Intestinal-enriched Krüppel-like Factor (Krüppel-like Factor 5) Is a Positive Regulator of Cellular Proliferation*

Received for publication, December 11, 2000 Published, JBC Papers in Press, January 10, 2000, DOI 10.1074/jbc.C000870200

Ronggai Sun†, Ximing Chen‡, and Vincent W. Yang§§

From the Departments of Medicine and Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Intestinal-enriched Krüppel-like factor (IKLF or KLF5) belongs to the family of mammalian Krüppel-like transcription factors. Previous studies indicate that expression of IKLF is enriched in the proliferating crypt epithelial cells of the intestinal tract. However, the biological function of IKLF is unknown. In the current study, we have shown that the level of IKLF mRNA was nearly undetectable in serum-deprived NIH3T3 fibroblasts but became acutely and significantly increased upon the addition of fetal bovine serum or the phorbol ester, PMA. This induction required protein synthesis because it was prevented by cycloheximide. Transfection of IKLF into NIH3T3 cells resulted in the formation of foci in a manner similar to that caused by the activated Ha-ras oncogene. Constitutive expression of IKLF in transfected NIH3T3 cells significantly increased the rate of proliferation when compared with cells transfected with an empty vector. The growth of IKLF-transfected cells was no longer inhibited by cell-cell contact or by low serum content. Moreover, these cells proliferated in an anchorage-independent fashion. We conclude that IKLF encodes a delayed early response gene product that positively regulates cellular proliferation and may give rise to a transformed phenotype when overexpressed.

Krüppel is a zinc finger-containing transcription factor that is responsible for segmentation of the Drosophila melanogaster embryo (1). In vertebrates, a large number of proteins have been identified that exhibit homology to Krüppel (2). One prominent example is Sp1 (3), a general transcription factor. Recently, a family of Krüppel-like factors (KLFs)† that are highly related to the mammalian Krüppel protein erythroid Krüppel-like factor (EKLF) (4) have been described (2, 5, 6). Many of these proteins were given a numerical designation by the Human Gene Nomenclature Committee (HGNC, Ref. 7), with EKLF designated as KLF1. The genes encoding many KLFs are expressed in a tissue-specific or selective manner. In addition, evidence suggests that KLFs collectively exert important regulatory functions in diverse biological processes such as growth, development, differentiation, and apoptosis.

One tissue in which a number of KLFs appear to play an important regulatory role is the intestinal epithelium. This tissue is a dynamic system in which proliferation of stem cells located in the crypts is intimately coupled to their differentiation into mature daughter cells once they exit the crypts (8, 9). Expression of the genes encoding two KLFs, gut-enriched Krüppel-like factor (GKLF or KLF4, Ref. 10, 11) and intestinal-enriched Krüppel-like factor (IKLF or KLF5, Refs. 12, 13), is particularly active in the intestinal epithelium. However, their patterns of expression appear to be complementary rather than redundant. Whereas GKLF is primarily expressed in the differentiated epithelial cells, away from the proliferating zone, IKLF is found mainly in the proliferating crypt cell population. The in vivo pattern of GKLF expression is mirrored by its in vitro pattern; it is found mostly in cells that are growth-arrested (10, 14). Moreover, constitutive expression of GKLF leads to the inhibition of DNA synthesis (10, 15). In contrast, the physiological function of IKLF is less clear, although it has been proposed to have an opposing effect to GKLF in regulating epithelial cell differentiation (12).

The present study seeks to characterize the effect of IKLF on cellular proliferation. We demonstrate that expression of IKLF in cultured cells responds transiently and acutely to growth stimuli. In addition, forced expression of IKLF in transfected cells results in an accelerated rate of proliferation and a transformed phenotype as evidenced by formation of foci, loss of contact inhibition, as well as acquisition of serum- and anchorage-independent growth. Our results indicate that IKLF has a proproliferative effect, which lends support to the previous hypothesis that it may counteract the function of GKFL.

EXPERIMENTAL PROCEDURES

Reagents—Cell culture media and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD) and Hyclone Laboratories (Logan, UT), respectively. Radiotopes were purchased from PerkinElmer Life Sciences. Phorbol 12-myristate 13-acetate (PMA) and cycloheximide (CHX) were purchased from Sigma. The monoclonal antibody directed against the hemagglutinin A (HA) epitope (F-7, sc-7392) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Expression constructs containing full-length IKLF and HA-tagged IKLF (pBK-CMV-IKLF and pBK-CMV-IKLF-PA, respectively) were kindly provided by Dr. Jerry Lingrel (12). The expression construct containing activated Ha-Ras was a generous gift of Dr. Raul Urrutia (16). The expression construct containing full-length GKL, FMT3-GKLF, was previously described (10).

Cell Culture—NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a 5% CO2 atmosphere. For experiments involving mitogenic stimulation, cells were first rendered quiescent by removal of serum from the medium for 24 h. Cells were then stimulated with fresh medium containing 15% FBS or PMA. Treatments were then continued for another 1 h with the respective mitogen and CHX.

Northern and Western Blot Analyses—RNA was isolated using the Trizol method (Life Technologies) and resolved by denaturing agarose gels.
gel electrophoresis followed by transfer to nylon membranes. Complementary DNA probes encoding IKLF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were labeled using [α-32P]dATP and the random-primed DNA labeling kit (Roche Molecular Biochemicals). Conditions of hybridization and washing were previously described (10). Western blot analysis was performed according to a previous protocol (10) using proteins extracted from stably transfected cells. The blots were probed with a monoclonal antibody directed against the HA epitope at a concentration of 200 ng/ml. Signals were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Focus Formation Assay**—Focus formation assays were performed according to a previously published protocol (17). Briefly, 5 μg of plasmid DNA containing the various effectors was transfected into NIH3T3 cells using LipofectAMINE (Life Technologies, Inc.). Cells were then maintained in DMEM and 10% FBS for 2–3 weeks at which time they were stained with methylene blue to demonstrate the foci. The number of foci in each dish was then manually counted.

**Establishment of Stable IKLF-expressing Cell Lines**—NIH3T3 cells were transfected with pBK-CMV-IKLF-HA or the pBK-CMV empty vector using LipofectAMINE. Two mg/ml G418 was added to the medium beginning 24 h after transfection to select for resistant clones, which were isolated 2 weeks later and expanded. The presence of HA-tagged IKLF was detected by Western blot analysis using the anti-HA monoclonal antibody.

**Cell Proliferation and Serum and Anchorage Dependence Assays**—Cell proliferation assays were performed by seeding the IKLF-HA- or empty vector-transfected cells at a density of 2 × 10⁵ cells per well in 6-well plates. Cells were fed DMEM with 10% FBS every other day, and the number of cells in the wells were manually counted with a hemocytometer daily for up to 5 days following seeding. For the serum dependence assay, cells were seeded at a density of 2 × 10⁵ cells per well in 6-well plates and maintained in DMEM with 1% FBS. Cells were counted every other day up to 6 days after seeding. Anchorage dependence assay was performed according to a published protocol (17). Ha-ras-, IKLF-HA-, or empty vector-transfected cells were seeded at a density of 5 × 10⁴ cells per 10-cm dish in a 0.33% agar dish in a 0.33% top agar suspension, which was overlaid onto a 0.5% agar bottom layer. Cells were fed DMEM, 10% FBS, and 2 mg/ml G418 every other day. Colonies developed in the agar suspension were examined 3 weeks following seeding, and the number was tabulated under an inverted phase-contrast microscope. Photodocumentation was accomplished with a Nikon digital camera.

**RESULTS**

**IKLF Encodes a Delayed Early Response Gene to Growth Stimulation**—To investigate the responsiveness of IKLF expression to growth stimulation, we conducted Northern blot analyses in cultured fibroblasts. NIH3T3 cells were first rendered quiescent by removing the serum from the medium and then stimulated by 15% FBS or the phorbol ester, PMA. As shown in Fig. 1, A and B, the level of IKLF transcripts was barely detectable in serum-starved, quiescent cells (time 0). Upon addition of FBS (Fig. 1A) or PMA (Fig. 1B), the levels of IKLF transcripts increased acutely and transiently, reaching a maximum after 2–3 h of treatment before returning to baseline levels. The enhancing effect of both stimuli was prevented in cells pretreated with the protein synthesis inhibitor, cycloheximide (Fig. 1C, CHX). These results indicate that IKLF is an early response gene to growth stimulation although this responsiveness requires protein synthesis. IKLF would therefore fall into the category of delayed early response genes (18, 19).

**Forced Expression of IKLF Causes Formation of Foci**—As a means to measure the effect of IKLF on cell proliferation, we conducted the focus formation assay previously described (17, 20). NIH3T3 fibroblasts were transfected with the various effectors constructs and foci scored 2–3 weeks after transfection. As shown in Fig. 2, an expression plasmid containing the HA-tagged IKLF produced ~30% of the number of foci caused by activated Ha-Ras. A second construct containing IKLF without the HA-tag gave rise to similar results (not shown). In contrast, an expression plasmid containing GKL failed to produce any foci, as were mock-transfected cells (Fig. 2). These results suggest that forced expression of IKLF causes focus formation in a manner similar to activated Ha-Ras, an activity that was not paralleled by GKL.

**Constitutive Expression of IKLF Causes Accelerated Cell Growth**—To further investigate the effect of IKLF on cell proliferation, we established several clonally derived NIH3T3 cell lines that had been transfected with pBK-CMV-IKLF-HA or the pBK-CMV empty vector and selected with the antibiotic, G418. Two independent clones from each construct were chosen.
IKLF

As shown, while the vector-transfected cells remained a monolayer, the transfected cells (Bars indicate S.E. each of IKLF monoclonal antibody (the content of IKLF are the mean cell numbers/well in log scale of were counted daily in triplicate, after seeding for up to 5 days. Shown with DMEM containing 10% FBS. Three wells of cells from each clone were counted daily in triplicate, after seeding for up to 5 days. Shown are the mean cell numbers/well in log scale of IKLF- and empty vector-transfected clones (I3 and 110 versus V3 and V5, respectively). In B, cells from each clone were seeded at a density of $2 \times 10^5$ cells/well in 6-well plates and fed every other day with DMEM containing 10% FBS. Three wells of cells from each clone were counted daily in triplicate, after seeding for up to 5 days. Shown are the mean cell numbers/well in log scale of IKLF- and empty vector-transfected clones (I3 and 110 versus V3 and V5, respectively). Bars indicate S.E. C, typical morphology of IKLF- and empty vector-transfected cells (left versus right, respectively) at 5 days after seeding. As shown, while the vector-transfected cells remained a monolayer, the IKLF-HA-transfected cells grew to multiple layers.

and examined. Fig. 3A shows that the two pBK-CMV-IKLF-HA- but not the two pBK-CMV-transfected clones (lanes 1 and 2 versus lanes 3 and 4, respectively) produced a full-length HA-tagged IKLF as detected by Western blot using a monoclonal antibody against the HA epitope. When the growth characteristics of these cells were examined and compared over a course of 5 days following seeding at a low density, it became apparent that the IKLF-HA-expressing cells proliferated at a much faster rate compared with the control, empty vector-transfected cells (Fig. 3B). Moreover, whereas the control cells ceased to proliferate after reaching confluency, the IKLF-HA-transfected cells continued to grow to several layers (Fig. 3C). These findings suggest that the growth of IKLF-HA-transfected cells was no longer subject to contact inhibition, providing further evidence for a proproliferative effect of IKLF.

IKLF Causes Serum- and Anchorage-independent Growth— The stably transfected NIH3T3 cells were subjected to additional and more stringent tests of cellular proliferation and transformation. In one, cells were seeded and maintained in medium containing only 1% FBS. Under these conditions, empty vector-transfected cells failed to proliferate. In fact, many perished because of the low-serum content. In contrast, IKLF-HA-transfected cells continued to proliferate at a relatively brisk rate albeit slightly slower than that when maintained in 10% FBS (compare Figs. 4A and 3B). A second test involved growth in soft agar (17). As seen in Fig. 4B, IKLF-HA-transfected cells formed colonies in an agar suspension as were Ha-ras-transfected cells at a ~1:3 ratio. In contrast, empty vector-transfected cells remained as single cells in the agar suspension without ever forming any colonies (Fig. 4C). The morphology and size of the colonies produced by IKLF-HA- and Ha-ras-transfected cells were very similar to each other (Fig. 4C).

COMPLEMENTARY DISCUSSION

Complementary DNA clones encoding mouse IKLF were initially identified because of sequence homology to IKLF (12). A human homolog was subsequently isolated based on its binding to a specific cis-sequence in the promoter of the lactoferrin gene (12). In situ hybridization studies of both adult (12) and fetal intestinal tissues (13) showed that expression of IKLF is concentrated in the base of intestinal crypts. The in vivo pattern of IKLF expression in the intestinal tract therefore correlates with a proliferative phenotype, although a direct effect of IKLF on cellular proliferation was not established by these studies. It should be noted that IKLF is identical to the previously isolated BTEB2 (22), which, because of a sequencing error, has a shorter open-reading frame than IKLF (13). In a rabbit model, expression of BTEB2/IKLF is induced in activated smooth muscle cells (SMCs) in the neointima of balloon-
injured aorta (23). Similarly, increased BTEB2 expression has been noted in proliferating SMCs at anastomotic vascular stricture (24) and this increased expression is a positive predictive factor for vascular restenosis in pathological conditions (25). Taken together, these studies suggest that activation of BTEB2/IKLF expression is correlated with a proliferative state.

The results of the current study indicate that expression of IKLF in cultured, quiescent NIH3T3 cells is acutely and transiently induced upon mitogen stimulation by factors such as serum and phorbol ester. This induction is dependent on new protein synthesis as it is abolished in the presence of cycloheximide. A similar inductive response of BTEB2/IKLF was noted in cultured rabbit aorta-derived SMCs treated with PMA or basic fibroblast growth factor (26). The latter study also showed that expression of the immediate early response gene, Egr-1, is highly up-regulated by PMA and that Egr-1 binds to and activates the promoter of the BTEB2/IKLF gene (26). It is therefore possible that Egr-1 is an immediate mediator of induction of IKLF during proliferative responses.

Despite evidence from in vitro and in vivo studies demonstrating a correlation between IKLF expression and proliferation, it is not clear whether IKLF directly regulates cellular proliferation. The results of the current study are the first to show that constitutive expression of IKLF increases proliferation. We also show that IKLF alone is sufficient to cause a transformed phenotype as assessed by focus formation (Fig. 2), loss of contact inhibition (Fig. 3C), and gain of serum- (Fig. 4A) and anchorage-independent growth (Fig. 4, B and C). These observations therefore suggest that IKLF is potentially a mediator of cellular proliferation in the various in vivo and in vitro systems described above. Whether IKLF directly participates in regulating the cell cycle or whether expression of IKLF is increased in pathological conditions such as neoplasm remains to be investigated.

Based on the opposing patterns of expression of GKL and IKLF in the intestine, Lingrel and co-workers (12) proposed that their gene products may have opposing effects in regulating proliferation and differentiation of the intestinal epithelium. Indeed, the antiproliferative effect of GKLF depicted by previous studies (10, 14) and the proproliferative effect of IKLF demonstrated by this study support their hypothesis. Biochemical evidence also suggests that the two proteins may have opposing functions. For example, the promoter of the SMC differentiation marker gene, SM22α, is repressed by GKL but activated by IKLF (27). Preliminary studies from our laboratory also indicate that GKL and IKLF regulate the GKLF promoter in an opposing manner.2 In view of the highly conserved sequence in the zinc finger region of the two proteins and the similar DNA sequences to which they bind (10, 11, 12, 14, 21), it would not be surprising that the two KLFs may coordinately regulate the expression of a group of genes through similar if not identical cis-elements. Further studies will demonstrate the biochemical mechanisms by which GKL and IKLF antagonize each other in the context of regulating complex biological processes such as cellular proliferation and differentiation.

Acknowledgments—We thank M. Conkright and Dr. J. Lingrel for providing pBK-CMV-IKLF-HA and pBK-CMV-IKLF (12) and Dr. R. Urudda for providing the activated Ha-Ras (16) expression construct.

REFERENCES

1. Schuh, R., Aicher, W., Gual, U., Cote, S., Preiss, A., Maier, D., Scifert, E., Nauber, U., Shroder, C., Kemler, R., and Jackle, H. (1986) Cell 47, 1025–1032
2. Turner, J., and Crossley, M. (1999) Trends Biochem. Sci. 24, 236–240
3. Kadonaga, J. T., Carner, K. R., Mastarz, F. R., and Tjian, R. (1987) Cell 51, 1079–1090
4. Miller, I. J., and Bieker, J. J. (1993) Mol. Cell. Biol. 13, 2776–2786
5. Philipsen, S., and Suske, G. (1999) Nucleic Acids Res. 27, 2991–3000
6. Dang, D. T., Pevener, J., and Yang, V. W. (2000) Int. J. Biochem. Cell Biol. 32, 1103–1121
7. White, J. A., McAlpine, P. J., Antonarakis, S., Cann, H., Eppig, J. T., Frazer, K., Frezel, J., Lancet, D., Nahmias, J., Pearson, P., Peters, J., Scott, A., Scott, H., Spurr, N., Talbot, C., Jr., and Povey, S. (1997) Genomics 45, 468–471
8. Stappenbeck, T. S., Wong, M. H., Saam, J. R., Mysorekar, I. U., and Gordon, J. I. (1998) Curr. Opin. Cell Biol. 10, 792–709
9. Gordon, J. I., and Hermiston, M. L. (1994) Curr. Opin. Cell Biol. 6, 795–803
10. Shields, J. M., Christy, R. J., and Yang, V. W. (1996) J. Biol. Chem. 271, 20009–20017
11. Garrett-Sinha, L. A., Eberzpaecher, H., Seldin, M. F., and de Crombrugghe, B. (1996) J. Biol. Chem. 271, 31384–31390
12. Conkright, M. D., Wani, M. A., Anderson, K. P., and Lingrel, J. B. (1999) Nucleic Acids Res. 27, 1283–1270
13. Ohnishi, S., Laub, F., Matsumoto, N., Asaka, M., Ramirez, F., Yoshida, T., and Terada, M. (2000) Dev. Dyn. 217, 421–429
14. Shie, J. L., Chen, Z. Y., Fu, M., Pestell, R. G., and Tseng, C. C. (2000) Nucleic Acids Res. 28, 2969–2976
15. Shie, J. L., Chen, Z. Y., O’Brien, M. J., Pestell, R. G., Lee, M. E., and Tseng, C. C. (2000) Am. J. Physiol. 279, G806–G814
16. Gebelein, B., Fernandez-Zapico, M., Imoto, M., and Uruutta, R. (1998) J. Clin. Invest. 102, 1911–1919
17. Cox, A. D., and Der, C. J. (1994) Methods Enzymol. 238, 277–294
18. Winkles, J. A. (1996) Prog. Nucleic Acid Res. Mol. Biol. 58, 41–78
19. Lanahan, A., Williams, J. B., Sanders, L. K., and Nathans, D. (1992) Mol. Cell. Biol. 12, 3919–3929
20. Clark, G. J., Cox, A. D., Graham, S. M., and Der, C. J. (1995) Methods Enzymol. 255, 395–412
21. Shi, H., Zhang, Z., Wang, X., Liu, S., and Teng, C. T. (1999) Nucleic Acids Res. 27, 4807–4815
22. Segawa, K., Imataka, H., Yamazaki, Y., Kusume, H., Abe, H., and Fujii-Kuriyama, Y. (1993) Nucleic Acids Res. 21, 1527–1532
23. Watanabe, N., Kurabayashi, M., Shinohara, Y., Kawai-Kowase, K., Yoshino, Y., Manabe, I., Watanabe, M., Aikawa, M., Kuruo, M., Suzuki, T., Yurase, Y., and Nagai, R. (1999) Circ. Res. 85, 182–191
24. Ogata, T., Kurabayashi, M., Kishino, Y.I., Sekiguchi, K.I., Ishikawa, S., Morishita, Y., and Nagai, R. (2000) J. Thorac. Cardiovasc. Surg. 119, 983–989
25. Hoshino, Y., Kurabayashi, M., Kanda, T., Hasegawa, A., Sakamoto, H., Okamoto, E.I., Kowase, K., Watanabe, N., Manabe, I., Suzuki, T., Nakano, A., Takase, S.I., Wilcox, J.N., and Nagai, R. (2000) Circulation 102, 2528–2534
26. Kawai-Kowase, K., Kurabayashi, M., Hoshino, Y., Ohyama, Y., and Nagai, R. (1999) Circ. Res. 85, 787–795
27. Adam, P. J., Regan, C. P., Hautmann, M. B., and Owens, G. K. (2000) J. Biol. Chem. 275, 37789–37806

2 C. Mahatan and V. W. Yang, unpublished observations.