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23. Areas of activation shown are those corresponding to the largest clusters. A complete list of activations can be obtained from the corresponding author. Within each cluster the coordinates of the voxel with the maximum FPQ are shown.
24. Supported by an Oxford University Pump Priming grant and the Bethlem and Maudsley Research Fund. E.T.B. and P.K.M. were supported by the Wellcome Trust, G.A.C. by the Medical Research Council of Great Britain, and P.W.R.W. by British Telecom. We are grateful to I. C. Wright and R. Howard and to two anonymous reviewers for their helpful comments on this manuscript.

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Repression of c-*myc* Transcription by Blimp-1, an Inducer of Terminal B Cell Differentiation

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Transcription of c-*myc* in plasma cells, which are terminally differentiated B cells, is repressed by plasmacytoma repressor factor. This factor was identified as Blimp-1, known for its ability to induce B cell differentiation. Blimp-1 repressed c-*myc* promoter activity in a binding site–dependent manner. Treatment of BCL_1 lymphoma cells with interleukin-2 (IL-2) plus IL-5 induced Blimp-1 and caused a subsequent decline in c-Myc protein. Ectopic expression of Blimp-1 in Abelson-transformed precursor B cells repressed endogenous c-Myc and caused apoptosis; Blimp-1–induced death was partially overcome by ectopic expression of c-Myc. Thus, repression of c-*myc* is a component of the Blimp-1 program of terminal B cell differentiation.

c-Myc functions at a critical decision point of cell growth to favor proliferation and to block terminal differentiation (*1*). c-Myc is present in dividing cells but is not expressed in quiescent or terminally differentiated cells (*2*); addition of exogenous c-Myc blocks terminal differentiation of several hematopoietic cell lines (*3*) and of myogenic cells (*4*), whereas inhibitors of c-Myc expression accelerate terminal differentiation of promonocytic HL60 cells (*5*), M1 leukemic myeloid cells (*6*), F9 teratocarcinoma cells (*7*), and human esophageal cancer cells (*8*).

Murine plasmacytomas are the transformed counterparts of plasma cells, which are terminally differentiated, nondividing B lymphocytes (*9*). Plasmacytomas have a characteristic reciprocal chromosomal translocation that juxtaposes one allele of the c-*myc* gene with an immunoglobulin heavy- or light-chain locus (*10*). The translocated c-*myc* allele is deregulated and overexpressed; however, the nontranslocated c-*myc* allele is transcriptionally silent (*1*). This state is thought to correspond to the silent state of the c-*myc* gene in normal plasma cells.

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A plasmacytoma-specific protein, plasmacytoma repressor factor (PRF), binds in the c-*myc* promoter 290 base pairs (bp) 5′ of the P1 transcriptional start site. PRF represses c-*myc* transcription in plasmacytomas and has not yet been cloned (*11*). The PRF binding site is similar in sequence to the interferon-stimulated response elements (ISREs) in many interferon-regulated genes (*12*) and to the positive regulatory domain 1 (PRD1) sequence of the human interferon-β (IFN-β) gene (*13*). Electrophoretic mobility shifts assays (EMSAs) with nuclear extracts from the plasmacytoma P3X63-Ag8 (P3X) and a c-*myc* promoter probe containing the PRF site confirmed that both ISRE and PRD1 oligonucleotides could compete for binding of PRF in this assay; PRD1 oligonucleotides competed more strongly than ISRE oligonucleotides (*14*).

PRD1-BF1 is a human zinc finger protein that was cloned by virtue of its ability to bind to the PRD1 site; PRD1-BF1 inhibits transcription of the IFN-β promoter (*13*). Recently the murine homolog of PRD1-BF1, Blimp-1, was identified as a protein that is induced upon stimulation of the BCL_1 B cell lymphoma line with interleukin-2 (IL-2) plus IL-5 (*15*). Ectopic expression of Blimp-1 can drive B cell terminal differentiation, and Blimp-1 is expressed only in plasmacytomas and mature B cells; however, its mechanism of action is not well understood (*15*). On the basis of cross-competition of their binding sites, common transcriptional repressor activity, and plasmacytoma-specific expression, we hypothesized that PRF might be identical to Blimp-1.

To test this hypothesis, we transfected 293T human kidney fibroblast cells with an expression plasmid encoding Blimp-1. An immunoblot developed with antiserum to Blimp-1 revealed that Blimp-1 was present in nuclear extracts from P3X plasmacytomas and in the transfected 293T cells but not in 18-81 precursor B cells (pre-B cells) or in mock-transfected 293T cells (Fig. 1). EMSAs were then done with these extracts with an oligonucleotide probe corresponding to the c-*myc* PRF site (Fig. 1). Complexes of identical mobility were observed for endogenous PRF in P3X cells and for the Blimp-1–transfected 293T cells, whereas no complex was detected for 18-81 or mock-transfected 293T cell extracts. The sequence specificity of these complexes was shown by the ability of PRF but not an unrelated sequence to compete the complexes. Finally, the complex from P3X extracts was completely ablated by antiserum against Blimp-1 but not by naïve antiserum. Thus, the protein in P3X cells that we identified as PRF is immunologically related to Blimp-1. The results obtained with EMSA and antibody ablation provide evidence that the c-*myc* repressor PRF is encoded by the *blimp-1* gene.

A site-directed mutation in the c-*myc* PRF site increases promoter activity 30-fold in plasmacytomas, which express PRF, but has no effect in fibroblasts and pre-B cells, which do not express the protein, showing that PRF represses c-*myc* transcription (*11*). To investigate the functional relation be-

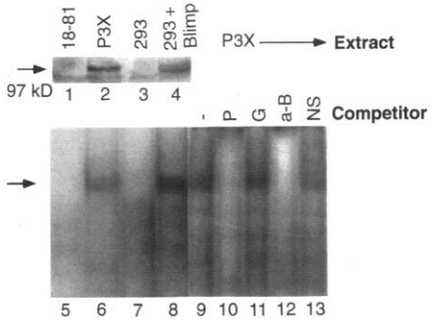


Fig. 1. Blimp-1 binds to the c-*myc* PRF site. (Top) Nuclear extracts from various cells were prepared as described (*26*) and subjected to immunoblot with antibody to Blimp-1; arrow indicates Blimp-1. Lane 1, 18-81 cells; lane 2, P3X cells; lane 3, mock-transfected 293T cells; lane 4, Blimp-1–transfected 293T cells. (Bottom) Lanes 5 to 8, the same extracts were used for EMSA with a 25-bp PRF oligonucleotide (*26*). Lanes 9 to 13, EMSA of P3X nuclear extracts with PRF oligonucleotide probe in the presence of no competitor (lane 9), PRF oligo tetramer (lane 10), GATA (nonspecific) tetramer (lane 11), rabbit antiserum to Blimp-1 (lane 12), and naïve rabbit serum (lane 13). Arrow indicates the protein-DNA complexes.

tween PRF and Blimp-1, we tested the effect of ectopically expressed Blimp-1 on the activity of the c-*myc* promoter. Reporters dependent on a c-*myc* promoter with either wild-type or mutated PRF sites were cotransfected with a Blimp-1 expression plasmid into the 18-81 and 300-18 pre-B cell lines (Fig. 2, A and B, respectively). In both pre-B cell lines, wild-type and mutant promoters had similar activity in the absence of Blimp-1. Expression of Blimp-1 repressed the wild-type promoter by 70% in both cell lines, whereas the promoter harboring a mutation in the PRF site was not repressed (Fig. 2, A and B). Thus, ectopic Blimp-1 represses c-*myc* transcription in pre-B cells, and the repression depends on the presence of the PRF site at −290 bp. Because the function of Blimp-1 is the same as that previously shown for PRF, these data, in conjunction with the in vitro binding studies, suggest that PRF is encoded by the *blimp-1* gene.

Blimp-1 is a nuclear protein with five zinc fingers in its COOH-terminus (*15*). Only two other target genes for Blimp-1 are known. Human Blimp-1 (PRD1-BF1) represses IFN-β transcription (*13*) and Blimp-1 activates J-chain transcription, although a binding site in the J-chain gene has not been identified (*15*). Transcriptional activation and repression in different gene contexts is frequently associated with zinc finger proteins including YY1 (*16*), Krüppel (*17*), and glucocorticoid receptor (*18*) and is often determined by binding of adjacent proteins. The Blimp-1 binding site in the c-*myc* promoter is near a YY1 binding site that functions as an activator site (*19*). Our preliminary data show that Blimp-1 and YY1 associate in vitro (*20*), and it may be that Blimp-1 and YY1 interfere with one another's activity in the c-*myc* promoter. However, further studies will be required to understand the mechanisms that determine the activity of Blimp-1 on this and other promoters.

BCL_1 is a mature B cell line that, upon stimulation with IL-2 plus IL-5, differentiates into a plasma cell–like state (*21*). Blimp-1 is induced early in BCL_1 differentiation (*15*). On the basis of the ability of transfected Blimp-1 to repress the c-*myc* promoter, we predicted that differentiation dependent on induction of Blimp-1 in BCL_1 cells would cause a decrease in endogenous c-Myc. Seventy-two hours after BCL_1 cells were treated with IL-2 plus IL-5, differentiation was verified by increased immunoglobulin secretion and changes in cell size as indicated by changes in forward versus orthogonal scatter (*14*). c-Myc amounts during this period were assessed by immunoblot (Fig. 3A). After a transient increase, c-Myc decreased by ~75% between 1 and 2 hours of IL-2 plus IL-5 stimulation and remained low for 72 hours. Northern (RNA) analyses showed that 1 hour after stimulation, *Blimp-1* mRNA increased approximately five times (*15*) (Fig. 3B). These data are consistent with the notion that Blimp-1 represses endogenous c-*myc* transcription, resulting in decreased c-Myc protein.

Because Blimp-1 drives terminal B cell differentiation, we reasoned that the c-*myc* gene would be an important target for Blimp-1–mediated repression. To test this hypothesis directly, we transfected 18-81 pre-B cells with combinations of a neo–Blimp-1 expression plasmid, or a Blimp-1 antisense control, and the pSV2 c-*myc* expression plasmid, or a pSV2 control. Few colonies were obtained with the Blimp-1 expression plasmid (Fig. 4), and 16 of 16 analyzed did not express Blimp-1 (*14*). However, transfections with varying ratios of Blimp-1 and c-*myc* plasmids gave 17 to 65% the number of control colonies; four of five colonies analyzed expressed Blimp-1 (*14*). Thus, ectopic c-Myc blocked the growth-suppressing effect of high Blimp-1 expression, suggesting that repression of c-*myc* transcription by overexpressed Blimp-1 is directly responsible for the death of Blimp-1–overexpressing cells.

To obtain direct evidence that ectopic

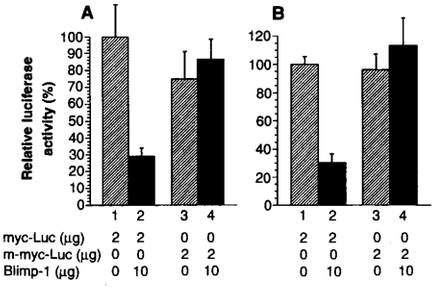


Fig. 2. Blimp-1 represses the c-*myc* promoter in a PRF site–dependent manner in pre-B cells. Two micrograms of a luciferase reporter fused with either a wild-type (myc-Luc) or PRF-deleted (m-myc-Luc) c-*myc* promoter including −1100 to +580 bp from P1 (*11*) with 10 μg of Blimp-1 expression vector (pBDP1-F) or expression vector control (pBDP1-B) (*15*) were used to transfect 18-81 or 300-18 pre-B cells. Log-phase cells (5×10^6) were collected by centrifugation and resuspended in 300 μl of culture medium, mixed with DNA, and subjected to electroporation at 960 μF and 240 V. Luciferase activity was measured 18 hours after transfection. Results show the average of at least three independent transfections. Error bars show standard deviations. (**A**) 18-81 cells. (**B**) 300-18 cells.

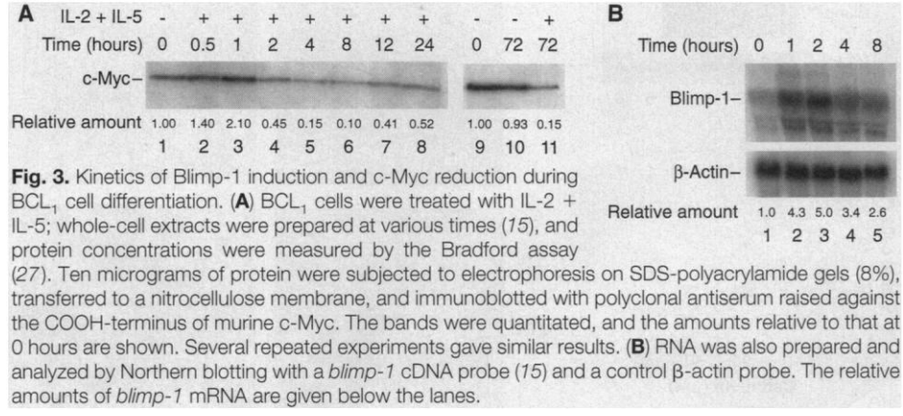
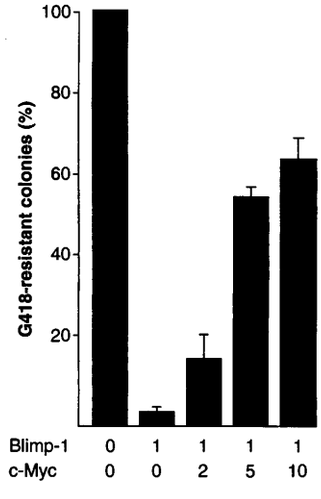


Fig. 3. Kinetics of Blimp-1 induction and c-Myc reduction during BCL_1 cell differentiation. (**A**) BCL_1 cells were treated with IL-2 + IL-5; whole-cell extracts were prepared at various times (*15*), and protein concentrations were measured by the Bradford assay (*27*). Ten micrograms of protein were subjected to electrophoresis on SDS-polyacrylamide gels (8%), transferred to a nitrocellulose membrane, and immunoblotted with polyclonal antiserum raised against the COOH-terminus of murine c-Myc. The bands were quantitated, and the amounts relative to that at 0 hours are shown. Several repeated experiments gave similar results. (**B**) RNA was also prepared and analyzed by Northern blotting with a *blimp-1* cDNA probe (*15*) and a control β-actin probe. The relative amounts of *blimp-1* mRNA are given below the lanes.

Fig. 4. c-Myc blocks the growth suppression effect of high Blimp-1 expression. A pBJ-neo plasmid containing either antisense or sense *blimp-1* cDNA was cotransfected into 18-81 pre-B cells with the pSV2-*myc* expression plasmid or a pSV2 control. Cells were diluted into 96-well plates, cultured with G418 (800 μg/ml), and resistant colonies were counted 10 days later. Colony numbers obtained for different ratios of Blimp-1 sense (S) or antisense (AS) to c-*myc* plasmids were as follows: (i) 1:2 control (5 μg of Blimp-1:10 μg of pSV2): S = 34 ± 7, AS = 1033 ± 46; (ii) 1:2 (5 μg of Blimp-1:10 μg of pSV2-*myc*): S = 347 ± 73, AS = 2011 ± 153; (iii) 1:5 (2 μg of Blimp-1:10 μg of pSV2-*myc*): S = 340 ± 11, AS = 599 ± 24; and (iv) 1:10 (2 μg of Blimp-1:20 μg of pSV2-*myc*): S = 446 ± 30, AS = 684 ± 19. A graphic representation was generated from these data; for each transfection with the Blimp-1 plasmid, the corresponding transfection with antisense Blimp-1 was taken as 100%.



Blimp-1 can repress the endogenous c-*myc* gene, we made stable B cell transfectants in which Blimp-1 was controlled by a metallothionein promoter. Cadmium treatment of 18-81 transfectants induced Blimp-1 mRNA more than 10 times relative to controls and reduced endogenous c-Myc by more than 90% with similar kinetics (Fig. 5A). This result shows that Blimp-1 directly represses c-Myc expression in vivo. In addition, 18-81 cells expressing Blimp-1 died by apoptosis, as shown by the decrease in viable cells (Fig. 5B) and by fragmentation of nuclear DNA revealed by gel electrophoresis (*14*) and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay (Fig. 5C). However, induction of Blimp-1 in BCL_1 transfectants revealed terminal differentiation including increased surface expression of Syndecan (*14*) and secretion of immunoglobulin M (IgM) (Fig. 5D). The reason Blimp-1 caused apoptosis rather than differentiation in 18-81 cells was probably because Abelson murine leukemia virus (AMuLv)–transformed pre-B cells cannot activate nuclear factor kappa B, which is required for immunoglobulin light-chain expression (*22*). Apoptosis induced by decreased c-Myc has also been reported in WEHI 231 (*23*) and Ramos (*24*) B cell lines.

Although c-Myc's role in blocking terminal differentiation has been well established, we have now shown that Blimp-1 specifically represses c-*myc* transcription as part of a program of terminal B cell differentiation. Our data show Blimp-1–dependent repression of c-*myc* and emphasize that growth and differentiation of B cells are exquisitely sensitive to c-Myc. It will be important to determine if Blimp-1 has other important targets or if blocking proliferation by way of c-*myc* repression is sufficient to trigger terminal B cell differentiation. It will also be interesting to determine if immunoglobulin gene sequences overcome Blimp-1–dependent repression of c-*myc* transcription in plasmacytomas where the Blimp-1 site is not removed by translocation.

The restricted pattern of Blimp-1 expression (*11*, *15*) suggests that the Blimp-1–mediated suppression of c-*myc* transcription may be unique to B lymphocytes. However, the human homolog PRD1-BF1 is induced upon viral infection of fibroblasts (*13*), and preliminary results show that Blimp-1–deficient mice die during embryonic development (*25*). Therefore, it will be important to determine if Blimp-1 also represses c-*myc* transcription in other lineages of terminally differentiated cells.

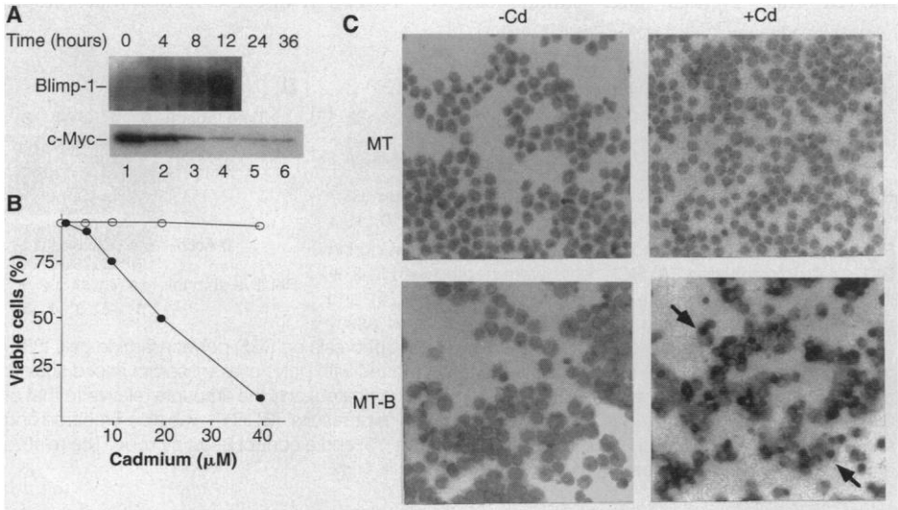


Fig. 5. Induction of Blimp-1 alters properties of 18-81 and BCL_1 cells. 18-81 and BCL_1 cells were stably transfected with an expression vector in which *blimp-1* expression was dependent on the sheep metallothionein promoter (MT-B) (*28*) or an empty control (MT). (**A**) (Upper panel) Northern blot of Blimp-1 mRNA after treatment of a MT-B 18-81 transfectant with 20 μM cadmium. (Lower panel) Immunoblot from the same cells showing c-Myc after treatment with 20 μM cadmium. (**B**) Viability of 18-81 MT-B transfectant (●) and MT control (○), determined by trypan blue exclusion, after growth in varying concentrations of cadmium for 36 hours. (**C**) Detection of Blimp-1–induced apoptosis in 18-81 transfectants by TUNEL staining (*29*). 18-81 cells transfected either by MT-B or MT control vectors were grown without (−Cd) or with (+Cd) 20 μM cadmium for 36 hours, centrifuged onto slides, and then fixed with 4% paraformaldehyde. The TUNEL staining was done according to the manufacturer's instructions (Boehringer Mannheim). Arrows indicate apoptotic cells. (**D**) Cells (10^5) from BCL_1 transfectants containing MT or MT-B (MT-B1 to 3) were treated with 20 μM cadmium for 72 hours, and IgM in the culture medium was measured by enzyme-linked immunosorbent assay (*15*). Untransfected BCL_1 ± IL-2/IL-5 served as controls.

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Maintenance of Acetylcholine Receptor Number by Neuregulins at the Neuromuscular Junction in Vivo

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ARIA (for acetylcholine receptor–inducing activity), a protein purified on the basis of its ability to stimulate acetylcholine receptor (AChR) synthesis in cultured myotubes, is a member of the neuregulin family and is present at motor endplates. This suggests an important role for neuregulins in mediating the nerve-dependent accumulation of AChRs in the postsynaptic membrane. Nerve-muscle synapses have now been analyzed in neuregulin-deficient animals. Mice that are heterozygous for the deletion of neuregulin isoforms containing an immunoglobulin-like domain are myasthenic. Postsynaptic AChR density is significantly reduced, as judged by the decrease in the mean amplitude of spontaneous miniature endplate potentials and bungarotoxin binding. On the other hand, the mean amplitude of evoked endplate potentials was not decreased, due to an increase in the number of quanta released per impulse, a compensation that has been observed in other myasthenic states. Thus, the density of AChRs in the postsynaptic membrane depends on immunoglobulin-containing neuregulin isoforms throughout the life of the animal.

The fidelity of neuromuscular transmission depends on the extraordinarily high density of AChRs in the postsynaptic muscle membrane. Developmental studies have pointed to the important trophic influence of the motor nerve in the regulation of endplate AChR density (*1*), an effect strong enough to override the suppression of AChR synthesis by muscle activity. Part of the nerve's local influence on AChR density is to promote the immobilization of AChRs, an effect mediated by the glycoprotein agrin (*2*). Another important local influence of the motor nerve is to increase the synthesis and insertion of AChRs into the postsynaptic membrane (*3*). In fact, endplate nuclei in developing and mature muscle are known to transcribe AChR subunit genes at a high rate as compared with that in nonsynaptic nuclei (*4*, *5*). The effect of synthesis on local receptor density remains evident in mice that lack the principal cytoplasmic AChR anchoring protein rapsyn (*6*).

The most likely candidate for mediation of the motor neuron's influence on endplate AChR synthesis is ARIA (for acetylcholine receptor–inducing activity), a protein purified from brain extracts on the basis of its ability to stimulate the synthesis of AChRs in cultured myotubes (*7*, *8*). ARIA is a member of the neuregulin family of ligands for the 185-kD transmembrane receptor tyrosine kinases ErbB2, ErbB3, and ErbB4 (also called Her2, Her3, and Her4), which are closely related to the epidermal growth factor (EGF) receptor (*9*, *10*). Neuregulins are potent activators of muscle AChR synthesis, with a median effective dose (ED_{50}) of 25 to 50 pM (*11*); neuregulin mRNA can be detected in embryonic motor neurons when motor axons first invade peripheral muscle masses (*12*, *13*) and is also abundant in adult motor neurons (*13*); neuregulin receptors are present in skeletal muscle cells and may be concentrated at the neuromuscular junction (*14–16*); neuregulin protein is concentrated in motor nerve terminals (*15–18*) and accumulates in the extracellular matrix of the synaptic cleft (*12*, *15*, *18*); and in mammalian muscle, neuregulin increases mRNA encoding ε (*17*, *19*), an AChR subunit that replaces the γ subunit during development. Neuregulins may therefore mediate the nerve-dependent maturation of junctional AChRs (*20*) as well as enhance overall AChR gene expression at developing and mature nerve-muscle synapses.

More direct evidence of the role of neuregulins at neuromuscular junctions would require the selective inhibition or elimination of neuregulin activity, or both. When mice are genetically altered so that exons encoding the EGF-like domain (*21*) or the immunoglobulin (Ig)-like domain (*22*) of neuregulin or the neuregulin receptors ErbB2 (*23*) and ErbB4 (*24*) have been deleted, homozygous animals die on or about embryonic day 10 (E10) with defects of the heart, cranial ganglia, and hindbrain. This is well before the formation of neuromuscular synapses begins on about E15 (*25*). Heterozygous animals appear normal and are fertile. This, of course, does not exclude a subtle defect at the neuromuscular junctions.

Although brain-purified ARIA contained Ig-domain amino acid sequences, Ig-containing isoforms may represent a small fraction (about 10 to 20%) of the total that are present in motor neurons (*13*, *26*). Other forms, in which a cysteine-rich region replaces the Ig-like domain (Fig. 1A), predominate. We studied mice deficient in Ig-containing neuregulins because these isoforms may be particularly important at the neuromuscular junction. The Ig-like domain binds heparin (*8*, *27*), an affinity that may be responsible for the observed association of neuregulin with the extracellular matrix of the synaptic cleft (*12*, *15*, *18*). Thus, by accumulating at the endplate, Ig-containing neuregulins may exert a major effect on endplate AChR synthesis. In fact, molecules associated with the synaptic basal lamina have been shown to promote the synthesis of AChRs in denervated adult muscle (*28*), and they appear to have a selective effect on the ε-containing (adult)

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