Journal of Cellular Biochemistry 86:624–629 (2002)

PROSPECT

Runx1, c-Myb, and C/EBPα Couple Differentiation to Proliferation or Growth Arrest During Hematopoiesis

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Abstract Immature hematopoietic precursors proliferate as they differentiate, whereas terminal differentiation is associated with cell cycle arrest. Stem cell lineage commitment and subseqent maturation is regulated predominantly by transcription factors. Runx1 and c-Myb act in early stage hematopoietic cells to both stimulate proliferation and differentiation, whereas C/EBPα, and perhaps other C/EBP family members, block progression from G1 to S and induce terminal maturation. Coupling of differentiation to either proliferation or growth arrest by transcription factors is likely an important regulatory mechanism in multiple developmental systems. J. Cell. Biochem. 86: 624–629, 2002. © 2002 Wiley-Liss, Inc.

Key words: Runx1; C/EBPα; c-Myb; hematopoiesis; cell cycle

RUNX1

Runx1(AML1) heterodimerizes with CBFB(CBFβ) to form core binding factor (CBF). Runx1 contacts both CBFB and DNA via its N-terminal Runt domain and can both activate and repress transcription. CBFB increases the DNA-affinity of Runx1. Runx1 is predominantly expressed in hematopoietic cells and is essential for early hematopoiesis, as Runx1 (−/−) mice lack all definitive hematopoietic lineages, including the lymphoid lineages [Friedman, 1999]. Runx1 has been implicated as a regulator of early lymphoid and myeloid differentiation by its ability to transactivate genes expressed specifically in these lineages, including the T-cell receptor δ and myeloperoxidase (MPO) genes [Redondo et al., 1992; Suzow and Friedman, 1993; Nuchprayoon et al., 1994].

In addition, several lines of evident indicate that CBF also stimulates G1 to S cell cycle progression. CBFB-SMMHC is an oncogene expressed in a subset of acute myeloid leukemia (AML) cases and acts as an inhibitor of endogenous Runx1 via its ability to complex with Runx1 [Friedman, 1999]. Induction of CBFB-SMMHC from the zinc-responsive metallothionein (MT) promoter in either Ba/F3 pro-B or 32D cl3 myeloblast cells slows G1 to S progression without inducing apoptosis [Cao et al., 1997]. Cell cycle inhibition was associated with increased hypo-phosphorylated retinoblastoma (Rb) protein relative to phosphorylated Rb, indicating a block at or prior to the restriction point. A second study demonstrated that neither the CBFB nor the SMMHC domains alone affected proliferation and that deletion of 10 CBFB residues required for interaction with Runx1 obviated the ability of CBFB-SMMHC to inhibit G1 progression [Cao et al., 1998]. These findings indicate that CBFB-SMMHC slows proliferation by inhibiting Runx1 and not by interactions mediated by its SMMHC domain. This conclusion is solidified by the finding that activation of an exogenous Runx1-ER fusion protein, using 4-hydroxytamoxifen (4HT), prevents cell cycle slowing by CBFB-SMMHC [Lou et al., 2000]. In addition, over-expression of Runx1 in 32D cl3 cells modestly stimulates G1 to S progression, a finding confirmed using Runx1-ER [Lou et al., 2000; Strom et al., 2000].

As an independent means to inhibit Runx1-regulated genes, we developed a fusion protein, KRAB-Runx1-ER, containing the KRAB trans-

Alan D. Friedman is a Scholar of the Leukemia and Lymphoma Society.

Grant sponsor: NIH; Grant sponsor: Children's Cancer Foundation..

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Received 7 June 2002; Accepted 11 June 2002

DOI 10.1002/jcb.10271

repression domain, the Runx1 DNA-binding domain, and the estradiol receptor ligand-binding domain. Activation of KRAB-Runx1-ER potently slows G1 progression in Ba/F3 or 32D cl3 cells. In 32D cl3 cells, activation of KRAB-Runx1-ER also prevents G-CSF-mediated induction of the endogenous MPO mRNA, demonstrating a simultaneous requirement for Runx1 to stimulate proliferation and differentiation [Lou et al., 2000]. Runx1-ETO, another CBF oncoprotein, acts in a manner analogous to KRAB-Runx1-ER as it contains the Runx1 DNA-binding domain linked to the ETO co-repressor [Friedman, 1999]. Two groups have shown that Runx1-ETO slows G1 to S progression in hematopoietic cells [Amann et al., 2001; Burel et al., 2001]. We have recently confirmed these findings by expressing Runx1-ETO from the MT promoter in Ba/F3 cells. In addition, mutating the Runx1-ETO DNA-binding domain obviates its ability to slow Ba/F3 proliferation, verifying that this effect occurs via repression of Runx1-target genes [Kummalue and Friedman, 2002].

The finding that KRAB-Runx1-ER or Runx1-ETO slow proliferation suggests that Runx1 transactivates one or more genes required for G1 progression. This conclusion is supported by the finding that the Runx1 transactivation domain is required for Runx1-ER to prevent CBFB-SMMHC-mediated cell cycle inhibition [Bernardin and Friedman, 2002]. Relevant Runx1 target genes remain to be identified. Retroviral expression of Runx1-ETO slows the growth of murine or human myeloid progenitors [Okuda et al., 1998; Rhoades et al., 2000; Mulloy et al., 2002]. However, the precise contribution of Runx1 activity to cell cycle progression in various hematopoietic stem/progenitor populations remains to be determined.

Inhibition of proliferation by CBFB-SMMHC and Runx1-ETO seems paradoxical in view of the ability of these oncoproteins to contribute to myeloid transformation. We have proposed that additional mutations which accelerate G1 enable these CBF oncoproteins to contribute to transformation by blocking differentiation, or perhaps apoptosis. In Ba/F3 cells, exogenous cdk4, cyclin D2, or c-Myc prevent cell cycle inhibition by CBFB-SMMHC or KRAB-Runx1-ER [Lou et al., 2000; Bernardin et al., 2002a]. To evaluate our model in vivo, we transduced wild-type murine marrow progenitors with a retroviral vector expressing both CBFB-SMMHC and E7, a viral protein which speeds G1 by inactivating Rb. In addition, $p16^{INK4a}p19^{ARF}$ (−/−) progenitors were transduced with CBFB-SMMHC alone. Transduced cells were injected into irradiated, syngeneic wild-type recipients. Compared to relevant control groups, a significantly increased rate of leukemia formation was seen when CBFB-SMMHC was combined with either E7 or loss of the over-lapping p16 and p19 genes [Yang et al., 2002]. Another CBF oncoprotein, TEL-AML1, also co-operates with loss of the p16p19 genes to induce acute leukemia in mice [Bernardin et al., 2002b].

c-MYB

c-Myb has an N-terminal DNA-binding domain and a centrally located transactivating domain. c-Myb is predominantly expressed in immature hematopoietic cells, and c-Myb (−/−) mice lack the lymphoid, myeloid, and erythroid lineages [Friedman, 2002]. Inhibition of c-Myb expression slows the proliferation of myeloid and lymphoid cells [Anfossi et al., 1989; Gewirtz et al., 1989], and over-expression of c-Myb prevents growth arrest associated with terminal erythroid or myeloid differentiation [Clarke et al., 1988; Bies et al., 1995]. The mechanisms responsible for cell cycle stimulation by c-Myb remain to be firmly established.

In addition to stimulating proliferation, c-Myb activates lineage-specific genes in immature hematopoietic cells. Notably, c-Myb and Runx1 co-operate to induce the T-cell receptor δ gene in T-cells, the MPO and neutrophil elastase (NE) genes in myeloblasts, and the Flt3 gene in pluripotent stem cells [Hernandez-Munain and Krangel, 1994; Nuchprayoon et al., 1994; Britos-Bray and Friedman, 1997; Qian et al., 1998]. In particular, Runx1 activation of the MPO promoter is increased approximately 10-fold by c-Myb's interaction with two nearby binding sites. As direct protein–protein interaction between c-Myb and Runx1 has not been demonstrated, this synergy is likely mediated by co-activators.

C/EBPα

C/EBPα, the founding member of the C/EBP family of bZIP transcription factors, binds DNA as an obligate dimer. Homo- or hetero-dimerization of C/EBPs occurs via their C-terminal leucine zipper domains, and DNA-contact is via the adjacent basic regions [Friedman, 2002]. Induction of the liver-specific gene encoding

serum albumin, via a binding site in its promoter, provided the first demonstration the C/EBPα possesses transactivation activity [Friedman et al., 1989]. In that same study, C/EBPα was shown to be expressed at much higher levels in quiescent hepatocytes compared with proliferating hepatoma cell lines, leading to the suggestion that C/EBPα stimulates hepatocyte differentiation while simultaneously inhibiting proliferation. The first direct evidence that C/EBP inhibits cell proliferation came serendipitously, as part of an experiment I conducted in which a C/EBPα basic region mutant was stably introduced into 3T3-L1 preadipocytes. While expression of this mutant did not inhibit 3T3-L1 differentiation, introduction of wild-type C/EBPα, as a control, greatly reduced the colony yield. Subsequent experiments using a C/EBPα-ER fusion protein demonstrated inhibition of 3T3-L1 cell cycle progression without induction of differentiation [Umek et al., 1991].

In hematopoietic cells, C/EBPα, C/EBPβ, and C/EBPδ are expressed predominantly in granulocytic and monocytic myeloid cells [Scott et al., 1992]. C/EBPα expression is highest in immature myeloid cells, whereas C/EBPβ, C/EBPβ, and C/EBPε expression increases during granulocyte or monocyte maturation [Scott et al., 1992; Hohaus et al., 1995]. C/EBPs regulate genes expressed in immature myeloid cells, including those encoding MPO, NE, and the M-CSF and G-CSF receptors [Nuchprayoon et al., 1994; Zhang et al., 1994; Ford et al., 1996; Oelgeschläger et al., 1996; Smith et al., 1996]. In addition, C/EBPα may activate the genes encoding the PU.1 and C/EBPε transcription factors as granulocytic cells mature [Wang et al., 1999; Wang and Friedman, 2002]. C/EBPs also regulate genes most highly expressed in mature myeloid cells, such as the lactoferrin gene [Khanna-Gupta et al., 2000].

C/EBPα (−/−) mice lack granulocytic cells but retain monocytes, whereas mice lacking both C/EBPβ and C/EBPδ apparently develop all of the hematopoietic lineages [Zhang et al., 1997]. Reintroduction of the G-CSF receptor into C/EBPα (−/−) progenitors restores the production of neutrophils [Zhang et al., 1998]. Compensatory effects from other C/EBP family members, each of which binds the same DNA consensus site, might enable granulopoiesis in this paradigm [Jones et al., 2002]. To globally inhibit C/EBP-regulated genes, we expressed KRAB-C/EBPα-ER, together with exogenous G-CSF receptor, in 32D cl3 cells. Activation of this inhibitory fusion protein prevented G-CSF-mediated granulopoiesis, indicating that C/EBPs regulate genes beyond the G-CSF Receptor critical for granulocyte development [Wang and Friedman, 2002]. The precise role of C/EBPs in monocyte commitment and maturation remains to be established. KRAB-C/EBPα-ER prevented the outgrowth of both granulocytic and monocytic colony-forming units, CFU-G and CFU-M, without affecting the development erythroid BFU-E from murine marrow or fetal liver. Similar findings were obtained with an alternative dominant-inhibitory C/EBPα [Iwama et al., 2002]. On the other hand, KRAB-C/EBPα-ER does not affect monocyte/macrophage maturation in cultured cell lines [Wang and A.D.F., unpublished].

When stably expressed in 32D cl3 cells, C/EBPα-ER, C/EBPβ-ER, or C/EBPδ-ER induce granulocytic maturation and an associated G1 cell cycle arrrest, events which also occur when parental 32D cl3 cells are cultured in G-CSF [Wang and Friedman, 2002]. Co-expression of bcr-abl(p210) prevented differentiation, but not C/EBPα-ER-mediated G1 arrest, indicating that C/EBPα has the capacity to arrest cell cycle progression in myeloid cells. In addition, C/EBPα-ER and C/EBPδ-ER markedly slow the proliferation of Ba/F3 cells without inducing their differentiation, while C/EBPβ-ER modestly retarded Ba/F3 proliferation, and the ER domain alone was ineffective [Wang et al., 1999; Wang and A.D.F., unpublished]. Thus, in contrast to Runx1 and c-Myb, C/EBPs simultaneously drive granulocytic differentiation and cell cycle arrest.

C/EBPα mediates cell cycle arrest via direct interaction with E2F and Cdk2/4 in several adherant cell lines [Porse et al., 2001; Wang et al., 2001]. Similarly, we have found that C/EBPα-ER mutants which cannot bind DNA retain the ability to slow G1 progression in 32D cl3 cells [Wang, Cleaves, and A.D.F., unpublished]. Notably, these lines do not differentiate in response to estradiol, indicating that cell cycle inhibition by C/EBPs is not sufficient for granulocytic maturation. On the other hand, cell cycle arrest is required for terminal granulopoiesis, perhaps reflecting a role for hypophosphorylated Rb in this process via direct interaction with PU.1 or C/EBPβ [Hagemeier et al., 1993; Chen et al., 1996].

SUMMARY

Figure 1 presents a model integrating the discussed effects of Runx1, c-Myb, and C/EBPs on hematopoietic proliferation and differentiation. Runx1 and c-Myb simultaneously stimulate cell cycle progression and lineage-specific gene expression in immature hematopoietic cells. A challenge for the future is to identify genes activated by Runx1 and c-Myb and required for cell cycle progression—perhaps such genes will be activated synergistically by these factors. C/EBPα is also present in immature myeloid cells, where it co-operates with Runx1 and c-Myb to activate several lineage-specific genes, including those encoding NE and the M-CSFR [Oelgeschläger et al., 1996; Zhang et al., 1996]. Yet, C/EBPα inhibits G1 to S cell cycle progression. Apparently the proliferative effects of Runx1 and c-Myb dominate the growth inhibitory effects of C/EBPα in immature myeloid cells. As myeloid progenitors mature, c-Myb levels fall, potentially allowing the anti-proliferative capacity of C/EBPα and other C/EBP family members to become evident. In keeping with falling c-Myb and perhaps Runx1, levels genes expressed in mature hematopoietic cells are not known to be activated by these factors. On the other hand, genes active in mature myeloid cells are typically regulated by C/EBP, PU.1, or both [Friedman, 2002]. Of note, activation of exogenous PU.1-ER does not affect 32D cl3 growth kinetics [Wang and Friedman, 2002].

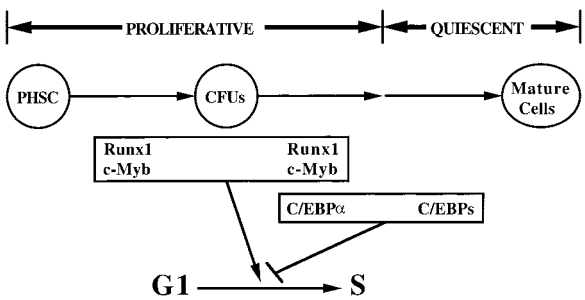

Fig. 1. Model integrating regulation of cell proliferation and differentiation by Runx1, c-Myb, and C/EBPs. Runx1 stimulates G1 to S cell cycle progression and differentiation of pluripotent hematopoietic stem cells (PHSC) and immature myeloid and lymphoid cells (e.g., CFUs). c-Myb acts similarly in immature myeloid, lymphoid, and erythroid cells. C/EBPs are present in both immature and mature granulocytic and monocytic myeloid cells, where they simultaneously inhibit G1 progression and induce differentiation. In immature myeloid cells, the proliferative stimulus from Runx1 and c-Myb dominates the inhibitory effect of C/EBPα, whereas mature myeloid cells become quiescent due to unopposed inhibition of the cell cycle by C/EBPs. Several genes expressed in immature myeloid cells are regulated co-operatively by Runx1 and/or c-Myb together with C/EBPα and/or PU.1, whereas genes active in mature myeloid cells are regulated by C/EBPs and/or PU.1, but not by Runx1 or c-Myb.

The concept that a subset of transcription factors that mediate differentiation also regulate proliferation, either positively or negatively, may be generally applicable to developing biologic systems. For example, MyoD both stimulates skeletal muscle-specific gene expression and inhibits cell proliferation [Crescenzi et al., 1990], analogous to the role we postulate for C/EBPs in granulocytes, hepatocytes, adipocytes, and perhaps other lineages. In contrast, Runx1 and c-Myb set a precedent for transcription factors capable of simultaneously stimulating proliferation and differentiation.

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