Identification of a Novel c-Myc Protein Interactor, JPO2, with Transforming Activity in Medulloblastoma Cells

Annie Huang,1,2 Cynthia S.W. Ho,5,6 Romina Ponzielli,1,6 Dalia Barsyte-Lovejoy,5,6 Eric Bouffet,3 Daniel Picard,1,7 Cynthia E. Hawkins,1 and Linda Z. Penn1,6

1Cancer Research Program; 2Arthur and Sonia Labatt Brain Tumor Research Centre; Divisions of 3Hematology, Oncology and 4Pathology, Hospital for Sick Children; 5Division of Cellular and Molecular Biology, Ontario Cancer Institute; and 6Department of Medical Biophysics, University of Toronto, Toronto, Canada

Abstract
c-myc oncogene activation is critical in the pathogenesis of a spectrum of human malignancies. The c-Myc NH2-terminal domain (MycNTD) is essential for cellular transformation, and mediates critical protein interactions that modulate c-Myc oncogenic properties. In medulloblastoma, the most common malignant pediatric brain tumor, deregulated c-myc expression is linked with poorer disease phenotypes and outcomes. The biological basis for these associations is, however, not well understood. To better understand mechanisms underlying Myc-mediated transformation of medulloblastoma, we sought to identify novel MycNTD protein interactors from a medulloblastoma cell line library using a unique two-hybrid system. We identified a novel MycNTD binding protein, JPO2, which shows nuclear colocalization with c-Myc, and interacts with c-Myc both in vitro and in mammalian cells. In Rat1a transformation assays, JPO2 potentiates c-Myc transforming activity, and can complement a transformation-defective Myc mutant. Immunohistochemical studies indicate tumor-specific JPO2 expression in human medulloblastoma, and an association of JPO2 expression with metastatic tumors. Significantly, JPO2 expression induces colony formation in UW228, a medulloblastoma cell line, whereas RNAi-mediated JPO2 knockdown impairs colony formation in UW228, and in Myc-transformed UW228 cells. These data provide evidence for biochemical and functional interaction between c-Myc and JPO2 in medulloblastoma transformation. JPO2 is closely related to JPO1, a Myc transcriptional target with transforming activity. As tumor-specific JPO1 expression in human and murine medulloblastoma has also been reported; these collective observations suggest important functional links between the novel JPO protein family and c-Myc in medulloblastoma transformation. (Cancer Res 2005; 65(13): 5607-19)

Introduction
c-myc oncogene activation is a critical transforming event in the pathogenesis of many human malignancies (1–4); including medulloblastoma, the most common malignant pediatric brain tumor (5, 6). Myc activation in medulloblastoma is evidenced by genomic amplification of c-myc and the closely related N-myc genes in primary human tumors (7–9) and expression of Myc protein in a proportion of tumors without gene amplification (10–13). Significantly, both the c- and N-myc genes have been identified as downstream mediators of WNT and Hedgehog signaling, two key pathways implicated in medulloblastoma development (14–17). Activated c-Myc promotes tumor formation and metastases by a number of different mechanisms including modulation of differentiation, cell adhesion, and tumor angiogenesis, and has been associated with particularly aggressive behavior in a number of human malignancies, including breast and colorectal cancer (1–3). In medulloblastoma, c-myc genomic amplification and mRNA expression is also associated with particularly aggressive histologic tumor subtypes and poorer patient survival (9, 18, 19). However, the biological basis for this correlation is unknown.

c-Myc is a conserved DNA binding transcription factor, with a COOH-terminal basic helix-loop-helix-leucine zipper region which obligately dimerizes with Max, another basic helix-loop-helix-leucine zipper protein required for all known Myc functions including gene activation and repression (20–22). The NH2-terminal domain of c-Myc (MycNTD) is not known to bind DNA but is also indispensable for all known Myc functions including cellular transformation, cell cycle regulation, apoptosis, and transcriptional activation and repression (3, 23–25). The MycNTD contains two evolutionarily conserved protein interaction domains, myc box I and II (MBI and MBII), which are unique to the Myc family. The MBI region (amino acids 45-63), which is frequently mutated in lymphomas (26), contain several in vivo phosphorylation sites implicated in complex regulation of Myc function and degradation (27–29). The MBII domain (amino acids 129-143), which is essential for Myc-mediated transformation, interacts with several different proteins which are components of large transcriptional complexes with histone acetylation and chromatin remodeling activities, including TRRAP (30, 31), BAF53 (32), and TIP60 (33). Other MycNTD interactors identified thus far include core transcriptional proteins such as TBP (34), as well as coregulatory proteins such as p107 (35).

Because c-Myc functions as a DNA-binding transcriptional regulator, Myc protein interactions are believed to critically influence Myc target gene selection. The large spectrum of Myc target genes identified by recent high-throughput studies (22, 36–43) suggest that an equally complex network of Myc protein interactions is likely (44, 45). Existing data indicates the MycNTD functions as a critical protein interaction hub that integrates the transcriptional and biological functions of c-Myc (45). Studies conducted by ourselves and others indicate that the functional organization of the MycNTD is complex (23–25), and is likely influenced by different protein interactors in specific cellular context. Thus, studies of c-Myc binding partners in a variety of cell backgrounds will be essential for comprehensive delineation of mechanisms underlying the oncogenic role of c-Myc in different human malignancies.
In this study, we were interested in identifying MycNTD protein interactors with potential roles in Myc-mediated transformation of medulloblastoma cells. As conventional yeast two-hybrid studies are complicated by the intrinsic transactivation properties of the MycNTD, we used a modified yeast two-hybrid assay for transcriptional activators, called the repressed transactivator assay (46), to identify MycNTD interactors from a medulloblastoma cell line library. We report here on the identification of a novel MycNTD binding partner, JPO2, which augments Myc-mediated transformation and can complement a transformation defective Myc mutant. JPO2 expression enhances anchorage-independent growth in UW228 medulloblastoma cells, and is increased in metastatic medulloblastoma tissues. We show that JPO2 knockdown impairs anchorage-independent growth in UW228 and Myc-transformed UW228 cells, thus pointing to an important role for this novel protein in Myc-mediated transformation of medulloblastoma.

Materials and Methods

Library construction and screening. Derivation of bait and prey vectors (pBDH1 and p6G, respectively), repressed transactivator assay library construction and screening were previously described (46). To make the D341-TUP1 fusion library, polyA+ RNA from 5 × 10⁶ cells was used to generate cDNAs for directional cloning into the pBDH1 vector (Quick Prep mRNA purification and Time Saver cDNA synthesis kit, Amersham BD Bioscience, Piscataway, NJ). Yeast MAV108 cells were sequentially transformed with DNA isolated from the D341 cDNA library in E. coli DH10B, and plasmid pGAL4 MycNTD, and plated on synthetic dropout media lacking histidine (his−) and tryptophan (trp−). Primary transformants (3 × 10⁵) were collected and stored at −70°C, and 1 × 10⁶ clones were replated from thawed aliquots onto try− his− met− (−methionine) medium containing 0.05% fluoroorotic acid (FOA) and grown at 30°C. At 3 to 5 days, colonies were picked and restreaked onto synthetic dropout try−, his−, met medium with (+) and without (−) 0.05% FOA. Clones with reproducible differential growth on FOA-selective media, were further tested by cotransformation of isolated plasmids with the MycNTD bait and appropriate controls. cDNA inserts from positive clones were PCR-amplified, sequenced, and tested for expression of appropriate TUP1-fusion cDNAs by immunoblotting with anti-TUP-1 antibodies (46).

Plasmid constructions. Full-length JPO2 cDNA was amplified from human medulloblastoma cell line, HL60 (ATCC) mRNA using gene-specific primers (National Center for Biotechnology Information sequence AH099352), sequenced and directionally cloned into pCMV10 (Sigma) or pcDNA3-T7 to generate JPO2 proteins with an NH₂-terminal 3× FLAG or T7 epitope. Clone identity was also confirmed by Northern blot detection of reproducible differential growth on FOA-selective media, corresponding to chromatin and nuclear fractions, respectively, were analyzed with cytosolic fractions by immunoblotting.

Immunofluorescence. Transiently transfected 293TV cells were fixed in 3% paraformaldehyde, permeabilized in 0.1% Tween 20/PBS, and incubated with anti-c-Myc (1:500, N-262, Sigma, anti-α-tubulin (Calbiochem, La Jolla, CA), anti-histone H3 (Santa Cruz), anti-GST (Sigma), and anti-JPO2. Anti-JPO2 antibodies were raised against a synthetic NH₂-terminal peptide SSARLQNEKKTI LER KKD (amino acids 175-195) and enriched by affinity purification of rabbit antiseraum with peptide coupled Sulfalink column (Pierce, Rockford, IL). Antibody specificity was determined by analyses of JPO2 expression in cell lines with RNai knockdown of exogenous FLAG-tagged and endogenous JPO2. For sequential probing of Western blots, membranes were routinely stripped with Restore Buffer (Pierce).

Chromatin association assay. 293TV cell transiently expressing CMV10-FLAG-JPO2 were processed essentially as per Wysocka et al. (48). Trypsinized cells were washed with PBS, resuspended in 10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.34 mol/L sucrose, 10% glycerol, and 1 mmol/L DTT. Cells were lysed with 0.1% Triton X-100 and centrifuged to obtain cytosolic supernatant fractions and nuclear pellets. Nuclear pellets were lysed with 3 mmol/L EDTA, 0.2 mmol/L EGTA, 1 mmol/L DTT, and resup. Resulting pellets and supernatants, corresponding to chromatin and nuclear fractions, respectively, were analyzed with cytosolic fractions by immunoblotting.

Cell culture, transfections, and stable JPO2 cell lines. Medulloblastoma cell lines D341, D384, and D425 (gifts from Dr. Bigner, Duke University) were maintained in Zinc Option medium (Life Technologies, Gaithersburg, MD) with 20% fetal bovine serum. UW228 (a gift from Dr. Silver, University of Washington), Daoy, TB671 (ATCC) cells were maintained in α-MEM with 10% FBS, 293TV and Phoenix Eco (ATCC) cells were maintained in DMEM (Life Technologies) with 10% FBS. For transient expression, 293TV cells were routinely transfected with 2 to 5 µg of plasmids using Fugene (Roche) and analyzed 24 to 48 hours later. For stable JPO2 expression, JPO2 cDNA was cloned into pBabeMires-GFP or pBabeMires-Lyt2 (pMNGFP or pHNLyt2) retroviral vectors (gift from Dr. G. Nolan, Stanford University). Retroviruses produced in Phoenix Eco cells were used to infect various Rat1a or UW228 cells using standard techniques. To generate Rat1a cell lines coexpressing JPO2 and different MycNTD proteins, cell lines derived in a prior study (23) were infected with pMN-Lyt2 JPO2 or pMN-Lyt2 vector. For the UW228 cells, ecotropic viral receptors were introduced by infection with protein (TNT T7 coupled transcription translation system, Promega (Madison, WI). Five microilters of in vitro translation mix was incubated with 10 µg of GST or equimolar amounts of GST fusion proteins, and 50 µL of Sepharose GL4B beads (Amersham) for 1 hour at 4°C. After PBS washes, pull-downs were analyzed by immunoblotting. For GST pull-down with FLAG-JPO2, 500 µg of nuclear lysates from 293TV cells expressing CMV10-FLAG-JPO2 were used.

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pMN-lyt2 retroviruses; cells were then infected with pMN-GFP-c-Myc or pMN-GFP-JPO2 or corresponding vector control. Fluorescence-activated cell sorting–enriched cells were expanded and protein expression verified by immunoblotting.

**Clinical material and immunohistochemistry.** A medulloblastoma tissue microarray was previously constructed with Institutional Research Ethics Boards approval (11). For immunohistochemical analyses of JPO2, array slides were baked overnight at 60°C, deparaffinized, and rehydrated using standard techniques. Tissue slides were blocked for endogenous peroxidase and bixin, incubated overnight with anti-JPO2 antibodies (1:50), and counterstained with hematoxylin. JPO2 staining was graded for strength (0-3), and distribution (<25%, 25-50%, >50% of tumor cells positive, relative to positive control thymic tissue and negative control fetal cerebellum by two individuals. Only tumors with strength >3 and distribution >25% were considered positive. Tumors with no visible staining in multiple tissue cores were scored as JPO2-negative. Histopathologic grading of tumors was conducted according to the criteria of Eberhart and Burger (49). The SPSS software was used for statistical analyses.

**Tumor RNA, RT-PCR, and Northern blot analyses.** Standard protocols for RNA isolation, RT-PCR, and Northern analyses were followed. For RT-PCR, JPO2 primers (forward, 5′-CGGGAAGATTTACGAC-3′; reverse, 5′-CTGACATCCCTCCCATAG-3′), and previously described 6384 were used (42). Murine tissues RNA blot (Clonetech, BD Biosciences, Mountain View, CA) was hybridized with murine JPO2 cDNA (ATCC, MGC11198) and processed as per the manufacturer’s recommendation.

**JPO2 knockdown with RNAi.** Protocols and plasmids for Lentivirus-based RNAi construction were kindly provided by S. Stewart (Whitehead Institute). Oligomers for JPO2 RNAi construction were: forward, 5′-CCGGAATGCTTGAGTCCCTAGGTTACACGAC-3′; and reverse, 5′-AATTGGGACTGATCGTCTTTGAATTCTGTCCGACGAC-3′. Oligomers were annealed and ligated into lentiviral vector, pLKO.1-puro; plasmids with inserts were used to produce JPO2 RNAi virus by cotransfecting 293TV cells with helper virus plasmids, pLentihair vector, pLKO.1-puro; plasmids with inserts were used to produce expression. Cells were selected with puromycin, and tested for JPO2 were used to infect UW228 cells with and without stable FLAG-JPO2 expression library made from D341, a metastatic medulloblastoma cell line (51), with a bait encompassing amino acids 1 to 262 of the MycNTD. We reasoned that c-Myc interactors identified from D341 cells, which have high c-Myc levels due to gene amplification, might be more relevant to the oncogenic function of c-Myc in medulloblastoma. From our initial screen, a 850-bp partial cDNA (designated #44) was most frequently identified, and hence, of interest for further characterization. To confirm specific interaction of #44 with the MycNTD bait, repeat cotransformation of combinations of empty bait (pDBH1) or prey (pG) vectors with either purified #44 or the MycNTD plasmids were done. Selective growth of transformants containing both #44 and MycNTD, but not of transformants containing either MycNTD or #44 with empty bait or prey vectors on (-)FOA and (+)-FOA media, indicated specific interaction of #44 and the MycNTD in yeast cells (Fig. 1B).

We used a GST pull-down assay to determine if #44/MycNTD interaction also occurred in vitro. Radiolabeled 35S-#44 protein synthesized in vitro was tested for binding to purified GST and a GST-MycNTD fusion protein. As shown in Fig. 1C, #44 bound to GST-MycNTD fusion protein, but not to GST alone, indicating physical association of #44 with the MycNTD.

**JPO2, a novel conserved ring finger protein, is closely related to a c-Myc transcriptional target, JPO1.** Sequence analyses of #44 identified it as a partial cDNA clone of the DKFZP762B0311/RAM2 locus on chr7p15. We renamed DKFZP762B0311/RAM2 as JPO2 due to its sequence homology with a known gene JPO1 (ref. 52; Fig. 1D). JPO2 is predicted to encode a transcript of 2.8 kb and a putative full-length protein of 454 amino acids (predicted molecular weight, 52-53 kDa). Clone #44 spans 285 amino acids of the central and COOH-terminal regions of full-length JPO2. BLASTP analyses showed that JPO2 was evolutionarily conserved, with significant sequence homology to JPO1, a putative transcription factor that is a direct Myc target (38, 52). JPO1 encodes two isoforms that differ in an alternative 80-amino acid NH2-terminal exon. The longer JPO1 protein (v1) is comparable in size to JPO2. Sequence alignment of human and mouse JPO2 and JPO1 proteins is shown in Fig. 1D. Pairwise comparison using the ALIGN algorithm (53) showed that the proteins shared 45% overall amino acid identity with greatest homology in two regions that are conserved from humans to Xenopus: a central 21 amino acid (region I), and a COOH-terminal 128-amino acid (region II) segment. Despite positional conservation across species of 5 isoleucine or leucine within region I, and 11 cysteine residues in region II, no identity to known functional domains were found in database queries (pfam/HFF Fam database). However, analyses with PROSITE—a motif prediction software, indicates a 79–amino acid segment (amino acids 350-429) within region II contains a putative ring finger domain. Positional conservation of leucines within region I (amino acids 213-235) is suggestive of a leucine zipper domain. The significant amino acid homology between JPO2 and JPO1 indicate that they are family members.

**JPO2 interacts with c-Myc in mammalian cells.** To determine if JPO2 interacts with c-Myc in vivo, we performed coimmunoprecipitation analyses of full-length FLAG-JPO2 and c-Myc after transient coexpression in 293TV cells. For coimmunoprecipitation assays, nuclear lysates were immunoprecipitated with two different anti-Myc antibodies (N-262 or C-33), followed by immunoblotting with anti-FLAG antibodies to detect coimmunoprecipitated FLAG-JPO2 protein. Controls included immunoprecipitations of lysates with exogenous Myc or FLAG-JPO2 only, and immunoprecipitations using normal rabbit IgG (data not shown). Western blots of nuclear lysates with FLAG or Myc antibodies were used to confirm equivalent protein expression. As shown in Fig. 2A, FLAG-JPO2 was specifically coimmunoprecipitated with Myc by two different c-Myc antibodies. To rule out potential artifacts associated with...
FLAG-epitope tagging, communoprecipitation experiments were also conducted with c-Myc and T7 epitope-tagged JPO2 (data not shown). All communoprecipitation results were verified by corresponding reciprocal immunoprecipitations with anti-FLAG or anti-T7, followed by immunoblotting with anti-c-Myc antibodies (data not shown). These results confirm the association of JPO2 and c-Myc in vivo.

To determine if JPO2 interacts with c-Myc at endogenous levels, nuclear lysates from 293TV cells expressing FLAG-JPO2 were immunoprecipitated with anti-c-Myc antibodies or normal rabbit serum, and subject to immunoblotting with anti-JPO2 antibodies. As shown in Fig. 2B, JPO2 was specifically brought down by anti-c-Myc antibodies but not by normal rabbit serum. These data show that JPO2 interacts with endogenous c-Myc in 293TV cells.

**JPO2 localizes with c-Myc to the nucleus and is associated with nuclear chromatin.** JPO2 protein sequence contains a putative nuclear localization signal (Fig. 1D), thus suggesting that it may encode a nuclear transcription factor. To investigate the subcellular location of JPO2, we conducted immunofluorescence studies on 293TV cells transiently expressing FLAG-JPO2. Cells were fixed and immunostained with polyclonal anti-Myc to localize endogenous c-Myc, and monoclonal anti-FLAG antibodies to detect JPO2. As shown in Fig. 2C, JPO2 and endogenous c-Myc colocalize diffusely in the nucleus. In addition, JPO2 and c-Myc colocalize to unidentified subnuclear structures in some 293TV cells. A similar pattern of endogenous Myc localization was seen in cells mock-transfected with reagent, or empty vector (data not shown), thus suggesting that the observed pattern is unlikely to be a transfection-related artifact.

To further define the subnuclear location of endogenous JPO2 and c-Myc; we conducted chromatin association assays on 293TV cells. Chromatin, nuclear and cytoplasmic fractions prepared from log growing 293TV cells were analyzed by immunoblotting with anti-c-Myc and anti-JPO2 antibodies. Western blots were also probed with antibodies to α-tubulin, a cytoplasmic protein, to monitor efficiency of cellular fractionation, and anti-histone H3 to confirm chromatin enrichment. As shown in Fig. 2D, endogenous JPO2 is most highly enriched with endogenous c-Myc in chromatin fractions. Collectively, these observations provide additional evidence of physical interaction between c-Myc and JPO2 in the nuclear compartment, and are consistent with the predicted putative function of JPO2 as a nuclear transcription factor.

Delineating binding regions of JPO2 and the MycNTD. The initial clone, JPO2 clone #44 (amino acids 155-430), encompassed both the conserved putative leucine zipper and ring finger domains, hence these represented potential Myc binding regions. To define Myc-binding region(s) on JPO2, various FLAG-JPO2 deletion mutants were generated and tested for communoprecipitation with c-Myc after transient expression in 293TV cells (Fig. 3). Nuclear extracts were immunoprecipitated with anti-c-Myc antibodies followed by immunoblotting with anti-FLAG antibodies to detect JPO2 mutants that retained Myc binding (Fig. 3A, top). Myc binding affinity of the different mutants was approximated relatively to control full-length JPO2. The efficiency of Myc immunoprecipitations was checked by probing of stripped blots with anti-Myc antibodies (Fig. 3A, middle). Appropriate expression of different JPO2 mutants was estimated by direct anti-FLAG immunoblot analyses of input lysate (bottom). As summarized in Fig. 3B, construct 1 encoding only the amino terminus (amino acids 1-209) did not bind c-Myc. Constructs 2 (amino acids 1-250) and 3 (amino acids 1-309), encompassing the putative leucine zipper domain, both bind c-Myc. However, in contrast to constructs 3 and 4 (originally clone #44, amino acids 155-430), which bind Myc comparably to full-length JPO2, construct 2 seems to bind Myc only weakly (compare lanes -C, 2, 3, and 4 in Fig. 3A, top). Construct 5 (amino acids 326-454), which spans the ring finger domain, do not seem to bind c-Myc. This is consistent with the relative lack of change in Myc binding seen with construct 6 (del 378-381), which contains a small deletion of conserved cysteines within the ring finger domain. Deletion of amino acids 352 to 356 within the ring finger domain also had no effect on Myc binding (data not shown). These data suggest that Myc binds to a region encompassing the putative leucine zipper domain, hence finer mutagenesis of this region was carried out. As seen in lanes 7 and 8, deletions of conserved leucines within constructs 7 and 8 (del 213-218 and 227-232, respectively), significantly diminishes Myc binding by JPO2. Collectively, these results indicate that the conserved leucine zipper domain (amino acids 213-235) of JPO2 is important for Myc binding.

Our bait for the initial repressed transactivator assay screen encompassed amino acids 1 to 262 of the MycNTD as current evidence suggested that this might preserve the most appropriate native conformation of the MycNTD (54). To further define MycNTD regions that mediate interaction with JPO2, different GST-MycNTD constructs were assayed for binding to FLAG-JPO2.
transiently expressed in 293TV cells in GST pull-down assays. Immunoblotting with anti-GST and anti-FLAG antibodies were used, respectively, to detect GST proteins and associated FLAG-JPO2. As shown in Fig. 3C, JPO2 bound to GST-MycNTD (amino acids 1-262) and to a fusion protein encompassing amino acids 1-69 of the MycNTD, but not to GST alone. JPO2 was also bound by GST-MycNTD fusion proteins with deletions of either the MBI (del 45-63) or MBII (del 128-143) regions, we deduced from these data that amino acids 1 to 40 of the MycNTD were involved in JPO2 binding.

JPO2 potentiates Myc-mediated transformation and complements a Myc transformation defective mutant. Many cellular activities of c-Myc, such as stimulation of proliferation, death, and transformation, map to the MycNTD (23, 45, 55). To determine the functional significance of JPO2/MycNTD interaction on Myc-mediated cellular phenotypes, we assessed the effect of constitutive JPO2 expression in Myc-null Rat fibroblast (56) and Rat1a fibroblast cells. Stable cell lines were generated by infection with pMN-GFP retroviruses containing FLAG-JPO2. Fluorescence-activated cell sorting–enriched cell pools with confirmed exogenous JPO2 expression were assayed for growth. Neither Myc-null cells nor Rat1a fibroblasts expressing exogenous JPO2 alone, or together with human c-Myc showed appreciable changes in growth rate (data not shown), although it is possible that these cell systems may not be optimal for assessing the cell cycle effects of JPO2.

The transforming effect of human c-Myc can be shown and quantified in Rat1a fibroblasts, which exhibits changes in anchorage-independent growth and forms colonies in soft agar when Myc is constitutively expressed (24, 57). We and others have shown that Myc-mediated transformation is significantly impaired by mutations of the conserved MBII region of the MycNTD (23, 24, 55). In order to assess if JPO2 had a role in cellular transformation, stable Rat1a cell line pools expressing JPO2 alone or together with wild-type Myc, or Myc mutants within the conserved MBI and MBII regions, were generated and tested for colony formation in soft agar. Colony-forming efficiency of wild-type Myc-expressing Rat1a cells. Figure 4A summarizes the results of five independent experiments. Ectopic JPO2 expression had no significant effect on Rat1a colony formation compared with vector controls. However, JPO2 coexpression with c-Myc significantly potentiated the colony-forming efficiency of wild-type Myc by up to 1.9-fold (mean, 1.5×, Student’s t test; P < 0.005). Rat1a-Myc and Rat1a-Myc + JPO2 colonies did not differ remarkably in size or time

Figure 2. JPO2 interacts with c-Myc in mammalian cells. A, JPO2 coimmunoprecipitates with c-Myc. Nuclear lysates from 293TV cells transiently transfected with CMV10-FLAG-JPO2 alone or with pcDNA3-Myc were immunoprecipitated with rabbit polyclonal (N-262) or mouse monoclonal (C-33) anti-c-Myc antibodies. Coimmunoprecipitated JPO2 was visualized after SDS-PAGE and immunoblotting with anti-FLAG M2 antibodies (top). Efficiency of c-Myc immunoprecipitation was determined by direct immunoblotting with anti-Myc antibodies of stripped blots (bottom); input controls (10%) shown in the last lane. Arrows, FLAG-JPO2, Myc protein and IgH bands. B, endogenous c-Myc binds JPO2. Nuclear lysates from 293TV cells transiently transfected only with CMV10-FLAG-JPO2 were immunoprecipitated with anti-Myc rabbit polyclonal antibodies (N-262) or a negative control normal rabbit serum (NRS). Immunoprecipitates were resolved on a 10% SDS-PAGE gel together with 10% input control lysate. Sequential immunoblotting with rabbit polyclonal anti-JPO2-specific antibodies and anti-c-Myc monoclonal antibodies (9E10) were used to visualize JPO2 and c-Myc proteins in the immunoprecipitates and in the control lysate. Arrows, JPO2 and c-Myc protein bands. C, JPO2 colocalizes with c-Myc in the nucleus. For immunofluorescence studies, 293TV cells were transiently transfected with CMV10-FLAG-JPO2 for 24 hours. Cells were fixed, stained with anti-FLAG monoclonal antibody (M2) and rabbit polyclonal anti-Myc antibodies (N-262), and processed for confocal imaging. Green immunofluorescence staining shows nuclear localization of JPO2 (left), similar localization of endogenous c-Myc shown by red fluorescence was seen (middle). Merged fluorescence images indicate nuclear localization of JPO2 and Myc (right). D, endogenous JPO2 is enriched with endogenous c-Myc in nuclear chromatin. Logarithmically growing 293TV cells were harvested and subcellular fractions isolated as described in Materials and Methods. Equivalent protein amounts of cytoplasmic, nuclear, and chromatin-enriched subfractions were separated on 10% SDS-PAGE gels, and then sequentially probed for immunoblotting with anti-JPO2 and anti-c-Myc antibodies (9E10). Stripped blots were subsequently probed with anti-histone H3 and anti-α-tubulin antibodies to confirm effective fractionation of subcellular compartments.
of appearance (data not shown). JPO2 coexpression had no significant effect on colony formation by Rat1a cells expressing the MBI or MBII deletion mutants. In contrast, JPO2 expression also significantly enhanced colony formation by Rat1a cells expressing a transformation-defective MBII point mutant, W135E (Student’s t test, P < 0.003; Fig. 4B). The W135E mutant contains a nonconserved amino acid substitution of a tryptophan residue at amino acid 135 that renders it profoundly transformation defective (23, 55). Intriguingly, similar rescue of W135E transformation defect by the closely related JPO1 protein has also been reported (52). Appropriate protein expression in all cell lines was verified by immunoblotting (Fig. 4C). Collectively, these observations indicate

Figure 3. Mapping binding domains of JPO2 and c-Myc. A, a putative leucine zipper region of JPO2 is involved in Myc binding. Nuclear lysates from 293TV cells transiently transfected with full-length JPO2 (+C) or various CMV10-FLAG-JPO2 deletion constructs (1-8), together with pcDNA3-c-Myc were immunoprecipitated with anti-Myc monoclonal antibodies (C-33, left) or with anti-Myc rabbit polyclonal antibodies (N-262, right). Immunoprecipitates were resolved on a 10% SDS-PAGE gel prior to immunoblotting with anti-FLAG monocular (top) or anti-Myc antibodies (9E10, middle). Direct FLAG Western blot of 10% input lysates (bottom). Arrows, the position of full-length JPO2, c-Myc, and IgH protein bands. +, mark a weak band corresponding to JPO2 construct 2 (lane 2). Position of molecular weight markers are indicated. B, myc binding activity of various JPO2 deletion constructs (1-8) are summarized. Construct 4 (*) corresponds to the original partial JPO2 clone #44. Boxes, location of the conserved leucine-rich and putative ring finger domains; numbers refer to amino acids; slashed lines indicate the COOH-terminal end of the different constructs. Slashed lines through the boxes indicate deletion (del) of amino acids within the leucine-rich or ring finger domains. C, GST-pull-down assays to map JPO2 binding domain on c-Myc is shown on top; a schema of different c-Myc GST-fusion proteins used in GST pull-down assays is shown on the bottom. For pull-down assays, nuclear lysates prepared from 293TV cells transiently transfected with full-length CMV10-FLAG-JPO2 were incubated with purified GST-fusion proteins of: MycNTD (amino acids 1-262), Myc amino acids 1-69, MycNTD with MB1(del amino acids 45-66) and MBII (del amino acids 128-143) deletion. Nuclear lysates incubated with GST protein, or beads served as negative controls. Bound proteins recovered with glutathione beads were resolved on an SDS-PAGE gel and immunoblotted with anti-FLAG monoclonal antibodies to detect bound JPO2 (arrow). Input lysates (10%) shown in the last lane. Stripped blots were reprobed with anti-GST antibodies to ensure expression (bottom).
a role for JPO2 in Myc-mediated transformation of Rat1a cells, and also suggest possible functional overlap of the JPO2 and PO1 proteins in Myc-mediated transformation. JPO2 expression is tissue-restricted and up-regulated in medulloblastoma tumors. To assess the relevance of JPO2 to tumorigenesis, we first investigated JPO2 expression in normal murine adult tissues by Northern analyses. JPO2 identifies a relatively low abundance transcript of >2.5 kb, which is present at highest levels in prostate, thyroid, thymus, and salivary glands, but is not detectable in total brain mRNA (Fig. 5A). We observed similar tissue-restricted JPO2 expression in semiquantitative RT-PCR analyses of select human tissues (data not shown). As we were interested in investigating a potential role for JPO2 in medulloblastoma transformation, we first surveyed JPO2 expression in a subset of human medulloblastoma tumor tissues and cell lines by semiquantitative RT-PCR analyses. As shown in Fig. 5B, JPO2 transcript was expressed in all medulloblastoma tumors and cell lines tested, hence, indicating that JPO2 expression is up-regulated in medulloblastoma tumors.

To confirm and extend these observations, we conducted immunohistochemical studies on normal human fetal and adult cerebellum (data not shown) and primary human tumors using anti-JPO2 antibodies. Figure 5C illustrates diffuse and punctate nuclear JPO2 staining in select positive control thymic tissue cells. Notably, JPO2 protein was undetectable in cells of the external granular layer, the presumed cell of origin for medulloblastoma in fetal cerebellum. We used a previously described medulloblastoma tissue microarray (11) to examine JPO2 expression in primary human tumors. JPO2 stainings were graded by two individuals with respect to intensity and distribution of staining in each tumor core (described in Materials and Methods). We evaluated a total of 50 tumors, 33 tumors had strong JPO2 staining whereas 17 tumors were clearly negative in multiple tissue cores, thus JPO2 is differentially expressed in medulloblastoma. Representative JPO2-negative and -positive tumors are shown in Fig. 5C.

As c-myc expression has been linked to tumor anaplasia and survival in medulloblastoma (9, 19), we investigated whether JPO2 expression correlated with tumor grade, MiB-1 expression, patient survival or metastatic status. No significant association was observed between JPO2 expression and tumor grade, MiB-1 expression or survival. However, statistical analyses (Fig. 5C) indicated a modest, but intriguing association of JPO2 expression with presence of metastatic disease (P = 0.049, one-sided Fisher exact test).

JPO2 induces colony formation, and contributes to c-Myc-mediated transformation of medulloblastoma cells. Collectively, the tumor-restricted expression of JPO2, the potentiating effect of JPO2 expression on Myc-mediated transformation, and the association of JPO2 expression with metastatic medulloblastoma suggested an oncogenic role for JPO2 in medulloblastoma. To determine if JPO2 has transforming activity in medulloblastoma cells, UW228 medulloblastoma cell lines with stable ectopic expression of JPO2 were generated and tested in soft agar colony-forming assays after verification of protein expression (Fig. 6A). We chose UW228 as it is reported to be derived from a primary nonmetastatic tumor (58), and has relatively low endogenous levels of JPO2 and c-Myc.

Colonies with presence of JPO2 expression were readily visible after 7 days.
and were ultimately much larger than UW228-JPO2 colonies (representative image in Fig. 6B). These results suggest that although JPO2 has transforming activity in UW228 cells, JPO2 overexpression does not entirely recapitulate a Myc transformation phenotype in these cells. To more directly assess the contribution of endogenous JPO2 to anchorage-independent growth of parental UW228 cells and UW228 cells with constitutive c-Myc expression, we used a lentivirus RNAi system (50) to stably knock down JPO2 expression in UW228 and UW228-Myc cells. Cell lines with confirmed JPO2 knock down (Fig. 6C) were analyzed by soft agar colony forming assays with corresponding control lines. As shown in Fig. 6D, colony formation in UW228 and UW228-Myc cells were consistently reduced by 25% to 30%. (Student's t test statistic, \( P = 0.01 \) and \( P = 0.04 \), respectively) with JPO2 knockdown. Taken together, these results indicate that JPO2 is necessary for complete manifestation of the transformed phenotype in UW228 cells, and also point to an important role for JPO2 in mediating the full transforming effect of c-Myc in medulloblastoma cells.

**Discussion**

c-Myc oncogene activation is associated with poorer disease phenotypes and outcomes in medulloblastoma, however, the molecular basis for this association is not well understood. Many oncogenic properties of the Myc molecule, in particular, the ability

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**Figure 5.** JPO2 expression is tissue-restricted and associated with metastatic medulloblastoma. A, JPO2 mRNA expression in normal adult mouse tissues was surveyed by probing a polyA+ RNA Multiple Tissue Northern blot (Clontech) with a murine JPO2 cDNA clone. A, JPO2 transcript band of >2.5 kb was seen at the highest levels in prostate, thyroid, thymus, and salivary glands (lanes 2, 4-6, respectively). Bottom, less JPO2 expression was seen in spleen, liver, kidney and testes (lanes 10, 12, 14, and 15, respectively). JPO2 expression was not evident in large intestine, stomach, uterus, heart, brain, lung or skeletal muscle tissues (lanes 1, 3, 7, 8, 9, 11, and 13). Control hybridization with \( h \)-actin (bottom). Approximate position of RNA molecular weight marker is indicated. B, JPO2 transcript expression in primary human medulloblastoma tumors (11-17), and medulloblastoma cell lines (Daoy, UW228, D341, D384) was assessed by semiquantitative RT-PCR analyses. RT-PCR analyses of the 36B4 gene were used to monitor RNA integrity and quantity. C, immunohistochemical analyses of JPO2 expression in normal and neoplastic human cerebellum using polyclonal anti-JPO2-specific antibodies. Specific nuclear staining of JPO2 in select cells in positive control thymic tissue is shown (arrow; \( \times 400 \)). JPO2 expression was not detected in the external (EGL) and internal granular layer (IGL) cells of a 50 weeks old human fetal cerebellum. Representative medulloblastoma without (−) and with (+) JPO2 expression are shown (vern 200). White arrows, JPO2 staining in tumor nuclei. Immunohistochemical analyses of 32 nonmetastatic (−mets) and 18 metastatic (+mets) tumors for JPO2 expression is summarized in the bottom figure. JPO2 expression was strong (++) in 33, and absent (−) in 17 of 50 tumors studied. Strong JPO2 expression showed an association with presence of tumor metastases at diagnosis (one-sided Fisher exact t test, \( P = 0.049 \)).
of c-Myc to induce anchorage-independent cell growth, a hallmark of cellular transformation, is dependent on critical protein interactions of the MycNTD (32, 47). In this study, we report on the identification and characterization of a novel MycNTD binding partner, JPO2, with close sequence relatedness to JPO1, a known c-Myc transcriptional target (38, 52). JPO2 expression potentiates Myc-mediated transformation of Rat1a fibroblasts, and can rescue a transformation-defective Myc mutant. JPO2 also exhibits transforming activity in UW228 medulloblastoma cells. Knockdown of endogenous JPO2 impaired colony formation in UW228 and UW228-c-Myc cells thus demonstrating a role for JPO2 in c-Myc-mediated transformation of UW228 cells. Significantly, although JPO2 is undetectable in the developing normal human cerebellum, we showed JPO2 expression in a proportion of medulloblastoma tumors and observed an association of JPO2 expression with metastatic medulloblastoma. Collectively, our data provides evidence of a role for JPO2 in Myc-mediated transformation of medulloblastoma and in medulloblastoma tumorigenesis.

Binding partners of the MycNTD identified to date (1, 45) include proteins with functions in transcription (34) or transcriptional modulation (35), and others, such as TRRAP (30) and BAF53 (31) with putative roles in chromatin remodeling. Common c-Myc-binding motifs are not apparent in the Myc-NTD partners reported thus far. Similarly, JPO2 is not related to any known MycNTD interactors, but shows strong homology to JPO1, a c-Myc transcriptional target, particularly within putative leucine zipper and ring finger domains that are remarkably conserved from humans to Xenopus. BLASTN analyses suggest that unlike JPO1, the JPO2 protein may only encode one protein isoform. Although the predicted molecular weight of JPO2 protein is about 51 kDa, a JPO2-specific antibody identifies exogenous and endogenous protein bands of about 75 kDa. Stable knockdown of JPO2 mRNA in cell lines correlates with loss of a corresponding 75 kDa band, thus suggesting that JPO2 may undergo significant posttranslational modification.

Myc binding maps to a putative leucine zipper in domain I of JPO2. As deletions of residues in domain I (amino acids 213-218 and 227-232) significantly impaired but did not eliminate Myc binding, our data cannot exclude contributions from other JPO2 regions. Indeed, gross quantitative differences in Myc binding by JPO2 constructs spanning amino acids 1 to 250 and 1 to 309 suggest that residues within amino acids 251 to 309 are needed for optimal Myc binding by JPO2. Localization of Myc binding to the highly conserved putative leucine zipper suggests that this region may characterize other MycNTD interactors. Indeed, we

Figure 6. JPO2 induces colony formation, and also contributes to c-Myc-mediated transformation of UW228 medulloblastoma cells. A, UW228 cells infected with FLAG-JPO2 or c-Myc-containing retroviruses were checked for protein expression by immunoblotting with anti-FLAG and anti-c-Myc antibodies. Actin was used as a loading control. Cell lines were then tested for colony formation in soft agar assays. Summary of three experiments with three replicas per data point is shown. Colony numbers are expressed relative to UW228 parental cell lines infected with vector alone. Exogenous JPO2 and c-Myc expression augmented colony formation in UW228 to comparable degrees. B, JPO2-induced colonies were intermediate in size between that seen in the parental cells and c-Myc-induced colonies, a representative soft agar growth assay is shown. C, UW228 and UW228-c-Myc cells with stable knockdown of JPO2 were generated by infection with pLKO lentivirus containing JPO2-specific RNAi. Western blot with anti-JPO2 and anti-c-Myc antibodies was used to confirm specific JPO2 knockdown without appreciable effects on endogenous and exogenous c-Myc levels. Actin was used as a loading control. D, cell lines were assayed for growth in soft agar in parallel with appropriate vector control lines. Colony-forming efficiency are calculated relative to control parental lines infected with pLKO vector only. Results of three experiments with three replicas per data point are shown. JPO2 knockdown significantly (*) reduced colony formation in UW228 and in Myc-transformed UW228 cells (Student’s t test, P < 0.05).
have observed that the closely related protein JPO1 is also a Myc-binding partner. Although leucine-rich motifs have been reported in at least one MycNTD interactor (59), no common interaction motifs are evident among other MycNTD interactors identified to date (45). Thus, common higher-order protein structures likely play a very important role in mediating MycNTD protein interactions.

The nuclear location of JPO2 and its association with chromatin is consistent with a predicted biochemical function for JPO2 as a transcription factor. These observations are also in accordance with the association between JPO2 and c-MycNTD shown in vitro and in vivo, and collectively support the role of JPO2 as a c-Myc protein binding partner. In 293TV cells, JPO2 predominantly localizes in a diffuse pattern overlapping with that of c-Myc in the nucleus. However, in a proportion of cells, both proteins also, intriguingly, localized to ring-shaped subnuclear structures. Similar localization of c-Myc to such nuclear bodies has been reported and proposed to function in c-Myc proteolysis (28); however, the precise identity of these structures and their relevance to c-Myc biology await elucidation.

Our limited analyses of domain I which encompasses the conserved putative ring finger, suggests that it is nonessential for Myc/JPO2 interaction. However, as domain II encompasses a fairly extensive conserved region, further analyses will be needed to determine its precise role in Myc binding. Domain II, like domain I does not identify significantly with known functional domains. However, BLASTP analyses of domain II alone shows limited relatedness to a novel cysteine-rich, ring finger motif implicated in DNA binding by the human transcription factor, NF-XI (60), and its Drosophila homologue, Ste (61). Thus, JPO2 may also have DNA binding activity. Indeed, we showed endogenous JPO2 protein in chromatin-enriched cell fractions in this study.

c-Myc functions that map to the MycNTD include cellular transformation, induction of apoptosis and gene activation and repression. Our GST pull-down data support the association of JPO2 with residues NH2-terminal to the MBI domain of c-Myc, however, involvement of other MycNTD regions in Myc/JPO2 interaction cannot be ruled out by these experiments. Indeed, binding to more than one MycNTD region has characterized a number of interactors identified to date such as TTRAP (47) and Bin-1 (62). Significantly, deletions of residues NH2-terminal to the MBI region have also been shown to substantially impair Myc transforming activity (30); hence, the association of JPO2 with this region would be consistent with a role for JPO2 in Myc-mediated transformation as we showed in this study. As a transactivation domain has also been identified within amino acids 1 to 41 of the MycNTD (63), it is tempting to speculate that the observed effect of JPO2 on Myc-mediated transformation may be linked to the transactivation function of c-Myc.

We showed that Myc transforming activity in Rat1a cells is augmented by JPO2 coexpression, these observations thus provide a functional basis for the observed biochemical interaction between the JPO2 and Myc proteins. Intriguingly, although JPO2 coexpression did not have any significant effects on transformation defects seen in the Myc MBI and MBII mutants; colony formation by the Myc W135E mutant was enhanced significantly by JPO2 coexpression. Notably, Prescott et al. (52) have observed similar complementation by JPO1 of W135E transformation defect in Rat1a cells. The mechanism of W135E rescue activity remains unclear but seems to be characteristic of the JPO1 and 2 proteins.

JPO2 expression is tissue restricted; notably, JPO2 expression was undetectable in human fetal cerebella, but was present in a number of medulloblastoma tumors and tumor cell lines. Taken together with JPO2 potentiation of Rat1a transformation by c-Myc, these data suggested a potential tumor-specific role for JPO2. Indeed, we observed that exogenous JPO2 expression enhanced the transformed phenotype in UW228 cells, and knockdown of endogenous JPO2 depressed colony formation in UW228 and UW228-Myc cells. Collectively, these data point to a role for JPO2 in medulloblastoma transformation, and suggest that the transforming effect of c-Myc in medulloblastoma is mediated in part by JPO2/Myc interaction. The observed transforming effect of JPO2 alone in UW228, but not Rat1a cells, suggests that other cooperating events are required for full JPO2 transforming activity. The mechanism by which JPO2 enhances the transformed phenotype is not entirely clear. We have observed that although JPO2 induced almost the same number of colonies as Myc in UW228 cells, the rate of colony development and ultimate colony size were consistently less robust in the JPO2 transformed cells. It is possible that these differences reflect the effect of c-Myc on UW228 proliferation; notably, we have not observed significant changes in growth rate in cell lines stably expressing JPO2. In this context, it is perhaps relevant that in our immunohistochemical analyses, we did not observe any correlation between JPO2, c-Myc, or MiB-1 expression in tumors. As MiB-1 and c-Myc are markers of proliferation, these observations reinforce our in vitro observations that JPO2 does not seem to function in Myc-mediated transformation by effects on cellular proliferation. Similarly, JPO1 has been reported to alter anchorage-independent growth without effects on cellular proliferation or death. Intriguingly, JPO1 expression has been linked to changes in cell adhesion induced by Myc overexpression in Rat1a cells (52). Given the identification of a number of genes involved in adhesion as putative Myc transcriptional targets (22, 36, 37, 39, 40, 42), it is tempting to speculate that JPO2 may contribute to anchorage independent growth by virtue of direct effect on transcription of adhesion molecules.

In summary, JPO2 is a novel Myc binding partner with functions in Myc-mediated transformation. We showed that JPO2 overexpression is transforming in medulloblastoma cells, and associated with tumor metastases. Significantly, the closely related JPO1 gene is also frequently overexpressed in human and murine medulloblastoma (64). Hence, this novel protein family likely has important roles in medulloblastoma transformation. Studies of JPO2 and JPO1 in normal and tumor tissues suggest that deregulated expression of these proteins have broader role in oncogenesis. Indeed, recent studies have linked JPO2 expression to late metastases in breast cancers (65), and have also identified JPO2 as a putative target gene of the pRB and E2F pathways (66). The striking structural and functional parallels between the JPO1 and 2 proteins, together with the remarkable conservation of a common putative Myc interaction domain, suggest the exciting possibility that we may have uncovered a novel network of MycNTD interactions with important contributions to the transforming function of Myc. Future investigations into this new protein family may provide insights into potential novel therapeutic avenues to abrogate the potent oncogenic effect of c-Myc in medulloblastoma and other human malignancies.

7 A. Huang et al., unpublished data.

8 R. Osthus et al., companion manuscript.
Note in Proof

While this paper was in review, Chen et al. (J Biol Chem 2005;280:12155-61) identified JPO/BA2/R1 as a DNA binding transcription factor.

Acknowledgments

References

Identification of a Novel c-Myc Protein Interactor, JPO2, with Transforming Activity in Medulloblastoma Cells

Annie Huang, Cynthia S.W. Ho, Romina Ponzielli, et al.


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