N-myc Regulation of Type I Insulin-like Growth Factor Receptor in a Human Neuroblastoma Cell Line

Daniel Chambery, Sarah Mohseni-Zadeh, Brigitte de Gallé, and Sylvie Babajko

ABSTRACT

Insulin-like growth factors I and II (IGF-I and IGF-II) stimulate proliferation and differentiation in many cell types, including cell lines derived from human neuroblastomas. Their effects are mediated via the IGF-I receptor (IGF-IR) that is essential for growth in these cells. Amplification of the N-myc oncogene is a marker for poor prognosis in neuroblastoma development, and it therefore seemed of interest to analyze the relationships that may exist between IGF-IR and N-myc. N-myc-deficient SK-N-SH neuroblastoma cells were used as an experimental model. After stable transfection with N-myc cDNA, Northern blotting revealed a marked increased in IGF-IR, IGF-II, IGF-binding protein (IGFBP)-2, and IGFBP-4 mRNA levels, whereas IGFBP-6 mRNA levels were clearly diminished. Western immunoblot analysis also demonstrated increased intact IGFBP-2 but decreased IGFBP-6 in the presence of N-myc oncogene. Parallel binding experiments using IGF-I missing the first 3 amino acids revealed a 47% increase in binding sites for IGF-I and an increase of at least 335% in DNA synthesis, as measured by labeled thymidine incorporation into DNA. s.c. injection of these cells into nude mice provoked xenograft development in 50–100% of cases (depending on the series of experiments). Control cells, in contrast, were not tumorigenic.

In cells transfected with hp ~420+/+60 of the human IGF-IR promoter controlling expression of the luciferase reporter gene, promoter activity was stimulated by a factor of 3.8 ± 0.6 (n = 6) in the presence of N-myc oncogene. This suggests transcriptional regulation of IGF-IR expression by N-myc. IGF-IR activity and N-myc amplification are two events that to date have been identified as independently instrumental in the etiology of human neuroblastoma. Our results provide the first evidence of a direct link between them and demonstrate the effects of the oncogene on components of the IGF system in neuroblastoma cell growth in vitro and in vivo.

INTRODUCTION

IGF-I and IGF-II play a crucial role in the metabolism, proliferation, differentiation, and transformation of numerous cell types, including neuronal cells (1, 2). The IGFs, especially IGF-II, are now known to be directly involved in tumorigenesis (3). Their effects are mediated via the IGF-IR, which possesses tyrosine kinase activity (4, 5). This accounts for nuclear transmission of the mitogenic and transforming actions of the IGFs via a cascade of phosphorylation reactions (6). Numerous studies have documented the involvement of IGF-IR in tumor cell proliferation and in protection from apoptosis as well as its mechanism of action (6–8). A recent report has indicated that a cDNA antisense to the IGF-IR abolishes the growth of neuroblastoma cells in nude mice (9). Another receptor exists that is capable of binding IGF-II with strong affinity. This is the cation-independent mannose-6-phosphate receptor, which appears to have no effect on cell proliferation but appears to be involved in clearance of the IGF-II...
nitrocellulose membranes presaturated with gelatin and then incubated with anti-IGFBP-2, anti-IGFBP-4, or anti-IGFBP-6 antibodies (Austral Biologicals, Tübingen, Germany) at a 1:600 dilution at 37°C for 1 h. The nitrocellulose membranes were rinsed and then incubated for 45 min with goat polyclonal antirabbit IgG antibody coupled to horseradish peroxidase (Sigma, St. Louis, MO) at a 1:1000 dilution. Horseradish peroxidase oxidation of luminol (Enhanced Chemiluminescence Western Blotting Detection System; Amersham) yields chemiluminescence from which the specific IGFBP-antibody complexes can be visualized.

Binding Assays. rh des (1-3) IGF-I was provided by GroPep (Adelaide, Australia). Peptides were iodinated by the chloramine T method (100 µCi/µg) and purified by gel filtration. Transfected SK-N-SH cells were cultured in 6-well plates (7 × 10⁵ cells/well) and incubated with 100,000 cpm of des (1-3) IGF-I (which does not bind or weakly binds IGFBPs) at 4°C for 4 h, with or without increasing concentrations of rh des (1-3) IGF-I (duplicate wells). The cells were then washed four times with Hank's solution (Sanofi, France) and dissolved in 0.06 M NaOH. The quantities of ¹²⁵I-peptide bound to the cells are expressed in relation to the protein content measured by the Bradford method (Bio-Rad, München, Germany). Nonspecific binding assessed in the presence of 400 nM unlabeled peptide was 0.4–0.7%. Specific binding in the absence of unlabeled peptide was 2.5–4%.

[^3H]Thymidine Incorporation Assays. Tests were carried out on two different clones that were stably transfected at 48, 72, and 96 h after serum deprivation. For the cells cultured in 24-well plates, 5 µCi of[^3H]thymidine (Amersham) were added for the final 16 h of culture with or without 1 µg/ml αIR-3 antibody (Calbiochem, San Diego, CA), which prevents IGF binding to IGF-IR. Cells were then rinsed and lysed using 200 µg of 0.06 M NaOH/well. Liquid scintillation counting was used to determine the amount of radioactivity incorporated into DNA, as described previously (27). For each time, results for N-myc-transfected cells were corrected for those obtained using N-myc/ΔXhoI-transfected cells (controls).

Tumors in Nude Mice. Stably transfected SK-N-SH cells (10⁶) were injected into the flanks of 6–8-week-old female nude mice (SP Swiss nude mice; origin, Elevage Janvier, Le Genest Saint Isle, France). For each set of experiments, the same number of mice was injected with test cells and control cells. The animals were examined regularly over a period of 4 months, and the tumors were measured with calipers using the National Cancer Institute formula:

\[
V = \frac{L (\text{cm}) \times I^2 (\text{cm})}{2}
\]

in which \(L\) is the largest diameter, and \(I\) is the smallest diameter of the tumor.

Luciferase Reporter Assays. At 48 h after transient transfection with the IGF-IR promoter, SK-N-SH cells transiently or stably transfected with either N-myc oncogene or the mutant N-myc/ΔXhoI were rinsed twice in a solution of 4 × PBS, centrifuged at 2,500 × g for 7 min at 20°C, and then taken up in 150 µl of 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 250 mM sucrose. They were then lysed by a series of rapidly alternating freezing and thawing. Cell extracts contained in the supernatant were recovered after centrifugation at 10,000 × g for 10 min at 4°C. Luciferase activity was measured in a luminometer (Lumat LB 9501; Lumat, Berthold, France) using 5-µl samples of cell extract diluted in 365 µl of 25 mM Gly-Gly, 15 mM MgSO₄, 4 mM EDTA, 10 mM DTT, 0.1 mg/ml o-luciferin (Sigma), and 4 mM ATP (Boehringer, Mannheim, Germany).

RESULTS

N-myc Modulation of the Expression of Components of the IGF System. Northern blotting revealed strongly increased levels of IGF-IR mRNA (+670 ± 60%; \(n = 6\)) migrating at 11 kb in the SK-N-SH cells stably transfected with N-myc cDNA (Fig. 1). The quantities of mRNA measured in N-myc-transfected cells were compared with those observed in cells transfected with a mutant form of the N-myc oncogene, which fails to bind DNA due to the XhoI site introduced into the coding sequence (N-myc/ΔXhoI). Under the same conditions, N-myc increased the levels of IGF-II (+840 ± 70%; \(n = 4\)), IGFBP-4 (+180 ± 30%; \(n = 4\)), and IGFBP-2 (+230 ± 10%; \(n = 4\)) mRNA but depressed IGFBP-6 expression (−93 ± 3%; \(n = 4\)). These are the only components of IGF system expressed by neuroblastoma cells. Similar results were obtained with SK-N-SH cells 48 h after transient transfection with N-myc or N-myc/ΔXhoI plasmids (data not shown).

N-myc Modulation of IGFBP Production by Stably Transfected SK-N-SH Cells. The IGFBPs secreted into the culture media by the neuroblastoma cells were analyzed by Western immunoblotting after serum deprivation for 48 h. The specific anti-IGFBP-2 antibody recognized intact IGFBP-2 migrating at 34 kDa and its proteolyzed form migrating at 20 kDa. The specific anti-IGFBP-6 antibody recognized IGFBP-6 migrating at 30–32 kDa. IGFBP-4 could not be detected because its expression is strongly inhibited in the absence of serum, and its levels were below the limits of detectability. In the presence of N-myc, intact IGFBP-2 was increased, and its proteolytic fragment was markedly reduced. IGFBP-6 levels were altered by the same proportions as the mRNA levels. They were depressed in the presence of N-myc and became difficult to detect in media conditioned by cells transfected with N-myc for 48 h (Fig. 2). Similar
results were obtained with SK-N-SH cells 48 h after transient transfection with N-myc or N-myc/ΔXhoI plasmids (data not shown).

**N-myc-induced Increase in IGF-IR Binding Sites.** Binding experiments performed 48 h after serum deprivation of stably transfected cells revealed an increase of 47 ± 2% in the number of binding sites for IGF-I in the presence of N-myc (Fig. 3). The binding was specific because it was totally displaced by increasing concentrations of unlabeled rh des (1-3) IGF-I, which does not bind IGFBPs. The hook effect observed in Fig. 3, despite the use of des (1-3) IGF-I, was unrelated to the presence of the IGFBPs. Scatchard analysis revealed an affinity constant for the receptor of 5.1 μM, which is in agreement with published values.

**N-myc Stimulation of DNA Synthesis.** [3H]Thymidine incorporation in cells stably transfected with N-myc was measured 48, 72, and 96 h after serum deprivation. At 48 h, [3H]thymidine incorporation in N-myc-transfected cells was increased by 335 ± 11% (n = 3) as compared with that in cells transfected with control plasmid. At 72 and 96 h, the corresponding increases were 389 ± 17% (n = 3) and 528 ± 26% (n = 3; Fig. 4). Similar results were obtained with transiently transfected SK-N-SH cells 48 h after serum deprivation. In cultures run in the presence of αIR-3 antibody (which blocks IGF binding to IGF-IR), N-myc failed to stimulate [3H]thymidine incorporation, but control cell growth was also inhibited, which confirms the essential role of IGF-IR in proliferation. Nevertheless, αIR-3 inhibition of DNA synthesis was stronger in N-myc-transfected cells (−88 ± 4%) than in controls (−38 ± 5%).

**Effects of N-myc on Xenograft Development in Nude Mice.** Under basal conditions, neither wild-type SK-N-SH cells nor cells stably transfected with N-myc/ΔXhoI mutant provoked tumor development in nude mice up to 6 months after injection. Three series of experiments were run, in which 10^7 cells of the different clones were injected s.c. into the flanks of nude mice. Each series comprised five controls (mice injected with cells stably transfected with N-myc/ΔXhoI or nontumorigenic wild-type SK-N-SH cells) and five test animals (mice injected with cells stably transfected with N-myc). Tumor size was measured with a caliper at regular intervals over a period of 90–130 days after injection. Test animals developed measurable and palpable tumors within 8–9 weeks, whereas no tumors were detected in controls. The incidence of tumor development in test animals was 50–100%, depending on the series of the experiment. Tumor growth in these animals is shown in Fig. 5.

**N-myc Modulation of the Transcriptional Activity of the IGF-IR Promoter.** Cells stably transfected with either N-myc or N-myc/ΔXhoI were then transiently transfected with the human IGF-IR promoter (−420/+60 bp) controlling expression of the luciferase gene. Luciferase activity, which reflects IGF-IR promoter activity, was measured 48 h after transfection and found to be 3.8 ± 0.6 times higher in N-myc-transfected cells than in controls transfected with N-myc/ΔXhoI (n = 4; Fig. 6). Similar results were obtained with wild-type SK-N-SH cells transiently transfected with 30 μg of IGF-IR prom/luc plasmid and 3 μg of N-myc plasmid, where promoter activity was increased 4 (± 0.9)-fold (n = 6).

**DISCUSSION**

It is now well established that the effects of IGF-I and IGF-II are associated with malignancy and that these factors stimulate tumor growth via the IGF-IR (3). The signaling pathways associated with this receptor have been studied extensively (5) in attempts to elucidate the events involved in cell proliferation and controlling apoptosis.

In neuroblastoma cells, growth has been linked to both the IGF system (18, 29) and the N-myc oncogene. In addition, c-myc levels are associated with malignancy and that these factors stimulate tumor growth. Nevertheless, the events involved in cell proliferation and controlling apoptosis.
conversely, N-myc is associated with tumorigenesis and cell proliferation (31, 32). We therefore thought it of interest to examine the possible relationships between the two effector systems.

Our results provide the first evidence of N-myc stimulation of IGF-IR messenger and IGF binding sites in a human neuroblastoma-derived cell line. Overexpression of the N-myc oncogene in SK-N-SH cells was found to have direct effects on proliferation in vitro. In view of the well-documented role played by IGF-IR in cell proliferation, it seemed possible that the effects observed in the presence of N-myc may at least, in part, reflect modulation of the IGF system. This notion was corroborated by the fact that cell growth was severely diminished after treatment with aiR-3 in both N-myc-transfected cells and control cells (transfected with N-myc/ΔXhoI). Because aiR-3 abolished N-myc stimulation of growth, IGF-IR mediation of the proliferative effects of N-myc could be considered as specific. The intensified proliferation in N-myc-transfected neuroblastoma cells is consistent with the results of other laboratories showing N-myc-induced inhibition of $\beta_1$ integrin expression, which would also contribute toward stimulating cell proliferation (33). Our experiments, in which nude mice were injected with neuroblastoma cells stably transfected with N-myc, provide confirmation in vivo of our results for cell proliferation in vitro; the tumorigenic potency of the cells is stronger in the presence of N-myc. This agrees with findings that neuroblastoma-derived cell lines expressing the most N-myc are also the most tumorigenic (34). Interestingly, wild-type SK-N-SH neuroblastoma cells, which do not express N-myc, are not tumorigenic, whereas IGR-N-91 cells, in which the N-myc oncogene is amplified and overexpressed, are tumorigenic (22). Our results are in agreement with those of Wang et al. (35), who used an approach similar to ours to show that knockout of N-myc in the WH4KA hepatoma cell line reduced its tumorigenic potency.

These observations may be compared with those for the IGF system, particularly for IGF-IR, which is intimately involved in cell proliferation.

Analysis of the promoter region of the IGF-IR gene has revealed a GC-rich structure lacking both TATA and CCAAT boxes (36), a basic structure common to numerous genes associated with proliferation. Computerized analysis was used to identify five potential binding sites (CACGTG) for N-myc or c-myc family members (depending on the culture conditions or cell type) in the proximal region of the human IGF-IR promoter (–300 to +100 bp from the transcription initiation site). Interestingly, it has been demonstrated that platelet-derived growth factor is capable of inducing IGF-IR gene expression and that the effect can be mediated by c-myc, which activates the IGF-IR proximal promoter (37). It is pertinent that N-myc and c-myc belong to the same family of oncoproteins and bind to the same DNA sequences. Several studies have shown that IGF-IR expression is modulated by hormones such as estradiol and thyroid hormone (38, 39), growth factors such as IGF-I (40), and, particularly, factors involved in tumor progression such as p53 and WT1 (41, 42). Our observation that IGF-IR promoter activity is stimulated by N-myc suggests transcriptional regulation of the IGF-IR gene in the proximal region of the promoter (nucleotides –420/+60), although we cannot exclude the possibility that N-myc may have posttranscriptional effects, as has been observed in the case of integrin $\beta_1$ subunit expression (43). It seems possible that N-myc may interact directly with the proximal promoter sequence in activating transcription of the gene. The mechanism of action of the N-myc oncogene elicited by these results would be the activation of IGF-IR expression and hence the proliferation of cells that would become more aggressive and promote tumor development.

Induced IGF-IR expression was found to be accompanied by increased IGF-II mRNA levels that have a direct effect on SK-N-SH cell proliferation (44). IGFBP-6 expression in N-myc-transfected cells was well below that in controls, and because IGFBP-6 has a strong affinity for IGF-II, its reduction would result in enhanced IGF-II bioavailability and mitogenic activity. Such findings suggest that IGFBP-6 is involved in the arrest of neuroblastoma cell proliferation, as reported previously (22). In addition, IGR-N-91 neuroblastoma cells, whose proliferative and tumorigenic potencies are greater than those of SK-N-SH cells, express larger quantities of IGF-II and no IGFBP-6 under basal conditions, providing further indirect evidence of their implication in neuroblastoma cell proliferation (22). Conversely, IGFBP-2 expression increased concomitantly with stimulated cell proliferation, confirming earlier findings in our laboratory that IGFBP-2 is associated with proliferation in neuroblastoma cells (20). N-myc reduced the amounts of fragment generated by limited proteolysis of IGFBP-2. It could be hypothesized that in the media conditioned by cells transfected with mutant N-myc, these fragments may contribute toward checking cell proliferation, as has been described for IGFBP-3, whose NH$_2$-terminal fragments block cell growth in MG63 cells (45). Further study will reveal the roles of these fragments, their possible intrinsic activities, and the IGF-dependent and -independent mechanisms by which IGFBP-2 proteolysis participates in the induction of cell proliferation and tumorigenesis.
Therefore, our results confirm the direct role of the IGF system and particularly of IGF-IR in neuroblastoma cell proliferation (24, 25) and agree with findings for other experimental models (3). It appears that modulation of IGF-IR expression by the N-myc oncogene is instrumental in stimulating tumor cell growth in neuroblastomas. The N-myc protein seems to be capable of altering IGF-IR expression, probably at the transcriptional level. Apart from confirming the crucial role of the IGF-IR and the IGF system in tumor development and growth (46), these findings throw some light on the mechanisms that regulate the expression of the IGF-IR gene.

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REFERENCES


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