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Inhibition of Cell Growth by Overexpression of the *ZPK* Gene

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ZPK is a recently described serine/threonine protein kinase that is thought to be involved in the regulation of cell proliferation and differentiation. To directly determine whether ZPK exhibits any effect on cell growth, NIH 3T3 fibroblasts were transfected with an expression vector harboring the murine *ZPK* cDNA. Stable expression of this construct led to a dramatic reduction in the proliferative capacity of these cells as measured by a colony formation assay in monolayer culture. By contrast, overexpression of a *ZPK* cDNA with a mutation in the ATP-binding domain did not affect clonal expansion of the transfected cells. These findings suggest that the *ZPK* gene may act as a negative regulator of cell growth and that this function may be mediated in part by the intrinsic kinase activity of the ZPK protein.

ZPK is a non-receptor serine/threonine kinase belonging to the mixed-lineage (MLK) family of protein kinases (1,2). The protein structure of the members in this subfamily is characterized by the presence of amino acid sequence motifs that are potentially involved in protein-protein interactions, such as leucine zipper domains and proline-rich regions. Originally, the *ZPK* gene was identified in a human teratocarcinoma cell line after a search for protein kinases differentially expressed during neuronal development (1). Recently, the mouse and rat counterparts of human ZPK have been cloned and partially characterized at the molecular level (3-5). Sequence analysis revealed extensive homology between the different species, and a conservation of the structural features of MLK family members.

Although no physiological function for ZPK has been clearly established, recent expression studies have suggested a role in the regulation of cell growth and differentiation. By *in situ* hybridization, *ZPK* transcripts have been detected in specialized cell populations of various adult mouse organs (3). In addition, during murine embryogenesis, the *ZPK* gene exhibits remarkable patterns of cell type- and developmental stage-specific expression in neuronal tissues, as well as in the epithelia of organs that rely on inductive interactions for their development (6). In these tissues, the *ZPK* mRNA was primarily localized in areas undergoing terminal cell differentiation.

To further investigate the role of ZPK in the control of cell growth and differentiation, we examined the effects of the constitutive overexpression of the *ZPK* gene in NIH 3T3 cells by means of a colony formation assay in monolayer culture. Our results revealed that deregulated expression of this gene leads to growth arrest.

MATERIALS AND METHODS

Plasmids. Wild-type and mutated mouse ZPK cDNAs (3) were subcloned under the transcriptional control of the Moloney murine sarcoma virus long terminal repeat in the previously described retroviral vector LXSN (7). The LXSN vector contains a neomycin (*neo*) resistance gene driven by the simian virus 40 (SV40) promoter. The ATP-binding mutant of ZPK, designated ZPK[K/R], was constructed by introducing a point mutation resulting in the conversion of a lysine to an arginine at amino acid 185 in the catalytic domain of the enzyme.

Cell transfection. Mouse NIH 3T3 cells, grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% Fetal Bovine Serum (FBS), were plated at a density of 7.5×10^4 cells/ml in 100-mm dishes and transfected 24 h later by calcium phosphate precipitation (8) with the indicated amounts of the plasmid LXSN, LXSN-ZPK or LXSN-ZPK[K/R]. After overnight incubation, the medium was removed and replaced with fresh medium. Forty-eight hours after transfection, 400 μg/ml G418 was added to the culture medium and drug selection was carried out for up to 2-3 weeks. At the end of the selection period, the plates of transfected cells were fixed and stained with 2% (w/v) crystal violet/1% (w/v) ammonium oxalate/20% ethanol.

RESULTS

In an effort to directly determine whether ZPK could have an effect on cell growth properties, an expression vector containing the entire coding region of mouse

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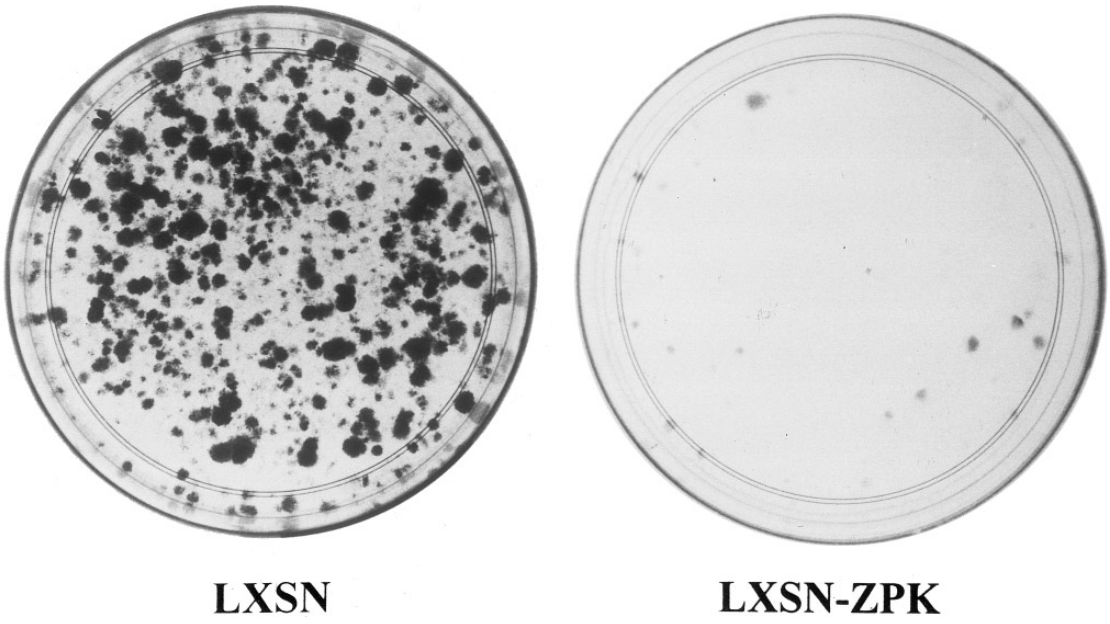

FIG. 1. Growth suppressive activity of ZPK in NIH 3T3 cells. NIH 3T3 cells, transfected with either the empty LXSN (20 μg) vector or the LXSN-ZPK (20 μg) expression construct, were cultured in selection medium containing G418 for three weeks and then stained with crystal violet.

ZPK cDNA was transfected into NIH 3T3 fibroblasts. After three weeks of selection in the presence of the antibiotic G418, the plates of transfected cells were stained with crystal violet and the number of neomycin resistant colonies was scored. As shown in Fig. 1, the yield of colonies transfected with the LXSN-ZPK expression vector was much lower than in cells transfected with an equal amount of the respective empty plasmid (LXSN). Growth inhibition, as measured by reduced colony yield, was seen with multiple independent preparations of LXSN-ZPK, as well as in similar experiments with the murine neuroblastoma cell line Neuro-2a (data not shown).

To examine whether the growth suppressive activity of the *ZPK* gene was dose-dependent, different concentrations of LXSN and LXSN-ZPK were transfected in NIH 3T3 cells and the number of G418-resistant colonies determined 15 days later. As shown in Fig. 2, the increase in the concentration of LXSN-ZPK DNA correlates with a decrease in the number of G418-resistant colonies in the culture. At the highest dose tested for each plasmid, the control LXSN gave rise to approximately 330 G418-resistant colonies per 100-mm-diameter dish, whereas LXSN-ZPK gave rise to only approximately 8 resistant colonies. These results indicate that overexpression of the *ZPK* gene has a negative effect on cell growth.

To determine whether the intrinsic kinase activity of the ZPK protein was required for growth suppression, we constructed a mutant ZPK cDNA in which the lysine codon (Lys 185) found in the ATP-binding site of protein kinases (9) was replaced by an arginine. This substitution was expected to result in loss of protein kinase activity as previously reported for several other members of this family (10,11). This mutant cDNA, designated ZPK[K/R], was inserted into the LXSN expression vector and then transfected into NIH 3T3 fibroblasts. In contrast with wild-type ZPK, NIH 3T3 cells transfected with LXSN-ZPK[K/R] gave rise to a number of G418-resistant colonies comparable to that

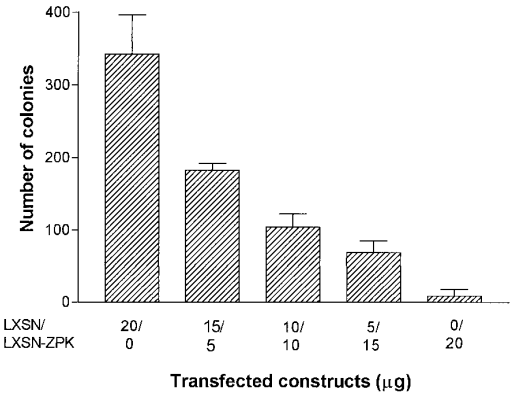

FIG. 2. Dose-dependent effect of ZPK on cell growth. NIH 3T3 cells were cotransfected with different amounts of LXSN and LXSN-ZPK expression vectors. The total amount of DNA was kept constant at 20 μg. Cells were cultured in selection medium containing G418 for two weeks and then stained with crystal violet to score the number of resistant colonies. Data represent the mean $\pm$ S.E. of two independent experiments.

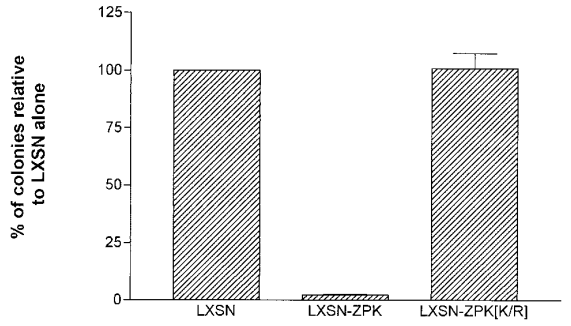

FIG. 3. ZPK requires an intact ATP-binding site to inhibit cell growth. NIH 3T3 cells were transfected with either the empty LXSN (20 μg) vector or the indicated expression constructs (20 μg) and cultured in selection medium containing G418 for three weeks. Cultures were then stained with crystal violet and the number of G418 resistant colonies was scored. The columns show the relative effect of the different expression constructs on colony formation expressed as percent of colonies obtained with LXSN alone. Data represent the mean ± S.E. of three independent experiments.

seen with the control empty vector (Fig. 3). Therefore, these findings suggest that the enzymatic activity of ZPK is critical to confer the potential to inhibit cell growth.

DISCUSSION

The control of cell growth is an orderly biological process that depends on the co-ordinate action of stimulatory and inhibitory signals. In a cell, growth inhibitory signals play crucial roles in the maintenance of homeostasis by inducing various growth arrest responses such as cell cycle delay, differentiation, apoptosis, and cell necrosis (12). Although much remains to be learned about the molecular mechanisms underlying growth arrest, it has become evident over the past decade that a network of genes play major roles in growth inhibition. Here, we show that the gene encoding the recently identified ZPK serine/threonine kinase (1,3) inhibits cell growth when overexpressed in NIH 3T3 cells. This growth inhibitory function can be totally abolished by mutation of the critical lysine codon believed to be essential for kinase activity. These results therefore suggest that the ZPK protein probably exerts its negative effect on cell proliferation by phosphorylating and activating downstream signalling molecules. Potential candidate factors might include members of the mitogen-activated protein kinase (MAPK) family since it has been recently demonstrated that the rat (MUK) and mouse (DLK) counterparts of human ZPK can activate c-Jun N-terminal kinase (JNK), which is also referred to as stress-activated protein kinase (SAPK), and p38 MAPK when overexpressed in transiently transfected NIH 3T3 cells (5,13). The JNKs/SAPKs and p38s are two newly discovered subfamilies of MAPKs capable of phosphorylating and regulating the activity of several transcription factors and other protein kinases (14). These kinases are potently and preferentially activated by inflammatory cytokines (e.g. tumor necrosis factor α, interleukin 1β) and various forms of cellular stresses (e.g. heat shock, hyperosmolarity, UV and ionizing irradiation) that are known to induce cell cycle delay, cellular repair, and/or apoptosis (14). Accordingly, it is tempting to suggest that the dramatic reduction in the number of G418 resistant colonies observed after transfection with the ZPK expression vector could be attributed to the activation of these kinases which, in turn, elicit growth arrest or cell death. It will be worth establishing cell lines expressing the *ZPK* gene under the control of an inducible promoter to determine whether the negative effect of the ZPK protein on cell growth reflects its true physiological role, and whether growth arrest is due to a block of the cell cycle or due to a loss of cell viability.

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REFERENCES

1. Reddy, U. R., and Pleasure, D. (1994) *Biochem. Bioph. Res. Commun.* **202,** 613–620.
2. Dorow, D. S., Devereux, L., and Simpson, R. J. (1994) *J. Protein Chem.* **13,** 458–460.
3. Blouin, R., Beaudoin, J., Bergeron, P., Nadeau, A., and Grondin, G. (1996) *DNA and Cell Biol.* **15,** 631–642.
4. Holzman, L. B., Merritt, S. E., and Fan, G. (1994) *J. Biol. Chem.* **269,** 30808–30817.
5. Hirai, S., Izawa, M., Osada, S., Spyrou, G., and Ohno, S. (1996) *Oncogene* **12,** 641–650.
6. Nadeau, A., Grondin, G., and Blouin, R. (1997) *J. Histochem. Cytochem.* **45,** 1–12.
7. Miller, A. D., and Rosman, G. J. (1989) *Biotechniques* **9,** 980–986.
8. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) *Mol. Cell. Biol.* **2,** 1044–1051.
9. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) *Science* **241,** 42–52.
10. Lin, A., Minden, A., Martinetto, H., Claret, F.-X., Lange-Carter, C., Mercurio, F., Johnson, G. L., and Karin, M. (1995) *Science* **268,** 286–290.
11. Xu, S., Robbins, D., Frost, J., Dang, A., Lange-Carter, C., and Cobb, M. H. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6808–6812.
12. Liebermann, D. A., Hoffman, B., and Steinman, R. A. (1995) *Oncogene* **11,** 199–210.
13. Fan, G., Merritt, S. E., Kortenjann, M., Shaw, P. E., and Holzman, L. B. (1996) *J. Biol. Chem.* **271,** 24788–24793.
14. Kyriakis, J. M., and Avruch, J. (1996) *BioEssays* **18,** 567–577.