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Phosphoinositide 3-kinases can act independently of $p27^{Kip1}$ to regulate optimal IL-3-dependent cell cycle progression and proliferation

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Abstract

We have examined the role of phosphoinositide 3-kinases (PI3K) in interleukin (IL)-3-dependent cell cycle progression and compared the effects of LY294002 with expression of a dominant negative form of p85, termed Δp85, which more specifically inhibits class I_A PI3Ks. Inhibition of PI3Ks in BaF/3 led to accumulation of cells in G1 and extension of cell cycle transit times. Biochemically, both LY294002 and Δp85 decreased levels of p107 and cyclins D2, D3 and E and reduced retinoblastoma protein (pRb) phosphorylation. Significantly, only LY294002 treatment increased expression of $p27^{Kip1}$. Interestingly, LY294002 decreased IL-3-induced proliferation of primary bone marrow-derived mast cells (BMMC) derived from both wild-type and $p27^{Kip1}$-deficient mice and importantly, LY294002 treatment failed to upregulate $p27^{Kip1}$ in wild-type BMMC. These data support a role for class I_A PI3K in regulating optimal cell cycle progression in response to IL-3 and demonstrate that upregulation of $p27^{Kip1}$ is not essential for attenuation of the cell cycle resulting from PI3K inhibition.

Keywords: Interleukin-3; Phosphoinositide 3-kinases; $p27^{Kip1}$; Cell cycle; Mast cells

1. Introduction

Interleukin (IL)-3 is a pleiotropic cytokine that has been shown to be important for the survival and proliferation of mast cells and basophils [1,2]. IL-3-binding to its high-affinity receptor activates a number of signaling cascades [3] that include Jak2/STAT5, the Ras/Raf/mitogen extracellular kinase (MEK)/mitogen-activated protein kinase (MAPK) module [4,5] and members of the class I_A phosphoinositide 3-kinase (PI3K) family [6,7]. PI3Ks are a family of lipid kinases, whose products, phosphoinositide (3,4) bisphosphate [$PI(3,4)P_2$] and phosphoinositide (3,4,5) triphosphate [$PI(3,4,5)P_3$] act as intracellular second messengers [8,9]. The class I_A family of PI3Ks comprises a regulatory subunit (p85) and a 110-kDa catalytic subunit [8,9]. Three forms of class I_A p110 (α, β, δ) have been identified, with the p110δ isoform being largely restricted in its expression to cells of the immune system [7,10]. Members of the distinct classes of PI3Ks have been implicated in the regulation of an array of physiological processes, notably the control of proliferation, cell survival, cell migration and trafficking [8,9].

Microinjection studies first suggested a role for class I_A PI3Ks in regulating cell proliferation [11–13] and defects in proliferation have been reported in mice bearing knockouts

Abbreviations: BMMC, bone marrow-derived mast cells; CFDA SE, carboxy-fluoroscein diacetate succinimidyl ester; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; ERK, extracellular-regulated kinase; FOXO, Forkhead family transcription factors; G1, Gap phase 1; G2, Gap phase 2; IL, interleukin; MAPK, mitogen-activated protein kinase; MEK, mitogen extracellular kinase; M phase, mitosis; PI, propidium iodide; PI3K, phosphoinositide 3-kinases; PKB, protein kinase B; pRb, retinoblastoma protein; S phase, DNA synthesis phase; SCF, stem cell factor; STAT, signal transducer and activator of transcription; Tet, Tetracycline; WT, wild type; XTT, sodium 3′ -[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-*bis*(4-methoxy-6-nitro)benzene-sulphonic acid hydrate.

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of either the regulatory or catalytic subunits of class I_A PI3Ks [14–18]. In addition, we have reported that specific inhibition of class I_A PI3Ks reduces IL-3-induced proliferation of BaF/3 cells [19]. Studies performed in a variety of cells have primarily located the requirement for PI3K activity to the G1 phase of the cell cycle [20] and treatment of cells with pharmacological inhibitors of PI3Ks, e.g., LY294002 or wortmannin, leads to partial cell cycle arrest, with cells typically accumulating in mid to late G1 phase [21–25].

Progression through the mammalian cell cycle is regulated by the sequential and cooperative activation of different classes of cyclin-dependent kinases (CDKs, reviewed in Ref. [26]), which in turn phosphorylate key cell cycle regulatory proteins. The activity of cyclin:CDK complexes are in turn regulated by members of the cyclin-dependent kinase inhibitor (CKI) family of proteins. Levels of the G1 cyclins, D1, D2 and D3 can be regulated in a PI3K-dependent manner in a variety of cell types [27–32] including T and B lymphocytes [33–35] and in IL-2 signalling, PI3K activity is required for maximal induction of cyclins D2, D3 and E, potentiating signals transduced via Stat5 [35,36] as well as for pocket protein phosphorylation [33]. Pharmacological inhibition of PI3Ks has, in many cases, also been shown to lead to increased levels of the CKI $p27^{Kip1}$ [21,22,33,37], which has been associated with both cellular quiescence (reviewed in Refs. [38,39]) as well as regulation of apoptosis of lymphocytes [39,40]. $p27^{Kip1}$ levels can be regulated in a PI3K-dependent manner via forkhead transcription factors in IL-3-dependent BaF/3 cells [37,41] and ectopic expression of $p27^{Kip1}$ has been reported to induce apoptosis of BaF/3 cells [37,42] implicating PI3K-dependent regulation of $p27^{Kip1}$ as an important regulator of cell survival. A common theme to have emerged from these studies is that PI3Ks are required for modulating the levels and activities of several key regulators of the G1 phase of the cell cycle.

We have reported previously that expression of dominant negative p85 (Δp85), which specifically targets class I_A PI3Ks, reduces IL-3-induced proliferation of BaF/3 cells [19] and decreases phosphorylation of protein kinase B (PKB), Bad [19], Mek, Erk1 and Erk2 [43]. To date, pharmacological inhibitors of PI3Ks, which target all three PI3K subfamilies to a greater or lesser extent, have been widely used to demonstrate a role for PI3Ks in regulation of the cell cycle. Our main interest is in the specific role of class I_A PI3Ks in IL-3 signalling. No direct comparative studies have been reported so we have carefully compared if more specific inhibition of class I_A PI3Ks, achieved by regulated expression Δp85, has similar effects on IL-3-controlled cell cycle regulation to treatment with the broad specificity PI3K inhibitor, LY294002. Our results support a role for class I_A PI3Ks in regulation of the IL-3-controlled cell cycle, but suggest that in certain cell systems $p27^{Kip1}$ does not play an essential role in PI3K-dependent regulation of the cell cycle.

2. Materials and methods

2.1. Cell culture

Murine IL-3-dependent BaF/3 cells and derivatives expressing the tetracycline transactivator (tTA) were a kind gift of DNAX, Palo Alto, CA [44]. The characterization of clones inducibly expressing myc-epitope tagged Δp85 (BaF/3Δp85) has been described previously [19]. All cells were maintained at 37 °C, 5% (v/v) CO_2 in a humidified incubator in RPMI 1640 medium supplemented with 10% (v/v) FBS (Sigma), 20 μM 2-mercaptoethanol, 100 u penicillin/streptomycin and 2 mM glutamine (RPMI media), with the addition of 10% (v/v) conditioned media from WEHI3B cells as a source of murine IL-3. In some experiments cells were cultured in AIM-V media (Invitrogen) containing 400 pg/ml rmIL-3 (R&D Systems, Oxon). Δp85 expressing clones were cultured in the presence of 2 μg/ml tetracycline.

2.2. Mice, genotyping and bone marrow-derived mast cells

The $p27^{Kip1}$-deficient mice [45] used in this study were maintained on a mixed C57BL/6×CBA genetic background. Heterozygous $p27^{Kip1+/-}$ knockout mice were intercrossed to generate wild-type, $p27^{Kip1+/-}$ heterozygous and $p27^{Kip1-/-}$ homozygous (null) offspring. $p27^{Kip1}$ null animals were genotyped by PCR using primers (described by Fero et al. [45]) that amplified a 500-bp fragment unique to the mutant allele (N1 forward primer, 5′-CCTTCTATGGCCTTCTTGACG-3′ and K3 reverse primer, 5′ TGGAACCCTGTGCCATCTCTAT′-3′, using conditions of 95 °C for 60 s, 60 °C for 60 s and 72 °C for 60 s for 36 cycles), while being deficient for a 1305-bp fragment specific to the wild type allele (K5 forward primer, 5′-GAGCAGACGCCCAAGAAGC-3′ and K3 reverse primer; using conditions of 93 °C for 30 s, 57 °C for 30 s and 65 °C for 2 min for 40 cycles). All mice were housed under standard conditions, as previously described [46]. Wild-type and $p27^{Kip1-/-}$ mice were sacrificed by cervical dislocation, and IL-3-dependent bone marrow-derived mast cells (BMMC) were derived and cultured as described previously [47]. Immunoblotting with anti-$p27^{Kip1}$ antibodies was performed to verify the genotype of the WT and $p27^{Kip1}$-deficient BMMC.

2.3. Cytokine-dependent proliferation assays

Sodium 3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-*bis*(4-methoxy-6-nitro) benzene-sulphonic acid hydrate (XTT) bioreduction assays to assess growth of BMMC in response to IL-3 or stem cell factor (SCF) were performed as previously described [19,48]. Five thousand BMMC per well were set up in triplicate in 96-well plates in the presence or absence of different doses of LY294002 (Calbiochem) or DMSO alone in media containing different