Regulation of Cell Proliferation by Interleukin-3-induced Nuclear Translocation of Pyruvate Kinase*1

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Akemi Hoshino, John A. Hirst, and Hodaka Fujii

From the Department of Pathology, New York University School of Medicine, New York, New York 10016

Extracellular signaling molecules bound to cell surface receptors can regulate nuclear function with consequences for cell proliferation, differentiation, and function. To regulate nuclear function, signals must be transduced across the nuclear envelope to propagate the signal from the cytoplasm to the nucleus. Therefore, many signaling responses induce the nuclear translocation of transcription factors, kinases, and others. By using inducible translocation trap, a reporter gene-based system to detect inducible nuclear translocation, we found that the M2 isoform of pyruvate kinase, a key enzyme in glycolysis, translocates into the nucleus by interleukin-3, but not by epidermal growth factor, stimulation. The C domain of the M2 isoform of pyruvate kinase was sufficient for interleukin-3-induced nuclear translocation. Interleukin-3-induced nuclear translocation of the M2 isoform of pyruvate kinase was dependent on the activation of Jak2. Overexpression of the M2 isoform of pyruvate kinase protein fused with a nuclear localization signal enhanced cell proliferation in the absence of interleukin-3, suggesting that the nuclear pyruvate kinase plays an important role in cell proliferation.

INTERLEUKIN-3 (IL-3)2 stimulation induces activation of Jak2 (1), and activated Jak2 phosphorylates and activates Stat5α/β (2–4). Accumulating evidence suggests critical roles of Stat5 activation in IL-3 signaling (5–7). On the other hand, Stat5-independent induction of target genes has also been reported (7), suggesting that molecules other than Stat5α/β may translocate into the nucleus by IL-3 stimulation. Here, we showed IL-3-induced nuclear translocation of the M2 isoform of pyruvate kinase (M2-PK) and examined its significance in cell proliferation.

EXPERIMENTAL PROCEDURES

Cell Lines—BB13 was derived from an IL-3-dependent hematopoietic cell line, Ba/F3 (8), and ectopically expresses human epidermal growth factor (EGF) receptor and an anti-apoptotic protein, Bcl-2 (9). BB13 proliferates in response to EGF even in the absence of IL-3. Bcl-2 expression is required for EGF-induced proliferation. In the absence of EGF and IL-3, BB13 dies by apoptosis, but cell death is markedly delayed compared with its parental cell line, which does not overexpress Bcl-2. LexA-d1EGFP (10) gene was transfected into BB13 to establish the BBLG cell line. LexA-d1EGFP reporter gene contains 8 × LexA binding elements, interferon β minimal promoter, and destabilized enhanced green fluorescent protein (GFP) (d1EGFP).

Plasmid Construction—For construction of a retroviral vector expressing GFP fused with the carboxyl (C)-terminal of the full-length M2-PK (M2-PK-GFP), the coding sequence of mouse M2-PK was amplified by PCR from the Ba/F3 cDNA library and ligated with GFP gene from pEGFP-N3 (Clontech) and pMX-neo vector. For construction of a retroviral vector expressing hemagglutinin (HA) epitope-tagged M2-PK fused with the nuclear localization signal (NLS) from SV40 T-antigen at its N terminus (HA-NLS-M2-PK), the coding sequence of M2-PK was amplified by PCR and ligated with a sequence encoding SV40 T-antigen NLS cleaved from pLGV-NLS-β-galactosidase (10) and pEF-HA (11). The HA-NLS-M2-PK sequence was cleaved from the resultant plasmid and ligated with pMXs-IG (12) (HA-NLS-M2-PK/pMXs-IG). For construction of a retroviral vector encoding HA-tagged M2-PK (HA-M2-PK/pMXs-IG), the coding sequence of M2-PK was amplified by PCR and ligated with HA sequence from pEF-HA into pMXs-IG.

Immunoblot Analysis—Preparation of nuclear extracts/whole cell lysates and immunoblot analyses were performed as described (10, 13). The antibodies (Abs) used in this study were anti-PK (Abcam and Serotec), anti-Stat5, anti-Stat5, anti-Stat5, anti-Rho-GDI (Santa Cruz Biotechnology), anti-GFP (Clontech), and anti-HA (Roche Applied Science). For inhibition of Jak2, AG-490 (BIOSOURCE) (50 μM) was added to growth factor-starved cells 30 min before IL-3 stimulation.

Preparation of Nuclei for Flow Cytometric Analysis—Cells were washed with ice-cold phosphate-buffered saline, suspended in 400 μl of A buffer (10 mm Hepes-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM dithiothreitol, protease inhibitor mixture (Complete, Mini, EDTA-free; Roche Applied Science), and 0.3% Nonidet P-40), and vortexed for 30 s. Nuclei were spun down at 5,000 rpm for 1 min at room temperature and suspended in 4% paraformaldehyde to be fixed for 10 min at room temperature. Fixed nuclei were washed with flow cytometry buffer (phosphate-buffered saline containing 0.5% bovine.
IL-3-induced Nuclear Translocation of Pyruvate Kinase

**RESULTS**

**Isolation of M2-PK cDNA in Screening of IL-3-induced Nuclear Translocating Molecules**—By using the inducible translocation trap system (10), we attempted to isolate cDNAs that encode proteins translocating into the nucleus by IL-3 stimulation. The inducible translocation trap system is based on expression of a fusion protein consisting of the LexA DNA-binding domain, the Gal4 transactivation domain, and the test protein encoded by cDNA subcloned downstream of the Gal4 transactivation domain (10). The fusion molecule is expressed in cells containing GFP reporter gene with multiple LexA binding sites in its promoter (LexA-d1EGFP). Following nuclear translocation of the fusion protein by ligand stimulation, the LexA DNA-binding domain targets the fusion protein to the LexA operator sites of the reporter gene and then the Gal4 transactivation domain activates the expression of GFP. Thus, nuclear translocation of the test protein is detected by the expression of GFP. LexA-d1EGFP gene was transfected into BB13 (9) to establish the BBLG cell line. BB13 is a Ba/F3-derived cell line ectopically expressing EGF receptor and anti-apoptotic protein Bcl-2. Parental Ba/F3 dies by apoptosis in the absence of IL-3, whereas BB13 proliferates even in the absence of IL-3 if EGF is present in the culture medium (9).

Screening of the cDNA library was performed as schematized in Fig. 1. The Ba/F3 cDNA library (48 μg) constructed in the pLG retroviral vector to express cDNA-encoded proteins fused with LexA DNA-binding domain and Gal4 transactivation domain (10) was transfected into 1.8 \times 10^7 of 293T cells with amphotropic helper plasmid to produce retrovirus particles. Two days after transfection, 2 \times 10^7 of BBLG were infected with the supernatant (60 ml) of the 293T containing virus particles.

Infection efficiency was estimated to be ~7% (data not shown). A day after infection, cells were resuspended in serum albumin (9) and suspended in flow cytometry buffer for flow cytometric analysis.

**Fluorescent Microscopy**—Ba/F3 expressing M2-PK-GFP were growth factor-starved and mock-stimulated or stimulated with IL-3 for 30 min. Cells were fixed with paraformaldehyde, counterstained with 4',6-diamidino-2-phenylindole to indicate the positions of the nuclei, and observed with a deconvolution microscope.

**Northern Blot Analysis**—Cells were growth factor-starved for 5 h and mock-stimulated or stimulated with EGF (10 ng/ml) or IL-3 (1 ng/ml) for the indicated time intervals. Total RNA (5 μg) were analyzed by Northern blot analysis with cis (14) and oncogen M (15) probes as described previously (16, 17).

**RESULTS**

**Isolation of M2-PK cDNA in Screening of IL-3-induced Nuclear Translocating Molecules**—By using the inducible translocation trap system (10), we attempted to isolate cDNAs that encode proteins translocating into the nucleus by IL-3 stimulation. The inducible translocation trap system is based on expression of a fusion protein consisting of the LexA DNA-binding domain, the Gal4 transactivation domain, and the test protein encoded by cDNA subcloned downstream of the Gal4 transactivation domain (10). The fusion molecule is expressed in cells containing GFP reporter gene with multiple LexA binding sites in its promoter (LexA-d1EGFP). Following nuclear translocation of the fusion protein by ligand stimulation, the LexA DNA-binding domain targets the fusion protein to the LexA operator sites of the reporter gene and then the Gal4 transactivation domain activates the expression of GFP. Thus, nuclear translocation of the test protein is detected by the expression of GFP. LexA-d1EGFP gene was transfected into BB13 (9) to establish the BBLG cell line. BB13 is a Ba/F3-derived cell line ectopically expressing EGF receptor and anti-apoptotic protein Bcl-2. Parental Ba/F3 dies by apoptosis in the absence of IL-3, whereas BB13 proliferates even in the absence of IL-3 if EGF is present in the culture medium (9).

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Infection efficiency was estimated to be ~7% (data not shown). A day after infection, cells were resuspended in medium containing EGF (10 ng/ml). Two days after infection, cells were stimulated with IL-3 (1 ng/ml) for 4 h, and GFP (+) cells were sorted and incubated in medium containing EGF (IL-3 (−)) for 4 days for down-regulation of GFP, and then GFP (−) cells were sorted. Three days after, cells were restimulated with IL-3, and GFP (+) cells were sorted. Nine rounds of GFP (+) sorting after IL-3 stimulation and subsequent GFP (−) sorting were performed. The percentage of GFP (+) cells did not increase by IL-3 stimulation 2 days after infection (mock-stimulation, 1.57%; IL-3 stimulation, 1.56%) (Fig. 2A). In contrast, after nine rounds of sorting, IL-3 stimulation significantly increased GFP (+) cells in the sorted population (mock-stimulation, 17.1%; IL-3 stimulation, 33.6%) (Fig. 2A). Then cells were subjected to single-cell sorting, and GFP induction by IL-3 was examined for each clone. About 240 of more than 600 clones showed IL-3-induced GFP expression. Representative data for clones that showed GFP induction by IL-3 are shown in Fig. 2B. PCR amplification using genomic DNA extracted from these clones and viral vector primers gave rise to several different patterns (Fig. 2C, a–d). Percentages of each pattern were 84% (a), 8% (b), 4% (c), 1% (d), and 3%, others. We concentrated our effort for the analysis of the cDNA inserts in the dominant pattern (a). The fact that three bands were amplified in the pattern (a) suggested that three different cDNA clones are integrated in the genome of this clone. To determine which cDNA insert is responsible for IL-3-induced expression of GFP, PCR products from the pattern (a) were

**FIGURE 1. Scheme of screening of cDNA library to identify proteins that translocate into the nucleus by IL-3 stimulation.**
subcloned into pLG and transduced into BBLG, and then GFP expression by IL-3 was analyzed. IL-3 stimulation significantly induced expression of GFP in BBLG expressing the fusion protein consisting of LG and the protein encoded by the 1.2-kb pair (kbp) insert (Fig. 2D). In contrast, IL-3 stimulation did not induce expression of GFP in BBLG expressing fusion proteins consisting of LG and proteins encoded by the other inserts (1.6- and 0.8-kbp) (Fig. 2D). These data indicate that the protein encoded by the 1.2-kbp insert of the pattern (a) translocates into the nucleus by IL-3. Sequence analysis revealed that the 1.2-kbp insert encodes the C-terminal 139 amino acid residues of mouse M2-PK (18) (Fig. 2E). PK is a key enzyme in the glycolytic pathway, and it catalyzes the transfer to ADP of the high energy phosphate group of phosphoenolpyruvate to generate ATP (19, 20). Four highly homologous isoforms, M1, M2, L, and R types, have been reported in mammalian cells (21–23). M2-PK is localized mainly in the cytoplasm and expressed in early fetal tissues as well as in most of the adult tissues (19, 20).

**Nuclear Translocation of M2-PK by IL-3**—To examine whether endogenous PK translocates into the nucleus by IL-3, Ba/F3 was growth factor-starved and stimulated with IL-3. Nuclear extracts were subjected to immunoblot analysis with anti-PK Ab, which detects M2-PK (see below). IL-3 stimulation

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**FIGURE 2. Molecular cloning of M2-PK cDNA by inducible translocation trap.** A, Ba/F3-derived cDNA library was transduced into BBLG cells. Transduced cells were maintained in medium containing EGF (10 ng/ml) (right panels) or stimulated with IL-3 (1 ng/ml) (left panels) for 5 h, and then GFP expression was examined. Upper panels, before sorting; lower panels, after nine rounds of sorting. B, IL-3-induced up-regulation of GFP in sorted clones. Cells were maintained in medium containing EGF (thin line) or stimulated with IL-3 (thick line) for 5 h. C, cDNA inserts amplified by PCR using genomic DNAs extracted from IL-3-responsive clones and viral vector primers. Percentages of each pattern (a–d) in the total of ~240 clones are indicated. D, IL-3-induced up-regulation of GFP in BBLG expressing a fusion protein consisting of LG and the protein encoded by the 1.2-kbp cDNA insert from the pattern (a). PCR products from the pattern (a) subcloned into pLG were transduced into BBLG. Cells were stimulated with IL-3 for 4 h, and then GFP expression was analyzed by flow cytometry. E, schematic view of M2-PK. The 1.2-kbp fragment encoded amino acids 393–531 of the C domain of M2-PK.
induced increases in the amounts of PK in the nucleus within 30 min (Fig. 3A, upper panel). The total expression levels of PK did not change by IL-3 stimulation (Fig. 3A, lower panel), suggesting that the increase in the amounts of PK in the nucleus is not caused by the increase in the expression levels of PK. In contrast to IL-3, EGF stimulation did not increase the amounts of PK in the nucleus (Fig. 3B).

To further confirm nuclear translocation of M2-PK by IL-3 stimulation, a retroviral vector expressing GFP fused with the C-terminal of the full-length M2-PK (M2-PK-GFP) was transduced into Ba/F3 cells. Expression of the M2-PK-GFP fusion protein was detected by immunoblot analysis with anti-PK Ab (Fig. 3C, left panel). GFP (+) cells were sorted, growth factor-starved, and then stimulated with IL-3 for 30 min. IL-3 stimulation induced increases in the amounts of M2-PK-GFP in the nuclear extracts (Fig. 3C, right panel, upper). The total expression levels of M2-PK-GFP did not change by IL-3 stimulation (lower panel), suggesting that the increase in nuclear amounts of M2-PK-GFP reflects its nuclear translocation. Next, Ba/F3 expressing M2-PK-GFP were IL-3-starved for 5 h and stimulated with IL-3 for the indicated time intervals. Nuclei were purified and subjected to flow cytometric analysis. IL-3 stimulation increased the fluorescence intensities of GFP (Fig. 3D). Furthermore, subcellular localization of M2-PK-GFP was examined by fluorescent microscopy. In the absence of IL-3, M2-PK-GFP was excluded from the nucleus (Fig. 3E). In contrast, M2-PK-GFP distributed ubiquitously in the cytoplasm as well as in the nucleus in IL-3-stimulated cells (Fig. 3E). Collectively, these analyses showed nuclear translocation of M2-PK by IL-3 stimulation. Treatment with AG-490, an inhibitor of Jak2 (24), completely inhibited nuclear translocation of PK by IL-3 stimulation (Fig. 3F), suggesting that IL-3-induced nuclear translocation of PK is dependent on Jak2 activation.

**Functional Role of Nuclear M2-PK in Cell Proliferation**—To examine the functional significance of IL-3-induced nuclear translocation of M2-PK, we generated constructs encoding HA-tagged M2-PK (HA-M2-PK) and HA-tagged M2-PK fused with NLS from SV40 T-antigen at its N terminus (Fig. 4A, HA-NLS-M2-PK). These constructs were inserted into the pMXs-IG retroviral vector containing an internal ribosome entry site–GFP sequence, which permits simultaneous expression of a cloned gene and GFP (12). Changes in the percentages of transgene (+) cells can be easily quantified by analyzing GFP (+) cells. The retroviral constructs were transduced into BB13 and cultured in RPMI medium containing EGF. As expected, in the absence of IL-3, HA-NLS-M2-PK was readily detected in the nucleus of GFP (+) BB13 cells, while marginal amounts of HA-M2-PK were detected in the nucleus (Fig. 4B).

Next, we examined the proliferation of BB13 constitutively expressing M2-PK in the nucleus by monitoring the percentages of transgene (+) cells in EGF-containing medium. The percentages of GFP (+) cells in cell populations transduced with HA-M2-PK/pMXs-IG or pMXs-IG decreased gradually (Fig. 4C), indicating that neither transgene enhances proliferation. In contrast, the percentages of GFP (+) cells in the cell population transduced with HA-NLS-M2-PK/pMXs-IG increased markedly in EGF-containing medium (Fig. 4C). After 30 days of culture, the per-
percentage of GFP (+) cells expressing HA-NLS-M2-PK increased 3-fold (Fig. 4D). These data indicate that nuclear M2-PK enhances EGF-induced proliferation. We also observed enhanced proliferation of clones expressing HA-NLS-M2-PK (data not shown). It is noteworthy that enhanced proliferation of cells expressing HA-NLS-M2-PK in the presence of IL-3 was not observed (supplemental Fig. S2), the nuclear localization of M2-PK alone is not sufficient for cell proliferation and survival.

**DISCUSSION**

Here, we showed that M2-PK translocates into the nucleus by IL-3 but not by EGF stimulation (Figs. 2 and 3). Because sequence identities of isoforms of mouse PK are high, it is likely that other isoforms (L, R, and M1) of PK can also translocate into the nucleus by IL-3. It has been recently shown that growth factors, including IL-3, influence cell growth and survival through effects on glucose metabolism (25). Our data show that growth factors also modulate localization of PK, a key enzyme in glycolysis, suggesting a novel link between growth factor stimulation and the glycolytic pathway.

PK proteins consist of the N-terminal domain, the A domain subdivided into A1 and A2 subdomains, the B domain, and the C domain (Fig. 2E) (26). The ADP/ATP binding site lies in subdomain A2 near the domain C (26). The binding site of phosphoenolpyruvate is located in the loops that connect subdomain A1 and B and strands in subdomain A2 (27). The active site lies in a pocket between domains A and B (27), where there is a high degree of identity among the 50 PK sequences of organisms from bacteria to human (26). In contrast, the C domain is variable among species (26). We showed that the C domain contains an inducible NLS(s) (Fig. 2). On-line motif analysis with the PSORT II program (psort.ims.u-tokyo.ac.jp) did not detect any classical and bipartite NLS rich in Arg and Lys (28) in the C domain of PK. Instead, their signal-induced conformational changes can be recognized by nuclear importer proteins such as importin/karyopherins. Nuclear translocation of PK may also be mediated through signal-induced conformational changes but not by classical/bipartite NLS.
We showed that IL-3-mediated nuclear translocation of PK is dependent on activation of Jak2 (Fig. 3F). At this stage, it is not clear how Jak2 activation induces nuclear translocation of PK. IL-3-induced activation of Jak2 induces activation of Stat5 as well as other signaling molecules such as Ras (30) and Akt (31, 32), and they may be involved in the regulation of nuclear translocation of M2-PK. Alternatively, Jak2 may directly phosphorylate PK to induce its nuclear translocation.

The nuclear localizing M2-PK significantly enhanced EGF-mediated cell proliferation in the absence of IL-3 (Fig. 4). It is not clear how nuclear M2-PK enhances cell proliferation. EGF-stimulated cells did not induce Stat5 target genes even when HA-NLS-M2-PK was expressed (Fig. 4E), excluding a possibility that nuclear M2-PK induces or enhances activation of Stat5. M2-PK may provide ATP for nuclear functions, including transcription. Alternatively, PK may function as a signaling molecule to modulate nuclear function. Further studies will be required to elucidate how nuclear M2-PK enhances cell proliferation.

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