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Characterization of a Neuregulin-Related Gene, *Don-1*, That Is Highly Expressed in Restricted Regions of the Cerebellum and Hippocampus

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Members of the epidermal growth factor family of receptors have long been implicated in the pathogenesis of various tumors, and more recently, apparent roles in the developing heart and nervous system have been described. Numerous ligands that activate these receptors have been isolated. We report here on the cloning and initial characterization of a second ligand for the erbB family of receptors. This factor, which we have termed Don-1 (divergent of neuregulin 1), has structural similarity with the neuregulins. We have isolated four splice variants, two each from human and mouse, and have shown that they are capable of inducing tyrosine phosphorylation of erbB3, erbB4, and erbB2. In contrast to those of neuregulin, high levels of expression of *Don-1* are restricted to the cerebellum and dentate gyrus in the adult brain and to fetal tissues.

Neuregulin (also called neu differentiation factor [NDF], heregulin, glial growth factor, and acetylcholine receptor-inducing activity [ARIA]) is a member of the epidermal growth factor (EGF) family, whose splice variants commonly consist of as many as six recognizable domains: an N-terminal domain, an immunoglobulin-like domain, a glycosylation-rich spacer, an EGF-like domain, a hydrophobic transmembrane sequence, and a cytoplasmic tail (14, 26, 34). Numerous isoforms of neuregulin exist (10, 19, 34) that result from alternate splicing of a single gene located on human chromosome 8 at position 8p12-21 (24). Although the mosaic structure is conserved between the many isoforms of neuregulin, the EGF-like domain alone is sufficient for receptor binding and for the stimulation of a range of cellular responses in vitro (14).

Various functions have been ascribed to neuregulin, including modulation of the number of acetylcholine receptors at the neuromuscular junction (10, 12), promotion of proliferation and survival of Schwann cells and their progenitors (9, 19, 22), and induction of oligodendrocyte maturation (32). In addition, it has been shown that neuregulin may act outside the nervous system to induce the growth and differentiation of epithelial cells (8, 20, 25).

The biological activity of neuregulin is mediated by the EGF receptor family of tyrosine kinase receptors. This family is composed of the EGF receptor (EGFR), erbB2, erbB3, and erbB4. These receptors are expressed in a variety of tissues and are often overexpressed in human tumor cells. Ligand-dependent signalling occurs through receptor homo- or heterodimerization, followed by activation of cytoplasmic protein tyrosine kinase domains through autophosphorylation. Historically, a great deal of interest centered around neuregulin as a potential ligand for erbB2, but although neuregulin is capable of stimulating the tyrosine phosphorylation of erbB2, it does not bind this receptor directly. Instead, it has been shown that neuregulin binds erbB3 and erbB4 (5, 25, 27–29) and that the activation of erbB2 occurs indirectly through heterodimerization of the various receptors (15). It has been suggested that erbB2 may act solely to potentiate the activity of the EGF family of ligands by heterodimerization with other receptors and that there may not be an endogenous ligand for erbB2 (15). The possibility of the existence of additional, undiscovered ligands for the erbB receptors was raised on the basis of the results obtained from a series of neuregulin-, erbB2-, and erbB4-targeted deletions in mice (11, 16, 17, 21). All mutant embryos died in utero around day 10 or 11, displaying similar heart malformations and defects in the developing nervous system. While neural defects in mice lacking neuregulin and erbB2 seemed comparable, the phenotype of the developing hindbrain of erbB4-deficient mice was dramatically different, leading the authors to propose that a different, related ligand for erbB4 existed in the developing rhombomeres during embryogenesis (11).

We have identified a novel growth factor that shows a high degree of homology to neuregulin. The expression pattern of this gene, which we termed *Don-1* (divergent of neuregulin 1); appears to be more tissue restricted than that of the neuregulin gene. In both mouse and human, *Don-1* is found predominantly in the cerebellum. Our results show that Don-1 is capable of activating the same receptors as neuregulin, although differing activity in in vitro bioassays together with its different expression pattern indicates that Don-1 may play a role distinct from that of neuregulin in vivo.

MATERIALS AND METHODS

Library construction and screening. A cDNA library of mouse choroid plexus was constructed as described previously (30). Random EST sequences of this library were searched against the GenBank nucleotide database and a combined database of Swiss-Prot, PIR, and the National Center for Biotechnology Genpept protein database by using BLASTN and BLASTX algorithms (1), which resulted in the identification of a novel homolog of neuregulin.

To isolate full-length sequences from both mouse and human, various libraries from Stratagene (La Jolla, Calif.) and Clontech (Palo Alto, Calif.) were screened with a radiolabeled 1.4-kb cDNA probe that was originally isolated from the choroid plexus library. Hybridization was performed as recommended by the manufacturer.

Mouse genomic clones were isolated by screening an E129 library, constructed in Lambda FixII (Stratagene) with the radiolabelled 1.4-kb murine *Don-1s* (mu-*Don-1s*) cDNA, as recommended by the manufacturer.

Northern blotting and in situ analysis. Mouse and human multiple-tissue Northern blots purchased from Clontech were hybridized to radiolabelled 1.4-kb

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mu*Don-1s* or 2.5-kb human *Don-1r* (hu*Don-1r*) cDNAs as described by the manufacturer.

For in situ analysis, the brains of 4- to 6-week-old C57BL/6 mice were removed and frozen on dry ice. Ten-micrometer-thick coronal sections of the brain were postfixed with 4% formaldehyde in 1× phosphate-buffered saline at room temperature for 10 min before being rinsed twice in 1× phosphate-buffered saline and once in 1 M triethanolamine-HCl (pH 8). Following incubation in 0.25% acetic anhydride–1 M triethanolamine-HCl for 10 min, sections were rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Tissue was dehydrated through a series of ethanol washes, incubated in 100% chloroform for 5 min, and then rinsed in 95% ethanol for 1 min and 100% ethanol for 1 min. Hybridizations were performed with ^{35}S-radiolabelled (5×10^7 cpm/ml) cRNA probes encoding a 500-bp segment of the 5′ end of the mu*Don-1m* gene (generated with PCR primers F, 5′-CTTAGGATCTGGCATGTACA, and R, 5′-CTGAAGAAGATGAAGAGCCA). Probes were incubated in the presence of a solution containing 600 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 0.01% sheared herring sperm, 0.01% yeast tRNA, 0.05% total yeast RNA type X1, 1× Denhardt's solution, 50% formamide, 10% dextran sulfate, 100 mM dithiothreitol, 0.1% sodium dodecyl sulfate (SDS), and 0.1% sodium thiosulfate for 18 h at 55°C. After hybridization, slides were washed with 2× SSC. Sections were then sequentially incubated at 37° in a solution containing 10 mM Tris-HCl (pH 7.6), 500 mM NaCl, and 1 mM EDTA (TNE) for 10 min; in TNE with 10 μg of RNase A per ml for 30 min; and finally in TNE for 30 min. Slides were then washed with 0.2× SSC at 60°C for 1 h. Sections were dehydrated rapidly through serial ethanol–0.3 M sodium acetate concentrations before being air dried and exposed to Kodak Biomax MR scientific imaging film for 7 days at room temperature. Controls for the in situ hybridization experiments included the use of a sense probe, which showed no signal above background, and RNase-treated tissue, which showed a significantly reduced signal.

Genetic mapping. The *Don-1* gene was mapped to the proximal end of chromosome 18 in the mouse by utilizing the *Mus spretus*/C57BL/6J backcross panel, as described previously (30). Primers were designed from the intronic sequence of mu*Don-1* and were as follows: F1, 5′-AGAGGAAGGCCAAAGTAGTG, and R2, 5′-GTGGACCACAAGGTAAACAG. Amplification conditions were 35 cycles of 95°C for 1 min, 57°C for 1 min, and 72°C for 45 s. Amplification products were run on a 10% SSCP gel at 2 W at 4°C overnight. Two primer sets from intronic sequences of the human gene were designed to map *Don-1* by using the Stanford Human Genome Center's G3 radiation hybrid panel and the Whitehead Institute/MIT Center for Genome Research's GENEBRIDGE4 radiation hybrid panel (Research Genetics, Huntsville, Ala.). The primers were as follows: F1, 5′-CCCTCAGAAACACTCCAAAG; R1, 5′-AGTGTGAAAAGTGTCCTGGG; F2, 5′-ATGACAGACTGGGACTAGAG; and R2, 5′-GGGACATGGAACTTCTAGCA. Amplification conditions were 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Amplification products were run on a 2% agarose gel.

Expression of *Don-1*. The regions encoding the EGF domain and flanking amino acids of muDon-1m were amplified by PCR, subcloned into the mammalian expression vector, PN8E, and then transfected into 293EBNA cells with Lipofectamine as detailed by Gibco-BRL (Gaithersburg, Md.). A signal sequence (13) was fused to the sequence corresponding to the N terminus of the EGF domain by PCR (MALPVTALLLPLALLLHAARP [signal]-LHVNSV [Don-1 5′]), and a FLAG epitope tag was placed on the C terminus (YMPD PK[Don-1 3′]-DYKDDDDK[FLAG]) prior to subcloning into PN8E. The secreted EGF domain of *Don-1* was purified from the conditioned media of 293EBNA cells by binding to an anti-FLAG M2 affinity resin. After the resin was washed with Tris-buffered saline, the protein was eluted with FLAG peptide (75 μg/ml) in Tris-buffered saline. To quantitate the amount of affinity-purified material obtained, various dilutions of the EGF domain were electrophoresed on an SDS-polyacrylamide gel electrophoresis (PAGE) gel with known concentrations of chemically synthesized Don-1 EGF domain peptide (Anaspec, San Jose, Calif.). Following silver staining, densitometry was performed to determine the concentration of the purified protein.

A version of mu*Don-1m* cDNA that encoded a protein with a truncated cytoplasmic tail and lacked a signal sequence (amino acids [aa] 38 to 447 in Fig. 1) was subcloned into pMet7 with a 5′ FLAG epitope (MDYKDDDDK[FLAG]-KLKKMK[muDon-1m]) and was then transfected into COS-7 cells with Lipofectamine as detailed by Gibco-BRL. Expression of FLAG–Don-1 constructs in transfected cells and their conditioned media was determined by immunoblotting of SDS-PAGE-separated protein extracts with anti-FLAG monoclonal antibody M2 (Eastman Kodak, Rochester, N.Y.) at a dilution of 1/500. The secreted form of muDon-1m was purified from the conditioned media of COS-7 cells as detailed above for the Don-1 EGF domain.

Immunoblotting assays. MDA-MB453, SKBR-3, and SKOV-3 cells (American Type Culture Collection, Rockville, Md.) were grown to 80% confluence in Dulbecco minimal essential medium supplemented with 10% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37°C. The cells were then replated in serum-free medium for 16 h before being exposed to neuregulin-α (100 ng/ml), EGF (100 ng/ml), or affinity-purified Don-1 EGF-like domain (10 ng/ml) for 15 min at 37°C. Cell lysates were prepared in buffer (1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 20 mM Tris [pH 8.0], 1 mM EDTA, 30 mM $Na_4P_2O_7$, 50 mM NaF, 0.1 mM Na_3VO_4, 10 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride), and comparable amounts of protein were separated on an SDS–7.5% PAGE gel. Following transfer to nitrocellulose, immunodetection of phosphorylated proteins was performed with the monoclonal antiphosphotyrosine antibody 4G10 (Upstate Biotechnology, Lake Placid, N.Y.), as described by the manufacturer, and by enhanced chemiluminescence detection (Amersham, Cleveland, Ohio). Recombinant neuregulin-α (EGF-like domain) and EGF were purchased from R&D Systems (Minneapolis, Minn.). For immunoprecipitation assays, 1 mg of cell extract was incubated with antibodies to erbB3 (RTJ.2; Santa Cruz Biotechnology, Santa Cruz, Calif.), erbB4 (C-18; Santa Cruz Biotechnology), or erbB2 (C-18; Santa Cruz Biotechnology) at a concentration of 1 μg/ml for 2 h at 4°C. Samples were then bound to protein G agarose for 12 h at 4°C before being washed four times in lysis buffer. Immunoprecipitated samples were resuspended in sample buffer and boiled for 5 min and were then electrophoresed through SDS-PAGE gels. Gels were then transferred and blotted as detailed above.

Bioassays. The effect of Don-1 on the growth of SKBR-3 cells was ascertained by seeding cells (10^4 cells per well) into 96-well plates in the presence of purified Don-1 EGF domain or recombinant neuregulin-α for 48 h. Ten micromolar bromodeoxyuridine was included for the final 24 h and incorporation into double-stranded DNA was quantitated by enzyme-linked immunosorbent assay as outlined by the manufacturer (Boehringer Mannheim, Indianapolis, Ind.).

ARIA was measured as previously described (10). Briefly, 7-day-old primary cultures of E11 chick pectoral muscle grown in 2% chicken embryo extract-minimal essential medium were incubated with test factors for 24 h. "Old" surface acetylcholine receptors were blocked with excess cold alpha bungarotoxin, and the emergence of "new" surface acetylcholine receptors was measured by labelling with radioiodinated alpha bungarotoxin (Amersham) for 5 h, followed by measuring radioactivity of whole-cell lysates on a gamma counter.

RESULTS

Cloning of *Don-1*. Using computer-based searching of EST sequences from a murine choroid plexus library, we isolated a clone that showed striking homology to the neuregulin family of growth factors. Further sequencing of this 1.4-kb clone (mu*Don-1s*) confirmed the homology over part of an immunoglobulin domain and an EGF-like domain. The open reading frame (ORF) of this cDNA terminated 41 codons after the EGF-like domain but did not extend as far upstream as an initiating methionine. Northern blot analysis of the expression of this clone identified murine brain and lung as potential sources of full-length clones. A second murine clone (mu*Don-1m*), of 2.5 kb, was isolated from a murine lung library, containing a single long ORF of 605 codons. This clone differed from the original isolate in that it encoded a predicted membrane-bound version of Don-1 (Fig. 1). Subsequently, two human homologs (hu*Don-1r* and hu*Don-1b*) were isolated from fetal brain libraries by hybridization to the murine clones. The cDNA for huDon-1b did not contain a termination codon, and the protein differed from huDon-1r by an 8-amino-acid insertion proximal to the transmembrane domain. The sequence for hu*Don-1r* was determined from two overlapping cDNA clones. Figure 1 shows that, like neuregulin, Don-1 is a mosaic protein containing an N-terminal domain, an immunoglobulin-C2 loop, an EGF-like domain, a transmembrane sequence, and a large cytoplasmic tail. No recognizable signal sequence is apparent, and a potential protease cleavage site is present proximal to the transmembrane domain (14). The glycosylation-rich extended spacer domain found in most isoforms of neuregulin is not present in any of the Don-1 isoforms.

Alignment of Don-1 to neuregulin-β reveals that the highest degree of homology extends over the transmembrane domain (92%); significant homology is also evident over the immunoglobulin (40%), EGF-like (49%), N-terminal (36%), and cytoplasmic (24%) domains. Interestingly, a small region of the cytoplasmic tail (residues 431 to 458 in Fig. 1) exhibited high homology (89%) over a stretch of 28 aa, suggesting a region of functional significance.

Closer scrutiny of the EGF-like domain of Don-1 and comparison with the sequences of other EGF domain-containing growth factors (Fig. 2) shows that, although overall homology was greatest between Don-1 and members of the neuregulin

```
                                                                      50
    hrg-b  MSERKEGRGK GKGKKKERGS GKKPESAAGS QSPALPPRLK EMKSQESAAG
  huDon-1r MSESRRRGR. GRGKKHPEGR KREREPDP.. GEKATRPKLK KMKSQTGQVG
  huDon-1b .......... .......... .......... .......... .MKSQTGQVG
  muDon-1m .......... .......... .......... .......... .MKSQTGEVG

           51    ^             ^                                  100
    hrg-b  SKLVLRCETS SEYSSLRFKW FKNGNELNRK NKPQNIKIQK KPGKSELRIN
  huDon-1r EKQSLKCEAA AGNPQPSYRW FKDGKELNR. SRDIRIKYGN GRKNSRLQFN
  huDon-1b EKQSLKCEAA AGNPQPSYRW FKDGKELNR. SRDIRIKYGN GRKNSRLQFN
  muDon-1m EKQSLKCEAA AGNPQPSYRW FKDGKELNR. SRDIRIKYGN VRKNSRLQFN
  muDon-1s                                   RIKYGN GRKNSRLQFN

           101         ^                                         150
    hrg-b  KASLADSGEY MCKVISKLGN DSASANITIV ESNEIITGMP ASTEGAYVSS
  huDon-1r KVKVEDAGEY VCEAENILGK DTVRGRLYV. .......... ..........
  huDon-1b KVKVEDAGEY VCEAENILGK DTVRGRLYV. .......... ..........
  muDon-1m KVRVEDAGEY VCEAENILGK DTVRGRLHV. .......... ..........
  muDon-1s KVRVEDAGEY VCEAENILGK DTVRGRLHV. .......... ..........

           151                                  *       *      * 200
    hrg-b  ESPIRISVST EGANTSSSTS TSTTGTSHLV KCAEKEKTFC VNGGECFMVK
  huDon-1r .......... .....NSVST TLSSWSGHAR KCNETAKSYC VNGGVCYYIE
  huDon-1b .......... .....NSVST TLSSWSGHAR KCNETAKSYC VNGGVCYYIE
  muDon-1m .......... .....NSVST TLSSWSGHAR KCNETAKSYC VNGGVCYYIE
  muDon-1s .......... .....NSVST TLSSWSGHAR KCNETAKSYC VNGGVCYYIE

           201      *  *         *                                250
    hrg-b  DLSNPSRYLC KCPNEFTGDR CQNYVMASFY KHLGIE.... .FME.AEELY
  huDon-1r GINQLS...C KCPNGFFGQR CLEKLPLRLY MPDPKQK... .....AEELY
  huDon-1b GINQLS...C KCPNGFFGQR CLEKLPLRLY MPDPKQKHLG FELKEAEELY
  muDon-1m GINQLS...C KCPNGFFGQR CLEKLPLRLY MPDPKQ...  .....AEELY
  muDon-1s GINQLS...C KCPNGFFGQR CLEKLPLRLY MPDPK*SVLW *DTPGTGVSSS*

           251                                                  300
    hrg-b  QKRVLTITGI CIALLVVGIM CVVAYCKTKK QRKKLHDRLR QSLRSERNNM
  huDon-1r QKRVLTITGI CVALLVVGIV CVVAYCKTKK QRKQMHNHLR QNMCPAHQN.
  huDon-1b QKRVLTITGI CVALLVVGIV CVVAYCKTKK QRKQMHNHLR QNMCPAHQN.
  muDon-1m QKRVLTITGI CVALLVVGIV CVVAYCKTKK QRRQMHHHLR QNMCPAHQN.
  muDon-1s *QWSTSPSTLD LN**

           301                                                  350
    hrg-b  MNIANGPHHP NPPPENVQLV NQYVSKNVIS SEHIVEREAE TSFSTSHYTS
  huDon-1r RSLANGPSHP RLDPEEIQM. ADYISKNVPA TDHVIRRETE TTFSGSHSCS
  huDon-1b RSLANGPSHP RLDPEEIQM. ADYISKNVPA TDHVIRRETE TTFSGSHSCS
  muDon-1m RSLANGPSHP RLDPEEIQM. ADYISKNVPA TDHVIRREAE TTFSGSHSCS
                                     ^      ^          ^^ ^ ^   ^
           351                                                  400
    hrg-b  TAHHSTTVTQ TP.....SHS WSNGHTESIL SESHSVIVMS SVENSRHSSP
  huDon-1r PSHHCSTATP TSSHRHESHT WSLERSESLT SDSQSGIMLS SVGTSKCNSP
  huDon-1b PSHHCSTATP TSSHRHESHT WSLERSESLT SDSQSGIMLS SVGTSKCNSP
  muDon-1m PSHHCSTATP TSSHRHESHT WSLERSESLT SDSQSGIMLS SVGTSKCNSP
        ^^ ^  ^      ^ ^  ^   ^ ^   ^ ^ ^    ^ ^   ^   ^
           401                                                  450
    hrg-b  T..GGPRGRL NGTGGPRECN SFLRHARETP DSYRDSPHSE RYVSAMTTPA
  huDon-1r ACVEARARRA AAYNLEERRR ATAPPYHDSV DSLRDSPHSE RYVSALTTPA
  huDon-1b ACVEARARRA AAYNLEERRR ATAPPYHDSV DSLRDSPHSE RYVSALTTPA
  muDon-1m ACVEARARRA AAYSQEERRR AAMPPYHDSI DSLRDSPHSE RYVSALTTPA
                                       ^   ^   ^  ^     ^  ^^
           451                                                  500
    hrg-b  RMSPVDFHTP SSPKSPPSEM SPPVSSMTVS MPSMAVSPFM EEERPLLLVT
  huDon-1r RLSPVDFHYS LATQVPTFEI TSPNSAHAVS LPPAAPISYR LAEQQPLLRH
  huDon-1b RLSPVDFHYS LATQVPTFEI TSPNSAHAVS LPPAAPISYR #.........
  muDon-1m RLSPVDFHYS LATQVPTFEI TSPNSAHAVS LPPAAPISYR LAEQQPLLRH
           ^                     ^   ^    ^
           501                                                  550
    hrg-b  PPRLREKKFD HHPQQFSSFH HNPAHDSNSL PASPLRIVED EEYETTQEYE
  huDon-1r PAPPGPGPGP GPGPGPGADM QRSYDSYYYP AAGPGPRRGT CALGGSLGSL
  muDon-1m PAPPGPGPGS GPG....ADM QRSYDSYYYP AAGPGPRRSA CALGGSLGSL
                                                               ^
           551                                                  600
    hrg-b  PAQEPVKKLA NSRRAKRTKP NGHIANRLEV DSNTSSQSSN SESETEDERV
  huDon-1r PASPFRIPED DEYETTQECA PPPPPRPRAR GASRRTSAGP RRWRRSRLNG
  muDon-1m PASPFRIPED DEYETTQECA PPPPPRPRTR GASRRTSAGP RRWRRSRLNG

           601                                                  650
    hrg-b  GEDTPFLGIQ NPLAASLEAT PAFRLADSRT NPAGRFSTQE EIQARLSSVI
  huDon-1r LAAQRARAAR DSLSLSSGSG GGSASASDDD ADDADGALAA ESTPFLGLRG
  muDon-1m LAAQRARAAR DSLSLSSGSG CGSASASDDD ADDADGALAA ESTPFLGLRA

           651                                                  700
    hrg-b  ANQDPIAV*. .......... .......... .......... ..........
  huDon-1r AHDALRSDSP PLCPAADSRT YYSLDSHSTR ASSRHSRGPP PRAKQDSAPL*
  muDon-1m AHDALRSDSP PLCPAADSRT YYSLDSHSTR ASSRHSRGPP TRAKQDSGPL*
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FIG. 1. Sequence comparison of mouse and human Don-1 to human neuregulin-β. The predicted amino acid sequence of two forms of murine Don-1 (muDon-1s and muDon-1m) and two forms of human Don-1 (huDon-1r and huDon-1b) are compared with the amino acid sequence of human neuregulin-β. Conserved cysteines and tryptophan residues within the immunoglobulin-C2 domain are indicated by a caret, and the six cysteines that form the EGF domain are marked with an asterisk. The transmembrane domain is underlined. Sequence digression in the muDon-1s clone has been italicized. #, clone with no termination codon.

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     don-1.b                                          KCPVGYTGDRCQQFAM
       don-1  GHARKCNETAKSYCVNGGVCYYIEGINQLS...CKCPNGFFGQRCLEKLP
       hrg-a  SHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCQPGFTGARCTENVP
         ndf  SHLIKCAEKEKTFCVNGGECFTVKDLSNPSRYLCKCQPGFTGARCTENVP
       hrg-b  SHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCPNEFTGDRCQNYVM
        aria  SHLTKCDIKQKAFCVNGGECYMVKDLPNPPRYLCRCPNEFTGDRCQNYVM
mouse hb-egf  KLRDPCLRKYKDYCIH.GECRYLQEFRTPS...CKCLPGYHGHRCHGLTL
human hb-egf  GKRDPCLRKYKDFCIH.GECKYVKELRAPS...CICHPGYHGERCHGLSL
   mouse egf  NSYPGCPSSYDGYCLNGGVCMHIESLDSYT...CNCVIGYSGDRCQTRDL
   human egf  NSDSECPLSHDGYCLHDGVCMYIEALDKYA...CNCVVGYIGERCQYRDL
   consensus        C       C   G C             C C     G RC
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FIG. 2. Alignment of the EGF-like domain of Don-1 with other known EGF motifs. Protein sequences correspond to human neuregulin-α (accession no. A43273), rat NDF (accession no. A38220), human neuregulin-β1 (accession no. B43273), chicken ARIA (accession no. A45769), mouse hb-EGF (accession no. L07264), human hb-EGF (accession no. A38432), mouse EGF (accession no. P01132), and human EGF (accession no. P01133). The sequence Don-1.b is derived from a predicted exon in the genomic sequence of mu*Don-1* and in the 3′ untranslated region of mu*Don-1s*.

family (49%), the spacing between the third and fourth cysteines of Don-1 was similar to that of factors known to bind the EGFR, including heparin-binding EGF (hb-EGF), EGF, transforming growth factor α, and amphiregulin.

Evidence of signaling via erbB receptors. We transiently transfected cells with constructs encoding either the EGF-like domain with an artificial signal sequence (to produce a soluble EGF-like domain) or a version of muDon-1 that has a truncated cytoplasmic domain similar in size to that of rat NDF (34) and that lacks an apparent signal sequence. Both constructs were FLAG epitope tagged. Western blot analysis showed that cells transfected with the transmembrane domain-containing muDon-1m synthesized a tagged protein of ~50 kDa that was associated with the cell (Fig. 3A, lane 2). This species was larger than the predicted molecular mass of 42 kDa. In addition, a protein of ~28 kDa was detected in the conditioned medium from these cells, indicating that the transmembrane domain-containing precursor of Don-1 was being processed and released from the cell membrane (Fig. 3A, lane 4). The difference between the predicted molecular mass of 20

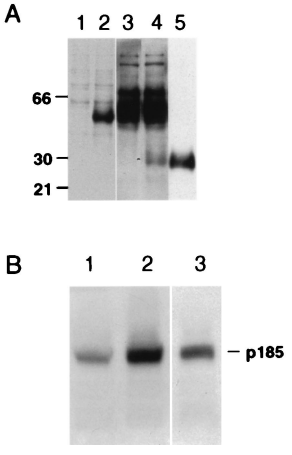

FIG. 3. Secretion of a biologically active form of Don-1. Transfection of COS-7 cells with the cDNA for muDon-1m results in the secretion of biologically active material. (A) The cell extract (lanes 1 and 2) and conditioned medium (lanes 3 and 4) of mock (lanes 1 and 3)- or muDon-1m (lanes 2 and 4)-transfected COS-7 cells were electrophoresed, transferred, and then blotted with anti-FLAG M2 antibody. A secreted form of Don-1 (~28 kDa) was released into the media (lane 4). This material was affinity purified (lane 5) and then used to stimulate MDA-MB453 cells. Numbers on the left are molecular masses, in kilodaltons. (B) Serum-starved MDA-MB453 cells were stimulated with buffer control (lane 1), neuregulin-α (100 ng/ml) (lane 2), or Don-1 (10 ng/ml) (lane 3) as described in Materials and Methods. Cell lysates were separated, transferred to nitrocellulose, and then blotted with antibodies to phosphotyrosine.

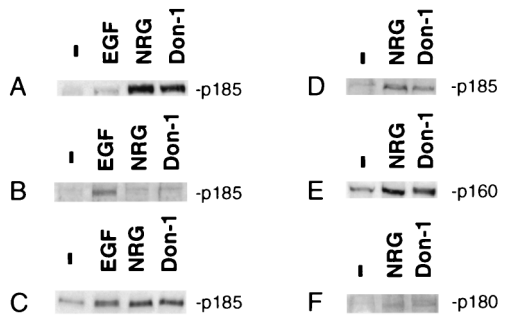

FIG. 4. Don-1-induced tyrosine phosphorylation. (A) Activity of the Don-1 EGF-like domain (Don-1) was ascertained by phosphorylation of a 185-kDa protein in the breast adenocarcinoma cell line MDA-MB453. Serum-starved cells were either untreated (−) or treated with EGF (100 ng/ml), neuregulin-α (100 ng/ml), and affinity-purified Don-1 EGF-like domain as described in Materials and Methods. Cell lysates were separated, transferred to nitrocellulose, and then blotted with antibodies to phosphotyrosine. (B and C) Serum-starved SKOV-3 cells (B) and SKBR-3 cells (C) were left unexposed (−) or were exposed to EGF (100 ng/ml), neuregulin-α (100 ng/ml), and affinity-purified Don-1 EGF-like domain (10 μg/ml). Cell lysates were analyzed as detailed above. Cell lysates from serum-starved MDA-MB453 cells that had been left unstimulated (−) or stimulated with neuregulin-α (100 ng/ml) or affinity-purified Don-1 EGF-like domain (10 μg/ml) were subjected to immunoprecipitation with antibodies against erbB2 (D) erbB3 (E), or erbB4 (F) before electrophoresis and blotting with antiphosphotyrosine. NRG, neuregulin.

kDa (FLAG and residues MKSQT. . . · LYQKR in Fig. 1) and the observed molecular mass of 28 kDa of the extracellular domain of muDon-1m is likely due to glycosylation. A number of potential N-linked and O-linked glycosylation sites exist in Don-1. The 28-kDa secreted form of muDon-1m was affinity purified and tested for its ability to induce phosphorylation in the breast tumor cell line MDA-MB453. These cells express erbB2, erbB3, and erbB4 (27, 31). Figure 3B shows that the secreted form of muDon-1m is capable of inducing tyrosine phosphorylation of a protein of ~185 kDa. Similar experiments with the purified EGF-like domain of Don-1 were also performed (Fig. 4A). These results show that, like that of neuregulin, the EGF-like domain of Don-1 alone is sufficient to exert a biological effect. In addition, Don-1 (10 ng/ml) was capable of eliciting similar levels of tyrosine phosphorylation at concentrations approximately 10-fold lower than neuregulin-α (100 ng/ml). Further assays were performed on two other cell lines, SKOV-3 and SKBR-3, and in contrast to the results obtained in the MDA-MB453 cells (Fig. 4A), no response was observed in the ovarian carcinoma cell line, SKOV-3, which overexpresses erbB2 but expresses little or no erbB3 or erbB4 (Fig. 4B). This finding is similar to results obtained with neuregulin (25). Stimulation of the SKBR-3 mammary carcinoma cell line (which expresses EGFR, erbB2, and erbB3) with Don-1 peptide resulted in increased phosphorylation of a protein of ~185 kDa (Fig. 4C). Immunoprecipitation of erbB2 (Fig. 4D), erbB3 (Fig. 4E), and erbB4 (Fig. 4F) from Don-1-stimulated MDA-MB453 cells, followed by antiphosphotyrosine blotting, revealed that all of these receptors are phosphorylated in response to Don-1.

Biological response of Don-1. After demonstrating that Don-1 can activate members of the erbB receptor family, we decided to see if Don-1 can stimulate a full biological response at the cellular level. We first determined whether Don-1 was capable of stimulating the proliferation of a breast tumor cell line that had previously been shown to proliferate in response to neuregulin (14). Purified recombinant Don-1 EGF-like domain was incubated at a range of doses with the SKBR-3 tumor cells for 48 h, and proliferation was assessed by measuring the amount of bromodeoxyuridine incorporated over the final 24 h. One nanomolar Don-1 EGF-like domain gave a significant increase in the rate of proliferation in the cells to a value 40% greater than controls (Fig. 5A). This effect was dose dependent, with a half-maximal stimulation of proliferation at approximately 6 pM. The EGF-like domain of an alpha neuregulin was found to be active in this assay as has been noted previously (14). However, half-maximal stimulation of proliferation was achieved only with doses in excess of 100 pM. As both alpha and beta isoforms of neuregulin have equivalent potency in this assay (14), we conclude that Don-1 is a more potent mitogen for this cell type than neuregulins.

Neuregulins also have ARIA, that is, they stimulate the rate

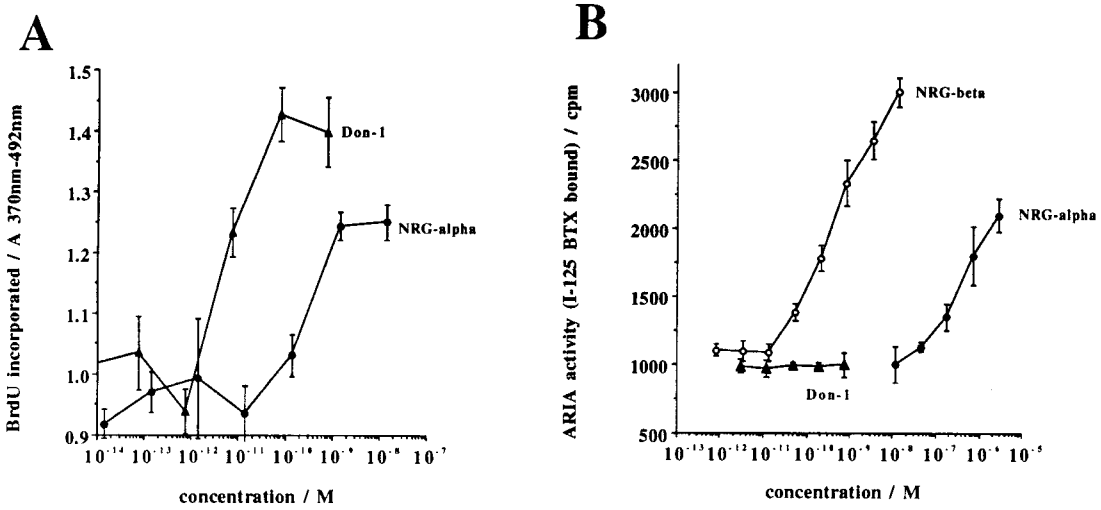

FIG. 5. Activity of Don-1 in vitro. (A) Stimulation of proliferation of SKBR-3 cells. Cells (10^4/well) were incubated with purified Don-1 EGF-like domain or neuregulin-α for 48 h, and bromodeoxyuridine incorporation into DNA was quantitated by enzyme-linked immunosorbent assay. Each point represents the mean ± standard deviation of triplicate wells. (B) Effect of Don-1 on ARIA in muscle cells. Primary chick muscle cells were incubated with purified Don-1 EGF-like domain, neuregulin-α, or neuregulin-β for 24 h before measurement of induction of acetylcholine receptor synthesis. Each point represents the mean ± standard deviation of quadruplicate wells. BrdU, bromodeoxyuridine; NRG, neuregulin.

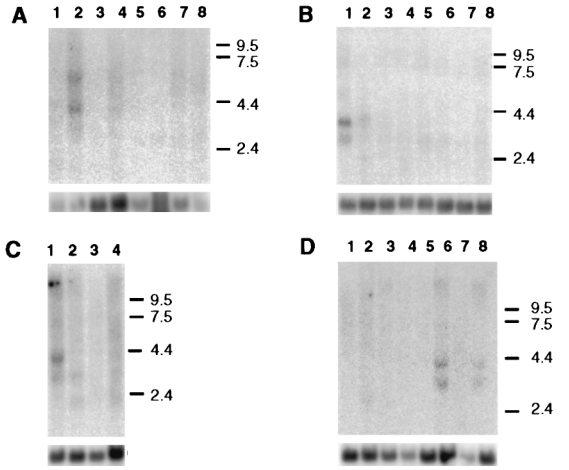

FIG. 6. Tissue distribution of mouse and human *Don-1*. Northern blots of poly(A)$^+$ RNA from various mouse and human tissues (Clontech) were probed with the mu*Don-1s* and hu*Don-1r* sequences, respectively. (A) Mouse multiple tissue: lanes 1 to 8, heart, brain, spleen, lung, liver, skeletal muscle, kidney, testis. (B) Human brain tissue: lanes 1 to 8, cerebellum, cerebral cortex, medulla, occipital lobe, frontal lobe, temporal lobe, putamen, spinal cord. (C) Human fetal tissue: lanes 1 to 4, brain, lung, liver, kidney. (D) Human cancer cell lines: lanes 1 to 8, promyelocytic leukemia HL-60, HeLa cells S3, chronic myeloid leukemia K562, lymphoblastic leukemia MOLT-4, Burkitt's lymphoma Raji, colorectal adenocarcinoma SW-480, lung carcinoma A549, and melanoma G361. Corresponding hybridizations to β-actin are shown for each blot. Numbers on the right of the gels are molecular sizes, in kilobases.

of nicotinic acetylcholine receptor synthesis in embryonic chick muscle (10). Thus, we compared the dose dependence of alpha and beta isoforms of neuregulin with that of the Don-1 EGF-like domain. Both neuregulin isoforms were active in the assay in a dose-dependent fashion, with the beta isoform being more potent by approximately 3 orders of magnitude (Fig. 5B). Don-1 displayed no ARIA at doses up to 1 nM, concentrations that are biologically active in the SKBR-3 proliferation assay.

Expression profile of Don-1. To determine the size and tissue distribution of both mouse and human *Don-1* mRNAs, Northern blot hybridizations were performed with the 1.4-kb mu*Don-1s* and the 2.5-kb hu*Don-1r* as probes. In adult murine tissues (Fig. 6A), expression was highest in the brain, with lower levels observed in the lung. Multiple transcripts of ~6, ~4, and ~3 kb were observed. These same transcripts were also evident when a smaller probe, covering just the EGF-like domain, was used.

In order to identify those cells that express *Don-1* mRNA, in situ analysis of murine adult brain sections with a probe directed against both the immunoglobulin and EGF-like domains was performed. High levels of *Don-1* expression were observed in the cerebellum and dentate gyrus of the hippocampus (Fig. 7). The uniform labelling of the cerebellar inner granule cell layer suggests that *Don-1* mRNA is expressed by the granule cells themselves, which are the major cell type densely packed into this layer. In contrast to neuregulin, however, *Don-1* is also expressed in the Purkinje cells (data not shown). Sense controls showed no signal over background, and RNase-treated sections showed a significant reduction in signal intensity. In contrast to the pattern of expression seen with neuregulin (7), no strong signal was observed in the ventral spinal cord (data not shown), suggesting that *Don-1* is not highly expressed in adult motor neurons. In addition, although the original *Don-1* clone was isolated from a choroid plexus library, no discernible signal could be detected in this region of the brain. In concordance with the expression patterns observed in the mice, *Don-1* expression in adult human tissues also appeared to be restricted to the cerebellum (Fig. 6B), where two predominant transcripts of ~4 and ~3 kb were observed. Additional regions of the brain (the thalamus, amygdala, caudate nucleus, corpus callosum, and substantia nigra) and other adult tissues (including the heart, lung, liver, skeletal muscle, kidney, and pancreas) showed no signal by Northern blotting (data not shown). Expression was, however, detected in human fetal brain and lung (Fig. 6C). As erbB receptors are often overexpressed in many carcinomas, we analyzed a panel

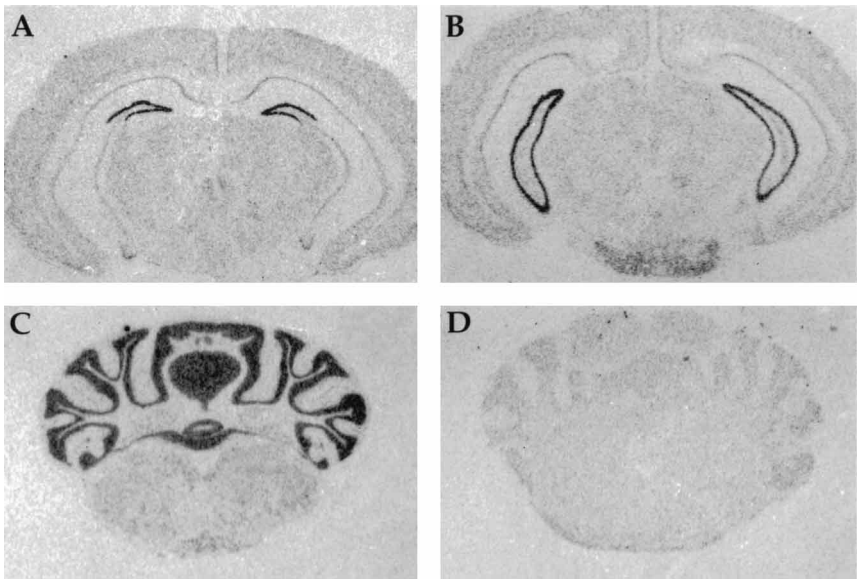

FIG. 7. In situ analysis of *Don-1* expression in mouse brain. In situ hybridization of a *Don-1* probe, covering the immunoglobulin and EGF-like domains of mu*Don-1m*. (A to C) Sections hybridized to antisense probes; (D) corresponding sense control. Control sections treated with RNase prior to hybridization also showed considerable attenuation of signal. Tissues that displayed strong signal were the dentate gyrus (A and B) and the cerebellum (C).

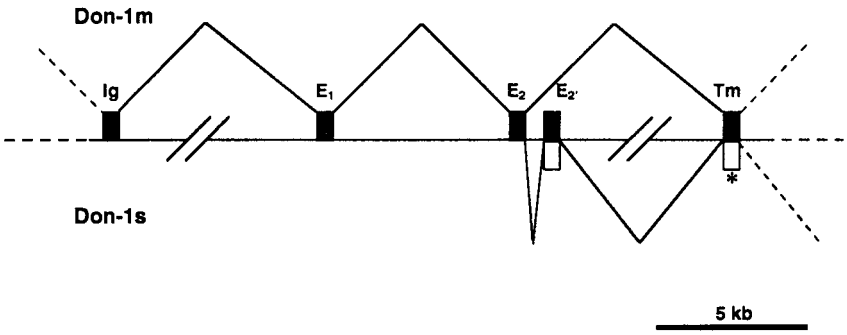

FIG. 8. Genomic organization of the region encoding the EGF-like domain in *Don-1*. A lambda FixII genomic DNA clone was isolated, and the region encoding the EGF-like domain was fully sequenced. The location of the EGF-like domain-encoding exons was determined by sequence comparison with the cDNA. The boundaries of the clone are shown with a double slash. E1 and E2 correspond to EGF-like domains, E2′ designates an exon encoding an alternate EGF-like domain, and Tm represents the exon encoding the transmembrane domain. Above the line are shown splicing events corresponding to the transmembrane form of *Don-1* (mu*Don-1m*). Below the line are shown alternate splicing events that lead to the formation of the truncated mu*Don-1*s. In-frame exons are depicted as black boxes, and out-of-frame exons are depicted as white boxes. The asterisk indicates the position of the termination codon in mu*Don-1s.*

of human tumor cell lines for the expression of *Don-1*. We detected *Don-1* transcripts in a colorectal carcinoma cell line (SW-480) and lower levels of *Don-1* in a melanoma cell line (G361) (Fig. 6D).

Genetic mapping and genomic structure of Don-1. Murine genomic clones were isolated by screening a lambda library with the 1.4-kb mu*Don-1s* probe. One of these clones contained two exons of the EGF-like domain and revealed the presence of an additional exon (E2′) that putatively encodes the last loop of an EGF domain (Fig. 8). This sequence, which we have termed Don-1.b (Fig. 2), was also observed out of frame in the truncated mu*Don-1s* clone, and we speculate that alternate splicing of this third EGF exon and that of the transmembrane domain, immediately after the second EGF exon (E2), introduce the termination codon in that clone. By analogy with neuregulin, it is possible that other splice variants exist that may utilize this exon (E2′) in frame to produce an alternate EGF domain whose tissue distribution or function may differ from that of Don-1. So far, we have not isolated any isoforms containing this putative exon in the ORF.

Utilizing the intronic sequence prior to the first EGF domain exon as a probe, we have determined that mu*Don-1* is located on the proximal end of chromosome 18, close to cdc25c, and between the markers D18Mit20 and D18Mit24. The human *Don-1* gene was localized to chromosome 5q by using radiation hybrid panels (data not shown).

DISCUSSION

The initial identification of a neuregulin homolog in a murine choroid plexus EST database has led to the discovery of a novel neuregulin-like gene. This gene, which we have named *Don-1*, has the mosaic structure of the neuregulin gene and encodes multiple splice variants. We have identified four alternate forms of Don-1: two murine and two human. Each of these forms contains the immunoglobulin-like and EGF-like domains. The two human clones differ by the insertion of 8 amino acids after the EGF-like domain in the huDon-1b clone. In addition, the N-terminal sequence of huDon-1r is longer. Analysis of the cDNA sequence upstream of the first ATG of hu*Don-1b* revealed no further homology to hu*Don-1r* and, furthermore, an in-frame stop codon was present upstream of the first ATG, confirming our assignment of that ATG as the encoder of the initiating methionine. We feel confident that hu*Don-1r* is also full length, given the homology of the 5′ end of this clone to the start of the neuregulin gene. It appears as though these clones represent isoforms with distinct N termini. As with most isoforms of neuregulin, no identifiable signal sequence was apparent. The mu*Don-1m* cDNA is most closely related to the hu*Don-1r* sequence, although the N terminus of muDon-1m is identical to that of huDon-1b (Fig. 1). muDon-1s appears to represent a totally distinct isoform. Although not full length, it is nonetheless intriguing, as it introduces a termination codon prior to the transmembrane domain. Whether this clone represents a splicing error or a distinct non-membrane-bound isoform of Don-1 remains to be determined. We have yet to isolate a corresponding variant from human tissue. Variants of neuregulin that contain no transmembrane domain also exist (14, 19, 23), although their biological role has yet to be ascertained. Other obvious similarities between neuregulin and Don-1 include an immunoglobulin-C2 loop and a characteristic EGF-like domain. Interestingly, although the immunoglobulin and EGF-like domains are the primary candidate regions for protein interactions, homology between Don-1 and neuregulin is highest over the transmembrane sequence (92% identical) and in small sections of the cytoplasmic domain (up to 89% identity). This may indicate an important role for the transmembrane domain and for certain regions of the cytoplasmic tail that may be involved in specific molecular interactions. The EGF-like domain of Don-1 has ~49% identity with that of neuregulin, although the separation of cysteines 3 and 4 is more characteristic of other members of the EGF family, such as EGF and transforming growth factor α. It has been shown that the replacement of the amino acids between the third and fourth cysteines of neuregulin with the smaller corresponding region of EGF does not appreciably alter the binding affinity of neuregulin (3). This data, combined with our observed erbB receptor activation profile of Don-1, suggest that the difference in size of the EGF-like domains of Don-1 and neuregulin does not alter receptor specificity. Assignment of hu*Don-1* to chromosome 5q clearly demonstrates that *Don-1* is a novel gene and not a further splicing variant of the neuregulin gene, which is located on chromosome 8p (24).

Analysis of Don-1 mRNA expression in both human and mouse tissues indicated the presence of multiple transcripts. The sizes of the predominant messages were ~3, ~4, and ~6 kb. As yet, no cDNA clones corresponding to these sizes have been isolated. The transcripts identified on the blot may differ from the isolated clones (2.5, 1.4, 1.8, and 1.6 kb) because of the presence of large untranslated regions, or they may repre-

sent larger splice forms that have yet to be isolated. mRNA encoding Don-1 was detected in both adult and fetal brain and lung. Don-1 was also expressed in a cell line derived from an adenocarcinoma. This cell line (SW-480) expresses the erbB2 and erbB4 receptors (data not shown). Numerous studies have implicated the erbB family of receptors in the biology of adenocarcinomas, particularly those of the breast (2). The presence of both receptor and ligand in these cells may point to an autocrine activation of the receptors, leading to pathogenesis.

The restricted tissue distribution of Don-1 differs substantially from that of neuregulin, whose transcripts can be detected in a broad range of adult tissues, including the heart, lung, liver, muscle, ovary, and brain (14, 34). Even within the brain, neuregulin is expressed in a multitude of regions, including the cerebellum, spinal cord, brain stem, hypothalamus, hippocampus, and basal forebrain (6, 7). In contrast to neuregulin, Don-1 expression is restricted to the cerebellum and dentate gyrus of the hippocampus. The patterns of neuregulin expression in both of these brain regions are similar in embryonic and postnatal brains, but expression decreases significantly upon maturation. In the adult, neuregulin expression changes to a punctate pattern in the inner granule cell layer, characteristic of Golgi type II cells (6). In the dentate gyrus, the neuregulin in situ labelling that is observed in the embryo is no longer detectable in the adult. These developmental changes in the expression of neuregulin in the hippocampus and cerebellum are quite striking. In adult mammals, the dentate gyrus is the predominant site of neurogenesis in the brain. The possible role of Don-1 in regulating proliferation or differentiation events in the dentate gyrus cells is currently under investigation.

Another intriguing possibility is that Don-1 may be involved in neural development throughout embryogenesis. Comparison of erbB4-deficient mice, which exhibit hindbrain misinnervation, with neuregulin-deficient mice, which do not, suggests that erbB4 may bind another structurally related ligand. Don-1 may act as an erbB4 ligand specifically at this time and place in development (11), a role that awaits confirmation by analysis of Don-1 knockout mice.

Recombinant Don-1 was able to induce the tyrosine phosphorylation of a protein corresponding to the molecular weight of the EGFR family members. Further characterization of receptor activation with different carcinoma cell lines that express various combinations of receptors (25, 31) leads us to believe that Don-1 binds to erbB3 and erbB4 and not to erbB2 or to the EGFR, although the possibility of a further, as-yet-unidentified receptor for Don-1 cannot be excluded. The observed phosphorylation of erbB3, erbB4, and erbB2 in MDA-MB453 cells following stimulation with Don-1 suggests that, like neuregulin, Don-1 is capable of inducing receptor heterodimerization (15, 28, 29, 31, 33). Interestingly, although Don-1 appears to activate the same receptors as neuregulin, different biological effects were observed in vitro. The EGF-like domain of Don-1 was proficient at inducing the breast carcinoma cell line SKBR-3 to proliferate at concentrations approximately 10- to 20-fold lower than neuregulins (14), indicating that Don-1 is a more potent mitogen for these cells. In contrast, however, at equivalent concentrations of Don-1, no ARIA was detected in primary muscle cells, whereas both beta and alpha isoforms of neuregulin gave robust inductions of ARIA. As muscle cells express EGFR, erB2, erbB3, and erbB4, the differential effect on breast tumor cells compared with that on muscle suggests that a further receptor signalling component that is present in SKBR-3 cells but absent from cultured muscle cells may be required for bioactivity. On the basis of the differences in activity observed between Don-1 and the neuregulins, coupled with the restricted expression of Don-1 in adult tissues, we predict that the biological roles of Don-1 and neuregulin in vivo are very different.

One strategy for the determination of the biological roles of Don-1 is its deletion by homologous recombination in mice. Additionally, it is possible that Don-1 is involved in a previously described mutation. We have mapped mu*Don-1* to the proximal end of chromosome 18. Within this region are a number of mouse mutations whose phenotypes are indicative of neurodegenerative disorders. Two candidates of particular interest that we are investigating more fully are the ataxia and bouncy mutations. Ataxic mice show a steadily increasing paralysis up to three months of age that is characterized by tremors and loss of coordination (18). Furthermore, in the central nervous system of these mice, the hippocampus and dentate gyrus are underdeveloped (4). Bouncy mice are characterized by a severe tremor and a bouncy gait. Both of these phenotypes are consistent with cerebellar dysfunction, either during development or in the adult. Further investigation of the role of Don-1 in the cerebellum and throughout embryogenesis in normal and mutant mice will yield important insights into the function of Don-1.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Bacus, S. S., C. R. Zelnick, G. Plowman, and Y. Yarden.** 1994. Expression of the erbB-2 family of growth factor receptors and their ligands in breast cancers. Implication for tumor biology and clinical behavior. Am. J. Clin. Pathol. **102:**S13–S24.
3. **Barbacci, E. G., B. C. Guarino, J. G. Stroh, D. H. Singleton, K. J. Rosnack, J. D. Moyer, and G. C. Andrews.** 1995. The structural basis for the specificity of epidermal growth factor and heregulin binding. J. Biol. Chem. **270:**9585–9589.
4. **Burt, A. M.** 1980. Morphologic abnormalities in the postnatal differentiation of CA1 pyramidal cells and granule cells in the hippocampal formation of the ataxic mouse. Anat. Rec. **196:**61–69.
5. **Carraway, K. L., III, and L. C. Cantley.** 1994. A Neu acquaintance for ErbB3 and ErbB4: a role for receptor heterodimerization in growth signalling. Cell **78:**5–8.
6. **Chen, M. S., O. Bermingham-McDonogh, F. T. Danehy, C. Nolan, S. S. Scherer, J. Lucas, D. Gwynne, and M. A. Marchionni.** 1994. Expression of multiple neuregulin transcripts in postnatal rat brains. J. Comp. Neurol. **349:**389–400.
7. **Corfas, G., K. Rosen, H. Aratake, R. Krauss, and G. Fischbach.** 1995. Differential expression of ARIA isoforms in the rat brain. Neuron **14:**103–115.
8. **Danilenko, D. M., B. D. Ring, J. Z. Lu, J. E. Tarpley, D. Chang, N. Liu, D. Wen, and G. F. Pierce.** 1995. Neu differentiation factor upregulates epidermal migration and integrin expression in excisional wounds. J. Clin. Invest. **95:**842–851.
9. **Dong, Z., A. Brennan, N. Liu, Y. Yarden, G. Lefkowitz, R. Mirsky, and K. R. Jessen.** 1995. Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat schwann cell precursors. Neuron **15:**585–596.
10. **Falls, D. L., K. M. Rosen, G. Corfas, W. S. Lane, and G. D. Fischbach.** 1993. ARIA, a protein that stimulates acetycholine receptor synthesis, is a member of the neu ligand family. Cell **72:**801–815.
11. **Gassmann, M., F. Casagrand, D. Orioli, H. Simon, C. Lai, R. Klein, and G. Lemke.** 1995. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature **378:**390–394.
12. **Goodearl, A. D. J., A. G. Yee, A. W. Sandrock, G. Corfas, and G. D. Fischbach.** 1995. Aria is concentrated in the synaptic basal lamina of the developing chick neuromuscular junction. J. Cell Biol. **130:**1423–1434.
13. **Ho, A. S. Y., Y. Liu, T. A. Khan, D.-H. Hsu, J. F. Bazan, and K. W. Moore.** 1993. A receptor for interleukin 10 is related to interferon receptors. Proc. Natl. Acad. Sci. USA **90:**11267–11271.

14. **Holmes, W., M. Sliwkowski, R. Akita, W. Henzel, J. Lee, J. Park, D. Yansura, N. Abadi, H. Raab, G. Lewis, M. Shepard, W.-J. Kuang, W. Wood, D. Goeddel, and R. Vandlen.** 1992. Identification of heregulin, a specific activator of $p185^{erbB2}$. Science **256:**1205–1210.
15. **Karunagaran, D., E. Tzahar, R. Beerli, C. Xiaomei, D. Graus-Porta, B. Ratzkin, R. Seger, N. Hynes, and Y. Yarden.** 1996. Erb-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. EMBO J. **15:**254–264.
16. **Kramer, R., N. Bucay, D. J. Kane, L. E. Martin, J. E. Tarpley, and L. E. Theill.** 1996. Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. Proc. Natl. Acad. Sci. USA **93:**4833–4838.
17. **Lee, K.-F., H. Simon, H. Chen, B. Bates, M.-C. Hung, and C. Hauser.** 1995. Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature **378:**394.
18. **Lyon, M. F.** 1955. Ataxia—a new recessive mutant of the house mouse. J. Hered. **46:**77–80.
19. **Marchionni, M. A., A. D. J. Goodearl, M. S. Chen, O. Bermingham-McDonogh, C. Kirk, M. Hendricks, F. Danehy, D. Misumi, J. Sudhalter, K. Kobayashi, D. Wroblewski, C. Lynch, M. Baldassare, I. Hiles, J. B. Davis, J. J. Hsuan, N. F. Totty, M. Otsu, R. N. McBurney, M. D. Waterfield, P. Stroobant, and D. Gwynne.** 1993. Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. Nature **362:**312–318.
20. **Marikovsky, M., S. Lavi, R. Pinkas-Kramarski, D. Karunagaran, N. Liu, D. Wen, and Y. Yarden.** 1995. ErbB-3 mediates differential mitogenic effects of NDF/heregulin isoforms on mouse keratinocytes. Oncogene **10:**1403–1411.
21. **Meyer, D., and C. Birchmeier.** 1995. Multiple essential functions of neuregulin in development. Nature **378:**386–390.
22. **Morrissey, T. K., A. D. O. Levi, A. Nuijens, M. X. Sliwkowski, and R. P. Bunge.** 1995. Axon-induced mitogenesis of human schwann cells involves heregulin and p185 erbB2. Proc. Natl. Acad. Sci. USA **92:**1431–1435.
23. **Mudge, A. W.** 1993. New ligands for neu? Curr. Biol. **3:**361–364.
24. **Orr-Urtreger, A., L. Trakhtenbrot, R. Ben-Levy, D. Wen, G. Rechavi, P. Lonai, and Y. Yarden.** 1993. Neural expression and chromosomal mapping of Neu differentiation factor to 8p12-p21. Proc. Natl. Acad. Sci. USA **90:**1867–1871.
25. **Peles, E., S. Bacus, R. Koski, H. Lu, D. Wen, S. Ogden, R. Levy, and Y. Yarden.** 1992. Isolation of the Neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. Cell **69:**205–216.
26. **Peles, E., and Y. Yarden.** 1993. Neu and its ligands: from an oncogene to neural factors. Bioessays **15:**815–824.
27. **Plowman, G. D., J. M. Culouscou, G. S. Whitney, J. M. Green, G. W. Carlton, L. Foy, M. G. Neubauer, and M. Shoyab.** 1993. Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. Proc. Natl. Acad. Sci. USA **90:**1746–1750.
28. **Plowman, G. D., J. M. Green, J.-M. Culouscou, G. W. Carlton, V. M. Rothwell, and S. Buckley.** 1993. Heregulin induces tyrosine phosphorylation of HER4/p180erbB4. Nature **366:**473–475.
29. **Sliwkowski, M. X., G. Schaefer, R. W. Akita, J. A. Lofgren, V. D. Fitzpatrick, A. Nuijens, B. M. Fendly, R. A. Cerione, R. L. Vandlen, and K. L. Carraway III.** 1994. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. J. Biol. Chem. **269:**14661–14655.
30. **Tartaglia, L. A., M. Dembski, X. Weng, N. Dend, J. Culpepper, R. Devos, G. J. Richards, L. A. Campfield, F. T. Clark, J. Deeds, C. Muir, S. Sanker, A. Moriarty, K. J. Moore, J. Smutko, G. G. Mays, E. A. Woolf, C. A. Monroe, and R. I. Tepper.** 1995. Identification and expression cloning of a leptin receptor, OB-R. Cell **83:**1263–1271.
31. **Tzahar, E., G. Levkowitz, D. Karunagaran, L. Yi, E. Peles, S. Lavi, D. Chang, N. Liu, A. Yayon, D. Wen, and Y. Yarden.** 1994. ErbB-3 and erbB-4 function as the respective low and high affinity receptors of all neu differentiation factor/heregulin isoforms. J. Biol. Chem. **269:**25226–25233.
32. **Vartanian, T., G. Corfas, Y. Li, G. D. Fischbach, and K. Stefansson.** 1994. A role for the acetylcholine receptor-inducing protein ARIA in oligodendrocyte development. Proc. Natl. Acad. Sci. USA **91:**11626–11630.
33. **Wallasch, C., F. U. Weib, G. Niederfellner, B. Jallal, W. Issing, and A. Ullrich.** 1995. Heregulin-dependent regulation of HER2/neu oncogenic signalling by heterodimerization with HER3. EMBO J. **14:**4267–4275.
34. **Wen, D., E. Peles, R. Cupples, S. Suggs, S. Bacus, Y. Luo, G. Trail, S. Hu, S. Silbiger, R. Levy, R. Koski, H. Lu, and Y. Yarden.** 1992. Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. Cell **69:**559–572.