The cdc2-related Kinase, PISSLRE, Is Essential for Cell Growth and Acts in G2 Phase of the Cell Cycle

Shu Li, Timothy K. MacLachlan, Antonio De Luca, Pier Paolo Cl audia, Gianluigi Condorelli, and Antonio Giordano


Abstract

Mammalian cell cycle progression is regulated by several protein kinases that are activated by cyclically expressed proteins called cyclins. These cyclin-dependent kinases, the prototype of which is the cdc2 mitosis-promoting kinase, are known to phosphorylate substrates the modified status of which is critical for the cell to progress through sequential phases of the cycle. Recently, a new cdc2-related protein kinase has been discovered, PISSLRE, named with respect to its homology to the cdc2 PSTAIRE amino acid domain. Here we report that by using both antisense and dominant-negative mutant constructs of PISSLRE when overexpressed in U2OS cells, a growth suppression is found. Furthermore, the dominant negative forms of PISSLRE halt cell cycle progression in G2-M. Therefore, PISSLRE is essential for cellular proliferation, and its effect is exerted in G2-M. This describes the first evidence since cdc2 of a cdc2-related kinase acting through G2-M.

Introduction

The model of the cell cycle first began in studies involving the yeast Saccharomyces cerevisiae and Schizosaccharomyces pombe. The protein kinase found to regulate both G1-S and G2-M in S. cerevisiae was termed cdc28 (1). The vertebrate cell cycle was found to be much more complex. The association of cyclin B1 with mammalian cdc2, in conjunction with several regulatory phosphorylations of cdc2, does in fact regulate the onset of mitosis. However, another member of the cdc2 family, cdk2, has been found to monitor the onset of DNA replication in separate complexes with cyclins A and E (2-5). The discovery of the D-type cyclins added to this family. These are expressed very early in the newly divided cell in response to growth factors and function in activating the cdk4 and cdk6 kinases. These complexes are involved in cell cycle progression by, among possibly several other events, phosphorylating the Rb protein, resulting in release of the E2F transcription factor (6-10). Over the past few years, the cell cycle has been heavily implicated in cellular events that lead to oncogenesis, particularly in the first growth phase of the cell, G1 (11, 12). G1 progression has been found over the last few years to be very highly regulated by, for example, the plethora of cdk inhibitors that act in G1 as the result of different cellular responses. These controls are important in keeping the cell from passing the G1 restriction point and replicating its genome if nongrowth signals exist (e.g., DNA damage or cell-cell contact; Refs. 12, 13). It has been suggested that similar controls are present in G2 to detect, for example, completion of DNA replication and DNA damage induced by ionizing radiation (14, 15). The effective monitoring of G2 progression will keep cells from dividing with mutations, with DNA damage, or before the completion of DNA replication, an event that could result in chromosomal breakage at metaphase. It is possible that, although the cyclin B1/cdc2 complex is the master kinase in mitotic initiation, there exist complex mechanisms regulating cdc2 and other proteins that only allow division at the perfect moment. Yet with the exception of the modifying phosphorylation of cdc2 throughout G2, no other kinases have been found to act throughout this phase.

Present in all cdc2-related kinases is a KLADFGGLAR amino acid sequence near residue 143, as is present in PISSLRE (KTADFGGLAR). (16). The aspartate amino acid within this conserved stretch of proteins has been hypothesized to be essential for the ATPase activity in many cdc2-related kinases. In support of this type of residue being necessary for phosphate transfer is the crystal structure of the cyclic AMP-dependent protein kinase. This information reveals that this Asp residue is likely using its negative charge to attract the Mg2+ in magnesium ATP and to orient the β and γ phosphates to the active site of the kinase (17). In the mammalian form of the cdc2 kinase, a point mutation changing this Asp residue to an Asn causes it to lose its ability to complement the yeast cdc28 mutation (18). Therefore, if this residue is solely responsible for ATPase activity, then the protein will be able to carry out all normal functions, specifically, binding other proteins, with the lone exception of phosphotransfer. Indeed, in the cdc2 kinase, this ATPase domain is well separated from the region responsible for cyclin binding (19). Previous work in this vein has demonstrated that this type of mutation is useful in determining specific qualities of kinases (e.g., dominant-negative Ras; Ref. 20), in particular, the cell cycle kinases cdc2, cdk2, and cdk3 (21). In the cell cycle, it is postulated that when overexpressed in cells, the dn form of these are able to sequester away the activating cyclins or other unknown activating subunits from their wild-type counterparts, resulting in a permanently inactive wild-type cell cycle kinase. If the particular being expressed as a mutant is essential for cell cycle progression, then when overexpressed, it will force a halt in the progression of the cell cycle, particularly at the temporal location where its wild-type counterpart acts. van den Heuvel and Harlow (21) first showed this using dn-cdc2, dn-cdk2, and dn-cdk3. dn-cdc2 forced an increase of cells in G2-M, likely by sequestering cyclin B; dn-cdk2-expressing cells halted at the G1-S border by binding available cyclin A; and dn-cdk3 also forced a G1 block, but it is unknown what cyclin is responsible for this act. Studies performed of the cdc2 and cdk2 kinases suggest that mutation of this Asp to an Asn generated a dn mutation. Overexpression of dn-cdk2 results in sequestering cyclins A and E from endogenous wild-type cycl2 and arrests the cells before S phase, likewise for dn-cdc2, which can bind all cyclin B1 present and increase the number of cells in G2-M.

We and others have cloned a cdc2-related kinase containing the PISSLRE amino acid motif (22, 23) and have mapped it to chromo-
some 16 at location q24, a region that is altered in several breast and prostatic carcinomas, possibly suggesting a role in oncogenesis (24). The M, 33,000 PISSLRE protein contains several residues that are conserved in known cdkks, including a similarly placed Thr 161 that in cdc2 is phosphorylated by the cdk7/cyclin H complex. In this study, we sought to determine where in the cell cycle, if at all, the active function of PISSLRE is required. A dn mutant form of the PISSLRE kinase was generated that is wild type in every sense with the exception of its ATPase domain. Therefore, this alteration will still allow the protein produced to bind all associated proteins but will not be able to carry out its catalytic activities.

Materials and Methods

**Plasmid Constructs.** The wild-type full length clone was inserted in the mammalian expression vector pcDNA3 (Invitrogen) either in the sense (pCMV-PISSLREs) or the antisense (pCMV-PISSLREas) orientation. The dn mutant (Asp → Asn at position 154; Refs. 22, 23) was generated by PCR and subcloned into pcDNA3 in the sense orientation (pCMV-PISSLREdn). The HA epitope (YPYDVPDYA) was placed at the COOH terminus of wild-type PISSLRE. The plasmid expressing the CD20 cell surface marker, pCMV-CD20, has been described previously (26).

**Cell Culture and Transfection.** The human tumor cell lines Saos-2 (osteosarcoma, inactive Rb protein), T98G (glioblastoma, p16/−), and U2OS (osteosarcoma, intact Rb protein) were used. U2OS cells were cultured into DMEM with the support of 10% fetal bovine serum, 5% glutamine and penicillin-streptomycin. The cell lines were transfected by the mammalian expression vector. These constructs were separately transfected into different tumor cells (U2OS, T98G, and Saos-2). After 2–3 weeks in medium selecting for the transgene, the cells were stained. A dramatic decrease in the growth of the tumor cells was observed in all three cell lines transfected when the dn form, or, to a lesser extent, the antisense form of PISSLRE (Fig. 1; only the results from U2OS cells are shown) was overexpressed compared to cells transfected with the vector alone. This result suggests that the active form of this kinase is indispensable for cellular proliferation. Conversely, when the wild-type forms of this kinase were overexpressed, there was no observable difference between the growth of the transfected cells and cells transfected with empty vector. This is consistent with presently characterized cell cycle kinases; it is the cyclin that binds the kinase, not the kinase itself, that is the rate-limiting step in kinase activity. Therefore, increased wild-type kinase present in the cell will not speed up growth as long as its activating cyclin remains at normal levels.

With this data, we sought to determine where exactly in the cell cycle PISSLRE may exert its effect. Therefore, we set out to potentially functionally knockout endogenous PISSLRE by overexpression of the dn mutant form. U2OS cells were cotransfected in suspension with 30 µg of either wild-type, antisense, or dn forms of the kinases or vector alone and 5 µg of the CD20 cell surface marker construct. These transient transfections were then run through fluorescence-activated cell sorting analysis for cell cycle distribution determination. Consistently, compared to cells transfected with empty vector, cells transfected with dn-PISSLRE (Fig. 2a) showed a significant increase in the number of cells in G2-M. There appeared to be no significant change in the cell cycle distribution in cells transfected with the wild-type form of the kinases, again consistent with the results obtained previously for characterized cell cycle kinases. As a control, dn-cdc2 (Fig. 2b) was transfected into the same cells and exerted its effects at its known temporal location of activity, G2-M. These results were found to be specific to the particular kinases because coexpression of the wild-type forms rescued the growth-suppressive effects of their respective mutant (data not shown).

**Results and Discussion**

By site-directed mutagenesis PCR, we obtained cDNAs for PISSLRE that carry the Asp → Asn mutation. The mutant, along with wild-type and antisense cDNAs, was subcloned into the pcDNA3 mammalian expression vector. These constructs were separately transfected into different tumor cells (U2OS, T98G, and Saos-2). After 2–3 weeks in medium selecting for the transgene, the cells were stained. A dramatic decrease in the growth of the tumor cells was observed in all three cell lines transfected when the dn form, or, to a lesser extent, the antisense form of PISSLRE (Fig. 1; only the results from U2OS cells are shown) was overexpressed compared to cells transfected with the vector alone. This result suggests that the active form of this kinase is indispensable for cellular proliferation. Conversely, when the wild-type forms of this kinase were overexpressed, there was no observable difference between the growth of the transfected cells and cells transfected with empty vector. This is consistent with presently characterized cell cycle kinases; it is the cyclin that binds the kinase, not the kinase itself, that is the rate-limiting step in kinase activity. Therefore, increased wild-type kinase present in the cell will not speed up growth as long as its activating cyclin remains at normal levels.

With this data, we sought to determine where exactly in the cell cycle PISSLRE may exert its effect. Therefore, we set out to potentially functionally knockout endogenous PISSLRE by overexpression of the dn mutant form. U2OS cells were cotransfected in suspension with 30 µg of either wild-type, antisense, or dn forms of the kinases or vector alone and 5 µg of the CD20 cell surface marker construct. These transient transfections were then run through fluorescence-activated cell sorting analysis for cell cycle distribution determination. Consistently, compared to cells transfected with empty vector, cells transfected with dn-PISSLRE (Fig. 2a) showed a significant increase in the number of cells in G2-M. There appeared to be no significant change in the cell cycle distribution in cells transfected with the wild-type form of the kinases, again consistent with the results obtained previously for characterized cell cycle kinases. As a control, dn-cdc2 (Fig. 2b) was transfected into the same cells and exerted its effects at its known temporal location of activity, G2-M. These results were found to be specific to the particular kinases because coexpression of the wild-type forms rescued the growth-suppressive effects of their respective mutant (data not shown).
To ensure that the wild-type form of PISSLRE did not have any effect on cell growth and cell cycle phase distribution, we wanted to confirm that wild-type PISSLRE was being overexpressed in this cell line. U2OS cells were transfected with a hemagglutinin epitope-tagged version of the wild-type PISSLRE kinase, distinguishing between endogenous and exogenously expressed forms of the kinase, subcloned into the pcDNA3 vector. Whole cell lysates were made from these cells, run on a 12% acrylamide gel, and transferred to a polyvinylidene difluoride membrane. This was then blotted with the antihemagglutinin 12CA5. Ample PISSLRE protein was detected overexpressed in these cells and remained unaffected in regard to cell cycle distribution (Fig. 3), consistent with known data regarding kinases that are only effective when bound to an activating subunit.

These results show that the cdc2-related kinase PISSLRE is essential for cellular proliferation and acts in a particular phase of the cell cycle. Moreover, PISSLRE acts in a manner much like kinases that are active only when complexed to a regulatory subunit (e.g., the cyclin-dependent kinases). Although this is true for many cdks, some in fact are not able to display this effect. For example, when dn forms of cdk4 or cdk6 are expressed in U2OS cells, no effect is seen on cell cycle distribution (21), regardless of the well characterized importance that has been shown for both kinases on G1 progression. This is probably due to the redundancy in function between the two, in that when one is expressed as a mutant, the other will compliment its function. Also, cdk5 and the cdc2-related PCTAIRE kinase are not able to affect cell cycle progression when overexpressed in mutant form. This is due to either a functional redundancy between them and some other unknown kinase as described above, a constitutive activation throughout the cell cycle, or a cell type-specific function that is not present in U2OS cells, as is probably the case with neural tissue-throughout the cell cycle, or a cell type-specific function that is not essential for cellular proliferation and acts in a particular phase of the cell cycle regulation that is monitored by wild-type PISSLRE.

Fig. 2. Cell cycle distribution of cells transfected with different forms of PISSLRE and cdc2. Cells transfected with the indicated plasmids, either a form of PISSLRE (A) or cdc2 (B), were run through a fluorescence-activated cell sorter, and DNA content was measured. □, G1; □, S; ■, G2-M.

Fig. 3. Expression of the wild-type PISSLRE transgene. Whole cell lysates on a polyvinylidene difluoride membrane from parental U2OS cells and U2OS cells transfected with wild-type PISSLRE were blotted with the antihemagglutinin (HA) mAb 12CA5. Ample exogenous PISSLRE was found expressed in the cells transfected with the wild-type-PISSLRE expression vector. kDa, molecular weight in thousands.
   G2 checkpoint function from individuals with familial cancer syndromes. Cancer
15. Kaufman, W. K. Cell cycle checkpoints and DNA repair preserve the stability of
   E., and Tsai, L. H. A family of cdc2-related protein kinases. EMBO J., 11: 2909–2917,
18. Mendenhall, M. D., Richardson, H. E., and Reed, S. I. Dominant negative protein
   kinase mutations that confer a G1 arrest phenotype. Proc. Natl. Acad. Sci. USA 85:
19. Ducommun, B., Brambilla, P., and Draetta, G. Mutations at sites involved in Suc1
20. Ferrari, U., and Greene, L. A. Proliferative inhibition by dominant-negative Ras
   rescues naive and neuronally differentiated PC12 cells from apoptotic death. EMBO
21. van den Heuvel, S., and Harlow, E. Distinct roles for cyclin-dependent kinases in cell
23. Brambilla, R., and Draetta, G. Molecular cloning of PISSLRE, a novel putative
   member of the cdk family of protein serine/threonine kinases. Oncogene, 9:
   Chiorazzi, N., Koff, A., Heubner, K., Croce, C. M., and Giordano, A. Chromosomal
   mapping of members of the cdc2 family of protein kinases, cdk3, cdk6, PISSLRE and
   PITALRE and a cdk inhibitor, p27Kip1, to regions involved in human cancer. Cancer
   Baserga, R. Gene transfer: DNA microinjection compared with DNA transfection
26. Claudio, P. P., Howard, C. M., Baldi, A., DeLuca, A., Fu, Y., Condorelli, G., Sun, Y.,
   Colburn, N., Calabretta, B., and Giordano, A. pRb2/p130 has growth-suppressive
   properties similar to yet distinct from those of retinoblastoma family members pRb
The cdc2-related Kinase, PISSLRE, Is Essential for Cell Growth and Acts in $G_2$ Phase of the Cell Cycle

Shu Li, Timothy K. MacLachlan, Antonio De Luca, et al.


**Updated version**
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/55/18/3992

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.