Farnesylation of Cenp-F is required for G2/M progression and degradation after mitosis

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Summary

Farnesyl transferase inhibitors induce G2/M cell cycle delays that cannot be explained by inhibition of the Ras GTPase. Recently, the kinetochore protein Cenp-F has been shown to be farnesylated. Here, we show that ectopic expression of the kinetochore targeting domain of Cenp-F delays progression through G2/M. Significantly, this is dependent on the CAAX farnesylation motif. We also show that localisation of Cenp-F to the nuclear envelope at G2/M and kinetochores in prometaphase is dependent both on its CAAX motif and farnesyl transferase activity. Strikingly, farnesyl transferase activity is also required for Cenp-F degradation after mitosis. Thus, these observations suggest that farnesylation of Cenp-F is required not only for its localisation to the nuclear envelope and kinetochores but also for timely progression through G2/M and its degradation after mitosis. In addition, these observations raise the possibility that the anti-proliferative effects induced by farnesyl transferase inhibitors may be due to inhibition of Cenp-F function and/or turnover.

Key words: Mitosis, Kinetochore, Cenp-F, Protein farnesylation, Farnesyl transferase inhibitor

Introduction

Protein farnesylation involves the covalent addition of a 15-carbon isoprenoid lipid moiety to the cysteine residue of a C-terminal CAAX motif (reviewed by Sinensky, 2000; Crul et al., 2001). Farnesylated proteins include the Ras GTPase, pre-lamin A, lamin B, fungal pheromones and rhodopsin kinase. Although most farnesylated proteins are found associated with membranes, farnesylation may also mediate protein-protein interactions. Because farnesylation is required for Ras signalling (Willumsen et al., 1984; Kato et al., 1992) and because activating mutations in Ras have been detected in many human tumours, farnesyl transferase inhibitors (FTIs) have been developed as potential anti-cancer agents. While FTIs can suppress growth of tumour cells with Ha-Ras mutations by inducing G1 growth arrest (Sinensky, 2000; Crul et al., 2001), many other tumour cells exhibit G2/M delays in response to FTIs (Vogt et al., 1997; Ashar et al., 2001; Crespo et al., 2001). The mechanisms underlying FTI-induced G2/M delays are unknown.

Chromosome segregation in mitosis depends on kinetochores, complex protein structures that assemble at the centromeres of chromosomes (Rieder and Salmon, 1998). Kinetochores not only tether and move chromosomes along microtubule fibres but they also play a central role in the checkpoint mechanism that delays anaphase onset until all the chromosomes achieve correct, bipolar attachments (Amon, 1999). Although a number of kinetochore components have been identified, their precise roles are only partially defined (Maney et al., 2000; Pidoux and Allshire, 2000). Cenp-F was first identified as a human autoantigen that localises to kinetochores during mitosis (Rattner et al., 1993). During interphase Cenp-F is part of the nuclear matrix but upon entry into mitosis Cenp-F is released into the cytosol and a sub-pool becomes detectable at kinetochores (Casiano et al., 1993; Liao et al., 1995; Zhu et al., 1995a). Cenp-F remains kinetochore associated until anaphase onset when it re-localises to the spindle midzone. Thus, Cenp-F belongs to the family of 'chromosome passenger' proteins (Earnshaw and Bernat, 1991). Cenp-F is a cell-cycle-regulated protein, reaching maximum levels in G2/M followed by rapid degradation after mitosis (Liao et al., 1995; Zhu et al., 1995a). The 3210 amino acid protein is mainly coiled coil sequence, containing two internal repeats, several leucine heptad repeats and a bipartite nuclear localisation sequence. Expression of epitope-tagged deletion mutants indicates that the kinetochore localisation domain resides in the C-terminal region of the protein (Zhu et al., 1995b). Yeast two hybrid screens show that the C-terminal domain of Cenp-F is also capable of interacting with itself, the kinetochore-associated kinesin-related motor protein Cenp-E, and the spindle checkpoint component Bub1 (Zhu et al., 1995b; Chan et al., 1998; Jablonski et al., 1998). Although these observations suggest that Cenp-F may play a role in kinetochore assembly and/or the spindle checkpoint, the function of Cenp-F remains obscure. Significantly, Cenp-F and Cenp-E end with CAAX farnesylation sequences and recently both have been shown to be farnesylated (Ashar et al., 2000). These observations raise the possibility that the G2/M effects induced by FTIs are due to inhibition of Cenp-E and Cenp-F.

To determine whether Cenp-F plays a role in the spindle checkpoint we generated cell lines expressing a C-terminal Cenp-F mutant. Rather than compromising spindle checkpoint function, expression of this mutant delays progression through G2/M. Significantly, this effect requires an intact CAAX farnesylation motif. We also show that the CAAX motif and farnesyl transferase activity are required for Cenp-F localisation to kinetochores, the nuclear envelope at the G2/M

transition and for its degradation after mitosis. Not only do these observations highlight new roles for farnesylation as a signal for cell-cycle-regulated protein localisation and degradation but they also suggest that FTI-induced G2/M delays may be due, at least in part, to Cenp-F dysfunction.

Materials and Methods

Cell culture

The retroviral packaging line Phoenix-Ampho was obtained from Gary Nolan (Stanford) via the ATCC. BHK, TA-HeLa cells and culture conditions were as described (Taylor et al., 2001). The FTI SCH 66336 was obtained from Bob Bishop (Schering-Plough, Kenilworth, NJ), dissolved in DMSO at 10 mM and used at a final concentration of 1 μM. Nocodazole (Sigma, 5 mg/ml in DMSO) was diluted in media and used at 0.2 μg/ml.

Retroviral plasmids

The retroviral plasmid pLPCX (Clontech) was modified to include the 5′ untranslated sequence from the human lamin A cDNA and an N-terminal myc tag (Taylor and McKeon, 1997) to create pLPCX-Myc. This plasmid was used as the empty vector control. cDNA fragments encoding C630 and the CAAX mutants were generated by PCR amplification of a Cenp-F cDNA, obtained from Don Cleveland (University of California at San Diego), cloned into pLPCX-Myc and sequenced. The GFP open reading frame was generated by PCR amplification of pGFPemd-N1[F] (Packard) and N-mBub1 was as previously described (Taylor and McKeon, 1997). All plasmids were purified by ion exchange chromatography (Qiagen).

Antibody production

To create the sheep polyclonal anti-Cenp-F antibody, SCF.1, a central portion of Cenp-F encoding amino acids 1363-1640 was cloned as a *Bam*HI/*Not*I fragment into pGex-4T-3 (Pharmacia) and transformed into the *E. coli* strain BL21. Expression and purification of the GST-fusion protein, sheep immunisation and affinity purification were all carried out as described (Taylor et al., 2001).

Transfections and retroviral infections

Transient transfections of BHK and HeLa cells were carried out as described (Taylor et al., 2001). For retroviral infections, approximately 1×10^7 Phoenix-Ampho cells were transfected in 10 cm dishes with 27 μg of the pLPCX-Myc-based vectors using the ProFection calcium phosphate transfection kit (Promega). DNA precipitates were washed away after 24 hours then, 48 hours post transfection, the tissue culture supernatant was harvested, filtered through a 0.22 μm filter (Millipore) and diluted 2:3. Approximately 1×10^6 TA-HeLa cells in 10 cm dishes were then infected for 24 hours in the presence of 8 μg/ml polybrene (Sigma). Virus containing media was then removed and, 48 hours after infection, the cells were expanded into three 10 cm dishes and cultured in the presence of 0.5 μg/ml puromycin (Clontech). After 4 days of selection this procedure typically resulted in 3×10^7 puromycin-resistant cells when using pLPCX-Myc. These cells were then expanded for immediate analysis or frozen. To determine the expression efficiency, puromycin resistant HeLa cells infected with control and GFP viruses were analysed by flow cytometry on a FACScan (Becton Dickinson).

Cell cycle analysis

To determine relative cell number, 1.25×10^4 cells were plated in 12-well dishes and then fixed in 4% formaldehyde at the times indicated. Following washes in PBS, cells were stained with 0.1% crystal violet for 30 minutes and then washed with several volumes of water. Bound dye was then extracted with 10% acetic acid and the optical density at 590 nm was determined. Within an experiment, each point was determined in triplicate and each growth curve was performed at least twice. Synchronisations, DNA content and mitotic index measurements were all carried out as described (Taylor et al., 2001).

Immunofluorescence and western blotting

Cell fixation, labelling and fluorescence microscopy were all carried out as described (Taylor et al., 2001) using the following primary antibodies: mouse anti-Myc tag (9E10, 1:300); mouse anti-tubulin (TAT-1, 1:100); sheep anti-Cenp-F (SCF.1, 1:2500); mouse anti-lamin B2 (Zymed, 1:50); rabbit anti-phospho-histone H3 (Upstate Biotech, 1:700). Secondary antibodies used were either Cy2- or Cy3-conjugated donkey anti-rabbit, anti-mouse or anti-sheep antibodies (Jackson Immunoresearch) diluted 1:1000. Preparation of cell extracts and western blotting were all carried out exactly as described (Taylor et al., 2001) using the following primary antibodies: sheep anti-Cenp-F (SCF.1, 1:2500); mouse anti-Myc tag (9E10, 1:300); mouse anti-tubulin (TAT-1, 1:100).

Results

Ectopic expression of C630 delays progression through G2/M

To determine whether Cenp-F plays a role in chromosome segregation and/or the spindle checkpoint, we ectopically expressed the C-terminal 630 amino acids of Cenp-F, referred to here as C630 (Fig. 1A), in HeLa cells. We reasoned that by competing for kinetochore-binding sites C630 might have dominant properties. Consistent with previous observations (Zhu et al., 1995b), C630 localises to the nucleus during interphase and to kinetochores in mitosis (Fig. 1B). Retroviruses encoding C630, a green fluorescent protein (GFP), N-mBub1 (Taylor and McKeon, 1997) and a control virus were used to separately infect HeLa cells. Puromycin-resistant cells were selected, expanded and then analysed within two weeks of infection (Fig. 2A). Following this strategy, typically 85-95% of the resistant cells infected with GFP viruses expressed GFP (Fig. 2B). However, in an asynchronous population, a significant fraction of cells did not appear to express C630 (not shown) suggesting that, like Cenp-F, C630 might be cell cycle regulated. To test this we analysed cells immediately after and 12 hours after release from a single thymidine block. Whereas very few G1/S cells expressed C630, the majority of G2 cells were C630 positive (Fig. 2C). This shows that the majority of puromycin-resistant cells do indeed express C630 and that C630 is cell cycle regulated. Significantly, cells expressing C630 grew poorly relative to control cells. To quantitate this, equal numbers of cells were plated and the relative cell number determined over time. Whereas control cells grew with normal kinetics, the growth of cells expressing C630 was retarded (Fig. 2D). Furthermore, DNA content histograms of asynchronous cultures showed a 10% increase in the G2/M phase of the cell cycle (Fig. 2E). To determine whether progression through G2/M was delayed we analysed the DNA content of cells every three hours for 18 hours following release from a G1/S block (Fig. 2F). Whereas progression through S phase appeared normal, between 12 and 18 hours after G1/S more of the control cells returned to G1 relative to cells expressing C630. These observations indicate that expression of C630

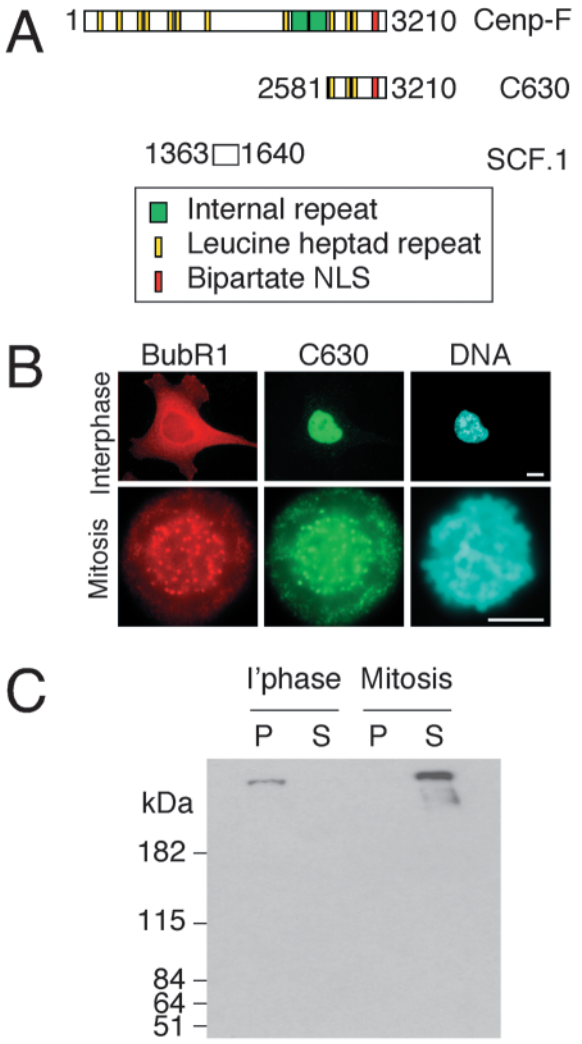


Fig. 1. The C-terminal 630 amino acids of Cenp-F localises to kinetochores. (A) Schematic of Cenp-F showing the domain encoded by C630 and the fragment used to generate the anti-Cenp-F antibody, SCF.1. (B) HeLa cells stained to detect endogenous BubR1 (red), myc-tagged C630 (green) and DNA (blue). During interphase C630 localises to the nucleus and in prometaphase it co-localises with BubR1 at kinetochores. Bars, 5 μm. (C) Immunoblot of pellets (P) and supernatants (S) following extraction of interphase and mitotic HeLa cells showing that SCF.1 recognises a single band significantly greater than 200 kDa.

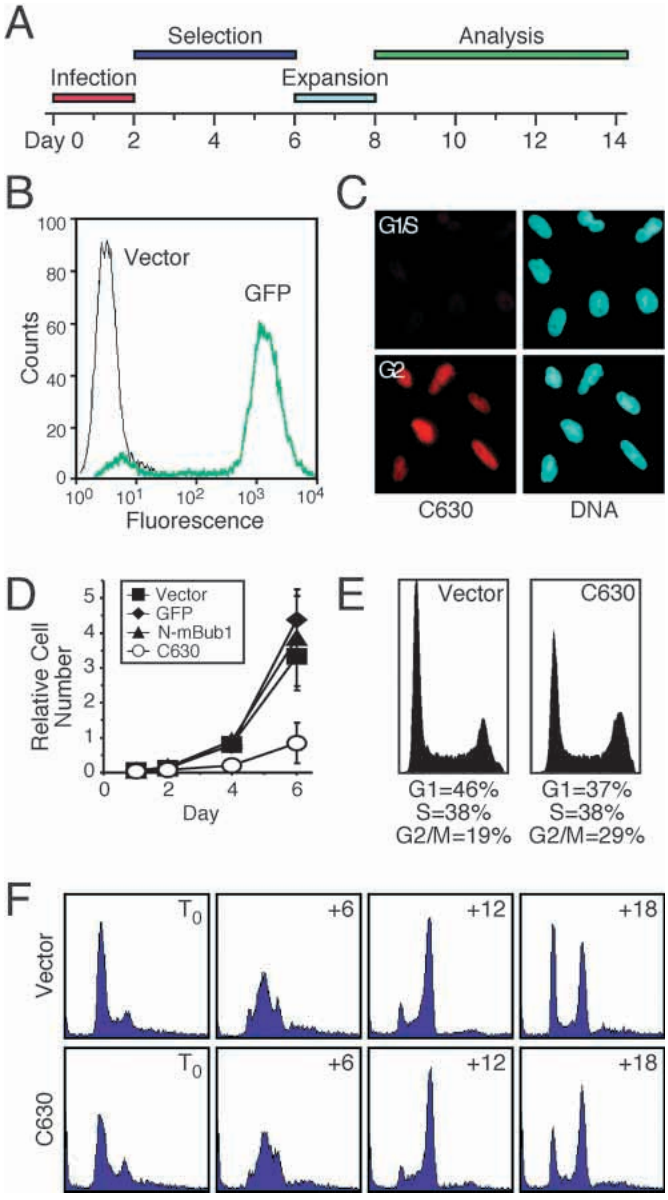


Fig. 2. Expression of C630 inhibits proliferation and delays progression through G2/M. HeLa cells were infected with either a control retrovirus or retroviruses encoding GFP, myc-tagged C630 and N-mBub1. Puromycin-resistant cells were selected, expanded then analysed. (A) Timeline used to generate cells expressing the cDNA of interest. (B) Fluorescence histograms showing that 95% of the cells infected with GFP retroviruses express GFP. (C) Immunofluorescence analysis of HeLa cells infected with retroviruses encoding C630 immediately after or 12 hours after release from a G1/S block indicating that expression levels of C630 are low at G1/S but high in late G2. (D) Growth curves showing that expression of C630 results in a growth defect.(E) DNA content histograms of asynchronous cultures plus the number of cells in each phase of the cell cycle. The mitotic index was determined by quantitating MPM-2-positive cells. The values represent the average of three independent cultures indicating that expression of C630 results in accumulation of cells in G2/M. (F) DNA content histograms of infected HeLa cells following release from a G1/S block showing that expression of C630 delays progression through G2/M. The cells were analysed every 3 hours but only the key time points are shown.

impairs cellular proliferation and delays progression through G2/M. In order to estimate the length of the G2/M delay induced by expression of C630 we measured the difference between the times taken for half of the cells to enter mitosis following release from a G1/S block (see below). Based on this analysis we estimate that expression of C630 delays entry into mitosis by approximately 1-1.5 hours. This delay does not appear to be sufficient to account for the reduced growth rate (Fig. 2D). Although the apoptotic index was not measured directly, over time an increasing fraction of cells in the C630 cultures display aberrant morphologies and appear to undergo cell death. Thus, the decreased growth rate of the C630 cultures may be due to a combination of the G2/M delay and an increase in cell death.

Expression of C630 delays entry into mitosis but does not prevent chromosome segregation

The observations described above show that expression of C630 delays but does not block G2/M progression. Because the mitotic index of the control and C630 cultures was not significantly different (not shown) we reasoned that C630 was inducing a G2 rather than a mitotic delay. To test this we measured the mitotic index of control and C630 cultures following exposure to the microtubule toxin nocodazole. We reasoned that if C630 delayed progression through G2, the accumulation of mitotic cells would be slower compared with that observed in a control population. In contrast, if expression of C360 had no effect on mitotic entry but delayed progression through mitosis, mitotic cells would accumulate at a similar rate in both control and C630 cultures. Fig. 3 shows that the mitotic index of either asynchronous or synchronised populations of cells expressing C630 is reduced relative to control cells in the presence of nocodazole (Fig. 3A,B), suggesting that expression of C630 does indeed delay progression through G2 rather than mitosis. To determine whether expression of C630 had an effect on spindle assembly or chromosome segregation we compared mitotic cells in both control and C630 cultures. The number of metaphases and anaphases did not appear different (Fig. 3C) and the spindle morphology did not appear different (Fig. 3D). While we cannot rule out the possibility that expression of C630 has a subtle effect on mitotic chromosome alignment and segregation, these observations are more consistent with the notion that C630 delays progression through G2 and/or the onset of mitosis.

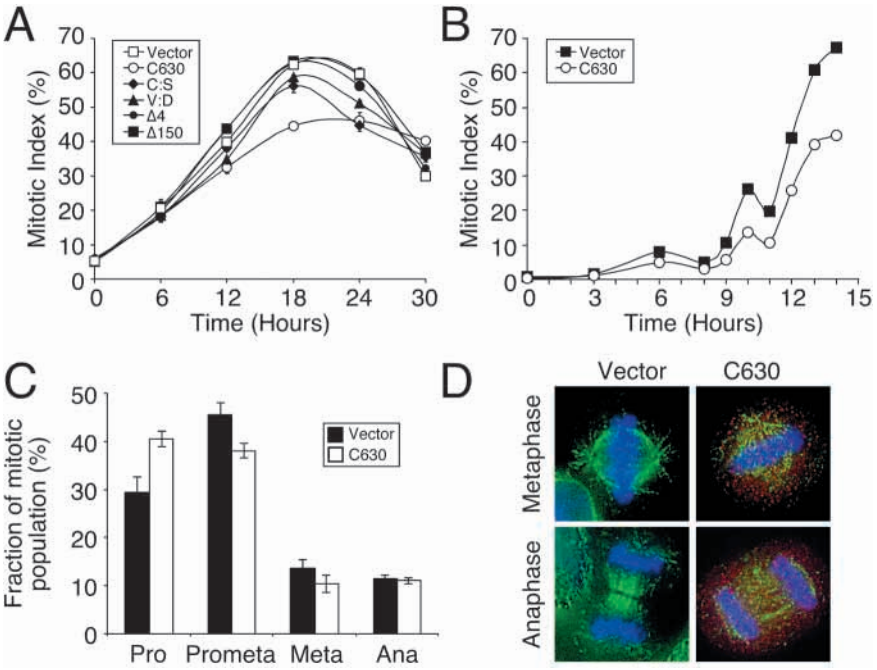
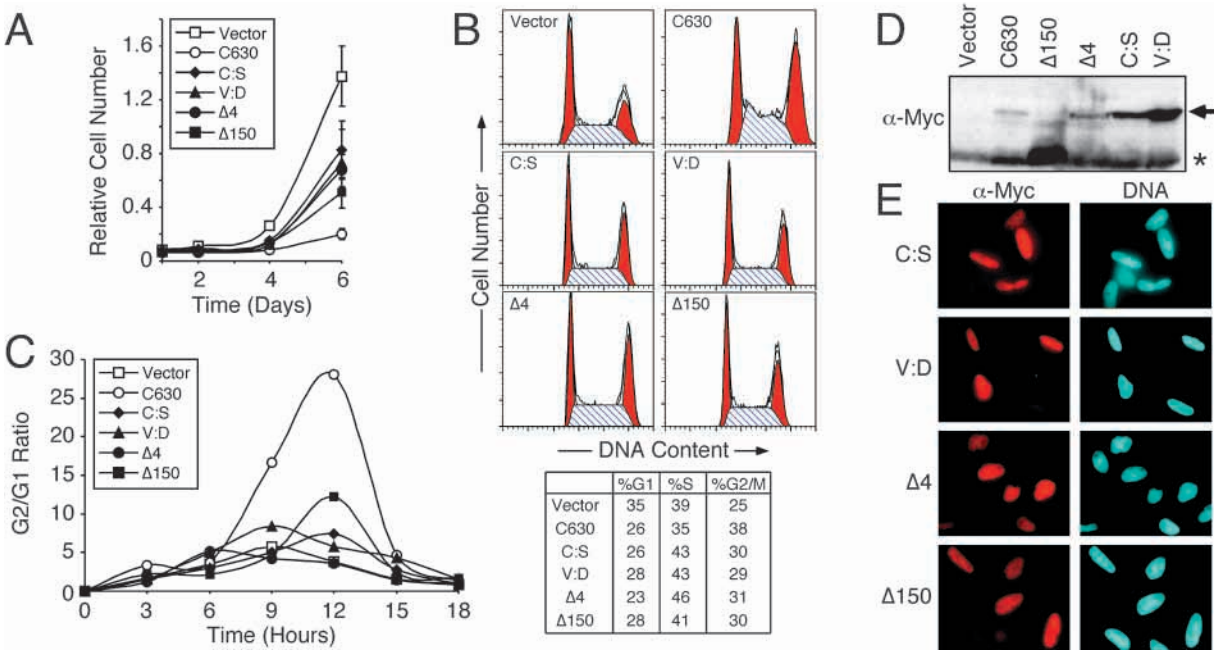


Fig. 3. Expression of C630 delays entry into mitosis. HeLa cells infected with either a control retrovirus or retroviruses encoding myc-tagged C630 were analysed by flow cytometry and fluorescence microscopy in the presence and absence of nocodazole. (A) Graph plotting the mitotic index following treatment of asynchronous populations with nocodazole for the times indicated showing that the C630 population accumulates mitotic cells slower than the control population. Also shown is data from cells expressing the four CAAX mutants, C:S, V:D, Δ4 and Δ150 (see text for details). Each point represents the mean and s.e.m. derived from three independent experiments. (B) Graph plotting the mitotic index following release of cells synchronised at G1/S into nocodazole showing that the C630 population accumulates mitotic cells slower than the control population. (C) Bar graph plotting the proportions of cells in each phase of mitosis showing that expression of C630 does not appear to effect progression through mitosis. Each value is expressed as a percentage of the number of cells in mitosis and represents the mean and s.e.m. derived from three independent experiments in which at least 50 mitotic cells were counted. (D) Representative immunofluorescence images of metaphase and anaphase cells typically observed in control and C630 cultures showing that expression of C630 does not appear to disrupt chromosome alignment or segregation. Tubulin is shown in green, myc-tagged proteins in red and the chromosomes in blue.

The CAAX motif of C630 is required for delaying progression through G2/M

Recently, Cenp-F has been shown to be farnesylated (Ashar et al., 2000). In addition FTIs inhibit proliferation and can delay G2/M progression in some cell lines (Vogt et al., 1997; Ashar et al., 2001). Based on these and our observations described above, we formulated the following hypothesis. We reasoned that perhaps Cenp-F binds to some unknown protein(s) and/or membrane structure in a farnesylation-dependent manner and that this is required for timely progression through G2/M. Thus, when farnesylation of Cenp-F is inhibited by FTIs this interaction is less efficient resulting in a G2/M delay. Likewise expression of C630, which contains the CAAX motif and should therefore be an efficient farnesylation substrate, should compete for this interaction, thus also delaying G2/M progression. This hypothesis predicts that if the CAAX motif in C630 is mutated to prevent farnesylation it should be a less efficient competitor and thus not delay G2/M.

Therefore, based on mutations that inhibit farnesylation of Ras (Kato et al., 1992) we mutated the CAAX motif in C630, replacing the cysteine and valine with a serine and aspartic acid, creating C630 C:S and C630 V:D, respectively. We also deleted the CAAX motif (C630 Δ4) and the C-terminal 150 amino acids (C630 Δ150). HeLa cells were then infected in parallel with viruses encoding C630, the four CAAX mutants and a control virus. Puromycin-resistant cells were selected, expanded and all six lines analysed in parallel. Significantly, cells expressing the CAAX mutants grew better than cells expressing C630 (Fig. 4A). Whereas cells expressing C630 doubled every 3 days, cells expressing the CAAX mutants doubled every 1.5 days and cells infected with the control virus doubled approximately every day. DNA content histograms of asynchronous cultures showed that cells expressing the CAAX mutants did not accumulate in G2/M to the same extent as those expressing C630 (Fig. 4B). To investigate cell cycle



	%G1	%S	%G2/M
Vector	35	39	25
C630	26	35	38
C:S	26	43	30
V:D	28	43	29
Δ4	23	46	31
Δ150	28	41	30

Fig. 4. The CAAX motif of C630 is required for delaying progression through G2/M. HeLa cells were infected with a control retrovirus, retroviruses encoding myc-tagged C630 and the four CAAX mutants, C:S, V:D, Δ4 and Δ150. Puromycin-resistant cells were analysed to determine protein expression, localisation, relative growth rates and cell-cycle timing. (A) Growth curves showing that the CAAX mutants induce a less severe growth defect relative to C630. (B) DNA content histograms of asynchronous cultures showing that the CAAX mutants induce a less profound G2/M accumulation relative to C630. (C) Graph plotting the 4n/2n DNA content ratio, as determined by DNA content flow cytometry, following release from a G1/S block. Unlike C630, the CAAX mutants do not induce a significant G2/M delay. (D) Immunoblot showing the relative expression levels of C630 and the CAAX mutants. The arrow shows the position of C630 while the asterisk indicates a nonspecific background band that serves as a convenient loading control. (E) Immunofluorescence analysis 12 hours after release from a G1/S block showing that the C630 CAAX mutants localise to the nucleus during interphase.

timing in more detail, cells were released from a G1/S block, harvested every 3 hours and then analysed by flow cytometry to determine DNA content. To facilitate comparison of all six lines we calculated the 4n/2n DNA content ratio at each time point. In all cases the 4n/2n ratio increased following release as the cells progressed into G2/M, and then decreased after 9-12 hours as the cells completed mitosis and returned to G1 (Fig. 4C). At 9 and 12 hours, the 4n/2n ratio of the C630 culture was markedly higher than that of the control cells, consistent with a G2/M delay. Significantly, however, the CAAX cultures exhibited 4n/2n ratios more typical of the control cells, indicating that expression of the CAAX mutants does not delay G2/M progression as markedly as C630. In addition, cells expressing the CAAX mutants behaved more like control cells rather than C630-expressing cells when exposed to nocodazole over a 30-hour period (Fig. 3A).

While these observations suggest that the phenotype induced by expression of C630 does indeed require an intact CAAX sequence, mutation of the CAAX motif does not appear to completely alleviate the C630 phenotype. This raises the possibility that mutation of the CAAX motif simply leads to lower expression levels and/or protein misfolding. However, immunoblotting of whole cell lysates prepared from all six lines shows that mutation of the CAAX motif does not result in lower expression levels (Fig. 4D). Furthermore, immunofluorescence of late G2 cells shows that the CAAX mutants localise to the nuclei in the vast majority of cells (Fig. 4E), demonstrating that mutation of the CAAX motif does not result in cytoplasmic aggregation of C630. Thus, as predicted by the hypothesis outlined above, these observations suggest that the phenotype induced by expression of C630 does indeed depend to a large extent on an intact CAAX motif.

Cenp-F localises to the nuclear envelope at the G2/M transition

To determine whether C630 disrupts the localisation of endogenous Cenp-F we first generated a polyclonal antibody, SCF.1, against a central portion of Cenp-F (Fig. 1A). Consistent with previous observations indicating that Cenp-F is part of the nuclear matrix in interphase but mainly cytosolic in mitosis (Casiano et al., 1993; Liao et al., 1995), SCF.1 detects a single protein significantly greater than 200 kDa that remains in the cell pellet following mild detergent extraction of interphase cells (Fig. 1C). Following similar extraction of mitotic cells this protein is present in the supernatant. SCF.1 stains the nucleus of some but not all interphase cells (Fig. 5A,B) consistent with cell cycle regulation of Cenp-F (Liao et al., 1995). In prometaphase, SCF.1 stains the cytoplasm and kinetochores (Fig. 5A,C). Taken together, these observations suggest that SCF.1 specifically recognises Cenp-F.

When detectable, Cenp-F localised exclusively to the nucleus in most interphase cells. However, in interphase cells that showed early signs of phospho-histone H3 staining, Cenp-F also localised to the nuclear periphery (Fig. 5B). In prophase cells this peripheral localisation was more pronounced (Fig.

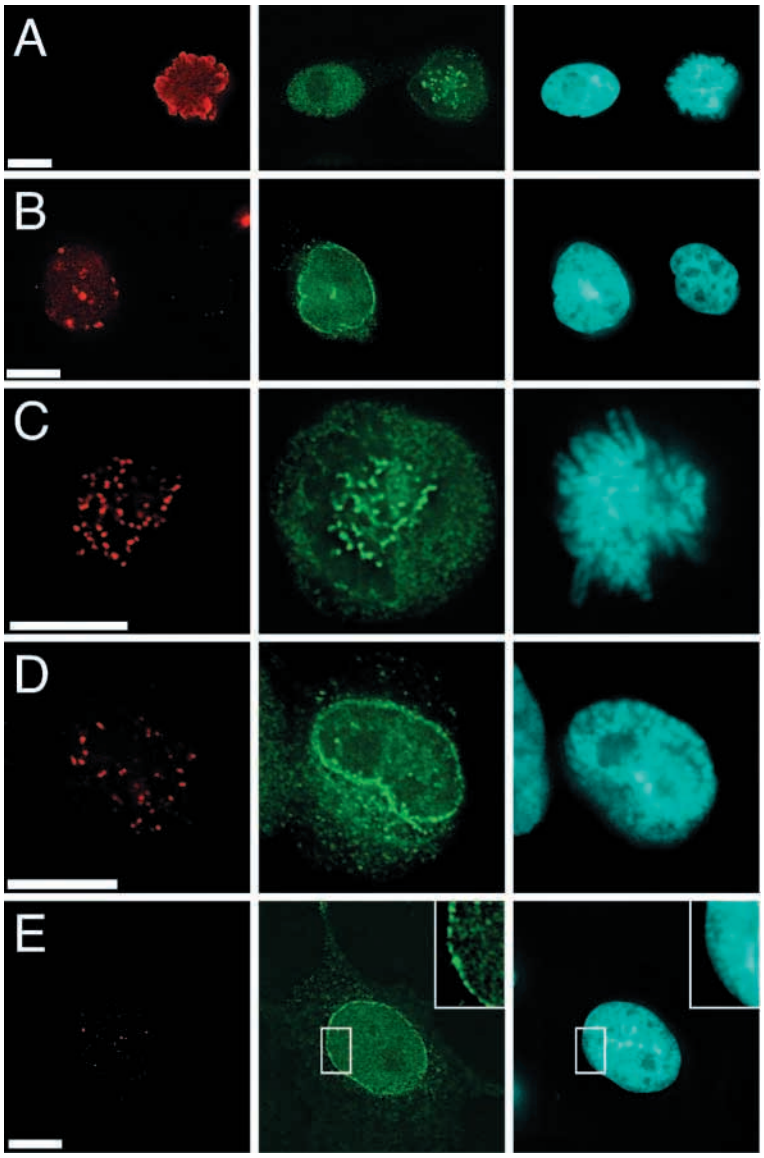


Fig. 5. Cenp-F localises to the nuclear periphery at the G2/M transition. Deconvolved images showing HeLa cells stained to detect Cenp-F (green) and DNA (blue). The cells were also stained to detect phospho-histone H3 (A,B) and Bub1 (C-E). Bars, 10 μm. (A) Interphase and prometaphase cells showing localisation of Cenp-F to the nucleus and kinetochores, respectively. (B) Two interphase cells; the one on the left is in late G2 and shows Cenp-F localising to the nucleus and the nuclear envelope. A small fraction is present in the cytoplasm. (C) Prometaphase cell showing colocalisation of Cenp-F and Bub1 at kinetochores. (D) Late prophase cell showing Cenp-F in the nucleus, cytoplasm and at the nuclear envelope. (E) A cell at the G2/M transition showing early signs of chromosome condensation (see inset), a few Bub1 foci and Cenp-F at the nuclear envelope.

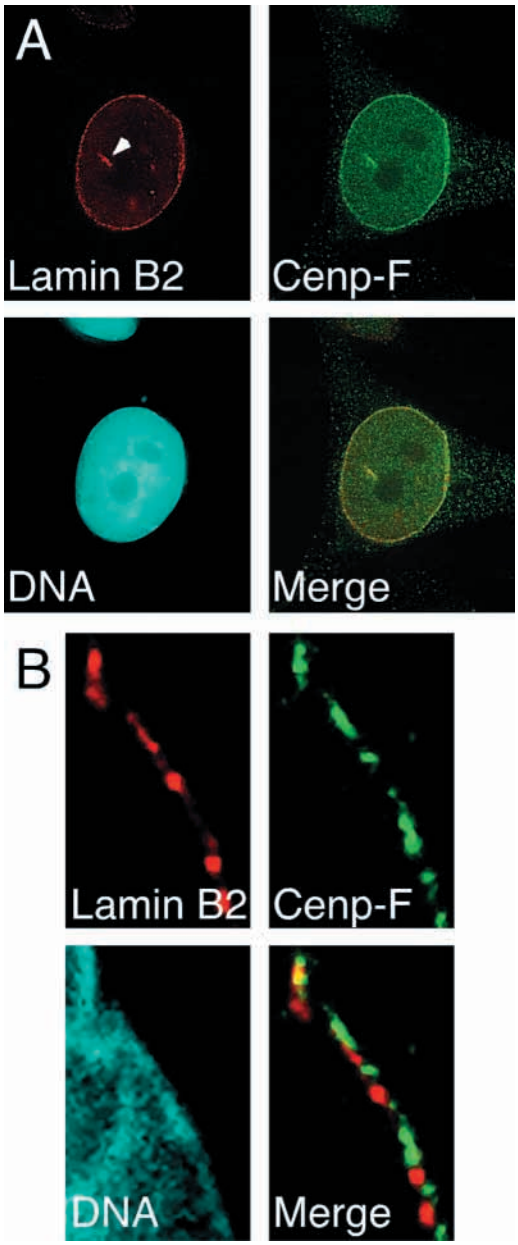


Fig. 6. Cenp-F localises to the nuclear envelope in G2. Deconvolved images showing HeLa cells stained to detect Cenp-F (green), lamin B2 (red) and DNA (blue). (A) G2 cell showing localisation of Cenp-F and lamin B2 to the nuclear envelope. Note that Cenp-F also localises to an invagination within the nucleus (arrowhead). (B) Enlarged view of a G2 cell showing that Cenp-F and lamin B2 localise to different regions of the nuclear envelope.

5D). Whereas previous reports have suggested that this nuclear rim staining is because soluble Cenp-F is excluded from the chromatin upon mitotic entry (Rattner et al., 1993; Liao et al., 1995), our observations are more consistent with the notion that Cenp-F localises to the nuclear envelope in late G2 and early mitosis. Indeed, in very early prophase cells where chromatin condensation is only just visible and only a few Bub1 foci are apparent, Cenp-F clearly localises to what appears to be the nuclear envelope (Fig. 5E). To confirm this we co-stained HeLa cells for Cenp-F and lamin B2. Fig. 6A shows that Cenp-F localises not only to the nuclear envelope around the nuclear periphery but also to invaginations within the middle of the nucleus. However, note that Cenp-F and lamin B2 do not appear to colocalise but rather localise to different regions of the nuclear envelope resulting in alternating red and green spots in the merged image (Fig.

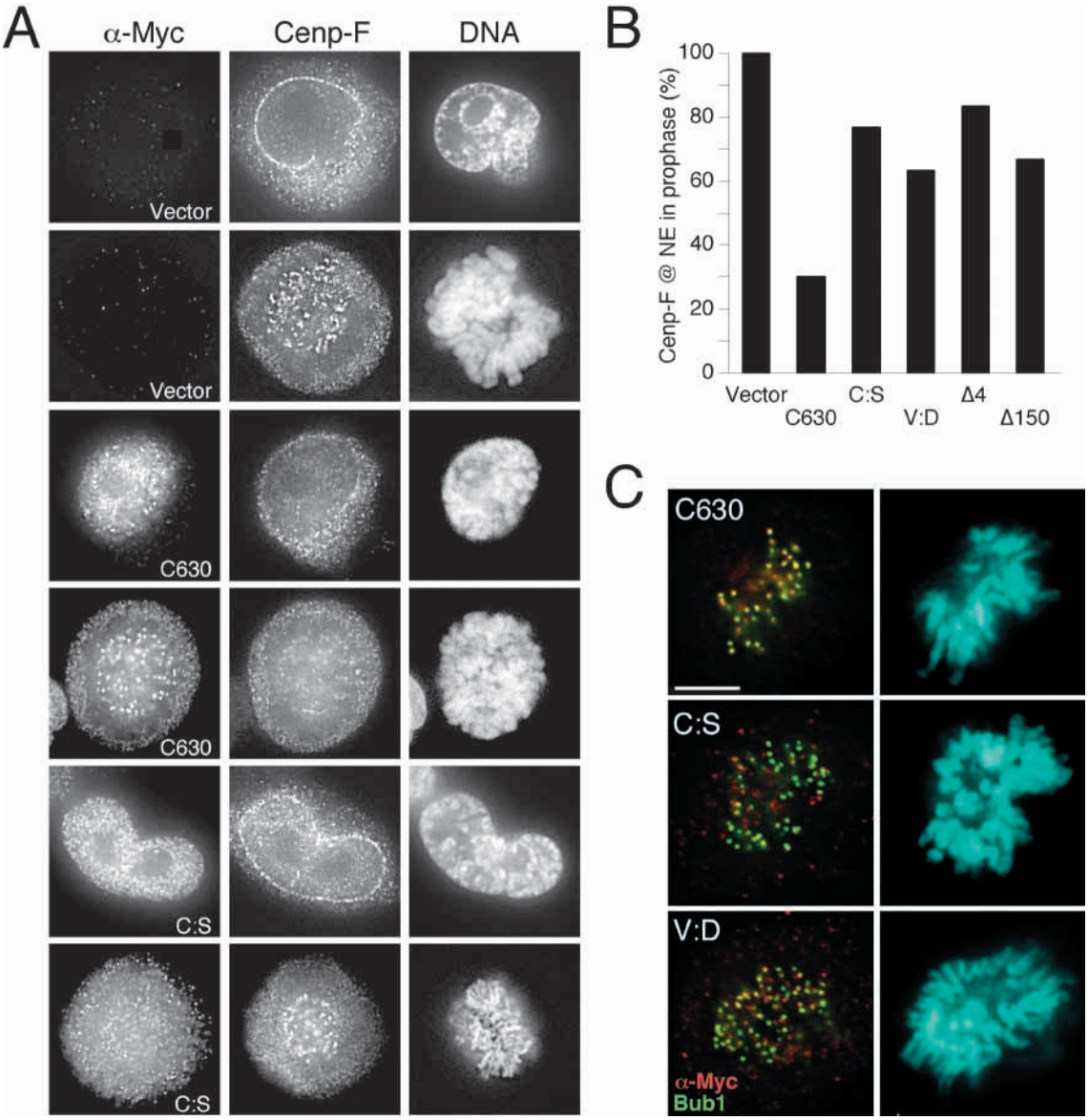


Fig. 7. The CAAX motif of Cenp-F is required for nuclear envelope and kinetochore localisation. (A) HeLa cells stained to detect myc-tagged proteins, Cenp-F and DNA as indicated. Nuclear envelope and kinetochore localisation of Cenp-F is diminished in the presence of C630 but is readily apparent when the C:S mutant is expressed. (B) Bar graph quantitating the number of cells with Cenp-F detectable at the nuclear envelope 12 hours after release from a G1/S block. Nuclear envelope staining is less frequent in cells expressing C630. (C) Projections of deconvolved image stacks showing HeLa cells stained to detect myc-tagged proteins (red), Bub1 (green) and DNA (blue). The C:S and V:D mutants localise poorly to kinetochores.

6A,B). Because there is no sign of chromosome condensation and the nucleoli are still intact, the cell shown in Fig. 6A must be in late G2. Therefore it seems unlikely that localisation of Cenp-F to the nuclear envelope is simply due to exclusion from the chromatin following breakdown of the nuclear matrix at the onset of chromosome condensation. Rather, a subpool of Cenp-F appears to concentrate at the nuclear envelope in late G2, through the G2/M transition and into early mitosis until envelope breakdown.

The CAAX motif of Cenp-F is required for nuclear envelope and kinetochore localisation

To determine whether C630 disrupts the localisation of endogenous Cenp-F, the six lines were stained to detect myc-tagged proteins and Cenp-F (Fig. 7A and data not shown). Cenp-F was readily detectable at nuclear envelopes and kinetochores in control cells but not in cells expressing C630. Significantly however, Cenp-F was frequently observed at nuclear envelopes and kinetochores in cells expressing the CAAX mutants (Fig. 7A,B). Furthermore, whereas C630 clearly localises to kinetochores, the C:S mutant appears not to (Fig. 7A). To confirm this, prometaphase cells were stained to detect myc-tagged proteins and Bub1 then analysed by deconvolution microscopy (Fig. 7C). C630 and Bub1 clearly co-localise to kinetochores resulting in yellow foci in the merged image. In contrast, the C:S and V:D mutants do not co-localise with Bub1 resulting in separate red and green foci in the merged image. Thus, this analysis suggests not only that an intact CAAX motif is required for kinetochore localisation of C630 but also that the ability of C630 to disrupt the localisation of endogenous Cenp-F depends on the CAAX sequence.

Farnesyl transferase activity is required for efficient localisation of Cenp-F to the nuclear envelope and kinetochores

The simplest explanation for the observations described above

is that the CAAX farnesylation motif of Cenp-F is required for both nuclear envelope and kinetochore localisation. To test whether farnesyl transferase activity is required for correct localisation, we analysed the effect of the FTI SCH 66336 (Ashar et al., 2000) on Cenp-F localisation. For these experiments we chose to use HeLa cells for the following reason. Consistent with previous observations (Ashar et al., 2001), we observed that culturing HeLa cells in SCH 66336 for more than 72 hours results in a G2/M cell cycle delay (not shown). However, the cell cycle effects of SCH 66336 following a 24 hour exposure are minimal (not shown). Therefore, by analysing HeLa cells that have been exposed to SCH 66336 for less than 24 hours we reasoned that we could analyse the effect of the FTI on Cenp-F localisation independently of any effects on cell cycle progression.

While Cenp-F was detectable at both nuclear envelopes and kinetochores in the presence of the FTI, the frequency and intensity of Cenp-F staining appeared diminished (not shown). To investigate this further, we analysed HeLa cells cultured with and without the FTI following release from a G1/S block (Fig. 8A). As cells progressed into G2 in the absence of the FTI, nuclear envelope staining became more frequent; after 10-12 hours, as the cells completed mitosis and returned to G1, nuclear envelope staining became less frequent. However, in the presence of FTIs the number of cells with Cenp-F detectable at the nuclear envelope was reduced by approximately 50%. To illustrate this, Fig. 8B shows two early prophase cells: the chromatin shows signs of condensation but the nucleoli are still distinct and the nuclear envelopes are intact as judged by the lamin B2 staining. However, while Cenp-F is detectable at the nuclear envelope in untreated cells, it is not apparent in the presence of the FTI (Fig. 8B). To investigate the effect on kinetochore localisation, FTI-treated HeLa cells were arrested in mitosis and then stained to detect Cenp-F and phospho-histone H3. In control cells, Cenp-F is detectable both in the cytoplasm and on the chromatin as discrete foci (Fig. 8C). In contrast, in the presence of the FTI, Cenp-F is only present in the cytoplasm and chromatin-associated foci are not apparent. Thus, these observations indicate that farnesyl transferase activity is required for efficient localisation of Cenp-F to both nuclear envelopes and kinetochores.

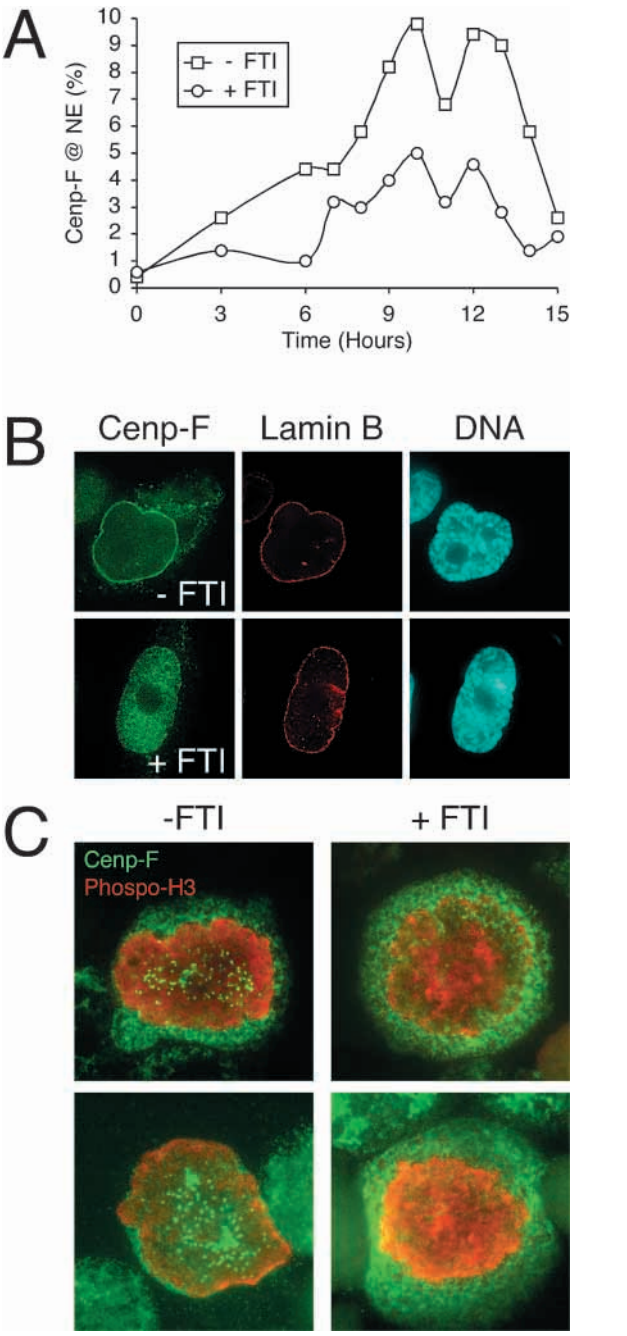


Fig. 8. Farnesyl transferase activity is required for Cenp-F localisation. (A) The percentage of cells with Cenp-F at the nuclear envelope in the presence or absence of SCH 66336 at various times following release from a G1/S block. (B) Early prophase HeLa cells cultured in the presence or absence of SCH 66336 stained for Cenp-F (green), lamin B2 (red) and DNA (blue) showing that nuclear envelope localisation of Cenp-F is not apparent in the presence of the FTI. (C) Nocodazole-arrested mitotic HeLa cells cultured in the presence or absence of SCH 66336 were isolated by shake off, centrifuged onto glass slides and then stained for Cenp-F (green) and phospho-histone H3 (red). Kinetochore staining is not apparent in the presence of SCH 66336.

Farnesyl transferase activity is required for degradation of Cenp-F after mitosis

Strikingly, after 24 hours in the presence of FTIs the vast majority of HeLa cells stained positive for Cenp-F (not shown) consistent with them being in G2 (Ashar et al., 2001). However, because a 24 hour exposure to SCH 66336 has only minimal effect on cell cycle progression in HeLa cells (see above), this observation raised the possibility that inhibition of farnesyl transferase activity may disrupt Cenp-F turnover. To test this, HeLa cells were released from a G1/S block in the presence and absence of SCH 66336. After 15 hours, most cells had completed mitosis and returned to G1 (Fig. 9A). In the absence of the FTI, Cenp-F levels were low in the vast majority of cells (Fig. 9B), consistent with Cenp-F being degraded after mitosis (Liao et al., 1995). The few Cenp-F-positive cells were typically phospho-histone-H3-positive, indicating that they were still in mitosis. In contrast, in the presence of the FTI the vast majority of interphase, phospho-histone-H3-negative nuclei contained Cenp-F (Fig. 9B). Furthermore,

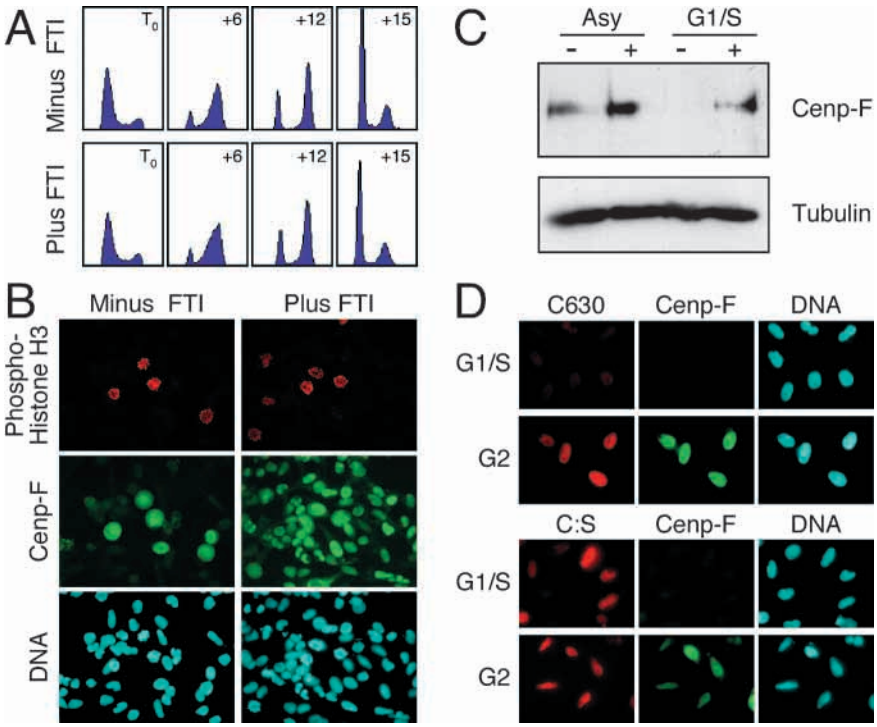


Fig. 9. Farnesyl transferase activity is required for Cenp-F degradation. (A) DNA content histograms of HeLa cells cultured in the presence or absence of FTIs following release from a G1/S block. (B) Immunofluorescence images of the cells 15 hours after release from G1/S stained to detect phospho-histone H3 (red), Cenp-F (green) and DNA (blue). In the presence of SCH 66336, Cenp-F is detectable in all the interphase nuclei. (C) Immunoblots of asynchronous or G1/S blocked HeLa cells showing that Cenp-F is present in G1/S cells only when cultured in the presence of SCH 66336. (D) HeLa cells infected with retroviruses encoding C630 or the C:S mutant stained to detect myc-tagged proteins (red), Cenp-F (green) and DNA (blue). The cells were synchronised at G1/S using a single thymidine block. The cells were then analysed immediately following release (i.e. at G1/S) or 12 hours after release (i.e. in G2). Like endogenous Cenp-F, C630 is only detectable in G2 cells, whereas the C:S mutant is also detectable in G1/S cells.

immunoblotting of total cell lysates prepared from HeLa cells synchronised at G1/S by a double thymidine block in the presence of the FTI contained high levels of Cenp-F protein (Fig. 9C). In contrast, in the absence of the FTI, Cenp-F was not detectable in G1/S suggesting that farnesyl transferase activity is indeed required for Cenp-F degradation after mitosis.

To determine whether the CAAX motif is required for degradation we examined the expression levels of C630 and the CAAX mutants following release from a G1/S block. Both Cenp-F and C630 were not detectable immediately after release from a G1/S block but were clearly present in G2 cells 12 hours after release (Fig. 9D). In contrast, the C630 C:S mutant was present both in G1/S and G2 suggesting that the CAAX motif is required for the cell-cycle-regulated turnover of C630. Whether the C630 C:S protein present in G1/S cells is due to post-mitotic persistence of protein synthesised in the previous cell cycle or the failure to degrade newly synthesised protein remains to be seen. However, taken together, these results suggest that farnesylation of Cenp-F is required for its degradation after mitosis.

Discussion

Farnesyl transferase inhibitors have previously been shown to delay progression through G2/M. However, the mechanisms underlying this phenomenon are unknown. Here, we show that expression of a C-terminal Cenp-F mutant delays progression through G2/M. Significantly, this effect is dependent on an intact CAAX farnesylation motif. We also show that the localisation of Cenp-F to the nuclear envelope at G2/M and to kinetochores in mitosis depends on both a CAAX motif and farnesyl transferase activity. We also show for the first time that cell-cycle-regulated degradation of Cenp-F requires both farnesyl transferase activity and a CAAX motif. Therefore, these observations suggest that farnesylation of Cenp-F is required for its timely progression through G2/M, its localisation and for its degradation. Furthermore, these observations raise the possibility that the anti-proliferative and cell cycle effects induced by FTIs may be due to Cenp-F dysfunction.

Farnesylation of Cenp-F is required for proliferation and progression through G2/M

Expression of C630 inhibits both cellular proliferation and progression through G2/M (Fig. 2). Previously, transient transfection of CV-1 cells with plasmids expressing C-terminal Cenp-F fragments resulted in the accumulation of cells in G2/M (Zhu et al., 1995a). However, because transient transfections are refractory to detailed cell cycle analysis, the significance of this observation was unclear. In addition, generation of stable cell lines was uninformative (Zhu et al., 1997), possibly because the anti-proliferative effects resulted in clones with very low expression levels. Here, we have used retroviral infections to overcome both of these limitations. Importantly, the strategy employed typically results in 85-95% of the cells stably expressing the cDNA of interest at high levels during the period in which the cell cycle analysis was performed (Figs 2-4). Using this strategy, we have shown that both the proliferation defect and the G2/M delay induced by expression of C630 depends on an intact CAAX farnesylation motif (Fig. 4). What can these observations tell us about the endogenous Cenp-F protein? Clearly, any interpretations must take into account our experimental strategy, namely the expression of truncation mutants. First, in the absence of a clearly defined function for Cenp-F it is not clear whether expression of C630 exerts a dominant negative or a dominant

active effect. In addition, owing to technical constraints we have been unable to determine the expression levels of C630 relative to endogenous Cenp-F. Furthermore, we cannot rule out the possibility that expression of C630 interferes with proteins other than Cenp-F, for example, by simply competing for farnesyl transferase activity. Second, although mutation of the CAAX motif alleviates the phenotype induced by expression of C630, we cannot rule out the possibility that these mutations simply result in misfolding of the C630 protein, effectively producing a null mutant. However, despite these caveats, the simplest explanation for our observations is that C630 competes with Cenp-F for one or more CAAX-dependent interactions, thus interfering with the function of the endogenous Cenp-F protein. The observations based on expression of C630 and the CAAX mutants are, however, only consistent with the notion that farnesylation of Cenp-F is required for timely progression through G2/M, its localisation and its degradation after mitosis. Nevertheless, the observations showing that FTIs interfere with G2/M progression (Vogt et al., 1997; Ashar et al., 2001), the localisation and degradation of Cenp-F (Figs 8, 9) lend further support to this hypothesis. Clearly, however, a more definitive test will require analysing Cenp-F in a more genetically tractable system.

Mutation of the CAAX motif in C630 does not completely abolish the proliferation and cell cycle effects suggesting that expression of C630 may exert its effects via two different mechanisms. While one mechanism is clearly CAAX dependent, the second mechanism appears to be CAAX independent. One possible explanation for the CAAX-independent mechanism comes from the observation that the C-terminal domain of Cenp-F has a large number of potential phosphorylation sites (Liao et al., 1995; Zhu et al., 1995a). In addition, Cenp-F has been shown to be phosphorylated in mitosis (Zhu et al., 1995a). Therefore, overexpression of C630, and the CAAX mutants that still have many potential phosphorylation sites, might compete for Cdk1 and/or other kinase activities, and thus reduce the efficiency of certain phosphorylation events required for mitotic entry. However, taking into account the observation that C630 is expressed at much lower levels relative to the CAAX mutants (Fig. 4D), the predominant mechanism by which C630 inhibits proliferation and cell cycle progression clearly depends on the CAAX motif.

FTIs can suppress tumour cell growth, both in cell culture and in animal models, and consequently several FTIs are undergoing phase I clinical trials (reviewed by Sinensky, 2000; Crul et al., 2001). However, the molecular mechanisms underlying the anti-proliferative effects of FTIs remain obscure. In particular, the cell cycle effects of FTIs are multi-phasic: while cells with Ha-Ras mutations accumulate in G1, other FTI-sensitive cells accumulate in G2/M (Vogt et al., 1997; Ashar et al., 2001). The observation that both the anti-proliferative and G2/M effects of C630 expression are CAAX dependent (Fig. 4) suggests that FTI-induced effects may be mediated, at least in part, by inhibiting farnesylation of Cenp-F. In some cells that exhibit G2/M delays in response to FTIs, chromosome alignment defects have been observed (Crespo et al., 2001). Thus, the G2/M delay may be due to spindle checkpoint activation resulting in a prolonged mitosis. In addition, taxol and FTIs can act synergistically to induce mitotic arrest (Moasser et al., 1998), suggesting that FTIs may inhibit proteins required for the correct attachment of kinetochores to microtubules. A key FTI target in these cells is likely to be the kinesin-related motor protein, Cenp-E, which has recently been shown to be farnesylated, and has previously been shown to be required for both tethering chromosomes to microtubules and spindle checkpoint signalling (Schaar et al., 1997; Wood et al., 1997; Chan et al., 1999; Abrieu et al., 2000; Ashar et al., 2000; Yao et al., 2000). Although inhibition of farnesyl transferase activity does not appear to alter kinetochore localisation of Cenp-E, it does diminish the affinity of Cenp-E for microtubules (Ashar et al., 2000; Crespo et al., 2001).

It is possible that FTI-induced chromosome alignment defects may be due to inhibition of Cenp-F. Indeed, Cenp-F localises to kinetochores and can interact with the spindle checkpoint protein Bub1. Furthermore, two Cenp-F related proteins, Okp1 and HCP-1, are required for chromosome segregation in *S. cerevisiae* and *C. elegans*, respectively (Moore et al., 1999; Ortiz et al., 1999). However, there is no evidence at present that Cenp-F plays a role in chromosome alignment in human cells. Indeed, we have not observed any chromosome segregation defects or mitotic delays in cells expressing C630. In contrast, when treated with nocodazole, lines expressing C630 accumulate mitotic cells slower than controls (Fig. 3) which, taken together with our cell cycle analysis (Fig. 2), is consistent with either a delay in G2 progression or in the G2/M transition. Further evidence that Cenp-F may play a role in G2 or the G2/M transition comes from our observations that Cenp-F localises to the nuclear envelope in late G2 (Fig. 5). This localisation appears to be dependent on an intact CAAX motif and farnesyl transferase activity (Figs 6, 7). Thus, in some lines FTI-induced G2/M delays may be due to inhibition of Cenp-F function in late G2 or at the G2/M transition.

Cenp-F, a link between kinetochores and the nuclear envelope

The CAAX motif of C630 is required for its ability to efficiently localise to kinetochores and its ability to efficiently displace endogenous Cenp-F from kinetochores (Fig. 7). In addition, inhibition of farnesyl transferase activity reduces the levels of Cenp-F at kinetochores (Fig. 8). However, it has previously been reported that Cenp-F remains present at kinetochores following exposure to FTIs (Ashar et al., 2000; Crespo et al., 2001). Indeed, at high antibody concentrations we can detect Cenp-F at kinetochores in FTI-treated cells (not shown). Furthermore, FTI treatment does not completely abolish nuclear envelope localisation of Cenp-F (Fig. 8). One reason why FTIs might have only partial effects is that Cenp-F could be alternatively prenylated in the presence of FTIs. However, this is unlikely because SCH 66336 appears to block prenylation of Cenp-F completely under conditions where K-sRas is alternatively prenylated (Ashar et al., 2000). Farnesylated proteins are typically associated with membranes and although Cenp-F localises to the nuclear envelope in late G2, there is no evidence for membranes at kinetochores (McEwen et al., 1998). Although we cannot rule out the possibility that Cenp-F needs to associate first with the nuclear envelope in order to subsequently target the kinetochore, we have no evidence that C630 localises to the nuclear envelope.

Yet, C630 does localise to kinetochores (Fig. 7), suggesting that nuclear envelope localisation is not a prerequisite for kinetochore localisation. Thus, taking these observations together, we suggest that farnesylation of Cenp-F is not absolutely required for kinetochore localisation but rather increases the affinity of the protein-protein interaction(s) responsible for Cenp-F localisation.

Interestingly, two nucleoporins, hNup133 and hNup107, localise to kinetochores in mitosis (Belgareh et al., 2001). In addition, the kinetochore attachment checkpoint components Mad1 and Mad2 localise to nuclear pores in interphase (Campbell et al., 2001). While the significance of these observations is unclear it is possible that, by first localising to the nuclear envelope, Cenp-F mediates the recruitment of these proteins to kinetochores upon entry into mitosis. Therefore, a key question is how is the localisation of Cenp-F coupled with cell cycle progression? Perhaps the tight binding of Cenp-F to the nuclear matrix may be a sequestration mechanism that prevents it from interacting with the nuclear envelope in interphase. Alternatively, Cenp-F is phosphorylated in G2/M (Zhu et al., 1995a) and whereas the C-terminal 215 amino acids contain nine SP/TP motifs, the remainder of the protein (2995 amino acids) has only four. Therefore, hyperphosphorylation may expose the C-terminus, thus promoting farnesylation-mediated interactions. In addition, the rapid degradation of Cenp-F after mitosis may then liberate binding partners thus allowing them to reassociate with the nuclear envelope in G1.

Farnesylation as a signal for cell-cycle-regulated proteolysis

Ubiquitin-mediated proteolysis is required to drive cell cycle progression. In mitosis, the anaphase-promoting complex or cyclosome (APC/C), an E3 ubiquitin ligase that targets anaphase inhibitors and mitotic cyclins for degradation, is activated by two additional subunits, Cdc20 and Hct1/Cdh1, which regulate early and late mitotic events, respectively (reviewed by Morgan, 1999). Previous studies show that Cenp-F is rapidly degraded shortly after mitosis (Liao et al., 1995). While the mechanism by which Cenp-F is degraded is unknown, induction of a Cdh1 transgene in human cells leads to loss of Cenp-F protein (Sorensen et al., 2000), suggesting that Cdh1-APC/C may target Cenp-F for ubiquitin-mediated proteolysis. Consistently, Cenp-F contains several KEN sequences that target proteins for Cdh1-APC/C-mediated degradation (Pfleger and Kirschner, 2000).

Our observations also suggest that farnesyl transferase activity is required for degradation of Cenp-F after mitosis (Fig. 9). While it is possible that this is an indirect effect due to inhibition of the proteolysis machinery, we feel this is unlikely as cell cycle progression is largely unaffected following treatment of HeLa cells with SCH 66336 for up to 24 hours (D.H. and S.S.T., unpublished). Our analysis of C630 suggests that farnesylation of Cenp-F may be directly required for its degradation. The expression profile of C630, which importantly contains two KEN sequences, mirrors that of endogenous Cenp-F. C630 levels are low during G1/S, peak in G2/M and then fall after mitosis (Fig. 2). Significantly, mutation of the CAAX motif renders C630 insensitive to this cell cycle regulation (Fig. 9). Farnesylation alone is unlikely to be a direct signal for degradation as other farnesylated proteins are relatively stable (Crul et al., 2001). Therefore, perhaps only in concert with other degradation signals, such as KEN motifs, does farnesylation contribute to targeting proteins for proteolysis.

Interestingly, the cell cycle effects induced by FTIs are often not immediate. Rather there is a gradual decrease in proliferation over 5-7 days and significant cell cycle effects are typically apparent only after 2-3 days (Ashar et al., 2001; Crul et al., 2001). Thus, it is possible that the proliferation defects caused by FTIs are not due to immediate dysfunction of farnesylated proteins but rather due to the failure to degrade proteins such as Cenp-F. Finally, Cenp-F has been used as a proliferation marker both in hematopoietic and solid tumours (Landberg et al., 1996). Our observations now open up a new opportunity for using Cenp-F accumulation as a marker to determine whether FTIs effectively inhibit farnesyl transferase activity in animal models and patients.

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