[CANCER RESEARCH 55, 3992–3995, September 15, 1995]

Advances in Brief

The cdc2-related Kinase, PISSLRE, Is Essential for Cell Growth and Acts in G_2 Phase of the Cell Cycle[1]

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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 into 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 G_2-M. Therefore, PISSLRE is essential for cellular proliferation, and its effect is exerted in G_2-M. This describes the first evidence since cdc2 of a cdc2-related kinase acting through G_2-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 G_1-S and G_2-M in *S. cerevisiae* was termed cdc28[3] (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, G_1 (11, 12). G_1 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 G_1 as the result of different cellular responses. These controls are important in keeping the cell from passing the G_1 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 G_2 to detect, for example, completion of DNA replication and DNA damage induced by ionizing radiation (14, 15). The effective monitoring of G_2 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 G_2, no other kinases have been found to act throughout this phase.

Present in all cdc-2 related kinases is a KLADFGLAR amino acid sequence near residue 143, as is present in PISSLRE (KTADFGLAR) (16). The aspartate amino acid within this conserved stretch 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 Mg^{2+} 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 G_2-M, likely by sequestering cyclin B; dn-cdk2-expressing cells halted at the G_1-S border by binding available cyclin A; and dn-cdk3 also forced a G_1 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 cdk2 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 G_2-M.

We and others have cloned a cdc2-related kinase containing the PISSLRE amino acid motif (22, 23) and have mapped it to chromo-

Received 7/19/95; accepted 8/4/95.

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[1] T. K. M. is supported by NIH Training Grant 1-T32-HL07780; A. D. L. is supported by a fellowship from Universita' di Bari (Dottorato di Ricerca in Morfologia Umana e Sperimentale); P. P. C. is on leave of absence from the Dipartimento di Chirurgia Maxillo-Faciale, Facolta' di Medicina, Universita' di Napoli, "Federico II," Italy and is supported by a fellowship from the American-Italian Cancer Foundation. This work was supported by the Sbarro Institute for Cancer Research and Molecular Medicine, NIH Grant RO1 CA60999-01A1, and the Council for Tobacco Research.

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[3] The abbreviations used are: cdc, cell division cycle; cdk, cyclin-dependent kinase; dn, dominant-negative.

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_r 33,000 PISSLRE protein contains several residues that are conserved in known cdks, 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 U20S (osteosarcoma, intact Rb protein) were used. U2OS cells were cultured into DMEM with the support of 10% fetal bovine serum L-glutamine and penicillin-streptomycin. T98G and Saos-2 cells were cultured into MEM with Earl's salt, with the support of 10% fetal bovine serum-L-glutamine-basal medium Eagle's vitamin solution-penicillin-streptomycin. The cell lines were transfected by the standard suspension calcium-phosphate precipitation method (25) or by lipofectamine for colony formation assays.

Colony Formation Assay. The colony formation assay was performed as described (26). Briefly, 3×10^5 cells were plated 1 day before transfection. Ten μg of each of the four plasmid DNAs, pcDNA3 (vector), pCMV-PISSLREs, pCMV-PISSLREas, and pCMV-PISSLREdn, were transfected with lipid reagents (Lipofectamine reagent, GIBCO-BRL) into the respective cells (Saos-2, U2OS, and T98G) and cultured onto 100-mm dishes. Twenty-four h after transfection, cells were replated onto 100-mm dishes with a 1:5 dilution for Saos-2 cells or a 1:10 dilution for U2OS and T98G cells and cultured in media containing 600 μg/ml G418 (GIBCO-BRL) for 2–3 weeks. The cells were washed in PBS and then fixed and stained with methylene blue in 50% ethanol for 30 min.

Flow Cytometry Analysis. Flow cytometry analysis was carried out according to the procedure described by Claudio *et al.* (26) with some modification. Five μg of pCMV-CD20 (a mammalian expression vector for the cell surface protein CD20) were cotransfected with 30 μg of each of the four plasmids, pcDNA3 (vector), pCMV-PISSLREs, pCMV-PISSLREas, and pCMV-PISSLREdn, into 1×10^6 U2OS cells by the suspension calcium phosphate precipitation method (25). Forty-eight h after the DNA precipitates were washed out, the cells were collected, fixed, and stained with propidium iodide (20). Flow cytometry was performed on a Coulter Elite and data from 5×10^3 to 1×10^4 CD20-positive cells were used for measurement of DNA content.

Western Blotting. Cell lysates were prepared by resuspending pelleted cells in 200 μl lysis buffer (50 mM Tris-5 mM EDTA-250 mM NaCl-50 mM NaF-0.1% Triton-0.1 mM Na_3VO_4 plus protease inhibitors). Fifty μg of protein were run on a 12% polyacrylamide gel. Protein within polyacrylamide gel was transferred to a polyvinylidene difluoride membrane (Millipore) in CAPS buffer (10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS)-20% methanol, pH 11). Membrane was then blocked with 5% milk in TBS-T buffer (2 mM Tris-13.7 mM NaCl-0.1% Tween 20, pH 7.6) and then washed in TBS-T. Primary antibody was then incubated with the membrane in 3% milk and then washed in TBS-T. Rabbit antimouse antibody coupled to horseradish peroxidase was then incubated with the membrane and then washed in TBS-T. Presence of secondary antibody bound to the membrane was detected using the enhanced chemiluminescence system (DuPont New England Nuclear).

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 from 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. 2*a*) showed a significant increase in the number of cells in G_2-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. 2*b*) was transfected into the same cells and exerted its effects at its known temporal location of activity, G_2-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).

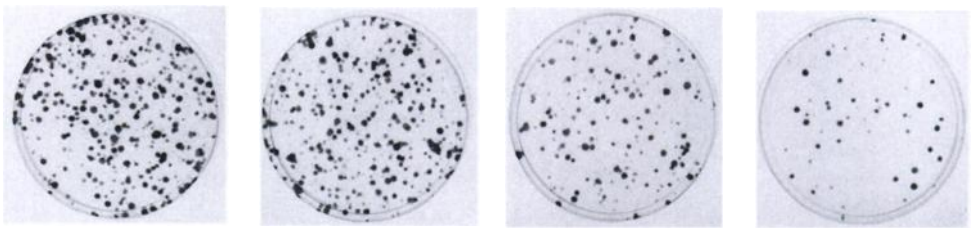

Fig. 1. Colony formation assay of U2OS cells transfected with different forms of PISSLRE. U2OS cells were transfected with the indicated plasmid. After 2–3 weeks of growth in G418-selecting medium, cells were stained in methylene blue.

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 G_1 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-specific cdk5. To date, only cdc2, cdk2, and cdk3 have been shown to have such a necessary effect on cell cycle regulation that when inactivated cause cell cycle arrest. Furthermore, cdc2 has been the only cdk known to act through G_2-M, primarily in mitosis initiation. We find that the kinase PISSLRE is essential in G_2-M, likely in progression of the phase. Over several experiments, our results show less dn-PISSLRE-transfected cells arrested in G_2 than in those transfected with dn-cdc2 (data not shown). This is likely due to the kinase acting earlier in G_2 than cdc2, possibly monitoring the completion of DNA replication. This action by PISSLRE would allow all cells to pass this restriction point, whereas they would not be able to in dn-cdc2 transfected cells. The knowledge of these and other kinases that act through G_2 phase will allow a better understanding of cell cycle regulation, specifically the mechanisms that monitor the fidelity of DNA replication and completion thereof.

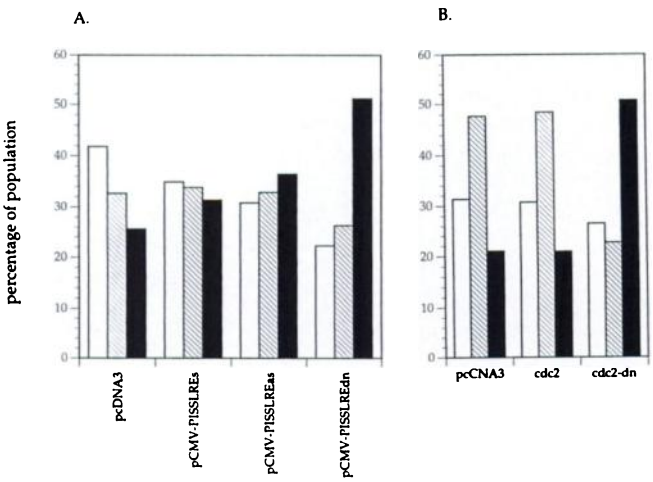

Fig. 2. Cell cycle distribution of cells transfected with different forms of PISSLRE and cdc2. Cells transfected with the indicated plasmids, either a form of PISSLRE (*A*) or cdc2 (*B*), were run through a fluorescence-activated cell sorter, and DNA content was measured. □, G_1; ▨, S; ■, G_2-M.

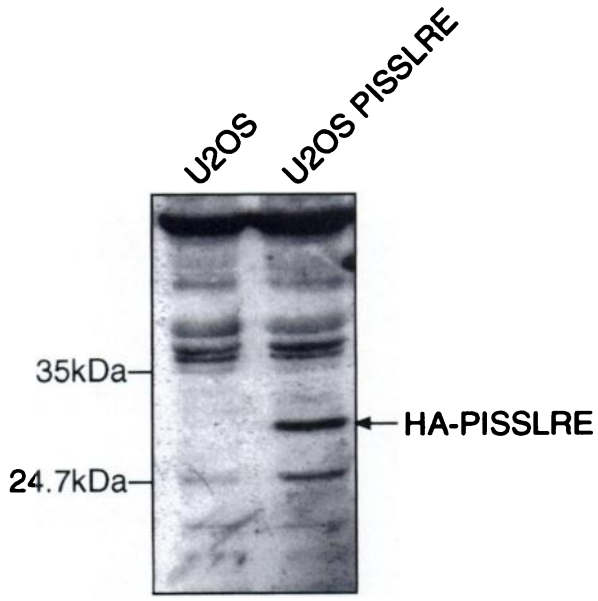

Fig. 3. Expression of the wild-type PISSLRE transgene. Whole cell lysates on a polyvinylidene difluoride membrane from parental U2OS cells and U2OS cells transfected with wild-type PISSLRE were blotted with the antihemagglutinin (*HA*) mAb 12CA5. Ample exogenous PISSLRE was found expressed in the cells transfected with the wild-type-PISSLRE expression vector. *kDa*, molecular weight in thousands.

Acknowledgments

The authors thank A. Baldi, P. Pertile, and N. Sang for helpful discussions and reviewing the manuscript. We also thank Ed Harlow for supplying dn-cdc2 and dn-cdk2 mammalian expression vectors.

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Cancer Res 1995;55:3992-3995.