with Xcdc25c *in vivo*, as suggested by Kumagai and Dunphy [3], it is plausible that a target for Fnk in this early function may be the Xcdc25c relative, Cdc25a, which is thought to be required for the initiation of the S phase [28]. Another intriguing possibility is that modification of Fnk affects the activity of one or more of the cdk inhibitor proteins. The timing of phosphorylation of Fnk in G_0/G_1 is more in line with this possibility. p27Kip would be one potential target, as this cell-cycle inhibitor is most responsive to the presence of growth factors [29] and its activity is regulated at least in part by phosphorylation [30].

Fnk protein abundance fluctuates throughout the cell cycle and is lowest in G_1 cells and reaches maximal abundance during mitosis. In cycling cells Fnk becomes phosphorylated as cells cross the G_2/M border and dephosphorylation occurs in late M phase. Our results indicate that the mitotic phosphorylation and thus perhaps the *in vivo* function of Fnk occurs subsequent to Cdc2 activation, which suggests that Fnk is not required for the onset of mitosis but rather for some later function. The phosphorylation of Fnk at the G_2/M border and its associated increase in kinase activity is strikingly similar to the mitosis-specific phosphorylation of Plk and suggests that Fnk may also provide a critical function during mitosis. *polo* has been shown to be important for progression through mitosis, as have the homologous genes in yeast [5,9,31]. Mutations in each of these genes leads to mitotic instability and results in cell-cycle arrest. Examination of the terminal phenotype of *polo* mutants in *Drosophila* suggests that *polo* has a role in centrosome maturation [31]. A similar role has been suggested for the mammalian homologue Plk and is supported by the Plk antibody injections performed in HeLa cells by Lane and Nigg [19]. There has also been a report that Plk associates with MKLP, a mitotic kinesin which is postulated to effect chromatid disjunction at anaphase B [15]. Based on the domain conservation of the polo enzymes one might suspect that they have similar regulators or substrates as has proven to be the case with the cyclin-dependent kinases. Thus it is possible that Fnk may interact with a set of motor proteins required for the successful completion of mitosis or to function in centrosome assembly. One possible role for Fnk that could account for its activity in both mitosis and entry into the cell cycle is the regulation of DNA replication. Such a role has been postulated for the polo homologue Cdc5p in *S. cerevisiae* [25]. Cdc5p has been shown by two-hybrid analysis to interact with Dbf4p [25], a protein required for DNA synthesis in yeast and a potential activator of the DNA replication complex or origin recognition complexes [32]. Perhaps Fnk is required in mitotic cells to initiate the conversion of the protein complex found at the origins of replication from the post-replicative state into the pre-replicative state required for the next round of DNA synthesis. One can envisage that the same type of conversion would be necessary on entry into the cell cycle from quiescence in preparation for the ensuing S phase.

Elucidating the roles for each of the mammalian polo homologues will probably be complex because of the high degree of similarity between the three known members and their overlapping cell-cycle expression patterns. Little is known about the third mammalian polo member, Snk, which is also encoded by an immediate-early gene and is induced on growth factor stimulation of quiescent cells. On the basis of the mRNA expression patterns of Snk and Fnk, we suspect that there will be overlap in the protein expression of these two members. Learning more about the cell-cycle expression pattern and activity of Snk and Fnk should give us helpful clues in determining functions for each of these family members.

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