Cell Cycle Analysis of the Activity, Subcellular Localization, and Subunit Composition of Human CAK (CDK-activating Kinase)

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Abstract. The activity of cyclin-dependent kinases (cdks) depends on the phosphorylation of a residue corresponding to threonine 161 in human p34^{cdc2}. One enzyme responsible for phosphorylating this critical residue has recently been purified from *Xenopus* and starfish. It was termed CAK (for cdk-activating kinase), and it was shown to contain p40^{MO15} as its catalytic subunit. In view of the cardinal role of cdks in cell cycle control, it is important to learn if and how CAK activity is regulated during the somatic cell cycle. Here, we report a molecular characterization of a human p40^{MO15} homologue and its associated CAK activity. We have cloned and sequenced a cDNA coding for human p40^{MO15}, and raised specific polyclonal and monoclonal antibodies against the corresponding protein expressed in *Escherichia coli*. These tools were then used to demonstrate that p40^{MO15} protein expression and CAK activity are constant throughout the somatic cell cycle. Gel filtration suggests that active

CAK is a multiprotein complex, and immunoprecipitation experiments identify two polypeptides of 34 and 32 kD as likely complex partners of p40^{MO15}. The association of the three proteins is near stoichiometric and invariant throughout the cell cycle. Immunocytochemistry and biochemical enucleation experiments both demonstrate that p40^{MO15} is nuclear at all stages of the cell cycle (except for mitosis, when the protein redistributes throughout the cell), although the p34^{cdc2}/cyclin B complex, one of the major purported substrates of CAK, occurs in the cytoplasm until shortly before mitosis. The absence of obvious changes in CAK activity in exponentially growing cells constitutes a surprise. It suggests that the phosphorylation state of threonine 161 in p34^{cdc2} (and the corresponding residue in other cdks) may be regulated primarily by the availability of the cdk/cyclin substrates, and by phosphatase(s).

THE cell cycle in all eukaryotes is governed by cyclin-dependent protein kinases (cdks)[1] (Pines and Hunter 1991a; Meyerson et al., 1992; Norbury and Nurse, 1992; Nigg, 1993; van den Heuvel and Harlow, 1993). These kinases are regulated by multiple mechanisms, including phosphorylation-dephosphorylation (For reviews see Fleig and Gould, 1991; Clarke and Karsenti, 1991; Solomon, 1993; Draetta, 1993), and the binding to other proteins, notably cyclin regulatory subunits (reviewed in Hunt, 1991; Sherr, 1993) and small inhibitory polypeptides (Xiong et al., 1993; Serrano et al., 1993; Gu et al., 1993; Harper et al., 1993; El-Deiry et al., 1993; Polyak et al., 1994). The complex regulation imposed on cdks is likely to reflect the fact that orderly progression through the cell cycle requires the integration of a multitude of positive and negative signals.

These are thought to include signals relayed from extracellular agents such as mitogenic hormones and growth factors, as well as signals generated by intracellular feedback mechanisms that monitor the quality of individual cell cycle events and their orderly succession (for review see Murray, 1992).

The prototypic and best characterized member of the cdk family, p34^{cdc2}, functions in association with cyclins of the A and B subfamilies to trigger entry into mitosis. Consistent with its function as an inducer of M phase, its activity increases sharply at the G2/M transition and drops abruptly as cells exit from M phase (reviewed in Murray and Kirschner, 1989; Maller, 1991; Norbury and Nurse, 1992). Several phosphorylation sites have been mapped within the p34^{cdc2} molecule (Gould and Nurse, 1989; Gould et al., 1991; Krek and Nigg, 1991a, 1992; Russo et al., 1992). In all species examined, p34^{cdc2} is phosphorylated on tyrosine 15 (Tyr15), a residue located within the ATP-binding site of the kinase, and multiple kinases (wee1, mik1) and phosphatases (cdc25, pyp3) acting on this residue have been characterized in several species (reviewed in Fleig and Gould, 1991; Labib and Nurse, 1993; Solomon, 1993). In both fission yeast and vertebrates, dephosphorylation of Tyr15 represents the rate-

1. *Abbreviations used in this paper*: cdk, cyclin-dependent protein kinase; PKA, cAMP-dependent protein kinase.

limiting step for activation of $p34^{cdc2}$ at the G2/M transition (Gould and Nurse, 1989; Krek and Nigg, 1991b; Norbury et al., 1991). It occurs in response to intracellular signalling pathways that monitor the completion of DNA replication, thereby preventing the premature initiation of mitosis (Enoch and Nurse, 1991; Smythe and Newport, 1992). The situation is somewhat different in budding yeast, where the temporal separation between DNA replication and certain aspects of mitosis is less distinct (Sorger and Murray, 1992; Amon et al., 1992). In vertebrates, additional phosphorylation of threonine 14 (Thr14) has been demonstrated (Krek and Nigg, 1991a; Norbury et al., 1991), and it is attractive to speculate that Thr14 may represent the sensor for a second checkpoint control pathway, monitoring the completion of another, as yet unidentified physiological event. Thr14 can be dephosphorylated by the same phosphatase (cdc25) that also acts on Tyr15 (Honda et al., 1993; Sebastian et al., 1993), but the kinase responsible for its phosphorylation has not yet been identified.

Not all phosphorylations occurring on $p34^{cdc2}$ lead to inactivation of the kinase. In particular, phosphorylation of Thr161 (Thr 167 in *Schizosaccharomyces pombe*) was shown to be positively required for the function of $p34^{cdc2}$: mutations of Thr161 to nonphosphorylatable residues (except Glu) inactivate $p34^{cdc2}$ protein kinase and interfere with cyclin binding (Gould et al., 1991; Ducommun et al., 1991; Solomon et al., 1992; Krek et al., 1992a). Furthermore, there is a temporal correlation between the phosphorylation of Thr161 in $p34^{cdc2}$ and the time when its cyclin partners are synthesized (Krek and Nigg, 1991a), and, conversely, dephosphorylation of Thr161 occurs concomitantly with $p34^{cdc2}$ inactivation upon exit from mitosis (Lorca et al., 1992). Phosphorylation of a corresponding residue is believed to be required for the activity of other cdks (Gu et al., 1992; Kato et al., 1994), although this has not yet been directly demonstrated for all members of the family. Also, corresponding residues have been shown to be phosphorylated in cAMP-dependent protein kinase (PKA) and in MAP kinases, and the recent x-ray crystallographic analyses of PKA (Knighton et al., 1991a, 1991b), cdk2 (De Bondt et al., 1993), and MAP-kinase (Zhang et al., 1994) provide plausible explanations for why attachment of a phosphate at this position may be critical for folding the various kinases in an active conformation (for reviews see Taylor et al., 1993; Morgan and De Bondt, 1994). Phosphorylation of this residue is considered to be essentially constitutive in the case of PKA (Taylor et al., 1993), but it clearly plays a key regulatory role in the activation of the MAP kinase cascade (reviewed in Pelech and Sanghera, 1992; Ahn et al., 1992; Nishida and Gotoh, 1993).

Recently, a kinase able to phosphorylate Thr161 in $p34^{cdc2}$ (and the corresponding residue in $p33^{cdk2}$) has been identified and termed CAK. Originally, CAK was meant to stand for cdc2-activating kinase (Solomon et al., 1992); however, as the same kinase phosphorylates also $p33^{cdk2}$ and perhaps other cdks, at least in vitro, it seems appropriate to interpret the term CAK in a broader sense (i.e., cdk-activating kinase; see also Poon et al., 1993). CAK isolated from starfish and *Xenopus* (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993) was shown to contain a 40-kD catalytic subunit that had previously been isolated under the designation MO15 (Shuttleworth et al., 1990). Highly purified active CAK from

starfish was found to contain a second polypeptide (Fesquet et al., 1993), but the identity of this potential regulatory subunit has not yet been elucidated. One of the key questions raised by the above findings is how the activity of CAK is regulated during the somatic cell cycle. To address this issue, we have cloned a cDNA for a human homologue of *Xenopus* $p40^{MO15}$, and we carried out a detailed analysis of $p40^{MO15}$/CAK expression, activity, and subcellular localization in exponentially growing human cells. Our results have implications for both the temporal and the spatial regulation of cdk activities during the cell cycle.

Materials and Methods

Cloning and Sequencing of a Human MO15 cDNA

10^6 plaque-forming units from a human placenta λ gt11 cDNA library (Clontech Laboratories, Palo Alto, CA), were screened by DNA hybridization. Plaques were transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH) that were treated with 1.5 M NaCl, 0.5 M NaOH for 5 min, then with 1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0, for 5 min, and finally with 2× SSC for 5 min (1× SSC is 0.15 M NaCl and 15 mM sodium citrate). Filters were baked at 80°C for 2 h and prehybridized at 42°C for 4 h in solution A (0.1 M Pipes, pH 7.0, 0.8 M NaCl, 0.1% N-lauroyl sarcosine, 50% formamide, 5× Denhardt's solution, and 200 μg/ml salmon sperm DNA (1× Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone). Filters were hybridized with ^{32}P-labeled MO15-specific probes (described below) at 42°C overnight in solution A, 2× Denhardt's solution, 100 μg/ml salmon sperm DNA, and 10% dextran sulfate. After washing for 2× 15 min at room temperature in 2× SSC and 0.1% SDS, and 4× 15 min at 50°C in 0.2× SSC, 0.1% SDS, filters were exposed to autoradiography film (X-OMAT; Eastman Kodak Co., Rochester, NY). Recombinant phages from positive plaques were purified and subjected to two additional rounds of purification.

A 198-bp partial human cDNA (originally designated as HsPK31), encoding a 66-amino acid polypeptide with 90.9% identity to the $p40^{MO15}$ protein kinase of *Xenopus laevis* (Shuttleworth et al., 1990), was originally isolated in the course of a PCR-based search for potential human homologues of the NIMA protein kinase, a cell cycle regulator in *Aspergillus nidulans* (Schultz and Nigg, 1993). To prepare a ^{32}P-labeled probe for hybridization, the 198-bp insert of the partial MO15 cDNA was excised from the original HsPK31 plasmid by digestion with PstI and KpnI. The fragment was gel isolated and labeled with [α-^{32}P]dATP using a random-primed DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Inserts were excised from recombinant λ phages by EcoRI digestion and subcloned into the EcoRI site of pBluescript II KS (Stratagene, La Jolla, CA). Plasmid DNAs were prepared using a Wizard Miniprep kit (Promega Corp., Madison, WI). Nested deletion templates for sequence analysis were generated using the Erase-A-Base kit (Promega Corp.). Double-stranded DNA sequencing was carried out for both strands by the dideoxynucleotide method.

For expression studies, we have also constructed an NH_2 terminally myc epitope-tagged version of $p40^{MO15}$ in the eukaryotic expression plasmid pRc/CMV (Invitrogen, San Diego, CA). The protein produced by this plasmid contains the peptide MEQKLISEEDLNMNF fused in frame to the initiator methionine of $p40^{MO15}$; thus, the anti-myc epitope mAb 9E10 (Evan et al., 1985) could be used for its detection. In vitro translation of $p40^{MO15}$ or myc epitope-tagged $p40^{MO15}$ (constructed in pBluescript; Schmidt-Zachmann and Nigg, 1993), was performed by priming the coupled transcription–translation reticulocyte lysate system (Promega Corp.) with the corresponding Bluescript plasmids, as described by the manufacturer.

Bacterial Expression of Human MO15 Protein and Production of Anti-MO15 Antibodies

A polyhistidine-tagged MO15 fusion protein was generated using the QIAexpress bacterial expression system (QIAGEN Inc., Chatsworth, CA). The plasmid pQE11-MO15 was constructed by excising a 820-bp BamHI fragment from the Bluescript plasmid containing the human MO15 cDNA and subcloned into the pQE11 vector. The expressed protein (221 residues) contains a poly-His tag fused to the 214-amino acid COOH terminus of $p40^{MO15}$. The protein was expressed in *Escherichia coli* strain M15[pREP4]

using super medium (25 g bacto-tryptone, 15 g bacto-yeast extract, and 5 g NaCl/liter). Recombinant protein was purified under denaturing conditions as described by the manufacturer (QIAGEN Inc.). Poly-His–tagged MO15 protein was finally eluted in buffer E (8 M urea, 0.1 M NaH2PO4, 10 mM Tris-HCl, pH 4.5) and stored at −20°C.

Rabbit antibodies against bacterially expressed MO15 protein were produced as follows: intramuscular injections were carried out every 4 wk with 280 µg of purified recombinant MO15 protein in buffer E emulsified in Freund's adjuvant. Complete adjuvant was used for the first injection, incomplete adjuvant for all subsequent injections. The polyhistidine-tagged MO15 fusion protein was also used to raise mAbs in mouse, as described previously (Lukas et al., 1992). The mAb MO-1.1 used throughout this study is an IgG2b.

Affinity Purification of the Rabbit Polyclonal Antibody and Preparation of Immunoglobulin Beads

For all experiments involving rabbit (R30) polyclonal anti-MO15 antibodies, these were affinity purified as described by Harlow and Lane (1988). Briefly, purified recombinant MO15 protein was subjected to polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane, and a nitrocellulose strip containing the MO15 protein was excised. After incubation with R30 anti-MO15 serum, this strip was extensively washed with PBS. Immunoglobulins were eluted with 0.1 M glycine, pH 2.5, neutralized with an equal volume of 1 M Tris-HCl, pH 8.0, and stored at 4°C. For coupling of antibodies to a solid support, affinity-purified rabbit immunoglobulins or hybridoma supernatants were incubated for 2 h at 4°C with protein A or protein G–Sepharose, respectively, equilibrated in 0.5 M Tris-HCl, pH 7.5. Immunoglobulin–protein A/G–Sepharose beads were washed with PBS and stored at 4°C.

GST-CDK2 Expression and Purification

GST-cdk2 in pGEX-2T (Tsai et al., 1991) was a generous gift from T. Hunt (Imperial Cancer Research Fund, South Mimms, U.K.). After transformation of *E. coli* strain BL21(DE3), 600-ml cultures were grown to an OD_{600} of ~1. Then, production of the recombinant GST-cdk2 protein was induced with isopropyl-β-D-thiogalactopyranoside (0.1 mM), and cells were lysed 4 h later with 35 ml of 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml aprotinin, and 2 mg/ml lysozyme at 4°C for 15 min. The lysate was sonicated and centrifuged at 10,000 g for 30 min. The supernatant was incubated for 1 h on ice with 0.5 ml glutathione–Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), equilibrated in buffer G (buffer G = PBS supplemented with 0.25 M KCl, 0.1% Tween-20, 1 mM DTT, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 10 µg/ml aprotinin). Then, the material was poured into a column, and the column was washed with 10 vol of buffer G, followed by 3 vol of PBS containing 1 mM DTT. Finally, GST-cdk2 was eluted with 5 mM reduced glutathione (Sigma Immunochemicals, St. Louis, MO) in 50 mM Tris-HCl, pH 8.0, 1 mM DTT.

Cell Culture and Metabolic Labeling

HeLa cells and the squamous carcinoma cell line UMSCC2 have been described previously (Krek and Nigg, 1991a; Palmero et al., 1993). IMR90 and WI38 diploid human lung fibroblasts were obtained from American Type Culture Collection (Rockville, MD). All cells were cultured at 37°C in a 7% CO2 atmosphere in Dulbecco's modified Eagle's medium, supplemented with 5% (for HeLa) or 10% fetal bovine serum (for other cell types) and penicillin-streptomycin (GIBCO BRL, Gaithersburg, MD). Where indicated, they were labeled with [35S]methionine/[35S]cysteine (Express35S35S; DuPont NEN, Regensdorf, Switzerland) as described previously (Krek and Nigg, 1991a).

Immunoprecipitation of Human p40MO15 and Kinase Assays

Asynchronously growing HeLa cells were lysed in radioimmunoprecipitation assay buffer containing both protease and phosphatase inhibitors (50 mM Tris-HCl, pH 8.0, 130 mM NaCl, 1% MP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF, and 10 µg/ml each of leupeptin, pepstatin, aprotinin, and 20 mM NaF, 0.3 mM Na3VO4, 20 mM β-glycerophosphate). The lysates were incubated with immunoglobulin–protein A/G–Sepharose beads (prepared as described above) for 2 h at 4°C on a rotating wheel. Immune complexes were washed four times with the above radioimmunoprecipita-

tion assay buffer, twice with PBS, and once with kinase assay buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM DTT).

Kinase assays were carried out in a total volume of 50 µl of the above assay buffer, supplemented with 4 mM ATP, 10 µCi of [γ-32P]ATP (Amersham Corp., Arlington Heights, IL), and 400 µg/ml of purified GST-cdk2 protein, as a substrate. After 30 min at 32°C, the reaction was stopped with 50 µl of 3× gel sample buffer. Phosphorylation of GST-cdk2 protein was analyzed by SDS-PAGE and autoradiography.

Gel Filtration

10^7 exponentially growing HeLa cells were homogenized in 1 ml of NP-40 buffer (1% NP-40 in 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 25 mM NaF, 1 mM PMSF, 10 µg/ml each of leupeptin, pepstatin, and aprotinin, and 30 µg/ml each of DNaseI and RNase A). After centrifugation (14,000 g for 10 min), proteins were precipitated with 70% ammonium sulfate and resuspended in 1 ml of column buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.5 mM EGTA, 10 mM MgCl2, 25 mM NaF). 200 µl of the lysate was applied to a Superose 6 FPLC column (Pharmacia Fine Chemicals). This column had been equilibrated in the above column buffer at a flow rate of 0.2 ml/min and calibrated with 200 µl of lysis buffer containing catalase (60 µg), aldolase (2 mg), ovalbumin (2 mg), and chymotrypsinogen A (0.5 mg).

Miscellaneous Techniques

HeLa cells were synchronized either by centrifugal elutriation (Draetta and Beach, 1988; Golsteyn et al., 1994) or using drug arrest-release protocols (Krek and Nigg, 1991a). The procedures for immunoblotting, indirect immunofluorescence microscopy, and enucleation have also been described previously (Krek and Nigg, 1991a, 1991b; Krek et al., 1992b). One-dimensional peptide mapping with V8 protease was carried out according to Cleveland (1983), and two-dimensional tryptic phosphopeptide mapping and phosphoamino acid analysis was conducted according to Boyle et al. (1991).

Results

Cloning of a Human MO15 cDNA and Identification of p40MO15 in Different Types of Human Cells

By screening a human placenta library with a PCR fragment isolated previously (originally termed HsPK31; Schultz and Nigg, 1993), 13 overlapping cDNAs coding for human p40MO15 were isolated. The nucleotide sequence of the longest cDNA (1,304 bp) and the corresponding translation product are shown in Fig. 1. The predicted human MO15 protein is composed of 346 amino acids, has an M_r of 39,123, and displays 86% identity with p40MO15 from *Xenopus laevis* (Fig. 1). A histidine-tagged fragment encompassing the COOH-terminal 214 amino acids of p40MO15 was expressed in *E. coli*, and the purified fusion protein was used to raise both polyclonal (rabbit) and monoclonal (mouse) antibodies. The specificity of these antibodies is illustrated in Fig. 2. As determined by immunoblotting, the R30 rabbit antibodies detected a single 40-kD protein in whole-cell extracts prepared from either human cancer-derived cell lines (HeLa and UMSCC2; Fig. 2, lanes 3 and 4) or cultures of nontransformed fibroblasts (IMR90 and WI38; Fig. 2, lanes 5 and 6). Interestingly, when comparable amounts of total protein were loaded in each lane (see the Coomassie blue staining profiles shown in Fig. 2, lanes 7–10), both cancer-derived cell lines expressed three to five times higher levels of p40MO15 than the nontransformed cell types (Fig. 2, lanes 3 and 4 vs lanes 5 and 6). The immunoreactive 40-kD protein comigrated exactly with the product obtained after in vitro translation of MO15 cDNA-derived RNA in a reticulocyte lysate (Fig. 2, lane 2). As expected, translation of a myc-

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              GGGCGGGGTTGGAGGCTTTAAGGTAGCTTTAAATTCGTGTTGTCCTGGGAGCTCGCCCTT
              TTCGGCTGGAGTCGGGCTTTACGGCGCCGGATGGCTCTGGACGTGAAGTCTCGGGCAAAG
HsMO15                                         M  A  L  D  V  K  S  R  A  K   10
XlMO15                      M  X  G  I  A  A  R  G  V  -  -  R  -  -  -  -   16
              CGTTATGAGAAGCTGGACTTCCTTGGGGAGGGACAGTTTGCCACCGTTTACAAGGCCAGA
HsMO15        R  Y  E  K  L  D  F  L  G  E  G  Q  F  A  T  V  Y  K  A  R   30
XlMO15        Q  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -   36
              GATAAGAATACCAACCAAATTGTCGCCATTAAGAAAATCAAACTTGGACATAGATCAGAA
HsMO15        D  K  N  T  N  Q  I  V  A  I  K  K  I  K  L  G  H  R  S  E   50
XlMO15        -  -  -  -  D  R  -  -  -  -  -  -  -  -  -  -  -  -  A  -   56
              GCTAAAGATGGTATAAATAGAACCGCCTTAAGAGAGATAAAATTATTACAGGAGCTAAGT
HsMO15        A  K  D  G  I  N  R  T  A  L  R  E  I  K  L  L  Q  E  L  S   70
XlMO15        -  N  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -   76
              CATCCAAATATAATTGGTCTCCTTGATGCTTTTGGACATAAATCTAATATTAGCCTTGTC
HsMO15        H  P  N  I  I  G  L  L  D  A  F  G  H  K  S  N  I  S  L  V   90
XlMO15        -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -   96
              TTTGATTTTATGGAAACTGATCTAGAGGTTATAATAAAGGATAATAGTCTTGTGCTGACA
HsMO15        F  D  F  M  E  T  D  L  E  V  I  I  K  D  N  S  L  V  L  T   110
XlMO15        -  -  -  -  -  -  -  -  -  -  -  -  T  -  -  -  -  -  116
              CCATCACACATCAAAGCCTACATGTTGATGACTCTTCAAGGATTAGAATATTTACATCAA
HsMO15        P  S  H  I  K  A  Y  M  L  M  T  L  Q  G  L  E  Y  L  H  Q   130
XlMO15        -  A  -  -  -  S  -  -  -  -  -  -  -  -  -  -  -  -  H   136
              CATTGGATCCTACATAGGGATCTGAAACCAAACAACTTGTTGCTAGATGAAAATGGAGTT
HsMO15        H  W  I  L  H  R  D  L  K  P  N  N  L  L  L  D  E  N  G  V   150
XlMO15        L  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -   156
              CTAAAACTGGCAGATTTTGGCCTGGCCAAATCTTTTGGGAGCCCCAATAGAGCTTATACA
HsMO15        L  K  L  A  D  F  G  L  A  K  S  F  G  S  P  N  R  A  Y  T   170
XlMO15        -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  I  -  -   176
              CATCAGGTTGTAACCAGGTGGTATCGGGCCCCCGAGTTACTATTTGGAGCTAGGATGTAT
HsMO15        H  Q  V  V  T  R  W  Y  R  A  P  E  L  L  F  G  A  R  M  Y   190
XlMO15        -  -  -  -  -  -  -  -  -  S  -  -  -  -  -  -  -  -  -  -   196
              GGTGTAGGTGTGGACATGTGGGCTGTTGGCTGTATATTAGCAGAGTTACTTCTAAGGGTT
HsMO15        G  V  G  V  D  M  W  A  V  G  C  I  L  A  E  L  L  L  R  V   210
XlMO15        -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -   216
              CCTTTTTTTGCCAGGAGATTCAGACCTTGATCAGCTAACAAGAATATTTGAAACTTTGGGC
HsMO15        P  F  L  P  G  D  S  D  L  D  Q  L  T  R  I  F  E  T  L  G   230
XlMO15        -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -   236
              ACACCAACTGAGGAACAGTGGCCGGACATGTGTAGTCTTCCAGATTATGTGACATTTAAG
HsMO15        T  P  T  E  E  Q  W  P  D  M  C  S  L  P  D  Y  V  T  F  K   250
XlMO15        -  -  -  -  -  -  -  -  G  -  S  -  -  -  -  -  -  A  -  -   256
              AGTTTCCCTGGAATACCTTTGCATCACATCTTCAGTGCAGCAGGAGACGACTTACTAGAT
XlMO15        S  F  P  G  I  P  L  H  H  I  F  S  A  A  G  D  D  L  L  D   270
XlMO15        -  -  -  -  T  -  -  -  L  -  -  I  -  -  -  -  -  -  X   276
              CTCATACAAGGCTTATTCTTATTTAATCCATGTGCTCGAATTACGGCCACACAGGCACTG
HsMO15        L  I  Q  G  L  F  L  F  N  P  C  A  R  I  T  A  T  Q  A  L   290
XlMO15        -  L  -  -  -  -  T  -  -  -  -  -  C  -  -  S  -  -  -   296
              AAAATGAAGTATTTCAGTAATCGGCCAGGGCCAACACCTGGATGTCAGCTGCCAAGACCA
HsMO15        K  M  K  Y  F  S  N  R  P  G  P  T  P  G  C  Q  L  P  R  P   310
XlMO15        R  X  R  -  -  -  -  -  A  -  -  -  -  N  L  -  -  -   316
              AACTGTCCAGTGGAAACCTTAAAGGAGCAATCAAATCCAGCTTTGGCAATAAAAAGGAAA
HsMO15        N  C  P  V  E  T  L  K  E  Q  S  N  P  A  L  A  I  K  R  K   330
XlMO15        -  -  S  I  -  A  -  -  -  -  Q  -  L  N  -  G  -  -  -  -   336
              AGAACAGAGGCCTTAGAACAAGGAGGATTGCCCAAGAAACTAATTTTTTAAAGAGAACAC
HsMO15        R  T  E  A  L  E  Q  G  G  L  P  K  K  L  I  F  *           346
XlMO15        -  -  -  G  M  D  -  K  D  I  A  -  -  -  S  -  *         352
              TGGACAACATTTTACTACTGAGGGAAATAGCCAAAAAGGCAAATAATGGAAAAATAGTAA
              ACATTAAGTAAATGCTGTAGAAGTGAGTTTGTAAATATTCTACACATGTAAAATATGTAA
              AACTATGGGTTATTTTTATTAAATGTATTTTAAAAAAAAAAAA
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Figure 1. cDNA and predicted amino acid sequences of human p40[MO15]. The human MO15 cDNA contains 1304 nucleotides. The longest open reading frame (nucleotides 91–1229) codes for a protein of 346 amino acids; the presence of an inframe stop codon upstream of the first ATG confirms that this open reading frame comprises the amino terminus of the MO15 protein. For comparison, the p40[MO15] protein sequence from *Xenopus laevis* (Shuttleworth et al., 1990) is shown beneath the human sequence. Note that no gaps were introduced in this alignment. Amino acids are indicated (in bold) only at positions where they diverge; identical residues are marked by dashes, and stop codons are indicated by stars. The human MO15 sequence has been submitted to the EMBL database (accession number X79193). Also, we note that complete sequences for human (L20320) and mouse (X74145) MO15 have been deposited independently by other workers.

tagged MO15 RNA yielded a slightly larger product of 42 kD (lane *1*). Several mAbs, e.g., MO-1.1 (Fig. 2, lane *II*), were also found to be highly specific for the 40-kD MO15 protein.

Expression of p40[MO15] Is Invariant throughout the Somatic Cell Cycle

To determine the steady-state levels of p40[MO15] during the cell cycle, HeLa cells were synchronized by centrifugal elutriation (Fig. 3). Aliquots of each sample, containing equal amounts of total protein, were then analyzed by immunoblotting using antibodies specific for p40[MO15]. For control, the same samples were also probed with anti-cyclin A and anti-PSTAIRE antibodies (a convenient tool to detect p34[cdc2] and other cdk family members; Yamashita et al., 1991), and a population of exponentially growing cells was

KD
97.4 -
66.2 -
45.0 -
31.0 -

1 2 3 4 5 6 7 8 9 10 11

α M015 -
α cycA -
α PSTAIRE -

Exp 1 2 3 4 5 6

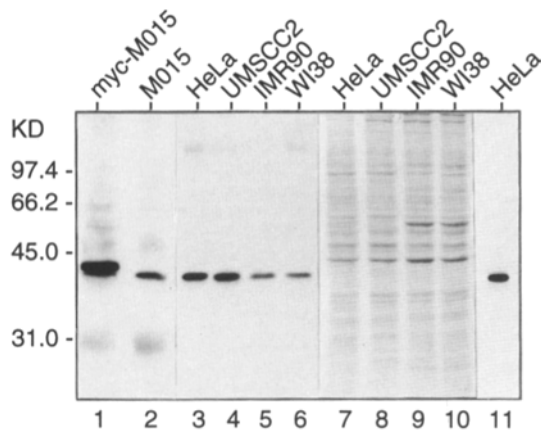|	Exp	1	2	3	4	5	6
% G1	77	99	96	93	56	34	19
% S	10	-	4	6	28	24	18
% G2 / M	13	-	-	1	17	42	63

Figure 2. Specificity of anti-MO15 antibodies and identification of p40^MO15 in different human cell types. Total cell lysates were prepared from exponentially growing HeLa and UMSCC2 carcinoma cells, as well as from two different types of diploid lung fibroblasts (IMR90 and WI38). Proteins were resolved on a 10% SDS-polyacrylamide gel, and were either stained with Coomassie blue (lanes 7–10) or transferred to nitrocellulose and probed with R30 anti-MO15 antibodies (lanes 3–6) or the monoclonal mouse antibody MO-1.1 (lane 11). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL), using appropriate secondary antibodies coupled to horseradish peroxidase. For comparison, plasmids encoding either full-length MO15 protein (lane 2) or a myc-epitope–tagged p40^MO15 (lane 1) were subjected to coupled in vitro transcription-translation, and the products of these reactions were analyzed in parallel, using the rabbit antibody for detection. Note that the only immunoreactive protein present in total cell lysates comigrated exactly with in vitro–translated p40^MO15; as expected, however, translation of the myc-epitope–tagged p40^MO15 yielded a slightly larger product (lane 1). The positions of molecular mass markers are indicated in kilodaltons.
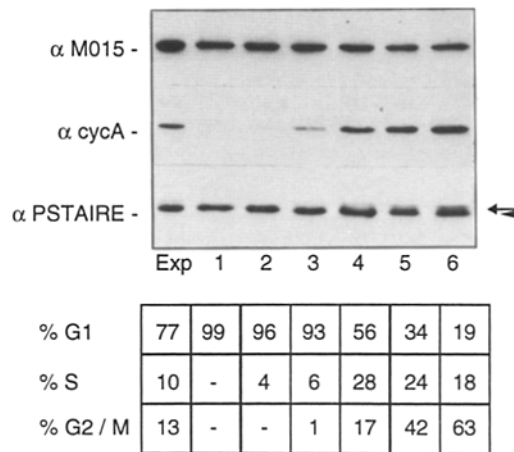
Figure 3. Steady-state levels of p40^MO15 are constant throughout the somatic cell cycle. HeLa cells were sorted by centrifugal elutriation, exactly as described previously (Golsteyn et al., 1994). For each cell population, one aliquot was used to determine its cell cycle position by FACS® analysis. Equal amounts of proteins were then resolved by gel electrophoresis and transferred to nitrocellulose. The same filter was probed with R30 anti-MO15 antibodies (top panel), followed by antibodies against cyclin A (Maridor et al., 1993; middle panel) and p34^cdc2 (anti-PSTAIRE; Yamashita et al., 1991; bottom panel). Note the typical appearance of differentially phosphorylated p34^cdc2 proteins in the lanes containing G2 phase cells (arrows); also, longer exposures revealed the expected reactivity of anti-PSTAIRE antibodies with p33^cdk2 (not visible in this figure). The lane marked Exp represents an extract prepared from exponentially growing cells; lanes 1–6 represent cells at progressive stages of the cell cycle. Below each lane, the contribution of G1, S, and G2/M phase cells is indicated in percentages.

analyzed in parallel (lane marked *Exp*). For each sample, a FACS® analysis (Becton Dickinson Immunocytometry Systems, Mountain View, CA) was carried out to determine the percentages of cells in G1, S, or G2/M phases (Fig. 3). Immunoblotting with R30 anti-MO15 antibodies revealed that the level of p40^MO15 stayed constant throughout the cell cycle (Fig. 3, *top panel*; see also Fig. 5 *A*). The control experiments yielded the expected results: cyclin A (Fig. 3, *middle panel*) was virtually undetectable during the G1 phase (Fig. 3, lanes *1* and *2*), but it accumulated as cells progressed towards S and G2 phases (Fig. 3, lanes *3–6*); in contrast, the PSTAIRE-positive signal (Fig. 3, *bottom panel*) was essentially invariant throughout the cell cycle, except for the appearance of a slower migrating PSTAIRE protein in S- and G2/M-phase cells, which is characteristic of tyrosine-phosphorylated p34^cdc2 (Solomon et al., 1992).

p40^MO15-associated CAK Activity is Constant throughout the Cell Cycle

To assay for CAK activity associated with p40^MO15, the protein was immunoprecipitated from asynchronous HeLa cells, using either mAb MO-1.1 or the R30 rabbit anti-MO15 antibody. After extensive washing, the immunoprecipitates were incubated with [γ-^{32}P]ATP and bacterially expressed GST-cdk2 as a substrate, as described by Poon et al. (1993). As shown in Fig. 4 *A*, GST-cdk2 was readily phosphorylated

when incubated with either type of MO15 immunoprecipitate (Fig. 4, *A*, lanes *2* and *3*), but not when exposed to mock immunoprecipitates carried out with protein G– or protein A–Sepharose beads, respectively (Fig. 4, *A*, lanes *1* and *4*). Tryptic phosphopeptide mapping and phosphoaminoacid analyses showed that GST-cdk2 was phosphorylated exclusively on threonine and on a single tryptic peptide (Fig. 4 *B*). These results are in full agreement with previous data showing that phosphorylation of GST-cdk2 by *Xenopus* CAK occurs exclusively on threonine 160 (Poon et al., 1993).

Using the above assay, we next investigated p40^MO15-associated CAK activity in HeLa cells that had been synchronized at various stages of the cell cycle. Cells were either arrested in mitosis using nocodazole, or at the G1/S boundary using aphidicoline, as described previously (Krek and Nigg, 1991a). Then, they were released from these blocks for various lengths of time, and p40^MO15 was immunoprecipitated. For each sample, the amount of p40^MO15 recovered was determined by immunoblotting, and p40^MO15-associated CAK activity was assayed as described above. In parallel, aliquots of cells were used for FACS® analyses to determine the proportions of cells at different stages of the cell cycle. In a first experiment, cells were released for comparatively short time intervals from a nocodazole-induced block in prometaphase (Fig. 5 *A*). Under these conditions, cells inactivate mitotic cdc2 kinase through destruction of B-type cyclins and complete mitosis; indeed, as assessed by microscopic monitoring of cell spreading to the substratum,
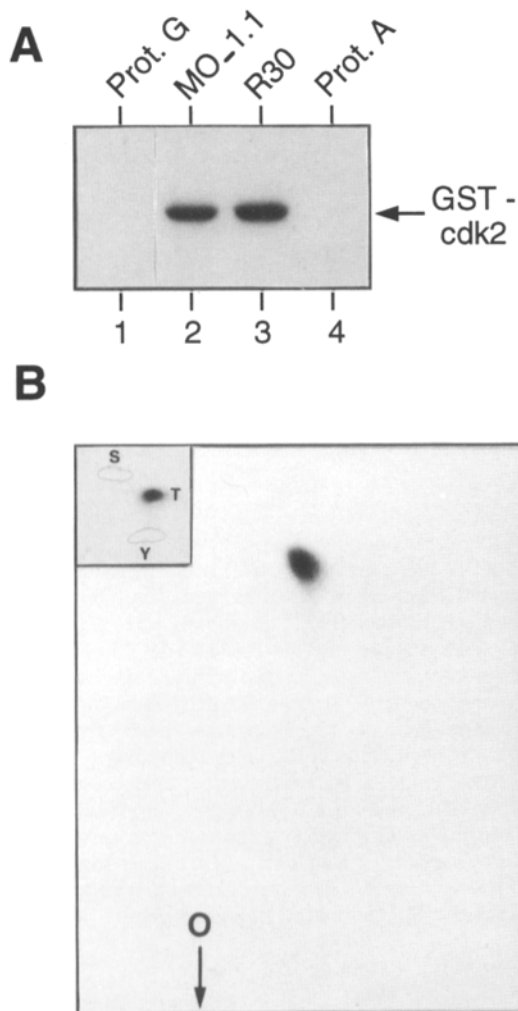
Nocodazole release

	% G1									
% G1	8/4	59/72	57/78	29/31	10/35	51/35	42/4	15/8	64/58	79/38
% S	13/17	15/5	34/18	60/30	38/10	36/46	55/46	74/9	25/14	17/33
% G2/M	79/79	26/23	9/14	11/41	52/55	13/19	3/50	11/83	11/28	4/29

Figure 5. p40[MO15]-associated CAK activity is constant throughout the cell cycle. (*A*) HeLa cells were arrested in prometaphase with nocodazole. Before release from the nocodazole block (0) or at the indicated time intervals after the release, p40[MO15] was immunoprecipitated from equal numbers of cells, using mAb MO-1.1. Each sample was assayed for CAK activity, using GST-cdk2 as a substrate (*upper part*), and recovery of p40[MO15] was controlled by immunoblotting using the R30 antibody (*lower part*). (*B*) HeLa cells were arrested either in prometaphase with nocodazole, or at the G1/S boundary with aphidicolin, exactly as described previously (Krek and Nigg, 1991a). Before release from these blocks, or at the indicated time intervals after the release, p40[MO15] was immunoprecipitated and its recovery and associated CAK activity were determined as described above (*A*). In parallel, aliquots of each sample were subjected to FACS® analyses. The specific CAK activities (i.e., phosphate incorporation into GST-cdk2, relative to the amounts p40[MO15] protein present in the immune complexes) were then determined by densitometric scanning of the data, and results from two independent experiments are shown in the form of a histogram. Below, the results of the FACS® analyses are summarized: numbers to the left refer to experiment 1 (*hatched bars*), numbers to the right to experiment 2 (*open bars*).

Figure 4. Human p40[MO15]/CAK phosphorylates GST-cdk2 on a single threonine residue. (*A*) p40[MO15] was immunoprecipitated from exponentially growing HeLa cells, using either mAb MO-1.1 (lane *2*) or the rabbit polyclonal antibody R30 (lane *3*). For control, mock immunoprecipitations were carried out using protein G beads (lane *1*) or protein A beads (lane *4*). Washed immune complexes were then assayed for kinase activity using GST-cdk2 as a substrate, as described previously (Poon et al., 1993). (*B*) Phosphorylated GST-cdk2 protein (obtained as described in *A*) was subjected to two-dimensional tryptic phosphopeptide mapping and phosphoamino acid analysis (Boyle et al., 1991). O marks the site of sample application, and the inset shows the result of the phosphoamino acid analysis (*S*, phosphoserine; *T*, phosphothreonine; *Y*, phosphotyrosine).

Figure 6. Gel filtration suggests the existence of a multiprotein CAK complex. A lysate (200 μl) was prepared from exponentially growing HeLa cells (see Materials and Methods) and applied to a Superose 6 FPLC column (Pharmacia Fine Chemicals). 500-μl fractions were collected, and 120 μl of each fraction were used to determine the abundance of p40[MO15], using mAb MO-1.1 for immunoblotting (*upper panel*). The remainder of each sample was subjected to immunoprecipitation with MO-1.1, and CAK activity was monitored with GST-cdk2 as a substrate (*lower panel*). Vertical bars mark the elution of molecular mass markers, catalase (232 kD), aldolase (158 kD), ovalbumin (43 kD), and chymotrypsinogen A (25 kD), used for calibration of the column.

daughter cells reentered G1 phase by ~4–6 h (data not shown). Fig. 5 *A* illustrates that the level of p40[MO15], as well as its associated CAK activity, remained virtually constant through mitosis and reentry into the subsequent G1 phase. Fig. 5, *B* and *C*, summarize the results obtained when similar drug-arrest/release experiments, using aphidicolin and nocodazole, respectively, were carried out over the entire cell cycle. Again, the specific activity of p40[MO15]-associated CAK was constant at all stages of the cell cycle. Yet, previous studies based on a virtually identical experimental approach had revealed striking cell cycle-dependent changes in the ac-
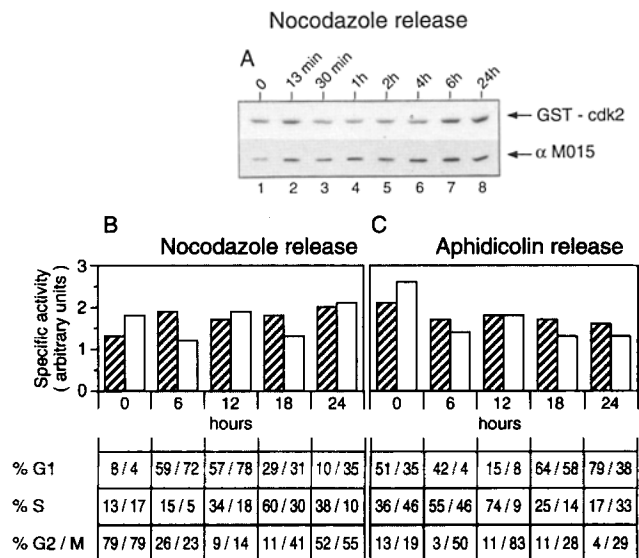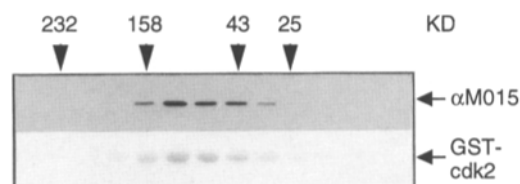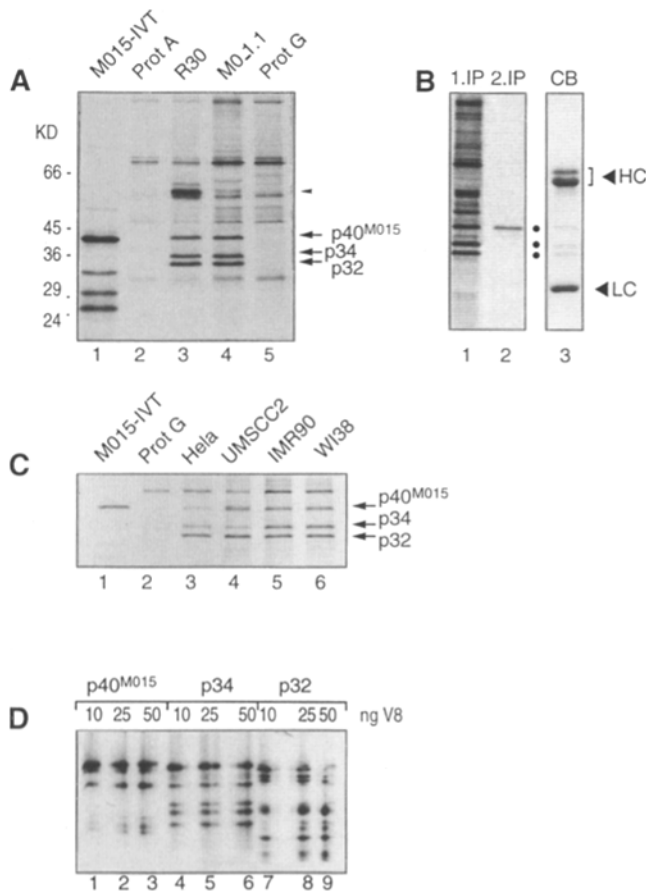
Figure 7. Identification of putative complex partners of p40^{MO15}. (*A*) Exponentially growing HeLa cells were labeled for 16 h with [^{35}S]methionine/cysteine (50 μCi/ml), and then p40^{MO15} was immunoprecipitated using either rabbit antibody R30 (lane *3*) or mAb MO-1.1 (lane *4*). Note that two proteins of 34 and 32 kD (p34 and p32) coimmunoprecipitate specifically with p40^{MO15} (*arrows*); a third protein of 57 kD (*arrowhead*) was also coprecipitated, but recovery of this protein was variable. For controls, the migration of in vitro–translated p40^{MO15} is shown in lane *1*, and the extent of nonspecific protein binding to beads is illustrated by mock immunoprecipitations using protein A–Sepharose (lane *2*) and protein G Sepharose (lane *5*). The positions of molecular mass markers are indicated in kilodaltons. (*B*, lanes *1* and *2*) An immune complex of p40^{MO15} obtained with MO-1.1 antibodies (lane *1*) was boiled for 7 min in 100 μl of 50 mM Tris-HCl, pH 8.0, 1% SDS, and 1 mM DTT. RIPA buffer conditions were then reconstituted by appropriate addition of detergents and salts to a final volume of 1 ml, and the sample was subjected to a second immunoprecipitation with MO-1.1 antibodies (lane *2*). Note that boiling disrupted the complex between p40^{MO15} and its putative partners. (Lane *3*) Coomassie blue staining of an immunoprecipitate obtained with mAb MO-1.1 from ∼4 × 10^6 cells. Note that p40^{MO15}, p34, and p32 are recovered in a ratio of ∼1:1:1, and that the identity of p40^{MO15} was confirmed by microsequencing (data not shown). Arrowheads indicate immunoglobulin heavy (*HC*) and light chains (*LC*). (*C*) p40^{MO15} immunoprecipitations were carried out from ^{35}S-labeled HeLa (lane *3*), UMSCC2 (lane *4*), IMR90 (lane *5*), and WI38 cells (lane *6*), as described above (*A*). For control, the migration of in vitro–translated p40^{MO15} is shown in lane *1*, and a HeLa cell lysate was treated with protein G Sepharose only (lane *2*). To facilitate the comparison of complexes between p40^{MO15} and p34/p32 in the different cell types, sample loadings for transformed and untransformed cells were adjusted to yield comparable levels of p40^{MO15}. (*D*) p40^{MO15}, p34, and p32 were subjected to one-dimensional

peptide mapping (Cleveland, 1983) using increasing amounts of V8 protease as indicated. Digestion products were analyzed by electrophoresis on a 15% SDS-polyacrylamide gel.

tivity of p34^{cdc2} (Krek and Nigg, 1991*a*). We are forced to conclude from these results that progression through the somatic cell cycle is not accompanied by any major changes in p40^{MO15}-associated CAK activity, at least as assayed in immunoprecipitates (see Discussion). In this context, we note also that p40^{MO15} is a phosphoprotein in vivo (Tassan, J.-P., and E. A. Nigg, unpublished results). Yet, CAK activity associated with p40^{MO15} immunoprecipitates was unaffected by the presence or absence of phosphatase inhibitors in lysis and washing buffers (data not shown).

Identification of Two Candidate Complex Partners of p40^{MO15}

In starfish and *Xenopus* eggs, p40^{MO15} appears to associate with other proteins to form active CAK (Fesquet et al., 1993; Poon et al., 1993). To determine whether p40^{MO15} forms complexes with other proteins in somatic cells, a gel filtration experiment was carried out (Fig. 6). HeLa cells were lysed in the presence of NP-40 detergent, and proteins were size fractionated on a Superose 6 column. Then, each fraction was subjected to immunoprecipitation with anti-MO15 antibodies, and immunoprecipitates were probed by immunoblotting for the abundance of p40^{MO15} (Fig. 6, *upper panel*). In parallel, they were assayed for CAK activity, using GST-cdk2 as a substrate (Fig. 6, *lower panel*). This experiment shows that the bulk of p40^{MO15} did not occur as a monomer but, instead, migrated in complexes with an apparent molecular mass of ∼60–120 kD. Interestingly, the CAK activity of each fraction paralleled the abundance of p40^{MO15}, indicating that, at least under the lysis conditions used here, the bulk of the p40^{MO15}-associated kinase was recovered in an active state.

To search for cellular proteins that might specifically associate with p40^{MO15}, immunoprecipitation experiments were carried out. As shown in Fig. 7 *A*, both polyclonal (lane *3*) and monoclonal (lane *4*) anti-MO15 antibodies specifically precipitated two polypeptides with molecular masses of 34 and 32 kD, in addition to p40^{MO15}. A third protein of ∼57 kD was also precipitated, particularly when using the rabbit anti-MO15 antibodies (Fig. 7 *A*, lane *3*; *arrowhead*); however, recovery of this 57-kD protein was variable (Fig. 7, *A* vs *B*), and its identity has not been further investigated. Importantly, none of these proteins was precipitated by protein A- or protein G–Sepharose (Fig. 7 *A*, lanes *2* and *5*). Also, when immune complexes were boiled and then subjected to a second immunoprecipitation with anti-MO15 antibodies, only p40^{MO15} was recovered (Fig. 7 *B*, lane *2* vs lane *1*). This indicates that none of the coprecipitating proteins were recognized directly by anti-MO15 antibodies.

As indicated by peptide mapping with V8 protease, the 34- and 32-kD proteins appear to be structurally distinct from each other, as well as from p40^{MO15} (Fig. 7 *D*). Also, the 34/32-kD proteins were not recognized by anti-PSTAIRE antibodies, indicating that they are not identical with p34^{cdc2} or p33^{cdk2} (data not shown), a conclusion supported further by microsequence analysis (Tassan, J.-P., S. Frutiger, G. J. Hughes, and E. A. Nigg, unpublished results). Complexes

65	39	5	1	% G1
4	60	58	5	% S
31	1	37	94	% G2 / M
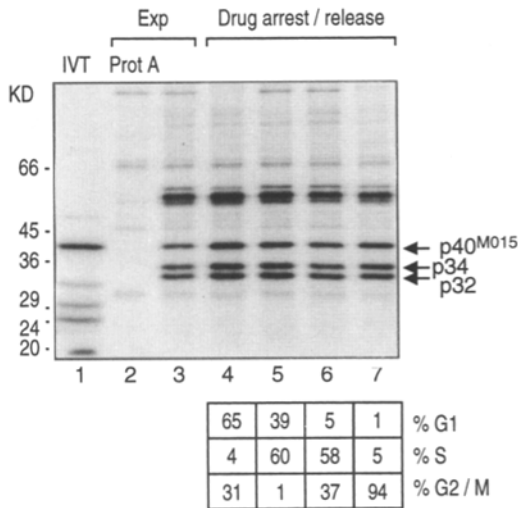
Figure 8. Association of p40^MO15^ with p34/p32 is constant during the cell cycle. Immunoprecipitations with rabbit R30 anti-MO15 antibodies were carried out from HeLa cells that had been labeled for 4 h with [³⁵S]methionine/cysteine (100 μCi/ml). Lysates were prepared from cells representing progressive stages of the cell cycle (Krek and Nigg, 1991a). Lanes 2 and 3, Exponentially growing cells (lane 2 shows a protein A–Sepharose mock immunoprecipitation for control); lane 4, cells predominantly in G1 phase (5 h after a release from a nocodazole block in prometaphase); lane 5, cells at G1/S phase (arrested with aphidicolin); lane 6, cells in S and G2 phase (5 h after release from the aphidicolin block); lane 7, cells in M phase (arrested with nocodazole). The contribution of G1, S, and G2/M phase cells to each population was determined by FACS® analysis (using duplicate plates), and the results are indicated below each lane.

between p40^MO15^ and the 34/32-kD polypeptides could be detected, not only in HeLa cells, but also in UMSCC2 carcinoma cells, and in two different types of nonimmortalized human fibroblasts (Fig. 7 C). Remarkably, p40^MO15^ and its putative 34/32-kD complex partners could be stained readily

by Coomassie blue (Fig. 7 B, lane 3), indicating that all three proteins coimmunoprecipitate in similarly large amounts and may, thus, form a near-stoichiometric complex.

To determine whether p40^MO15^ might undergo cell cycle–dependent complex formation, immunoprecipitation experiments were carried out from HeLa cells that had been synchronized by drug-arrest/release protocols and pulse labeled with [³⁵S]methionine/cysteine at different stages of the cell cycle (Krek and Nigg, 1991a). As shown in Fig. 8, no significant cell cycle-dependent changes could be detected in either the accumulated levels of p40^MO15^ or its association with the 34/32-kD proteins.

Human p40^MO15^ Protein Localizes to the Cell Nucleus

Indirect immunofluorescence microscopy was used to examined the subcellular localization of p40^MO15^ (Fig. 9). After incubation with R30 anti-MO15 antibodies, interphase HeLa cells displayed a strong staining of nuclei, with negligible staining of the cytoplasm (Fig. 9, a, f, and g). Uniform results were obtained for all cells in exponentially growing cultures, indicating that p40^MO15^ does not undergo any significant redistributions during interphase of the cell cycle. In mitotic cells, p40^MO15^ was diffusely distributed, with no evidence for a specific association with either condensed chromosomes or spindle microtubules (Fig. 9, c and f). Virtually identical results were obtained when analyzing nonimmortalized IMR90 human fibroblasts (Fig. 9 h) or when using monoclonal antibodies for detection of p40^MO15^ (Fig. 9 f). Also, results were indistinguishable regardless of whether cells were fixed and permeabilized using paraformaldehyde detergent (Fig. 9, a, g, and h) or organic solvents (Fig. 9 f). Finally, when HeLa were transfected with a cDNA encoding a myc epitope–tagged version of p40^MO15^, anti–myc epitope antibodies also stained the nuclei of transfected cells, indicating that the exogenously expressed MO15 protein localized to the same compartment as endogenous p40^MO15^ (Fig. 9 i).
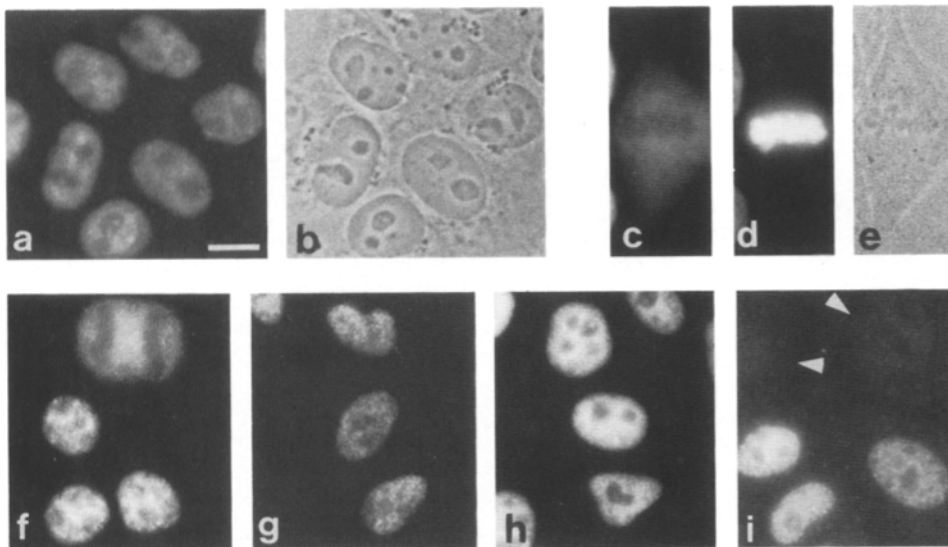

Figure 9. Immunofluorescent localization of p40^MO15^. Endogenous p40^MO15^ was visualized by indirect immunofluorescence microscopy, using either R30 antibodies (a, c, f, and g) or mAb MO-1.1 (h). Both HeLa (a–e, f, and h) and IMR90 cells (g) were examined. Fixation-permeabilization was achieved using either paraformaldehyde–Triton X-100 (a–e and g–i) or methanol/acetone (f), with virtually identical results. Finally, HeLa cells were transiently transfected with a pCMV plasmid coding for a myc-epitope-tagged p40^MO15^, and the subcellular localization of the ectopically expressed protein was visualized using the anti-myc tag mAb 9E10 (i; arrowheads indicate untransfected cells). Note the exclusive nuclear localization of p40^MO15^ in interphase cells (a vs b; phase contrast). In mitotic cells (c–e), p40^MO15^ was diffusely distributed and showed no obvious association with condensed chromosomes (d; DNA staining with Hoechst 33258; e; phase contrast). Bar in a, 10 μm.
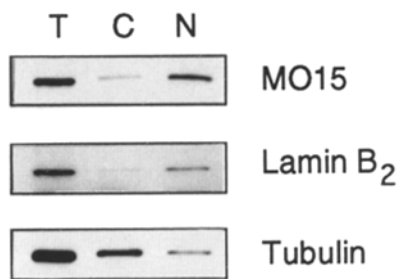
T C N

MO15

Lamin B$_2$

Tubulin

Figure 10. Localization of p40^MO15 by subcellular fractionation. Exponentially growing HeLa cells were enucleated as described previously for chicken DU249 cells (Krek et al., 1992*b*; Hennekes et al., 1993). Nuclear (*N*) and cytoplasmic (*C*) fractions, as well as total cellular lysates (*T*), were then analyzed by immunoblotting using antibodies against p40^MO15. In parallel, appropriate antibodies were used to monitor the partitioning of lamin B$_2$ (a marker for the nucleus) and tubulin (a marker for the cytoplasm).

To corroborate these immunocytochemical results by independent biochemical evidence, enucleation experiments were carried out. HeLa cells were grown on round coverslips and then fractionated by centrifugation in the presence of cytochalasin D (Krek et al., 1992*b*; Hennekes et al., 1993). This procedure yields nuclei with some contamination by cytoplasm, and cytoplasts with a minimal contamination by intact cells (Gordon et al., 1981; Bauerle and Baltimore, 1988). After separation of nuclei and cytoplasts, the partitioning of p40^MO15 was examined by immunoblotting. For control, the distributions of a nuclear marker (lamin B$_2$) and a cytoplasmic marker (tubulin) were assayed in parallel. As shown in Fig. 10 (*upper panel*), p40^MO15 was recovered almost exclusively from the nuclear fraction; the minimal amount of p40^MO15 detectable in cytoplasts (9%) can readily be attributed to contamination by intact cells because a similar proportion (7%) of nuclear lamin was also detectable in the cytoplasts (Fig. 10, *middle panel*). Conversely, tubulin was present predominantly in the cytoplasts, as expected (Fig. 10, *lower panel*). Based on the results of both immunocytochemistry and biochemical analysis of fractionated cells, we thus conclude that at all interphase stages of the cell cycle p40^MO15 is a predominantly nuclear protein.

Discussion

The p34^cdc2 protein kinase and structurally related cdks play a pivotal role in promoting cell cycle progression in all eukaryotes. In addition to cell cycle–dependent changes in the subunit composition of cdk/cyclin complexes, the reversible phosphorylation of p34^cdc2 and the other cdks has been recognized as a major mechanism for regulating their activities (for references see Introduction). Since the phosphorylation state of cdks appears to be controlled by external signals and/or internal checkpoint controls, it is important to identify the kinases and phosphatases acting on cdks, and to determine how their activities are integrated with intracellular signal transduction pathways. Important progress in this field has been made recently with the identification of p40^MO15 as the catalytic subunit of CAK, the enzyme responsible for phosphorylating p34^cdc2 on Thr161 (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993). This enzyme is also able to phosphorylate a corresponding residue in p33^cdk2 and possibly in other cdks, but its precise target range has not yet been determined (for reviews see Draetta, 1993; Solomon, 1994).

In this study, we describe a molecular characterization and cell cycle analysis of human p40^MO15/CAK. We have cloned, from a human placenta library, a cDNA coding for a 40-kD protein that displays 86% sequence identity to p40^MO15 from *Xenopus laevis*. Independently, partial cDNAs for human and mouse p40^MO15 have been isolated in other laboratories (Ershler et al., 1993; Cance et al., 1993). Using a CAK assay described by Poon et al. (1993), we further show that antibodies raised against bacterially expressed human p40^MO15 readily immunoprecipitate CAK activity from cultured human cells. From these results, we conclude that we have isolated a functional homologue of the *Xenopus* MO15 gene. Highly specific poly- and monoclonal antibodies were then used to study the expression and subcellular localization of p40^MO15, as well as the activity and subunit composition of CAK, during the human somatic cell cycle.

Perhaps the most unexpected result of the present study is that no cell cycle–dependent changes could be detected in either the expression of MO15 protein or its associated CAK activity. Previous studies had failed to reveal any significant variations in CAK activity during the early embryonic development of *Xenopus laevis* (Solomon et al., 1992; Poon et al., 1993). However, because cell growth is uncoupled from cell division in these early cleavage embryos, the embryonic cell cycles lack bonafide G1 and G2 phases, and at least some checkpoint controls are not operational (for review see Murray and Hunt, 1993). It was clearly important, therefore, to study the expression and regulation of CAK activity during progression through a somatic cell cycle. On the basis of the results obtained here and results obtained independently by Poon and colleagues (1994), it is difficult to escape the conclusion that CAK activity is constant not only in early embryonic cell cycles, but also in exponentially growing somatic cells. This conclusion, however, is subject to one important caveat: it is virtually impossible to exclude the possibility that p40^MO15 might be regulated within living cells by rapidly reversible posttranslational modifications, or by labile interactions with other proteins. Such modifications or weakly interacting proteins might be lost during immunoprecipitation of p40^MO15, and changes in CAK activity might thus go undetected in standard in vitro assays. In spite of this caveat, however, the available evidence suggests that the phosphorylation state of Thr161 in p34^cdc2 (and of the corresponding residues in other cdks) may be regulated primarily, and perhaps exclusively, at the level of the substrate and/or the corresponding phosphatase(s). This implies that cdks are likely to be phosphorylated by a constitutively active CAK as soon as they become available in a suitable conformation.

We emphasize that our study focused on CAK activity in exponentially growing cells. We have not described results on CAK expression or activity in Go arrested cells, but Poon et al. (1994) recently found that p40^MO15 is absent from serum-starved mouse 3T3 cells. This result is not unexpected, and it falls in line with the observation that cdk-cyclin complexes, the targets of CAK, are themselves absent from Go-arrested cells (e.g., Lee et al., 1988; Matsushime et al., 1994). Also, we emphasize that the results described here do not exclude that CAK may be responsive to external growth regulatory signals, or to internal checkpoint controls moni-

toring the quality of cell cycle progression. However, they do strongly indicate that CAK activity is not subject to regulation as part of a program intrinsic to every cell cycle.

Immunofluorescence microscopy, as well as biochemical fractionation experiments, unequivocally demonstrate that $p40^{MO15}$ is nuclear throughout interphase of the cell cycle. This result also constitutes a surprise. Although several potential targets of CAK do occur within the nucleus, e.g., the complexes between cdks and cyclin A (Pines and Hunter, 1991b; Girard et al., 1991; Pagano et al., 1992; Zindy et al., 1992; Maridor et al., 1993), cyclin D1 (Baldin et al., 1993), or cyclin E (Lehner, C. F., personal communication), the virtually exclusive nuclear localization of $p40^{MO15}$ is difficult to reconcile with the cytoplasmic localization of some of the purported CAK substrates, notably the $p34^{cdc2}$/cyclin B complexes (Pines and Hunter, 1991b; Gallant and Nigg, 1992; Ookata et al., 1992). These complexes are presumed to be phosphorylated in the cytoplasm, before they translocate to the nucleus at the onset of mitosis. To resolve this apparent paradox, one has to argue that either cytoplasmic cdk/cyclin complexes are substrates for CAKs containing as yet unidentified catalytic subunits distinct from $p40^{MO15}$, or, alternatively, that some of the protein complexes must cross the nuclear envelope. Considering that nucleocytoplasmic interactions are much more dynamic than suggested by the analyses of steady-state protein distributions (for discussion see Nigg, 1992), it is possible that $p40^{MO15}$/CAK or $p34^{cdc2}$/cyclin B complexes, or both, might shuttle between nucleus and cytoplasm (Schmidt-Zachmann et al., 1993). Furthermore, considering that $p34^{cdc2}$ occurs in excess over cyclins, it is attractive to speculate that $p34^{cdc2}$ protein might be phosphorylated by CAK within the nucleus, and then be captured by a B-type cyclin after its diffusion to the cytoplasm. It appears doubtful that free monomeric $p34^{cd2}$ could efficiently be phosphorylated by CAK (Solomon, 1994), but one could invoke a model in which a chaperone-like protein might bind to nuclear $p34^{cdc2}$, thereby conferring a conformation appropriate for the phosphorylation of Thr161. The fact that a GST-cdk2 fusion protein can readily serve as a substrate for CAK (Poon et al., 1993, and this study) illustrates that cdk phosphorylation can occur in the absence of cyclin binding. Perhaps the GST fusion moiety confers to cdk2 a conformation that would normally result from the binding of a cyclin or a (hypothetical) chaperone.

Previous work strongly suggests that the starfish oocyte MO15 protein may form a stoichiometric complex with a second, somewhat larger polypeptide (Fesquet et al., 1993). Although no information has yet been reported on the molecular identity of this putative regulatory subunit, there is additional evidence to support the idea that CAK may be a multiprotein complex. Gel filtration of starfish and Xenopus oocyte extracts revealed two complexes containing MO15 protein, only one of which, migrating at 180–220 kD, displayed the bulk of the CAK activity (Solomon et al., 1992; Poon et al., 1993). Also, bacterially expressed Xenopus MO15 protein was reported to be inactive unless it was incubated with cellular extracts, suggesting that either binding of another protein or a posttranslational modification of $p40^{MO15}$ was required for kinase activation (Poon et al., 1993). Given the structural similarity between $p40^{MO15}$ and cdks (Shuttleworth et al., 1990), it is tempting to speculate that $p40^{MO15}$ might require a cyclin-like partner. It is per-

haps significant, therefore, that the closest relative of $p40^{MO15}$ in S. cerevisiae, the product of the KIN28 gene, has recently been shown to associate with a cyclin-like protein (Valay et al., 1993). However, as yet, there is no direct evidence for a functional homology between the KIN28 and the MO15 gene products.

In this study, we provide evidence for the existence of a CAK multiprotein complex in somatic human cells. As determined by fractionation on a Superose 6 column, both $p40^{MO15}$ protein and CAK activity eluted as a broad peak with an apparent molecular mass of ~60–120 kD. In direct support of a multiprotein CAK complex, immunoprecipitation of $p40^{MO15}$ from human cells allowed the identification of two potential complex partners. Using two different antibody preparations, two proteins of 34 and 32 kD were found to coimmunoprecipitate invariably with $p40^{MO15}$ and CAK activity from all cell types analyzed and at all stages of the cell cycle. Binding of these proteins to $p40^{MO15}$ appeared to be of high affinity because the complexes remained intact in the presence of mixed detergents (1% NP-40, 0.5% deoxycholate, 0.1% SDS; see Fig. 7), as well as in high ionic strength solutions (2 M NaCl; Tassan, J.-P., and E. A. Nigg, unpublished results). Furthermore, all three coimmunoprecipitated proteins could readily be visualized in similar quantities by Coomassie blue staining, indicating that they may form a heterotrimeric complex. Alternatively, it is possible that our antibodies immunoprecipitate a mixture of several complexes; one such complex might then contain $p40^{MO15}$ and only the 34-kD protein, another $p40^{MO15}$ associated only with the 32-kD protein. Whatever the precise stoichiometry of these complexes, we note that human CAK is abundant in somatic cells, unlike CAK in Xenopus eggs (Solomon et al., 1993): on the basis of partial purification experiments, we estimate that $p40^{MO15}$ and its two putative complex partners may each be present at ~1–5 × 10^5 copies per HeLa cell, and at only three to five times lower levels in nontransformed human fibroblasts.

At present, we have no definitive information on the molecular identity of the 34- and 32-kD proteins. Preliminary results from microsequence analyses did not reveal any significant similarities to protein sequences presently available in databases, but they confirm that the two proteins are distinct from each other and from $p40^{MO15}$ (Tassan, J.-P., S. Frutiger, G. J. Hughes, and E. A. Nigg, unpublished results). Also, we note that the two proteins identified here differ in molecular weight from the single putative MO15 complex partner purified from starfish oocytes (Fesquet et al., 1993). Clearly, the molecular cloning of these putative $p40^{MO15}$ partners will be indispensable for a better understanding of CAK function in different cell types.

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