Lack of Correlation between N-myc and MAX Expression in Neuroblastoma Tumors and in Cell Lines: Implication for N-myc-MAX Complex Formation

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ABSTRACT

Detectable levels of MAX messenger RNA were found in a set of human neuroblastoma tumors and established cell lines. MAX mRNA levels were independent of tumor stage and N-myc genomic amplification. By contrast, N-myc mRNA transcripts were detectable only in tumors with amplification of N-myc gene and in cell lines. Analysis by reverse transcriptase polymerase chain reaction and hybridization to specific oligodeoxynucleotide probes revealed approximately equal amounts of two MAX transcripts in all cases analyzed. Immunoprecipitations with a specific antibody to MAX detected two proteins of Mr 21,000 and 22,000 in all neuroblastoma lines regardless of N-myc amplification and/or expression. On the other hand, protein binding to the myc DNA consensus sequence correlated with N-myc expression in neuroblastoma cells. Thus, N-myc expression might be a limiting factor in the formation of the N-myc-MAX heterodimer in neuroblastomas.

INTRODUCTION

Neuroblastoma is the most frequent noncranial solid tumor of childhood with an occurrence of 10 per million children yearly. Deletion of the short arm of chromosome 1 (2) and the amplification and consequent overexpression of the N-myc oncogene (3) are common genetic features of this neoplasia. Clinical studies have demonstrated a correlation between N-myc amplification, advanced tumor stage, and poor outcome (3). Recently, we described an association between the expression of N-myc and that of the proliferation-associated genes histone H3, c-myc and p53 (4).

TREATMENT

N-Myc and MAX Complex Formation

In this study, we compared the steady-state levels of MAX and N-myc mRNAs in a set of human neuroblastomas and cell lines. In addition, we detected comparable levels of MAX proteins in neuroblastoma lines and assessed the ability of whole cell extracts from two cell lines with opposite levels of N-myc expression to interact differentially with a myc binding consensus sequence. Our findings indicate that MAX expression is independent of N-myc, suggesting that the absence of simultaneous regulation of N-myc and MAX expression affects heterodimer formation in neuroblastoma cells.

MATERIALS AND METHODS

Tumors and Cell Lines. Primary tumor and metastasis samples were obtained at diagnosis and staged according to the Evans system (14). Biopptic material used for molecular analyses was subjected to histological analysis to separate normal from tumor tissue. The IMR32 (15) and LAN-5 (5) cell lines were grown in RPMI 1640 (Sigma, St Louis, MO) supplemented by 15% fetal bovine serum (Sigma). The SK-N-SH cell line (16) was grown in minimal essential medium (GIBCO, Grand Island, NY) with 10% fetal bovine serum. RNA Extraction and DNA Analyses. Total RNA was extracted as described (17). cDNA was size-fractionated on agarose-formaldehyde denaturing gels and transferred to nylon membranes as described (4). Hybridization to molecular probes was carried out using high stringency conditions (4). Genomic DNA was extracted according to Blin and Stafford (18). Southern blot analysis was carried out as described (4). The N-myc probe was NB 19–21, kindly provided by Dr. F. Alt (Columbia University, New York, NY). The human c-myc probe was pMYC7.4 obtained by Dr. S. Ferrari (Modena University, Modena, Italy).

Immunoprecipitations. Cell lines were metabolically labeled for three hours with [35S]methionine. Immunoprecipitations were carried out essentially as described by Wenzel et al. (13) using a specific anti-MAX polyclonal antibody obtained by Dr. Edward B. Ziff (New York University, New York, NY). The antibody was raised against a synthetic peptide corresponding to a COOH-terminal sequence of MAX: LQTNYPSSDNSLYTNAKGGTIS. Immunoprecipitates were separated on a 15% polyacrylamide gel.

RT-PCR Analysis. Unmodified oligodeoxynucleotides were synthesized and purified as described (19). The sequence of the 5' MAX primer (MAX-1) was 5'-GGGCCGTAGGAAATGAGCGATAAC-3'. The 3' primer (MAX-2) was 5'-CGAGTGCTAGCTGCGGATAC-3'. The MAX-3 primer was 5'-CCGAGTGGCFFAGCFFGCCTCCAT-3'.

Received 10/20/93; accepted 2/17/94.

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1 This work was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC). S. P. is supported by an AIRC fellowship. B. C. is a scholar of the Leukemia Society of America.

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3 The abbreviations used are: RT, reverse transcriptase; PCR, polymerase chain reaction; SSC, standard saline-citrate (1 X SSC is 0.15 M NaCl-0.015 M sodium citrate); SDS, sodium dodecyl sulfate; cDNA, complementary DNA.

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RESULTS

Construction of a MAX-specific Probe by RT-PCR. A 5'-primer spanning the region from nucleotides -12 to 12 and a 3'-primer complementary to the sequence from nucleotides 442 to 465 of the MAX cDNA sequence reported by Blackwood and Eisenman (10) were synthesized. Total RNA from neuroblastoma cell line IMR32 was reverse-transcribed using the 3' MAX primer, and the resulting cDNA was used as template for PCR. The PCR product was purified by chromatography through a G-50 column. Probe DNA (10 nM) was incubated in a final volume of 20 μl with 40 mM KCI, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.9), 1 mM EDTA, 0.5 mM diithiothreitol, 5% glycerol, 0.1 μg/μl polydeoxyinosinate-polydeoxycytidylate, 5 ng/μl of single-stranded unrelated oligonucleotide YY1 (5'-CCGAGCCCGCTCAAAATGGAGACCCTC-3'), and 10 to 20 ng of whole cell extract for 15 min at room temperature in the presence or in the absence of an excess of unlabeled competitor (Emax). In nonspecific competitions 1 pmol of unlabeled double stranded YY1 oligonucleotide was added. In experiments designed to compete out the DNA-protein interaction, 0.5 or 1 μg of a specific anti-N-myc monoclonal antibody (Oncogene Science, Uniondale, NY) or an anti-mouse H-2Kd monoclonal antibody (Pharmingen, San Diego, CA) were added to the cell extracts at room temperature 10 min before the addition of the 32P-labeled probe. The mixtures were separated on a native polyacrylamide gel (5%), run on 0.5 X Tris-borate-EDTA (1 X is 0.090 M Tris borate-0.002 M EDTA) for 2 h, 30 min at 100 V at room temperature. The gel was dried and exposed to an X-ray film.

Table 1 N-myc amplification and clinical stage of the neuroblastomas analyzed

<table>
<thead>
<tr>
<th>No.</th>
<th>Stage</th>
<th>N-myc copy no.</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>IV</td>
<td>63</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>&lt;3</td>
</tr>
<tr>
<td>3</td>
<td>IV (Metastasis)</td>
<td>&lt;3</td>
</tr>
<tr>
<td>4</td>
<td>IV</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>I</td>
<td>&lt;3</td>
</tr>
<tr>
<td>6</td>
<td>IV</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>IV (Metastasis of no. 6)</td>
<td>29</td>
</tr>
<tr>
<td>8</td>
<td>IV-S</td>
<td>&lt;3</td>
</tr>
<tr>
<td>9</td>
<td>IV</td>
<td>&lt;3</td>
</tr>
<tr>
<td>10</td>
<td>III</td>
<td>&lt;3</td>
</tr>
<tr>
<td>11</td>
<td>IV-S</td>
<td>&lt;3</td>
</tr>
<tr>
<td>12</td>
<td>III</td>
<td>&lt;3</td>
</tr>
</tbody>
</table>

Cell line<br>IMR 32 25 (22)<br>LAN-5 50 (22)<br>SK-N-SH <3 (22)

Fig. 1. Northern blot analysis of neuroblastomas RNAs. A, hybridization of 10 mg of total RNA to the MAX-specific probe (exposure, 18 h). B, hybridization of the same filter to the N-myc-specific probe (exposure time, 18 h) after removal of the previous probe. C, ethidium bromide staining of the gel. Arrows, position of the 28S and 18S ribosomal RNAs. Numbers 1 to 12 correspond to the neuroblastomas listed in Table 1.

transcripts in only the three primary tumors at stage IV and the metastasis (Fig. 1B, Lanes 1, 4, 6, and 7) with amplified N-myc gene and in the N-myc amplified cell line IMR32 (Fig. 1B). Hybridization of the same RNAs to a human c-myc probe did not reveal any detectable signal (not shown).

Detection of Two MAX Transcripts in Neuroblastoma. To determine whether the heterogeneous MAX product detected by RT-PCR in IMR32 cells reflected the presence of more than one transcript, RT-PCR was carried out with the tumor RNAs from the Northern analysis with the primers used to construct the MAX-specific probe. RT-PCR products were transferred to a nylon membrane in duplicate and hybridized with two MAX probes: MAX-3 which is identical to 20 of the 27 nucleotides inserted 5' of the basic region in several cDNAs of Manca cells mRNA (10) and able to
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Fig. 2. Detection of MAX transcripts and proteins by RT-PCR and immunoprecipitation analyses. A, RT-PCR products were hybridized to probe MAX-3 after transfer to a nylon membrane (exposure time, 1 h). B, hybridization to probe MAX-4 (exposure time, 1 h). C, ethidium bromide staining of a 6% polyacrylamide gel of the RT-PCR products. Numbers 1 to 12 are as in Fig. 1. Lymph and Fibroblasts are RNA from normal resting lymphocytes and normal proliferating fibroblasts respectively. Arrows in B, close doublet revealed by probe MAX-4. In D, MAX proteins were immunoprecipitated from [35S]methionine-labeled IMR32, LAN-5, and SK-N-SH cells. As control, LAN-5 whole cell lysate was treated with preimmune serum (Lane C). Left, the position of p21—22 MAX doublet. Right, number indicates the position of a 19-kDa molecular mass marker.

Two MAX Proteins are Expressed in Neuroblastoma Cells. To investigate if MAX protein(s) expression correlates with the presence of the MAX mRNAs in neuroblastomas, immunoprecipitations were carried out using a specific antibody to MAX on whole-cell lysates prepared from [35S]methionine-labeled neuroblastoma cell lines LAN-5 which has a 50-fold N-myc amplification (22) and expresses N-myc mRNA high levels (5), IMR32 and SK-N-SH. A closely spaced doublet banding at Mr 21,000—22,000 was detected in all samples (Fig. 2D) in agreement with the reported molecular weight for MAX proteins (12). Immunoprecipitation of the cell extract from LAN-5 cell line with preimmune serum did not detect any band in the Mr 21,000—22,000 region (Fig. 2D).

Fig. 3. In A gel mobility shift assays used whole extracts from LAN-5 (Lanes 1—5) or SK-N-SH (Lanes 6—8) and the Epro oligonucleotide as a probe. Lanes 2 and 7, specific competitions using 1 pmol of Epro double-stranded oligonucleotide. Lanes 3 and 8, nonspecific competitions with the YY1 oligonucleotide. Lanes 4 and 5 the shifts were carried out using 10 μg of LAN-5 extracts; in all the other lanes 20 μg of extracts were used. Arrow, the distinctive band in LAN-5 shifts (see text for comments). Autoradiogram was exposed for 18 h. B, gel mobility shift assay in presence of anti a N-myc antibody. Whole extracts from LAN-5 (Lanes 2 and 3) and SK-N-SH cells were preincubated for 10 min at room temperature before electrophoresis mobility shift assay with 500 ng (Lanes 2 and 6) or 1 μg of a specific anti-N-myc antibody (Lanes 3 and 7). In Lanes 4 and 8, samples were preincubated with 1 μg of an unrelated monoclonal antibody. In Lanes 1 and 5 samples were not preincubated. Exposure, 18 h.
genomic DNA was run on an agarose gel and transferred to a nylon membrane. A, for which no RNA was available for Northern and RT-PCR analyses. Neuroblastomas listed in Table 1. Samples 15 and 16 are from two primary neuroblastomas for which no RNA was available for Northern and RT-PCR analyses.

Lack of correlation between N-myc and MAX expression

To determine whether the presence or the absence of the N-myc protein in neuroblastoma cells results in different binding patterns to the max consensus sequence, we carried out electrophoresis mobility shift assay experiments using whole cell extracts from LAN-5 (high level of N-myc expression) and SK-N-SH (no detectable expression of N-myc). Whole cell extracts were prepared in high salt conditions to increase the solubility of myc proteins. Different band shift patterns were obtained using LAN-5 and SK-N-SH extracts (Fig. 3A). LAN-5 shifts showed an upper band (Fig. 3A, arrow) which was not present in SK-N-SH. Although also the ubiquitous transcription factor binds to the same DNA sequence recognized by myc proteins, it seems likely that the upper band seen in LAN-5 extracts derives from the binding of N-myc-MAX heterodimers to the oligonucleotide probe which does not occur in SK-N-SH extracts due to the lack of N-myc expression. To determine whether the upper band seen in Fig. 3A depended on N-myc expression in LAN-5 cells, total extracts from LAN-5 and SK-N-SH cells were preincubated with a monoclonal antibody to N-myc before electrophoresis mobility shift assay. Preincubation with the N-myc antibody reduced the density of the upper band seen with LAN-5 cell extracts (Fig. 3B, Lanes 2 and 3), whereas the pattern seen with the SK-N-SH extracts was not modified by the treatment. In control experiments, preincubation with an unrelated monoclonal antibody did not affect the intensity of the upper band seen with LAN-5 cell extracts (Fig. 3B, Lane 4).

Southern Blot Analysis of Neuroblastoma Genomic DNA. Hybridization of EcoRI-digested genomic DNA from five primary neuroblastomas and a metastasis of one of them to the MAX-specific probe revealed several bands ranging from more than 9 kilobases to less than 1 kilobase, a pattern that was invariant in all samples (Fig. 4A). Analysis of gene dosage, after normalization of the amount of DNA for each sample, revealed no genomic amplification of the MAX gene (not shown), suggesting that the transcription of MAX does not depend on amplification of the gene. Hybridization of the same filter to N-myc-specific probe, demonstrated amplification of N-myc in two tumors at stage IV and in one metastasis (Fig. 4B, Lanes 1, 6, and 7).

DISCUSSION

The association in vivo of N-myc oncoprotein with MAX phosphoprotein has been described in human neuroblastoma (13). This result suggests a role of the N-myc-MAX dimer in this neoplasia. Using a probe obtained by RT-PCR, we have detected MAX mRNA in 10 primary neuroblastomas, two metastases, and two cell lines, at comparable levels in all samples and independent of tumor stage or N-myc genomic amplification. Anti-MAX antibody immunoprecipitated two MAX proteins in all neuroblastoma cell lines tested. Thus, it seems unlikely that MAX expression is controlled in neuroblastoma by a posttranscriptional mechanism preventing translation of MAX mRNAs. By contrast, N-myc mRNA was present only in samples with amplified N-myc gene, suggesting that the latter is a limiting factor in the formation of the N-myc-MAX dimer in neuroblastoma. Unlike N-myc, the MAX levels of transcripts do not appear to depend on genomic amplification.

The detection of MAX transcripts in neuroblastomas that do not express N-myc raises the question of the function of MAX gene. It has been demonstrated in humans and mice that MAX can form an homodimer (10—12) the role of which in the cell might differ from that of the heterodimer. MAX protein(s) lacks the transactivation domain (22), yet MAX-MAX homodimers bind the same CACGTG sequence recognized by myc genes (10—12). Expression of MAX in excess over myc genes can shift the equilibrium in favor of homodimers, which in turn compete out the transactivating myc-MAX heterodimers (12), keeping the controlled genes in a state of transcriptional repression (23).

In neuroblastoma, the amplification and overexpression of N-myc is a marker of tumor aggressiveness (3). We can speculate that in tumors where only MAX expression is detectable, those genes under the transcriptional control of N-myc are silenced by MAX homodimers. The lack of expression of these genes could somehow decrease the tumor aggressiveness. The identification of neuroblastomas without N-myc amplification and overexpression, yet highly aggressive, might be explained with the activation of different pathways independent from those associated with N-myc-MAX expression. Alternatively, in tumors that do not express N-myc, the MAX protein could be in an inactive form. In light of the presence of a consensus signal for casein kinase II 3' of the leucine zipper of MAX (10) it is possible that the activity of MAX protein is related to the phosphorylation state.

All of the neuroblastoma samples analyzed revealed the presence of two MAX transcripts, one of which derives from an in-frame insertion of 27 nucleotides in a region 5' to the basic region. Since mutations outside the basic region helix-loop-helix-leucine zipper domain do not alter the ability of MAX to dimerize and bind to DNA (10, 11), it is conceivable that the presence of two MAX forms able to dimerize with N-myc serves to broaden the range of the gene activities controlled by the N-myc-MAX complex.

ACKNOWLEDGMENTS

We thank Dr. Edward B. Ziff for the gift of the anti-MAX antiserum.

REFERENCES

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