Expression of Insulin-like Growth Factor-binding Protein 5 in Neuroblastoma Cells Is Regulated at the Transcriptional Level by c-Myb and B-Myb via Direct and Indirect Mechanisms*

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Neuroblastoma (NB), a malignant childhood tumor deriving from the embryonic neural crest, is sensitive to the growth-stimulating effects of insulin-like growth factors (IGFs). Aggressive cases of this disease often acquire autocrine loops of IGF production, but the mechanisms through which the different components of the IGF axis are regulated in tumor cells remain unclear. Upon conditional expression of c-Myb in a NB cell line, we detected up-regulation of IGF1, IGF1 receptor, and insulin-like growth factor-binding protein 5 (IGFBP-5) expression. Analysis of the IGFBP-5 promoter revealed two potential Myb binding sites at position −59 to −54 (M1) and −429 to −424 (M2) from the transcription start site; both sites were bound by c-Myb and B-Myb in vitro and in vivo. Reporter assays carried out using the proximal region of the human IGFBP-5 promoter demonstrated that c-Myb and B-Myb enhanced transcription. However, site-directed mutagenesis and deletion of the Myb binding sites coupled with reporter assays revealed that M2 but not M1 was important for Myb-dependent transactivation of the IGFBP-5 promoter. The double mutant M1/M2 was still transactivated by c-Myb, suggesting the existence of Myb binding-independent mechanisms of IGFBP-5 promoter regulation. A constitutively active AKT transactivated the IGFBP-5 promoter, whereas the phosphatidylinositol 3-kinase inhibitor LY294002 suppressed it. Moreover, the kinase dead dominant negative K179M AKT mutant was able to inhibit transcription from the M2 and M1/M2 IGFBP-5 mutant promoters. Deletion analysis of the IGFBP-5 promoter revealed that the AKT-responsive region lies between nucleotides −334 and −83. Together, these data suggest that the Myb binding-independent transactivation of the IGFBP-5 promoter was due to the activation of the phosphatidylinositol 3-kinase/AKT pathway likely mediated by IGF1 receptor-dependent signals. Finally, IGFBP-5 was able to modulate proliferation of NB cells in a manner dependent on its concentration and on the presence of IGFs.

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1 Neuroblastoma (NB)1 accounts for 7–10% of the cancers of childhood with an annual incidence of ~1/100,000 children under the age of 15 years (1, 2). Although spontaneous regression frequently occurs in patients with localized disease (3), the prognosis of tumor stage 3 and 4 (4), which make up about 50% of the total cases at diagnosis, is generally unfavorable. NB cells derive from the neural crest and maintain gene expression profiles and differentiation potentials that are reminiscent of their embryonic origin (5). A distinctive feature of NB is the expression of trk receptors on the cell surface that mediate the differentiation signals sent by neurotrophins (6). Expression of trkA (7) and trkC (8) is associated with good prognosis, whereas that of trkB is an indicator of unfavorable outcome (9). As for many other neoplasms, the molecular mechanisms of NB aggressiveness are not fully understood. Amplification of the proto-oncogene MYCN and loss of genetic material at chromosome 1p36 are powerful markers of poor outcome (10), but the mechanisms through which these alterations exert their detrimental effects in tumor cells are largely unknown. The growth of NB cells is dependent on the supply of certain growth factors that, in the most aggressive forms, are frequently produced in an autocrine fashion. NB metastases occur in tissues where a paracrine support of IGF2 is available (11). Non-IGF2-producing tissues are invaded only by NBs that can autonomously produce IGF2 (12, 13). Thus, the inappropriate expression of some oncogenes can conceivably lead to the development of growth factor independence via activation of growth factors or growth factor-regulated pathways.

The biological activity of IGF1 and IGF2 is mediated by a large group of proteins that constitute the so-called IGF axis composed of receptors, intracellular substrates (IRS-1 and IRS-2), and transducers that relay the signal to the nucleus (14). In the extracellular compartment, another family of proteins, the IGF-binding proteins (IGFBPs), modulates the action of IGFs. IGFBPs can function as carriers to transport IGFs in the bloodstream in a ternary complex of 150 kDa with the acid labile protein (15). In the interstitial space, IGFBP-3 and -5 can bind to the extracellular matrix, decrease their affinity for the IGFs, and exchange them with the IGF1R (16). Together, these data suggest that the Myb binding-independent transactivation of the IGFBP-5 promoter was due to the activation of the phosphatidylinositol 3-kinase/AKT pathway likely mediated by IGF1 receptor-dependent signals. Finally, IGFBP-5 was able to modulate proliferation of NB cells in a manner dependent on its concentration and on the presence of IGFs.

1 The abbreviations used are: NB, neuroblastoma; IGF, insulin-like growth factor; IGF1R, IGF1 receptor; IGFBP, insulin-like growth factor-binding protein; PI3K, phosphatidylinositol 3-kinase; DOX, doxycycline; EMSA, electrophoretic mobility shift assay; wt, wild-type.
IGFBP-5 was also described as an IGF-independent growth factor in bone cells (21), acting through its putative receptor (22). The regulation of IGFBP-5 expression is finely tuned by a network of stimuli acting through different transcription pathways (23–26). Noticeably, in smooth muscle cells, IGF1 is a potent stimulant of IGFBP-5 synthesis through a transcriptional mechanism involving a signal transduction cascade dependent on the activation of IGF1R and PI3K/PIK-AKT (27, 28). The nuclear effector(s) and the DNA sequences on the IGFBP-5 promoter involved in the PI3K/PIK-AKT-dependent transactivation of IGFBP-5 expression are unknown, but it has been hypothesized that an important role is played by a GC-rich region located between nucleotides −147 and −134 from the transcription start (27, 28).

Myb genes (A-myb, B-myb, and c-myb) are a family of transcription factors whose expression has been implicated in the control of proliferation and differentiation of normal and transformed cells (29, 30). For example, c-Myb has been shown to be essential for the proliferation of normal and leukemic hematopoietic cells (31) and for the proliferation and survival of lymphoma cells (32, 33). Although the expression of c-Myb is predominant in the hematopoietic system, it is also expressed in other normal tissues (34) and in nonhematopoietic tumors (35, 36). Interestingly, the expression of IGF1 and IGFR1 is stimulated by c-Myb at the transcription level in fibroblasts (37). The expression of A-Myb and B-Myb occurs in cells of different embryonic origin (38). In neuroblastoma cells, c-Myb and B-Myb expression is down-regulated during in vitro differentiation (39, 40). Suppression of c-Myb expression leads to the inhibition of cellular proliferation (41). Expression of all members of the myb family is frequent in NB (36), but only that of B-myb appears to be associated with poor clinical outcome independent of MYCN amplification and age at diagnosis (36). Together, these findings suggest that genes of the myb family are important in the establishment and maintenance of the tumorigenic state of NB cells. However, information on the cellular targets through which myb genes exert their function is still scarce. The antiapoptotic ApoJ-1 protein appears to be a direct transcriptional target of B-Myb in proliferating neuroblastoma cells (42). Conversely, an atypical role of c-myb and B-myb in evoking cell death has been recently described in postmitotic neuronal cells (43). Thus, normal and transformed neural cells express myb genes that may have different effects, depending on the physiological or pathological context.

In this study, we investigated whether myb genes regulate the expression of members of the IGF axis in neuroblastoma cells. Using a model of c-Myb conditional expression, we found that c-Myb up-regulated the expression of IGFBP-1, IGF1R, and IGFBP-5. c-Myb and B-Myb transactivated the IGFBP-5 promoter through binding-dependent and -independent mechanisms. These findings are discussed in the light of the effects of IGFBP-5 on the proliferation of NB cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**

Cell lines were cultured in RPMI 1640 medium (LAN-5 cells) (40) (Euroclone) or in Dulbecco’s modified Eagle’s medium (N1E-115 cells) (45) supplemented with 10% fetal calf serum (Hyclone), penicillin and streptomycin (100 μg/ml each), and 2 mM l-glutamine at 37 °C, 5% CO2. For stable transfections, LAN-5 cells were grown at 70% confluence. Cells were transfected with the tet-responsive vector pHystr1 (46) containing full-length human c-myb cDNA using the calcium phosphate precipitation technique as described previously (45). Puromycin (Sigma) selection (1 μg/ml) and doxycyline (DOX) (Sigma) treatment (5 ng/ml) was started 48 h after transfection. Single clones were expanded after 21 days and tested for inducible c-Myb expression after removal of DOX.

For transient transfection, marine N1E-115 cells were transfected with pcDNA3 expression plasmids containing full-length c-myb or B-myb cDNAs or with the empty vector. 48 h later, cells were harvested, and RNA was extracted for analysis. Luciferase assays were carried out co-transfecting LAN-5 cells with reporter vectors pGL3-IGFBP-5-prom wt, M1mut, M2mut, and M1’ M2mut (2 μg) and pcDNA3-c-myb or -B-myb (1 μg unless otherwise indicated) in the presence or absence of the mutant K179M AKT plasmid (1 μg) using the calcium phosphate precipitation technique. The amount of DNA transfected in each plate was kept constant by adding the appropriate quantity of empty vector (pcDNA3). 6 h before transfection, RPMI 1640 medium was replaced with Dulbecco’s modified Eagle’s medium. After transfection, fetal calf serum was reduced to 2%, and luciferase assays were performed 36 h later using a detection kit (Promega).

**Plasmids**

**DBD-c-myb-pcDNA3**—The DNA binding domain of human c-myb was obtained by PCR using a set of primers containing the initial ATG (upstream primer) and a stop codon (downstream primer) after the hemagglutinin tag in-frame with the myb sequence. The PCR product was then cloned in the EcoRI site of the pcDNA3 vector.

**pGEX-2T-B-myb**—The DNA binding domain of B-myb was produced in bacteria from vector pGEX-2T-B-myb (42) using standard techniques.

**pBPSTR1-c-myb—A XhoI/SacII fragment of 3.4 kb containing the entire coding sequence of human c-myb was obtained from vector pMMb I/DHFR-c-myb (47) and cloned in the multiple cloning site of the tet-regulated vector pBSTR1 (46) using standard techniques.

**pGL3-IGFBP-5-prom**—The region from nucleotide −459 to +59 from the transcription start was amplified from human genomic DNA by PCR. The 518-bp product was cloned at the Smal site of the pGL3-basic vector (Promega). The sequence of the closed region was controlled by dyeoxy sequencing.

**pGL3-IGFBP-5-prom-D3, -D2, and -D1**—Deletion mutants spanning the regions from nucleotides −334 to +59 (D3), −209 to +59 (D2), and −83 to +59 (D1) were generated using the pGL3-IGFBP-5-prom as template and the following primers: D3 upstream, 5'-GTGGTCCACCT-GCTCCGAAGA-3'; D2 upstream, 5'-AAGGAGAAGGGCCTGT-3'; and D1 upstream, 5'-GGTGGAGCCTCAAATGCGACG-3'.

The common downstream primer for the three deletion mutants was 5'-TCAGCGATAGAAAGGCCC-3'. This primer anneals to nucleotides 121–141 in the pGL3-basic vector. The PCR-amplified products were digested with HindIII restriction enzyme and cloned in a HindIII/Smal-digested pGL3-basic vector using standard cloning procedures. The sequence of each construct was checked by dyeoxy sequencing.

**pGL3-IGFBP-5-prom-M1mut, pGL3-IGFBP-5-prom-M2mut, and pGL3-IGFBP-5-prom-M1mut/M2mut**—The three mutants in the M1 and M2 sites were obtained by using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The mutations produced in each site were the same present in the M1 and M2 oligonucleotides used in EMSA. The sequence of the mutants was controlled by dyeoxy sequencing.

**Dominant Negative K179M AKT and Constitutively Active Myr-AKT**—The kinase dead dominant negative inhibitor (K179M) of wt AKT (48) and the constitutively active Myr-AKT (49) were kindly obtained by Dr. Tischia (Thomas Jefferson University, Philadelphia, PA).

**RNA Analysis**

Total RNA was prepared by Trizol extraction (Invitrogen). Carryover DNA contamination was eliminated by treatment of the total RNA with the DNA-free kit (Ambion) according to the manufacturer’s instructions. RNA was reverse-transcribed with the first-strand cDNA synthesis kit for reverse transcription-PCR (Roche Molecular Biochemicals) using an input of 500 ng for each reaction. Subsequent PCRs were carried out for the indicated number of cycles at the appropriate annealing temperature for each pair of primers. Northern blot analysis was carried out as described previously (50) using standard techniques.

**Plasmids Containing the Sequences Used as Probes for Northern Blot Analysis**

Probes for IGFBP-5 and IGF1R were obtained by PCR amplification of human genomic DNA and cloning the amplified products in the pGEM-T vector (Promega). The primers used to amplify IGFBP-5 were as follows: upstream primer, 5'-TAGTGCCCTCAACTCTCTGG-3'; and downstream primer, 5'-GGACGATCATCAGGCG-3'. The primers used to amplify IGF1R were as follows: upstream primer, 5'--AACAAC-TACGCCCTGTGCATC-3'; and downstream primer, 5'-TGGCGA-
CACCATTGTTCTC-3'. The probe for c-myc was obtained from vector Bluescript-c-myc, in which the first 1200 nucleotides from the 5'-end of human c-myc cDNA had been cloned previously in our laboratory. The IGBP2 probe was obtained from vector pβ4 (51). The IGBP1 probe was obtained from vector pBluescript II (a gift of Dr. Maria Giulia Rizzo, Regina Elena Institute, Rome, Italy) containing the coding region of the gene. The β-actin probe was obtained from vector HgA-1 (52).

**Protein Analysis**

Cellular proteins were extracted as described previously (45). Briefly, cells were lysed on ice in 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton X-100, 0.1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. Lysate was then centrifuged at 14,000 rpm for 10 min at 4°C, the supernatant was collected, and protein concentration was determined using a colorimetric assay (BioRad). Protein extraction from the culture supernatants was carried out as follows: cells were plated in normal medium, and 24 h later, the medium was replaced with 2.5 ml of Dulbecco's modified Eagle's medium without fetal calf serum. After keeping the cells in culture for 40 h, the supernatant was collected, supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, and 10 mg/ml aprotinin, and centrifuged for 10 min at 1500 rpm at 4°C. The supernatant was transferred to a Centricon 30 (Amicon) and centrifuged for 1 h and 15 min at 5000 rpm at 4°C. The concentrated sample was collected by inverting the Centricon 30 device and centrifuging for 2 min at 3500 rpm at 4°C. Protein separation on SDS-polyacrylamide gels and Western blot analysis were carried out as described previously (45). Detection was performed by using the ECL Plus kit (Amersham Biosciences) following the manufacturer's instructions.

**Antibodies**

Polyclonal anti-B-Myb (c-20) and anti-IGFBP-5 (c-18) antibodies were purchased from Santa Cruz Biotechnology. Monoclonal anti-c-Myc antibody (clone 1-1) was purchased from Upstate Biotechnology; monoclonal anti-hemagglutinin antibody (clone 12CA5) was purchased from Roche Molecular Biochemicals; monoclonal anti-β-actin (clone AC-15) was purchased from Sigma.

**EMSA and Chromatin Cross-linking Immunoprecipitation**

Murine neuroblastoma N1E-115 cells transfected with the expression vector containing the DNA binding domain of human c-Myc or the empty vector were collected and lysed in 20 mM Hapes, pH 7.9, 0.4 mM NaCl, 25% glycerol, 1 mM EDTA, 2.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride on ice for 20 min, frozen, thawed, and centrifuged for 10 min at 13,000 x g at 4°C. Supernatants were collected and stored at -80°C until use. EMSAs were carried out as described previously (40). Briefly, binding reactions were done in 10 μl of 20 mM Tris-HCl, pH 7.5, 3 mM MgCl2, 50 mM NaCl, 15% glycerol, 0.2 μg/μl poly(dI-dC), 15 μg of cellular extract, and 1 x 106 cpm of double-stranded oligonucleotide (see below). In competition experiments, 5 pmol of the double-stranded competitor oligonucleotides were added to the mix. The reactions were incubated for 10 min on ice and loaded on a 6% polyacrylamide gel in 0.25× Tris-borate EDTA at 140 V. EMSAs to detect B-Myb binding were carried out as described above using 300 ng of the recombinant glutathione S-transferase-B-Myb protein.

The MBS-1 oligonucleotide containing the consensus Myb recognition element was 5’-CTCTACACCCAATCTACAGCATTT-3’. The M1 oligonucleotide was 5’-AATGCAGCTACAAAATCTGCTGGCAGCACC-3’. The mut M1 in which the M1 binding site was mutated (underlined) was 5’-AATTCGCAGCTACAAAATCTGCTGGCAGCACC-3’. The M2 oligonucleotide was 5’-GACGTTGATATCCGATGGCATTAGAAA-3’. The mut M2 in which the M2 binding site was mutated (underlined) was 5’-GACGTTGATATCCGATGGCATTAGAAA-3’.

Chromatin cross-linking immunoprecipitation was carried out essentially as described previously (53), with few modifications. We started with 20 x 106 cells for each experimental point. Sonication was carried out at 4°C with 12 pulses of 2 s each (35% amplitude) using a Sonics Vibracell 500 W equipped with a microtip. DNA size in the range from 200 to 600 bp was monitored on an agarose gel. Immunoprecipitations using protein A-Sepharose (Roche Molecular Biochemicals) were carried out using 4 μg of each antibody for each experimental point. At the end of the procedure, DNA from each point was amplified by PCR using the following primers: upstream M1 primer, 5’-CCGCGTTGGAGGTGTTACGTG-3’; and downstream M1, 5’-CAACGTCTTGATGTCGTTTTT-3’. The PCR annealing temperature was 53°C. Reactions were carried out in the presence of 5% Me2SO in PCR mix. This set of primers amplifies the region from nucleotide -135 to +59. Upstream M2 primer was 5’-GCTTAGGAAGATCTCTGGC-3’. Downstream M2 primer was 5’-GCTACCCAATGTTGCCCAGAG-3’. This set of primers amplifies the region from nucleotide -459 to -266. Amplification products were run on an agarose gel and transferred to nylon membrane (Hybond N+; Amersham Biosciences). Membranes were hybridized as described previously (41) using the following 32P-labeled probes: M1 probe, 5’-GGGAGCTCTAAATTGCAGCT-3’, spanning the region between nucleotides -81 and -61; and M2 probe, 5’-CCCTCATGGTGTCACCTGCT-3’, spanning the region between nucleotides -341 and -321.

**Proliferation Assays**

Cell proliferation was measured using the colorimetric cell proliferation kit II (WST-1; Roche Molecular Biochemicals) based on the colorimetric detection of a formazan salt. In each well, 4 x 104 cells were seeded in RPMI 1640 medium supplemented with 2% heat-inactivated fetal calf serum. Recombinant IGBP5 (Upstate Biotechnology, Inc.) and IGBP2 (Sigma) were added at the indicated concentration, and the colorimetric reading at 450 nm was carried out after 40 h according to the manufacturer's instructions. Background absorbance of each sample at 630 nm was subtracted from the readings at 450 nm.

**RESULTS**

**IGFBP-5 Expression Increases in Neuroblastoma Cells Expressing c-myc and B-Myb**—The human neuroblastoma cell line LAN-5 has been studied extensively for its ability to differentiate along a neural pathway upon exposure to all-trans-retinoic acid (40). These cells produce low but detectable amounts of both c-Myc and B-Myb, whose expression is down-regulated during all-trans-retinoic acid-induced differentiation (39, 40). LAN-5 cells were used to generate stable transfectants conditionally expressing c-Myc, c-myc full-length cDNA was cloned into the tet-regulated plasmid pBPS3R1 (46), and the resulting construct was used to transfect LAN-5 cells. After puromycin selection, resistant clones were selected for their ability to express increased amounts of c-Myc after withdrawal of the tetracycline homologue DOX from the media. One clone, referred to hereafter as LAN-5-c-myc, expressed high levels of c-Myc in the absence of DOX. Expression of some members of the IGF axis, whose activation is highly relevant for survival of neuroblastoma (11, 54), was tested in the presence or absence of DOX. Upon c-Myc expression, there was an increase in the mRNA levels of IGF1, IGFIR, and IGBP5 (Fig. 1A). Up-regulation of IGF1 and IGFIR was in part expected because it has been demonstrated previously that c-Myc induces an increase in the expression of these genes in fibroblasts (37). On the contrary, the strikingly elevated expression of IGBP5 represented a novel and unexpected finding. Of interest, the electrophoretic pattern of IGBP2 RNA differed in the presence and absence of DOX (Fig. 1A). IGBP2 is transcribed from distinct promoters giving rise to transcripts of different lengths ranging from 2.2 to 6.0 kb (55). In the absence of c-Myc induction (+DOX), there is expression of the 6.0- and 4.8-kb mRNA forms that arise from use of the F2 and F3 promoters, respectively (Fig. 1A). Upon c-Myc induction (−DOX), it appears that there is a preferential use of the F3 promoter from which the 4.8-kb RNA arises (Fig. 1A). Thus, IGBP2, compared with IGBP5, IGF1, and IGFIR, is not up-regulated by c-Myc expression in neuroblastoma cells.

To demonstrate that the c-Myc-induced increase of IGBP5 mRNA levels correlated with enhanced protein expression, culture supernatants were prepared from LAN-5-c-myc cells in the presence or absence of DOX and tested by Western blot analysis for IGBP5 expression. As shown in Fig. 1B, an increased amount of IGBP5 protein was detected in the supernatant of LAN-5-c-myc cells after c-Myc induction (Fig. 1B), in good correlation with the increased expression of c-Myc in the corresponding cellular extracts (Fig. 1B).

One possible problem with these results is that they could reflect the clonal nature of the LAN-5-c-myc cell line. In other
words, the observed differences could be intrinsic to this particular cell clone. Thus, we transiently transfected the murine neuroblastoma cell line N1E-115 with expression vectors for c-myb or B-myb. 40 h after transfection, cells were collected, and total RNA was prepared. Identical amounts of RNA from each experimental point were reverse-transcribed, and cDNAs were PCR-amplified for 25, 30, and 35 cycles using specific primers to detect c-myb, B-myb, and IGFBP-5 (Fig. 2A). After normalization for β-actin levels, the intensity of each band was analyzed and expressed in densitometric units, taking the intensity of the control as 1 (Fig. 2B). Cells transfected with c-myb and B-myb expressed approximately double the amount of IGFBP-5 expressed by control cells transfected with the pcDNA3 vector alone. It should be noticed that the increase in IGFBP-5 expression is probably underestimated because the transfection efficiency of N1E-115 cells was ~30% (data not shown). Thus, the increase of IGFBP-5 expression in neuroblastoma cells expressing c-Myb and B-Myb is not restricted to a particular cell clone.

c-Myb and B-Myb Transactivate the Proximal Region of the Human IGFBP-5 Promoter—Analysis of the proximal region of the human IGFBP-5 promoter revealed two bona fide Myb binding sites at positions −59 to −54 (hereafter termed M1) and −429 to −424 (M2). It is noteworthy that a potential Myb/E-box site, involved in transcriptional inhibition by cortisone in bone cells, was mapped in the mouse promoter at a position corresponding to M1 (23). A similar sequence was also found in the rat IGFBP-5 promoter, where it mediated responsiveness to osteogenic protein-1 (57). In the human promoter, the first base at the 5′-end of the core M1 sequence is a purine (A) instead of a pyrimidine as in the canonical Myb recognition element. The M2 site contains a perfect core consensus element in opposite orientation with respect to the direction of the transcription. The region spanning nucleotides −459 to +59 of the human IGFBP-5 promoter was cloned in the reporter vector pGL3-basic upstream of the luciferase gene (pGL3-IGFBP-5-prom), and functional assays were carried out in LAN-5 cells transfected with a fixed amount (2 μg) of the reporter vector (pGL3-IGFBP-5-prom) and increasing amounts (from 0.5 to 2 μg) of expression vectors coding for human c-myb or B-myb. Both genes transactivated the IGFBP-5 promoter, albeit with different efficiency (Fig. 3, A and B). B-Myb was more effective than c-Myb when transfected in amounts up to 1 μg. Thus, the human IGFBP-5 promoter is transactivated by c-Myb and B-Myb.

**Fig. 1.** IGFBP-5, IGFR1, and IGFI are up-regulated after c-Myb induction in human neuroblastoma cells. A, Northern blot analysis of LAN-5-c-myb cells before and after DOX withdrawal. The blotted was hybridized with probes specific for the genes indicated on the left. The size of the predominant mRNA species is indicated on the right. Western blot analysis of LAN-5-c-myb cells before and after DOX withdrawal. The top blot represents an equal amount of culture supernatant after protein concentration probed with a specific anti-IGFBP-5 antibody. Bottom blots were carried out on cellular extracts using specific anti-c-Myb and anti-β-actin antibodies.

**Fig. 2.** IGFBP-5 induction by c-Myb and B-Myb in mouse neuroblastoma cells. A, semiquantitative reverse transcription-PCR on RNA from N1E-115 cells transfected with empty vector or c-myb- and B-myb expression vectors. Reverse transcription-PCRs were carried out 48 h after transfection using a pair of primers specific for each of the genes indicated at the bottom panel for the indicated numbers of PCR cycles. B, quantitative densitometric measurements of IGFBP-5 mRNA expression in empty vector-, c-myb-, and B-myb-transfected N1E-115 cells after 30 cycles of PCR. Expression of IGFBP-5 in empty vector-transfected cells was arbitrarily set to 1.
Transcriptional Control of IGFBP-5 by Myb

**Fig. 3.** c-Myb and B-Myb transactivate the human IGFBP-5 promoter. Human neuroblastoma LAN-5 cells were transfected with a fixed amount of the reporter vector pGL3-IGFBP-5-prom (2 μg) and increasing amounts (0.5, 1, and 2 μg) of c-myb (A) or B-myb (B) expression vectors. Each experiment was carried out in triplicate. Bars, ±S.D.

**Fig. 4.** c-Myb and B-myb bind in vitro to M1 and M2 sites in human IGFBP-5 promoter. Band-shift experiments were carried on NIE-115 cellular extracts transfected with c-myb expression vector in the presence of 32P-labeled wt M1 and M2 double-stranded oligonucleotides. Competitions were performed using a 300-fold excess of cold wt and mutant double-stranded oligonucleotides. An oligonucleotide containing a canonical Myb binding site (MBS-1) was used as a positive control. Specific retarded bands (a–c) are indicated to the left of each blot.

**Fig. 5.** c-Myb and B-Myb bind in vivo to M1 and M2 sites in human IGFBP-5 promoter. Chromatin cross-linking immunoprecipitation was carried out on proliferating LAN-5 cells using antibodies specific to c-Myb (left panels) or B-Myb (right panels). No Ab, immunoprecipitations in the absence of antibodies; Mock, reactions in the absence of chromatin and antibodies. PCRs on the immunoprecipitated chromatin were carried out for 25 cycles, and amplification products were blotted and hybridized with specific probes for the amplified regions.

Transactivation of IGFBP-5 by Myb Occurs through Two Distinct Mechanisms—To assess the function of M1 and M2 sites in IGFBP-5 transcriptional control, we generated by site-directed mutagenesis three mutant forms of the pGL3-IGFBP-5-prom: mut M1 (in which the M1 site was mutated), mut M2 (in which the M2 site was mutated), and mut M1/M2 (in which both M1 and M2 were mutated). In addition, we also generated three deletion mutants of the IGFBP-5 promoter. A schematic representation of the IGFBP-5 promoter with the most relevant sites, the mut M1, mut M2, and mut M1/M2 and the deletion mutants Δ1, Δ2, and Δ5 are shown in Fig. 6A. These constructs were used in functional assays carried out co-transfecting the LAN-5 cell line with fixed amounts of each reporter (2 μg) and the expression vector (1 μg) coding for c-Myb or B-Myb. The transactivation of the wt and mutant IGFBP-5 promoters by c-Myb or B-Myb was normalized with respect to their controls (i.e., co-transfections of the reporter and the empty vector). Compared with the effect of c-Myb and B-Myb on the wt IGFBP-5 promoter, the M2 mutation caused a decrease in reporter gene transactivation, whereas the M1 mutant was transactivated more efficiently (Fig. 6B). This result suggests that the binding of Myb to the M2 site has a stimulatory effect on IGFBP-5 transcription. In contrast, Myb binding to M1 seems to cause transcriptional repression. The mut M1/M2 promoter was transactivated as effectively as the wt promoter (Fig. 6B). Because we found an increase in the expression of both IGFI and IGFIR after c-Myb induction (Fig. 1A), the up-regulation of IGFBP-5 in neuroblastoma cells could occur at least in part through an indirect mechanism involving activation of IGF1R and its downstream effectors PI3K/AKT (28). To test this hypothesis, we transfected LAN-5 cells with the wt IGFBP-5 promoter and a constitutively active form of AKT (myr-AKT) (49). Transcription from the IGFBP-5 promoter was increased ~2-fold by myr-AKT (Fig. 7A). On the contrary, the specific PI3K/AKT pathway inhibitor LY294002 was able to inhibit transcription below the basal level (Fig. 7A).

Thus, we tried to uncouple direct and indirect effects of Myb on transactivation of the IGFBP-5 promoter using a kinase dead dominant negative inhibitor (K179M) of wt AKT (48). In this case, functional assays were carried out in a neuroblastoma cell line constitutively overexpressing c-Myb. Cells were transfected with a fixed amount of the reporter vector (1 μg) with or without the K179M AKT plasmid (3.5 μg). When nec-
necessary, the amount of transfected DNA was kept constant by adding an appropriate amount of the pcDNA3 empty vector. The transactivation activity of each reporter construct was set to 100 (Fig. 7B, I). The relative residual activity of each reporter construct in presence of the dominant negative K179M AKT plasmid was calculated accordingly (Fig. 7B, □). Interestingly, the reporter activity driven by the mut M2 (with the intact M1 site) or the double mutant mut M1/M2 promoter was markedly reduced upon expression of the K179M AKT plasmid. On the contrary, the effect of the K179M AKT plasmid on the wt or on the mut M1 promoter (with the intact M2 site) was less dramatic. Together, these data indicated that M2 is the only site required for Myb-dependent transactivation of the IGFBP-5 promoter. Moreover, the drastic decrease in transactivation in the presence of the K179M AKT plasmid using the mut M1/M2 reporter strongly suggests that Myb transactivation of the IGFBP-5 promoter is partly dependent on an indirect mechanism mediated by AKT.

To determine which regions of the IGFBP-5 promoter are directly involved in the AKT-dependent transactivation, three deletion mutants spanning nucleotides −334 to +59 (Δ3), −209 to +59 (Δ2), and −83 to +59 (Δ1) were generated in pGL3-basic reporter vector. A graphic representation of these constructs is shown in Fig. 6A. LAN-5 cells were transfected with these mutants and c-Myb expression vector. Transactivation by c-Myb is attenuated in luciferase assays with the Δ3 promoter, which lacks the functional M2 site (Fig. 7C). In addition, c-Myb is no longer able to transactivate Δ2 and Δ1 truncated promoters (Fig. 7C). In parallel experiments, we studied the effect of constitutively active AKT (myr-AKT) on the same mutants (Fig. 7C). The Δ3 promoter was transactivated by AKT as effectively as the wt promoter. Of interest, the Δ3 mutant was similarly transactivated by AKT and by c-Myb, suggesting that in the absence of the M2 site, transactivation by c-Myb depends largely on AKT stimulation. A significant loss in AKT responsiveness was observed in luciferase assays carried out with the Δ2 mutant, whereas the Δ1 mutant was no longer responsive to AKT. Thus, the AKT-responsive element(s) is located in the region spanning the Δ3 and Δ1 IGFBP-5 promoter.

IGFBP-5 Protein Affects Proliferation of Neuroblastoma Cells—In bone and vascular smooth muscle cells (18–20), IGFBP-5 modulates cellular proliferation. To assess the relevance of IGFBP-5 expression in neuroblastoma cells, experiments based on a colorimetric assay were carried out to measure proliferation in the presence of increasing amounts of recombinant IGFBP-5. IGFBP-5 Protein Affects Proliferation of Neuroblastoma Cells

| IGFBP5 (ng/ml) | $A_{450}$ (nM) | Δ  | p       |
|----------------|---------------|----|---------|
| 0              | 0.581 ± 0.016 | 0  | <0.05   |
| 0.1            | 0.592 ± 0.031 | 2.0| 6.0     |
| 1              | 0.617 ± 0.014 | 6.3| 3.8     |
| 100            | 0.581 ± 0.009 | 0.1| 3.2     |
| 200            | 0.519 ± 0.014 | −10.7| 3.4 | <0.01 |

Effect of IGFBP-5 on proliferation of LAN-5 human neuroblastoma cells

The experiment was carried out in triplicate cultures (40,000 cells/well). Values are means ± S.D. A represents the percentage difference in proliferation with respect to that calculated in the absence of recombinant IGFBP-5. Statistical significance (p) with respect to the value in the absence of recombinant IGFBP-5 was calculated by the two-tailed Student’s t-test.
TABLE II
Effect of IGFBP-5 in presence of ectopically added IGF2 in LAN-5 cells

The experiment was carried out in sextuplicate cultures (40,000 cells/well). Values are means ± S.D. Δ represents the percentage of difference in proliferation with respect to that calculated in the absence of recombinant IGFBP-5. Statistical significance (p) with respect to the value in the absence of recombinant IGFBP-5 was calculated by the two-tailed Student’s t test.

| IGF2 (ng/ml) | IGFBP5 (ng/ml) | A_{450}/A_{430} | Δ | p          |
|-------------|----------------|-----------------|---|------------|
| 100         | 0              | 0.534 ± 0.029   | 9.2 ± 8.8 | <0.00005   |
| 100         | 0.01           | 0.581 ± 0.035   | 2.7 ± 10.6|            |
| 100         | 0.1            | 0.553 ± 0.048   | 2.7 ± 16.6|            |
| 100         | 1              | 1.652 ± 0.199   | 209.7 ± 44.9|           |
| 100         | 100            | 0.587 ± 0.138   | 10.0 ± 26.5|            |

at a 1 ng/ml concentration of IGFBP-5, followed by a decrease (−10.7 ± 3.4%) at 200 ng/ml; both variations were statistically significant. We also cultured the cells in the presence of a fixed quantity of recombinant IGF2 (100 ng/ml), the most relevant IGF in neuroblastoma (11), together with an increasing concentration of IGFBP-5 (0.1–100 ng/ml). A dramatic increase in proliferation (209.7 ± 44.9%) was observed at 1 ng/ml IGFBP-5 (Table II). The proliferation returned at a level similar to that of control cells at a concentration of 100 ng/ml IGFBP-5. Thus, IGFBP-5 is effective in modulating proliferation of neuroblastoma cells.

DISCUSSION

Previous studies have implicated IGFs in the growth and survival of NB cells (11, 54). The role of myb genes in controlling the expression of IGF1 and IGF1R has been demonstrated in fibroblasts (37). Using a NB cell line conditionally expressing c-Myb, we show here that the c-Myb-dependent up-regulation of IGF1 and IGF1R is not limited to fibroblasts. More importantly, we have identified a new Myb target gene, IGFBP-5, which belongs to the group of proteins that bind IGF with high affinity (60, 61). Myb proteins appear to control IGFBP-5 expression at the transcriptional level. This conclusion is corroborated by several observations. The analysis of the proximal region of the human IGFBP-5 promoter revealed two potential Myb binding sites at positions −59 to −54 (M1) and −429 to −424 (M2) from the transcription start site. The most proximal site, M1, is located in close proximity to a TATA-like sequence in a region with a high degree of conservation among different species. In humans, M1 is mutated in the first base of the core sequence, which is a purine instead of a pyrimidine as in the canonical Myb binding site. M2 is located in a less conserved region, although a similar site in the same orientation was found at nucleotides −351 to −346 of the mouse gene. c-Myb and B-Myb were able to bind to both M1 and M2 sites in vitro and in vivo, as revealed by EMSA and by chromatin cross-linking immunoprecipitation. In vitro, Myb binding to M2 was stronger than Myb binding to M1, suggesting a partial impairment in binding due to the above-mentioned mutation in the first base of the core sequence of M1. In vivo, we were unable to detect a similar difference in binding to M1 and M2. This discrepancy could be due to insufficient discrimination of high and low affinity binding sites of our chromatin cross-linking immunoprecipitation procedure or to different binding affinities of Myb proteins in vitro and in vivo. In fact, it has been reported that transcription factors that interact weakly with binding sites in vitro can bind with higher affinity in vivo (53, 62).

In functional assays, c-Myb and B-Myb were able to transactivate the IGFBP-5 promoter in NB cells. B-Myb was a better transactivator than c-Myb, possibly because of its ability to act through a binding-independent mechanism in cooperation with the Sp1 factor (63). In agreement with the latter hypothesis, potential Sp1 binding sites were described in the proximal region of the IGFBP-5 promoter (27, 28). Mutations of the M1 and M2 sites and use of deletion mutants revealed a high complexity in the Myb-dependent regulation of the IGFBP-5 promoter. In this regard, mutation in M2 brought about a decrease in transactivation by Myb proteins, and the deletion mutant Δ3, which lacks the region containing M2, was less responsive to c-Myb transactivation. In contrast, mutation of the M1 site revealed an increased transactivation by c-Myb or B-Myb. Moreover, c-Myb transactivation was also abrogated using the deletion mutants Δ2 and Δ1, in which only the M1 site was still present. These results suggest functionally different roles for the M1 and M2 sites. M2 acts as a positive cis-regulatory element. In contrast, M1, located in close proximity to a TATA-like sequence (−34 to −25 from the transcription start) in the IGFBP-5 promoter, does not act as an activator site upon Myb binding. Of interest, a Myb binding site overlapping the TATA box in the c-erb-2 promoter was shown to cause transcriptional repression upon c-Myb or B-Myb binding (65). The ability of c-Myb to transactivate the mut M2 and the mut M1/M2 promoters suggested the existence of Myb binding-independent mechanisms of IGFBP-5 promoter regulation. Because Myb expression stimulated IGF1 and IGF1R in NB, and the PI3K/AKT pathway appears to play a role in IGFBP-5 transactivation, part of the effect induced by c-Myb on the IGFBP-5 promoter could be through the IGF1R via the PI3K/AKT pathway (28). In agreement with this hypothesis, the IGFBP-5 promoter was transactivated by a constitutively active AKT (49), whereas expression of a dominant negative AKT mutant (48, 66) markedly suppressed Myb-dependent transactivation of the mut M2 and mut M1/M2 promoters. Of interest, dominant negative AKT was markedly less effective on the wt and mut M1 promoters that retained a functional M2 site activated by c-Myb via a direct binding-dependent mechanism. The wt and the Δ3 deletion promoter (which lacks the M2 site) were comparably activated by AKT, further suggesting
that the effect of AKT is independent of the functional Myb binding site. The responsiveness to AKT was significantly decreased in the Δ2 promoter and was completely lost in Δ1. Taken together, these results suggest that the AKT-responsive region is located between nucleotides −334 and −83 in the human IGFBP-5 promoter. Thus, transcription of IGFBP-5 appears to be subjected to direct regulation via Myb binding and to Myb-dependent indirect activation through the AKT pathway.

Besides IGF1/IGF1R (28), interleukin 6 with its soluble receptor (67), prostaglandin E2 (26), and retinoic acid (24) stimulate the expression of IGFBP-5 messenger RNA. Conversely, osteogenic protein-1 down-regulates the transcription of IGFBP-5 in primary culture of fetal rat cardiomyocytes via a 21-bp control element that includes the rat homologue of the IGF axis (17, 22). It should be stressed that our findings on Myb-dependent transcription activation of IGFBP-5 promoter apply, at present, only to NB cells, given the well known tissue-specific regulation of IGFBPs in non-NB cells, given the well known tissue-specific regulation of IGFBPs in non-NB cells.
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