Id2 Is Dispensable for Myc-Induced Lymphomagenesis

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ABSTRACT

The Eμ-Myc transgenic mouse appears to be an accurate model of human Burkitt’s lymphoma that bears MYC/Immunoglobulin gene translocations. Id2, a negative regulator of basic helix-loop-helix transcription factors, has also been proposed as a Myc target gene that drives the proliferative response of Myc by binding to and overriding the checkpoint functions of the retinoblastoma tumor suppressor protein. Targeted deletion of Id2 in mice results in defects in B-cell development and prevents the development of peripheral lymphoid nodes. In precancerous B cells and lymphomas that arise in Eμ-Myc transgenic mice and in Burkitt’s lymphomas, Id2 is overexpressed, suggesting that it plays a regulatory role in lymphoma development. Surprisingly, despite these connections, Eμ-Myc mice lacking Id2 succumb to lethal B-cell lymphoma at rates comparable with wild-type Eμ-Myc transgenics. Furthermore, precancerous splenic B cells lacking Id2 do not exhibit any significant defects in Myc-induced target gene transactivation and proliferation. However, due to their lack of secondary lymph nodes, Eμ-Myc mice lacking Id2 rather succumb to disseminated lymphoma with an associated leukemia, with pronounced infiltrates of the bone marrow and other major organs. Collectively these findings argue that targeting Id2 functions may be ineffective in preventing Myc-associated malignancies.

INTRODUCTION

The Myc family of oncogenes (c-Myc, N-Myc, and L-Myc) is activated in most malignancies, either directly through chromosomal amplifications or translocations or through indirect means, by mutations in cancer cells that alter signaling pathways or tumorsuppressors that normally control or restrict Myc gene expression (1). The net result of these events is Myc overexpression, and the strong selection for Myc activation in cancer in part reflects its essential role as a master transcriptional regulator of cell cycle traverse and/or cell growth. For example, the deletion of c-Myc in mice leads to cell cycle arrest (in G1; ref. 2), whereas Myc overexpression is sufficient to provoke the entry of quiescent cells into S phase (3) and tumor angiogenesis (4, 5) and prevents withdrawal from the cell cycle and terminal differentiation (6–9). However, in normal cells Myc overexpression is generally a lethal event, because Myc triggers the apoptotic program (10, 11), and bypass of this checkpoint is necessary in most cell contexts as a forerunner to overt transformation (12).

All of the biological effects of Myc rely on its ability to function as a transcription factor (13, 14). Myc oncoproteins are basic helix-loop-helix-leucine zipper transcription factors that belong to a larger family of related basic helix-loop-helix-leucine-zipper factors, the so-called Max network (15). Max is cast as the central player of this network, because it functions as a shared dimerization partner for all of the members and is required for their binding to their recognition elements, the E-boxes CAYGTG (16–19). Max partners serve one of two functions, as activators of gene transcription, a role played by the Myc proteins, or as transcriptional repressors, a function carried out by the Mad family of proteins (Mad1, Mxi1, Mad3, and Mad4) or by Mnt or Mga (15). Until quite recently it was thought that Mad and Mnt functioned as antagonists of Myc by effectively competing with Myc for Max. However, recent data obtained by both knockout and knockdown approaches rather indicate that Myc functions as an antagonist of Mnt functions and activates its target genes by relieving Mnt-mediated transrepression (20, 21). The significance of the other transrepressors of the network is presently unclear but may include roles in the growth arrest phase that precedes differentiation (22).

Regardless of the precise mechanisms involved, given the diverse array of biological processes regulated by Myc it is not surprising that it regulates a large cast of genes. Indeed, recent studies using gene expression profiling and scanning chromatin immunoprecipitation analyses indicate that as many as one-tenth of all genes may be bound by Myc (23–26). Whether all of these are direct Myc targets remains unclear, particularly given the relatively low abundance of Myc protein expressed in proliferating cells (1,000 to 2,000 molecules per cell; refs. 27–29). Although the complete transcriptional output likely dictates the cellular outcome of Myc expression, several studies have addressed the role of individual targets in Myc responses. For example, genetic approaches using mouse embryonic fibroblasts from knockout mice have suggested that Cyclin D2, E2f1, Cyclin-dependent kinase 4 (Cdk4), and Inhibitor of differentiation 2 (Id2) are necessary for Myc-induced proliferation and/or transformation (30–34).

Id2 belongs to a dedicated family of helix-loop-helix proteins that cannot bind to DNA but rather dimerize with basic-helix-loop-helix transcription factors, such as the E proteins and MyoD, and prevents their binding to DNA and their ability to direct differentiation programs (35). Id2 is particularly important for the correct development of the lymphoid system, because Id2 overexpression blocks the development of dendritic cells and T and B lymphocytes in favor of production of natural killer cells (36, 37). Conversely, loss of Id2 compromises the formation of secondary lymphoid organs, such as the lymph nodes and Peyer’s patches, and blocks natural killer cell development (38). More subtle effects of Id2 loss are also manifest, as B-cell maturation and IgE-mediated class switching are enhanced in Id2−/− mice (39, 40).

Overexpression studies have suggested that Id2 functions as an oncogene, where it can drive cell proliferation and trigger apoptosis (41). This property of Id2 but not of its related cousins Id1, Id3, and Id4 is associated with its ability to bind to pocket proteins such as the retinoblastoma protein (pRb) and to overcome pRb-mediated growth arrest (42). Links of Id2 to Myc have also been reported, where Id2 has been proposed as a Myc target gene that mediates the ability of Myc to drive the cell cycle through disruption of the pRb checkpoint (32). Initial studies in neuroblastomas having amplified MYCN suggested that these tumors also overexpress Id2 (43), yet this finding has been contested (44, 45). Therefore, we addressed whether a Myc-to-Id2 pathway was important for tumor development using mice lacking Id2 and Eμ-Myc transgenic mice, a mouse model of human Burkitt’s lymphoma (46). Indeed, although Id2 is overexpressed in the B-cell lymphomas that arise in these mice and in human Burkitt’s lymphoma...
mas, we show that Id2 is not a direct target of Myc in B-cells and that Id2 is dispensable for Myc-induced lymphomagenesis.

MATERIALS AND METHODS

Mice and Tumor Analyses. Id2 knockout mice (on a mixed background; ref. 47) were bred with E Myc transgenic mice on C57BL/6 background (46). F1 E Myc/Id2 mice were bred and F1 offspring of E Myc/Id2 transgenic hemizygous littermates. Animals were observed daily for signs of morbidity and tumor development. Sick animals were sacrificed, and tumors and lymphoid organs were analyzed by histology and immunohistochemistry.

With Institutional Review Board approval and after informed consent, RNA was extracted from tumors of 14 Burkitt’s lymphoma patients using the RNA/DNA kit from Qiagen.

Cell Culture. Mouse embryo fibroblasts deficient in Id2 were obtained from embryonic day E13.5 embryos generated from matings of Id2 males and females and were prepared as described previously (48). Primary bone marrow-derived pre-B cells were generated from 6-week-old C57BL/6 as described (49). After 2 weeks in culture on S17 stromal cells, the established B-cell culture was infected with the MSCV-Myc-ER internal ribosomal entry site-green fluorescence protein retrovirus or with a control MSCV-internal ribosomal entry site-green fluorescence protein retrovirus (50). Myc-ER is a chimeric fusion of human c-Myc and the estrogen binding domain of the estrogen receptor modified such that it only binds to the estrogen receptor agonist 4-hydroxytamoxifen; in the absence of 4-hydroxytamoxifen this fusion protein is sequestered in heat shock complexes within the cytoplasm, but after the addition of 4-hydroxytamoxifen Myc-ER rapidly localizes to the nucleus where it activates the Myc transcriptional response (51). The MSCV-Myc-ER internal ribosomal entry site-green fluorescence protein retrovirus also harbors the gene for green fluorescence protein, which is expressed by virtue of an internal ribosome entry site. This allowed for the selection for infected cells by fluorescence-activated cell sorting for green fluorescence protein. To evaluate consequences of Myc activation cells were treated with 1 μmol/L 4-hydroxytamoxifen and harvested for RNA preparation.

Fluorescence-Activated Cell Sorting and Magnetic-Activated Cell Sorting of B Cells. Rates of proliferation of B220+ IgM+ and B220+ IgM- cells were determined using a Flow kit as described by the manufacturer (BD Biosciences PharMingen, San Diego, CA). Animals were i.p. injected with 100 μL of 10 mg/mL bromodeoxyuridine (BrdUrd) in sterile PBS. Animals were sacrificed 12 hours after injection, and bone marrow and spleen were harvested. One million cells were used for the BrdUrd proliferation assay, by incubation with antibodies against B220 (allophycocyanin-conjugated) and IgM (phycoerythrin-conjugated), followed by washes. Labeled cells were additionally processed and stained with FITC and BrdUrd antibody, washed, and analyzed by fluorescence-activated cell sorting.

The remainder of the bone marrow and spleen cells was incubated with beads conjugated to a B220 antibody (Miltenyi Biotech, Auburn, CA) and enriched by magnetic-activated cell sorting for B cells according to the manufacturer’s instruction. The same procedure was used to obtain splenic B cells as controls for Western blot.

RNA Preparation and Analyses. RNA was prepared from cultured or magnetic-activated cell sorting B cells using the RNeasy kit (Qiagen, Valencia, CA). For Affymetrix analyses, cRNA was synthesized using the One-Cycle Target Labeling and Control Reagent package (Affymetrix Inc., Santa Clara, CA), and the reaction was probed to the 430A mouse Affymetrix chip. The scanned data output was imported into the Spotfire software. After normalization, selected probe sets for genes indicated in Fig. 2 were clustered using the hierarchical clustering function of Spotfire. Statistical analysis was performed in Spotfire with the Anova function.

For real-time PCR, cDNA was prepared from 1 μg of RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR was performed using an iCycler machine (Bio-Rad) and the iTaq SYBR green kit. Data analyses were performed by comparing threshold cycle values with a control sample set as 1. Sequences for primers are available upon request.

Western Blot Analyses. Extracts from magnetic-activated cell sorting B cells, mouse embryo fibroblasts, and lymphomas from E Myc mice were prepared as described previously (52). Protein (100 μg/lane) was separated on a 15% SDS-PAGE gel, transferred to membranes (Protran, Schleicher & Schuell, Keene, NH), and blotted with antibodies specific for Id2 (C-20, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and β-Actin (AC-15, Sigma Chemicals, St. Louis, MO).

RESULTS

Id2 Overexpression Is a Hallmark of E Myc and Human Burkitt’s Lymphomas. The importance of Id2 to Myc-induced tumorigenesis is unresolved. For example, some studies have suggested that Id2 is a direct transcriptional target induced by c-Myc (32) and is downstream of N-Myc in human neuroblastoma (43), whereas others have failed to show correlations between Myc and Id2 expression and tumorigenesis (44, 45, 53). To address this issue we initially examined Id2 expression in lymphomas from patients suffering from Burkitt’s lymphoma that bear MYC immunoglobulin translocations (54) by real-time PCR and compared it to levels of Id2 expressed in CD19+ peripheral blood-derived B lymphocytes. Interestingly, 12 of 14 primary Burkitt’s lymphoma tumors displayed elevated levels of Id2 transcripts (Fig. 1A), indicating that Id2 may play a role in tumor development.

Although Myc transgenic mice carry a c-Myc transgene driven by the E heavy-chain enhancer (46), a scenario that essentially recapitulates the effects of the MYC immunoglobulin translocations that occur in Burkitt’s lymphoma. In E Myc transgenics c-Myc is selectively expressed in the B-cell compartment (46), and the mice undergo a rather protracted course of disease that involves high proliferative rates that are initially offset by a high apoptotic index. However, with time, secondary changes occur that allow for the development of aggressive, clonal, and lethal lymphomas, and the mice usually succumb to B-cell lymphomas by 3 to 4 months of age. Strikingly, similar to observations with human Burkitt’s lymphoma, E Myc lymphomas (8 of 8) expressed very high levels of Id2 protein relative to levels of Id2 expressed in B cells of wild-type littermates (Fig. 1B). Therefore, Id2 overexpression is a hallmark of Myc-driven lymphomas in mice and humans.

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**Id2 Is Not a Direct Myc Target in Primary B Cells.** The elevated levels of Id2 observed in lymphomas that arise in Eµ-Myc transgenics could simply reflect secondary consequences of transformation. To address this issue we evaluated the expression of Id family members in precancerous splenic B220+ B cells from three weanling-aged Eµ-Myc transgenics and compared these with their expression in B cells of three wild-type littermates. As expected, expression profiling demonstrated that precancerous Eµ-Myc B cells expressed elevated levels of c-Myc and Myc transcription targets ornithine decarboxylase (Odc; ref. 55) and E2f1 (ref. 56; Fig. 2A). Surprisingly, however, levels of Id2 (and Id3 and Id4) transcripts were comparable in wild-type and Eµ-Myc B-cells (Fig. 2A). In contrast, levels of Id1 transcripts were reduced in the B cells isolated from all three of the Eµ-Myc transgenic mice (Fig. 2A). Real-time PCR confirmed some of these findings (see below). Finally, analyses of Id2 protein levels in precancerous B cells demonstrated that B220+ B cells of Eµ-Myc transgenics expressed markedly reduced levels of Id2 protein relative to levels of Id2 observed in lymphomas that arise in these mice (Fig. 1B). Therefore, enforced expression of c-Myc in B cells does not induce Id2 expression in vivo.

Myc has been reported to activate Id2 expression in fibroblasts (32). Therefore, we also tested whether inducible activation of Myc, using the conditional Myc-ER transgene (51), was capable of inducing Id2 expression in primary pre-B cells. Myc-ER is a fusion protein of c-Myc and the ligand-binding domain of the estrogen receptor modified to bind the estrogen receptor agonist 4-hydroxytamoxifen. In the absence of agonist, Myc-ER is sequestered in the cytoplasm in heat-shock complexes, yet after the addition of 4-hydroxytamoxifen the protein rapidly relocates to the nucleus where it dimerizes with Max and induces the transcription of Myc target genes (51). Bone marrow was isolated from wild-type weanling-aged mice and cultured on S17 stromal cells in medium containing interleukin 7; within 2 weeks of culture 95% of cells were pre-B cells on the basis of expression of B220 and IgM (49). These cells were then infected with MSCV-Myc-ER-integrated ribosomal entry site-green fluorescence protein retrovirus or with a control MSCV-integrated ribosomal entry site-green fluorescence protein retrovirus, and green fluorescence protein-positive cells were expanded in culture. Cells were then treated with 4-hydroxytamoxifen to activate the Myc-ER transgene and RNA isolated at specific intervals to address whether Myc activation induced Id2 expression. Surprisingly, rather than induce Id2 mRNA, activation of Myc actually resulted in a slight decrease of Id2 transcript levels (Fig. 2B). By contrast, as expected, activation of Myc-ER lead to a robust induction of carbomyl-aspartate synthetase (cad) transcripts and to more modest increase in odc mRNA (Fig. 2B), both of which are direct targets induced by Myc (57, 58). Therefore, the ability of Myc to induce Id2 expression is, at a minimum, cell context specific, and the elevated levels of Id2 observed in tumors arising in Eµ-Myc transgenics mice are likely an indirect consequence of transformation, environmental factors, cell-cell contact, and/or angiogenic stimulation.

**Id2 Is Dispensable for Lymphoma Development in Eµ-Myc Transgenic Mice.** Despite the failure of Myc to induce expression of Id2 in B cells, the high levels of Id2 protein detected in Eµ-Myc lymphomas still suggested that Id2 could play a role in lymphoma development. To test this issue we bred Eµ-Myc mice to Id2 heterozygous (Id2+/−) mice to generate Eµ-Myc;Id2+/− animals. These animals were bred back to Id2−/− mice to generate all of the Id2 genotypes and to avoid the sterility issues associated with Id2 loss (47). Mice obtained from this cross were monitored for disease, and the time of disease was plotted against the percentage of animals still alive (Fig. 3). Strikingly, despite Id2 overexpression in lymphomas arising in Eµ-Myc transgenics (Fig. 1B), heterozygosity or loss of Id2 had essentially no effect on tumor incidence or survival, as Eµ-Myc;
Id2+−/− and Eμ-Myc;Id2−/− mice had mean survival times comparable with wild-type transgenics (of 111, 109, and 107 days for wild type Eμ-Myc, Eμ-Myc;Id2−/−, and Eμ-Myc;Id2+/− mice, respectively; Fig. 3).

A notable feature of lymphomas of Eμ-myc;Id2−/− mice was their presentation. Palpable lymphomas typically seen in Eμ-Myc mice (46) were lacking. Rather, the onset of disease was evident as a marked apathy and/or the presence of a swollen skull (see below). Only 2 of 19 Eμ-Myc;Id2−/− mice developed visible tumor masses at necropsy, but all had enlarged spleens (data not shown), a phenotype typical of Eμ-Myc transgenics (46). A lack of nodal lymphomas was confirmed by histopathological analyses (Fig. 4, A–C) and was apparently due to a the lack of peripheral lymph nodes in Id2−/− mice (47). Indeed, the two tumor masses that arose in Eμ-myc;Id2−/− were located within muscles.

FULL necropsy and histopathological examination of sick Eμ-Myc;Id2−/− mice revealed infiltrates of malignant B cells at multiple sites in the body, including the liver, kidney, spleen, skeletal muscles (including the periocular muscles of the eye), lungs, and the meninges of the brain (Fig. 4, A, E, and F, shows tumor masses in the meninges and skeletal muscles, respectively). Because Id2−/− mice are defective in peripheral lymphoid organ development, they also show lymphocyte infiltration into nonlymphoid organs such as the liver and lung (Yokota Y., unpublished data), akin to the phenotype of lymphotoxin β receptor-deficient mice, which also exhibit lymphoaplasia (59). Therefore, it is conceivable that this is the reason for the massive infiltration of leukemic cells in many organs of Eμ-Myc;Id2−/− mice. Besides these sites, the bone marrow of Eμ-Myc;Id2−/− mice was extensively infiltrated with neoplastic lymphocytes, resulting in a displacement of normal hematopoietic cells (Fig. 4D). Although these sites have been observed previously in Eμ-Myc mice (46), overall the data indicate that Eμ-Myc;Id2−/− mice succumb to disseminated lymphoma with an associated leukemia. Finally, immunohistochemistry demonstrated that the lymphomas were of B-cell origin, as they stained positive for B220, IgM, and the κ light chain (Fig. 4, G–I), but were negative for terminal deoxytransferase (data not shown).

Lymphoma development in Eμ-Myc mice is driven by high rates of cell proliferation but is held in check by apoptotic programs triggered by Myc that are bypassed during tumor development (12). In fibroblasts Id2 has been suggested to play an essential role in the proliferative response of Myc (43). Therefore, we also assessed the effects of Id2 loss on the proliferative rates of precancerous B cells of Eμ-Myc transgenic mice by injecting weaning-aged wild-type and Id2-deficient Eμ-Myc mice with BrdUrd. As expected, splenic and bone marrow-derived IgM+ and IgM− B cells from Eμ-Myc mice proliferated at a higher rate than B cells from wild-type littersmates. However, in contrast to the effects of loss of another Myc target E2f1, which compromises accelerated rates of growth of Eμ-Myc transgenic B-cells (30), loss of Id2 had essentially no effect on the hyperproliferative nature of IgM+ and IgM− Eμ-Myc B cells, irrespective of their origin from spleen or bone marrow (Fig. 5A).

Because Id2 has been proposed to regulate the proliferative response of Myc by disabling the pRB checkpoint (32, 42, 43), we reasoned that Id2 loss could affect target genes downstream of Myc or E2f1. To test this notion RNA was prepared from B220+ B cells from the bone marrow or spleen samples used in the BrdUrd assays, and the expression of c-Myc, Cad, E2f1, Odc, Id2, and Ccnel (encoding Cyclin E1) was analyzed by real-time PCR. As expected from expression profiling and ex vivo experiments with B-cells engineered to express Myc-ER (Fig. 2), we again failed to observe an induction of id2 mRNA levels upon Myc overexpression (Fig. 5B). Although some exceptions were evident among individual mice, these analyses generally indicated that the level of c-myc expressed in these B-cell compartments of wild-type Eμ-Myc versus Eμ-Myc Id2−− mice was the determining factor dictating the outcome of target gene expression, because the steady state levels of these transcription targets essentially followed that of c-myc (Fig. 5B).

DISCUSSION

The data presented herein challenge the notion of Id2 as a universal target gene of Myc and more importantly establish that Id2 functions...
are dispensable for Myc-induced pathways provoking accelerated rates of cell growth and transformation. Specifically our results show that Myc does not induce Id2 expression in B cells in vivo or ex vivo and that Id2 loss has no affect on the ability of Myc to accelerate B-cell growth or to provoke the development of lymphomas. Indeed, the only profound effect of Id2 on lymphoma development centered on where these lymphomas arise, and because Id2/H11002/H11002 mice lack peripheral lymph nodes (47) the locale of these tumors was sometimes rather unusual (e.g., the meninges in the brain or within muscles). Nonetheless, this did not impair the overall progression of this disease, because the survival of Eμ-Myc:Id2−/− mice was essentially equivalent to that of wild-type transgenics, nor did Id2 loss affect the target cell transformed by Myc, because the lymphomas that arose in Id2-deficient Eμ-Myc transgenics were still composed of malignant immature and mature B cells. Therefore, in this mouse model of Burkitt’s lymphoma, Id2 does not contribute to any aspect of lymphoma development that can be measured.

These findings contrast those of others suggesting that Id2 is a direct Myc transcription target that is essential for Myc biological effects (32, 43), but are rather totally concordant with the recent study of Murphy et al. (53) that has established that Id2 is also dispensable for Myc-induced skin tumorigenesis. Given the prevailing lack of connections between Myc and Id2 in these two in vivo studies, we suggest that attempts to prevent tumor formation by targeting the “Myc/Id2/Rb” pathway, as proposed previously (43), may be ineffective. Furthermore, high levels of a gene product in tumors, such as Id2, may not necessarily mean that this gene product is essential for a preferred pathway, because its deletion did not lead to any delay in tumorigenesis. However, these findings do not rule out that Id2 may play some role in tumor maintenance, which could be the area of future investigation. Thus, it is still feasible that targeting this pathway may be of some therapeutic benefit.

Our findings also do not preclude the possibility that Id2 induction/function plays some tissue-specific roles in the Myc response. Whereas the knock-in of N-Myc into the c-Myc gene in mice formally established that N-Myc and c-Myc functions are redundant (60), it remains possible that Id2 may be a target specifically of N-Myc in neuroblastoma but not of c-Myc in lymphomas or in papillomas. Along this line, at least two target genes, α-Prothymosin and Eca39, have been reported to be tissue-specific targets that are selectively

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**Fig. 5.** Loss of Id2 does not prevent Myc’s ability to induce B cell hyperproliferation and select E2f and Myc target genes. (A) BrdUrd assay on FACS-sorted bone marrow-derived (BM) or splenic B cells derived from weanling-aged mice of the indicated genotypes. Black bars depict IgM− B cells and white bars indicate IgM+ B cells. (B) SYBR green real-time PCR analysis of B220+ cells from spleen (striped bars) and bone marrow (gray bars) of weanling-aged littermates of the indicated genotypes, using primers directed against c-myc, cad, odc, id2, ccne1, and e2f1 mRNAs. Gene expression levels in triplicates are shown as fold changes of expression in Eμ-Myc B cells relative to expression of those expressed in B cells derived from wild type littermates.

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induced by c-Myc (61). Whether Id2 may be a specific target of N-Myc awaits the analysis of mouse models of neuroblastoma and/or the analyses of other cell types engineered to express comparable levels of either c-Myc or N-Myc.

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