Transmembrane Neuregulins Interact with LIM Kinase 1, a Cytoplasmic Protein Kinase Implicated in Development of Visuospatial Cognition*

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The neuregulins are receptor tyrosine kinase ligands that play a critical role in the development of the heart, nervous system, and breast. Unlike many extracellular signaling molecules, such as the neurotransphins, most neuregulins are synthesized as transmembrane proteins. To determine the functions of the highly conserved neuregulin cytoplasmic tail, a yeast two-hybrid screen was performed to identify proteins that interact with the 157-amino acid sequence common to the cytoplasmic tails of all transmembrane neuregulin isoforms.

This screen revealed that the neuregulin cytoplasmic tail interacts with the LIM domain region of the non-receptor protein kinase LIM kinase 1 (LIMK1). Interaction between the neuregulin cytoplasmic tail and full-length LIMK1 was demonstrated by in vitro binding and co-immunoprecipitation assays. Transmembrane neuregulins with each of the three known neuregulin cytoplasmic tail isoforms interacted with LIMK1. In contrast, the cytoplasmic tail of TGF-α did not interact with LIMK1. In vivo, neuregulin and LIMK1 are co-localized at the neuromuscular synapse, suggesting that LIMK1, like neuregulin, may play a role in synapse formation and maintenance. To our knowledge, LIMK1 is the first identified protein shown to interact with the cytoplasmic tail of a receptor tyrosine kinase ligand.

The neuregulins (NRGs) were originally identified in searches for ligands of the receptor tyrosine kinase erbB2 (1-5) and as neuronally produced factors that stimulate the synthesis of muscle acetylcholine receptors (acetylcholine receptor protein kinase LIMK1 (29-32) interacts with the NRG cytoplasmic tail physically associate in vitro and in vivo studies now implicate the NRGs in the regulation of a large number of biological processes (7-10). Known functions of NRGs include regulation of synapse formation and maintenance, cell proliferation, apoptosis, differentiation decisions, and neuronal migration. Transgenic knockout mice lacking NRG have nervous system developmental defects and die at mid-embryogenesis (embryonic days 10-11) due to abnormalities in heart development (11, 12).

At least 15 NRG protein isoforms are produced from a single NRG gene (6, 13-16). Most of these NRG isoforms are synthesized as transmembrane proteins (see Fig. 1). Full-length transmembrane (TM)-NRG is found on the cell surface of TM-NRG-expressing cells (17), and TM-NRG can be proteolytically processed to release the NRG extracellular domain into the medium (17, 18). Thus, TM-NRG may act both as a juxtacrine (direct cell-cell contact) signaling protein (19-21) and as the precursor for a diffusible, paracrine signaling molecule.

The extracellular epidermal growth factor-like domain of TM-NRGs activates the receptor tyrosine kinases erbB2, erbB3, and erbB4. Most prior NRG studies have focused on the interaction of the NRG extracellular domain with these receptor tyrosine kinases (RTKs) and the biological consequences of erbB2/erbB3/erbB4 activation by NRG. In contrast, this study focused on the long intracellular region of TM-NRG isoforms (see Fig. 1). The high degree of amino acid sequence conservation of this intracellular region (4) suggests that it has important biological functions. Grimm and Leder (22) recently reported that one form of the NRG cytoplasmic tail (the b-tail) can activate apoptosis in TM-NRG-transfected HEK 293 cells. Two other potential biological functions of the NRG cytoplasmic tail are regulation of NRG protein trafficking and of proteolytic release of the NRG ectodomain into the extracellular space (see Refs. 13, 17, 23, and 24). Another intriguing possibility is that transmembrane NRG may function not only as a receptor ligand but also as a receptor and that the NRG cytoplasmic tail mediates outside-in signal transduction. If NRG acts as a “receptor” and the RTKs erbB2, erbB3, and erbB4 are its “ligand,” bi-directional signaling could occur between cells expressing TM-NRG and cells expressing the RTKs erbB2, erbB3, and erbB4. The idea of bi-directional signaling between cells expressing an RTK TM ligand and cells expressing the cognate RTK was first suggested by Pfeffer and Ullrich (25), and recent in vivo and in vitro studies of the interaction between the TM ligand LERK-2 and the RTK Nuk (26-28) have strongly supported this hypothesis. Thus, several potential biological roles for the cytoplasmic tails of NRG and other RTK TM ligands are supported by experimental evidence; however, no proteins interacting with these cytoplasmic tails have been molecularly identified.

As an approach to assessing these potential functions of the NRG cytoplasmic tail and to determine the mechanism by which these functions are carried out, we have used the yeast two-hybrid system to isolate brain proteins that interact with the cytoplasmic tail of NRG. We report evidence that the non-receptor kinase LIMK1 (29-32) interacts with the NRG cytoplasmic tail. We show that LIMK1 and the NRG cytoplasmic tail physically associate in vitro and in cultured cells. In vivo,
NRG and LIMK1 have overlapping expression patterns in the mammalian nervous system, and we show that these proteins are co-localized at the neuromuscular synapse. Although the cellular functions of LIMK1 remain unknown, LIMK1 hemizygosity has been implicated in the pathogenesis of the visuospatial constructive cognitive defect of Williams syndrome (33–35). Our findings suggest the possibility that the interaction of LIMK1 with NRG may play a role in the formation of neuromuscular synapses and of neuronal circuitry that mediates specific cognitive functions.

**EXPERIMENTAL PROCEDURES**

**Nomenclature Note**—Throughout this paper, neuregulin and the abbreviation NRG refer only to the proteins encoded by the first discovered NRG gene (1–6). These proteins might now be considered forms of NRG1 in light of the recent discovery of related proteins encoded by two other NRG family genes. These NRG1-related proteins have been dubbed NRG2 (or Don-1) (36–38) and NRG3 (39). All of the protein isoforms that are the subject of this study are produced from transcripts of the NRG1 gene.

**Reagents**—The cDNA clones encoding rat NRG isoforms are described in Ref. 13. The cDNA clone that encodes the full-length murine LIMK1 was a gift from E. Robertson (Department of Molecular and Cellular Biology, Harvard University) (31). The yeast two-hybrid bait vector pBTM116 and prey vector pVP16 were provided by S. Hollenberg (Fred Hutchinson Cancer Research Center, Seattle, WA) (40). The mouse brain library in pVP16, the PER bait, and the PER prey were provided by C. Weitz and N. Gekakis (Harvard Medical School). Polyclonal antibody 1310, raised against the common region of the NRG cytoplasmic tail, and the immunizing peptide were a gift from T. Burgess (Amgen, Inc.) (immunizing peptide, CNSFLRHARETPDSDYRDS) (17). Antibody sc-537, also recognizing the common region of the NRG cytoplasmic tail (immunizing peptide, FLRHARETPDSDYRSYPHER) and anti-Myc mouse monoclonal antibody 9e10 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody sc-537 was used for the immunohistochemical experiments because at the time these experiments were conducted, little Ab 1310 remained. Antibodies sc-537 and 1310 have given similar results in our experiments. Mouse monoclonal antibody 7D5, directed against the NRG ectodomain, was purchased from NeoMarkers (Fremont, CA). Anti-SV2 hybridoma supernatant was a gift of Dr. Kathy Buckley (Harvard Medical School) (41). The anti-FLAG mouse monoclonal antibody M2 was purchased from NeoMarkers (Fremont, CA) and a mouse monoclonal antibody for Western blot and immunofluorescence experiments were purchased from International Biotechnologies, Inc. (New Haven, CT). All secondaries for Western blot and immunofluorescence experiments were purchased from Amersham Pharmacia Biotech.

**Transfection of COS-7 Cells**—Culture conditions for COS-7 cells were as follows: 37 °C; 8% CO2; medium consisting of Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 2 mm-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Plasmid DNA for transfections was purified using Qiagen Plasmid Maxi kits. COS-7 cells were transfected using DEAE-dextran. Briefly, 5 × 105 cells were plated in each 100-mm dish 24 h prior to transfection. The DNA transfection solution was prepared by adding 10 µg of plasmid DNA, 30 µl of 50 mg/ml DEAE-dextran (Sigma), and 6 µl of 100 mM chloroquine (Sigma) to 6 ml of Dulbecco’s modified Eagle’s medium containing 10% Nu-Serum (Collaborative Biomedical Products). Cells were rinsed with PBS (Life Technologies, Inc.) or medium without serum and then incubated with DNA transfection solution (6 µl/dish) for 4 h in the incubator. Cells were then shocked with 10% MeSO4 in serum-free Dulbecco’s modified Eagle’s medium (6 µl/dish) for 3 min. The MeSO4-containing medium was replaced with normal growth medium, and the dishes were returned to the incubator.

**Plasmid Constructs**—Details of plasmid construct structure are provided in the legend to Fig. 2. To make yeast bait constructs, the target sequences (NRG and TGF-α cytoplasmic tails) were amplified by polymerase chain reaction (PCR). The PCR products were gel purified and subcloned into pBTM116 using the EcoRI and BamHI sites. As illustrated in Fig. 2, the bait proteins were fusions of the LexA DNA binding domain (N-terminally fused) and the bait sequence (C-terminally fused).

Myc/pcDNA3.1, a mammalian expression vector designed to express recombinant proteins with an N-terminal Myc tag, was prepared by ligating a Myc-tag encoding cassette into pcDNA3.1(+). Invitrogen, San Diego, CA) using the BsrXI and NotI sites. The cassette was made by annealing the two oligos, 5′-CTGGTATG GGG GAG CAA CAG AAA TTC ATC TCT GAG GAG GAT CTG GC-3′ and 5′-GCCCAGC GAG ATC TTC TTC AAG GAT TTT CTT TCC CCC CAT GATCCAGCACA-3′. The same strategy was used to generate flag/pcDNA3.1, a mammalian expression vector designed to produce N-terminally FLAG-tagged recombinant proteins. The FLAG-tagged cassette was prepared by annealing the oligos 5′-GAATCCATG GAC TAC AAG GAC GAC GAT GAC GAG G-3′ and 5′-AATTCT GTC ATC GTC TTC GTT GCA TGT AAG GAT GAT-3′. This cassette was ligated between the EcoRI and BamHI sites of pcDNA3.1(+) (+).

To create the Myc-tagged full-length LIMK1 construct, a NotI site was introduced into the LIMK1 cDNA immediately downstream of the initiating ATG using a multi-step strategy (details available upon request). This modified LIMK1 cDNA was then ligated into Myc/pcDNA3.1. As illustrated in Fig. 2, the resulting construct encodes a fusion protein (flag-NRGe-tail, flag-NRGb-tail, and flag-NRGa-tail) with a FLAG epitope tag appended onto the N-terminal end of full-length LIMK1.

To create FLAG-tagged NRG cytoplasmic tails, insert sequences from the appropriate yeast two-hybrid bait plasmid (NRGa-bait, NRGb bait, or NRGc-bait) were ligated into the flag/pcDNA3.1 vector. As illustrated in Fig. 2, the resulting construct encodes a fusion protein (flag-NRGe-tail, flag-NRGb-tail, and flag-NRGa-tail) with a FLAG epitope tag appended onto the N-terminal end of each cytoplasmic tail form. These FLAG-tagged NRG cytoplasmic tail proteins do not include the constitutively or transiently expressed domain of NRG and are therefore expected to be soluble cytoplasmic proteins.

To make expression vectors for full-length rat NRGs, the cDNAs R22 (NDF β2a; GenBank accession number U02318), R19 (NDF β2b; accession number U02316), and R44 (NDF α2c; accession number U02224) were subcloned into pcDNA3.1(+) (+) using the NotI and EcoRV sites.

To generate GST fusion protein constructs, the yeast two-hybrid bait constructs NRGas-bait and NRGc-bait were digested with EcoRI and SalI, and the cytoplasmic tail-encoding fragment was ligated in-frame into the vector pGEX-4T-1 (Amersham Pharmacia Biotech).

All constructs were verified by restriction digestion and by automated dye terminator cycle sequencing (Applied Biosystems). Western blot analysis confirmed that proteins of the expected size were produced in COS-7 cells transfected with each of the mammalian expression constructs. Expressed TM-NRGs sometimes appeared as a doublet on Western blots, presumably due to heterogeneous glycosylation (cf. Fig. 6A).

**Yeast Two-hybrid Screening and Assays**—The yeast two-hybrid screening reported here employed the bait plasmid pBTM116, the prey plasmid pVP16, and the yeast strain L40 (40). The bait construct (NRGc bait) encodes a fusion protein consisting of: 1) a nuclear localization sequence, 2) the VP16 transactivation domain, and 3) the protein encoded by a brain cDNA (see Fig. 2A). The prey library was prepared from poly(A)+ mRNA obtained from a 3-week-old mouse. The first strand cDNA synthesis was random primed to minimize bias toward C-terminal sequences and was size-selected for a length of 300–800 base pairs. This length is sufficient to encompass individual protein domains but, in many cases, may encode only a portion of a protein. The partial-length prey resulting from this size selection may be advantageous in allowing identification of interactions between the bait protein and proteins for which a full-length prey protein would not interact in a two-hybrid assay, either because the full-length protein is membrane-associated or because it contains a regulatory domain that blocks interaction with the bait. The library had 2 × 109 primary recombinants.

For screening, library plasmids were transformed into L40 yeast that had previously been transformed with the NRGc bait plasmid. The version of the yeast two-hybrid system used employs two independent reporter genes, HIS3 and LacZ. Colonies that grow on medium lacking histidine and that produce β-galactosidase are considered primary screen positives. Prey plasmids isolated from colonies positive in the primary screen were further tested in a secondary screen: 1) to confirm that the prey plasmid isolated from the initial positive interacts with the LexA bait, and 2) to test the specificity of the interaction with the NRGc bait.

For the specificity control, the candidate prey plasmid was transformed into L40 yeast containing a bait plasmid that encodes the PAS domain of the Drosophila periodic protein PER. This protein sequence has no known similarity to NRG. Only prey showing no interaction with the PER bait were further evaluated.
For the β-galactosidase filter assay, colonies of transformed yeast were picked and patched in triplicate on medium lacking tryptophan, leucine, uracil, and lysine. The yeast plates were transferred onto nitrocellulose filters, and the β-galactosidase assay was performed as described elsewhere (40). Strength of interaction was scored as described in the legend of Fig. 3. The screening from the β-galactosidase filter assay was performed as described elsewhere (43). β-Galactosidase unit activity was calculated using the formula: activity = 1000 × A_{420}/(time in min) × (volume of culture in ml) × A_{650}.

Production of GST Fusion Proteins and in Vitro Binding Assay—The GST expression vector pGEX-4T-1 and GST fusion protein constructs GST-NRGas-tail and GST-NRGc-tail were transformed into the bacterial strain BL21. An overnight culture in 2×YT medium was diluted 1:50 into 50 ml of fresh 2×YT and incubated at 37 °C in a shaking incubator for 90 min. Isopropyl-1-thio-β-D-galactopyranoside was then added to the culture to a final concentration of 0.1 mM, the culture was incubated for an additional 4 h, and then the bacteria were pelleted at 2500 × g. The pellet was washed once with 7 ml of STE buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5), suspended from 5 ml of cold STE containing 100 μg/ml lysozyme and incubated on ice for 15 min. Five hundred microliters of 100 mM dithiothreitol and 1 ml of 10% sarkosyl w/v were added, and the volume was brought to 10 ml with cold STE. The bacteria were lysed by freezing and thawing five times in a dry ice-ethanol bath. The lysate was cleared by centrifugation at 16,000 × g for 20 min at 4 °C. The supernatant was transferred to fresh tubes, and Triton X-100 was added to a final concentration of 2% v/v. The lysate (~10 ml) was then incubated with 100 μl of glutathione-agarose beads (Amersham Pharmacia Biotech) for 1 h at 4 °C. The beads were settled by centrifugation at 7000 × g for 15 min at 4 °C. For the in vitro binding assay, COS-7 cells were transfected as described above with the Myc-LIMK1 expression construct. The cells were lysed 60 h after transfection with 400 μl of TENT buffer per 100-mm dish. The cell lysate was cleared by centrifugation at 7000 × g for 15 min at 4 °C. One milliliter of the cell lysate was incubated overnight at 4 °C with GST fusion protein immobilized on glutathione-agarose beads (20 μl). The beads were washed three times with TENT buffer and then resuspended in 50 μl of 2× SDS sample buffer with dithiothreitol and heated at 95 °C for 5 min. For Western blot analysis, 15 μl of this sample was loaded on a 10% SDS-polyacrylamide gel. Myc-tagged proteins and precipitated lysates were analyzed by Western blot as described above.

Antibody Production—Anti-LIMK1 antibody was produced by immunizing a rabbit with the synthetic peptide α-acetyl-KETYRRGESSLPAHPERV. The underlined amino acids correspond to the 18 C-terminal amino acids of the mouse and rat LIMK1 proteins (31, 45). For immunizations, the peptide was conjugated to horseshoe crab hemocyanin (Sigma) using glutaraldehyde. Immunizations and harvesting of serum were performed by Covance Research Products (Denver, PA). Serum from the rabbit was analyzed by Western blot and affinity purified using the immunizing peptide conjugated to Affi-Gel 15 (BioRad) according to the manufacturer’s instructions. The affinity-purified antibody preparation had an immunoglobulin concentration of 1.5 mg/ml and was used at a dilution of 1:200.

Immunohistochemistry—An adult rat was anesthetized with 4% chloral hydrate and perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Tissues were dissected and immersed in 15% sucrose/0.1 M phosphate buffer until they sank, then transferred to 30% sucrose/0.1 M phosphate buffer for 2–3 days and frozen in OCT compound using liquid N2. 10-μm frozen sections were cut in a cryostat and dried onto SuperfrostPlus slides (Fisher Scientific). For immunohistochemistry, tissue was processed as follows: 1) washed three times with histology PBS (100 mM sodium phosphate, pH 7.4, 150 mM sodium chloride), 2) blocked for 1 h at room temperature in PBS with 0.3% Triton X-100, 0.5% BSA, and 0.3% Triton X-100, 3) incubated overnight at 4 °C in primary antibody, 4) washed in PBS, 5) incubated for 1 h at room temperature with secondary antibodies in PBS blocking buffer, 6) washed with histology PBS, and 7) coverslipped with Vectashield Mounting Medium (Vector Laboratories).

Antibody concentration/dilutions used are as follows: α-SV2 hybridoma supernatant, 1:50; α-NRG cytoplasmic tail antibody sc-537, 1.0 μg/ml; α-LIMK1, 1.5 μg/ml; LiRG-conjugated donkey anti-rabbit, 1:200; and FITC-conjugated donkey anti-mouse, 1:200. The specificity of anti-LIMK1 antibody was confirmed by preabsorbing the antibody to the immunizing peptide (15 μg/ml) for 2 h at room temperature. The specificity of antibody sc-537 labeling was confirmed by preabsorbing the antibody to the Ab 1310 immunizing peptide (10 μg/ml), the sequence of which overlaps the sequence of the sc-537 immunizing peptide (see under “Reagents” above). The specificity of anti-SV2 labeling was assessed by preabsorbing the ExPasy anti-SV2 antibody. Experiments using each primary antibody alone with both secondary antibodies demonstrated the absence of bleedthrough and cross-labeling. Sections were viewed through a Zeiss Axiophot microscope using a ×100 oil immersion lens. Images were recorded with an MTI 300T-RC CCD camera using the NIH Image software.

RESULTS

The LIM Domain Region of LIMK1 Interacts with the Neuregulin Cytoplasmic Tail in the Yeast Two-hybrid System—The sequence of the NRG cytoplasmic tail is highly conserved between mammals and birds (Fig. 1), suggesting that this region of the NRG protein serves one or more important biological functions. We are particularly interested in the possibility that transmembrane NRGs may serve as cell surface receptors—i.e. the NRGs are receptors in addition to being ligands for the erbB2/erbB3/erbB4 receptor tyrosine kinases. If so, one role of the NRG cytoplasmic tail may be to transduce signals mediated through the NRG extracellular domain. To test the hypothesis that the NRG cytoplasmic tail is involved in signal transduction, we conducted a yeast two-hybrid screen to identify proteins that interact with the NRG cytoplasmic tail.

A region of the NRG cytoplasmic tail common to all transmembrane NRGs (NRGc bait; Fig. 2) was used to screen a
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**Fig. 1. Schematic representation of a transmembrane NRG isoform.** As a consequence of alternative splicing, multiple NRG isoforms are synthesized. Most are transmembrane proteins. All contain an epidermal growth factor-like domain in the extracellular portion of the protein. This domain is necessary and sufficient for activation of the receptor tyrosine kinases erbB2/erbB3/erbB4. Due to alternative splicing, transmembrane NRGs with three different cytoplasmic tail forms are synthesized. The TM-NRG isoform illustrated has the longest form of the tail (a-tail). This a-tail consists of the 157 amino acids common to all NRG cytoplasmic tails (# to *, solid line) and a 217 amino acid sequence unique to the a-tail (* to C terminus, broken line). The shortest variant of the NRG cytoplasmic tail (c-tail) consists only of the 157-amino acid common region (i.e. ends at the *). The b-tail isoform consists of the 157 amino acid common region plus 39 amino acids unique to the b-tail (not shown here). For each domain of the TM-NRG isoform illustrated, the percentage of amino acids identical between rat and chicken TM-NRG is shown. Also shown is the number of amino acids in each domain. Note the high conservation of amino acid sequence for the cytoplasmic tail. Proteolysis of the extracellular domain of the TM-NRG isoform illustrated, the percentage of amino acids identical between rat and chicken TM-NRG is shown. Also shown is the at the * cognate RTKs on cells contacting the NRG-expressing cell. This direct cell-cell contact mode of signaling has been called “juxtacrine” signaling (19, 20). This diagram is adapted from Ref. 2. A rat NDF 2a isoform is illustrated (GenBank accession number U02318). Ig-like, immunoglobulin-like domain; EGF-like, epidermal growth factor-like domain; TM, transmembrane domain.

To assess the specificity of the interaction between JX55–80 (hereafter referred to as LIMK1-1dr (LIMK1 LIM domain region)) and the NRGc bait, we tested the interaction of the LIMK1-1dr with the PAS domain of PER and with the cytoplasmic tail of TGF-α, a RTK transmembrane ligand structurally related to NRG. Neither interacted with the LIMK1-1dr (Fig. 3B). Thus, although the 71 kDa LIMK1 is of a size roughly similar to a kinase activity that associates with the cytoplasmic tail of TGF-α in Chinese hamster ovary cells (Refs. 50 and 51; see under “Discussion”), it is unlikely that LIMK1 is this kinase.

LIMK1 Interacts with the Cytoplasmic Tail of Neuregulin in Vitro and in Cultured Cells—The LIMK1-1dr prey used in the two-hybrid assays includes only the LIM domain region of LIMK1. We used an in vitro binding assay and a co-precipitation assay to determine whether full-length LIMK1 binds to the NRG cytoplasmic tail common region. For the in vitro binding assay, COS-7 cells were transfected with an expression construct encoding full-length LIMK1. A lysate prepared from these transfected cells was incubated with GST fusion proteins attached to glutathione-agarose beads (Fig. 4). LIMK1 bound to the GST-NRG-e-tail protein, which consists of the entire NRG cytoplasmic tail common region, but not to GST alone. LIMK1 also did not bind to a fusion protein containing the 217-amino acid sequence unique to the longest NRG cytoplasmic tail isoform but lacking the NRG cytoplasmic tail common region (GST-NRGas-tail; cf. Fig. 2B). These results demonstrate that full-length LIMK1 binds specifically to the NRG cytoplasmic tail common region. The GST-NRGas-tail result also indicates that the interaction of the NRG a-tail isoform with LIMK1 (see below) appears to be restricted to the portion of the a-tail common to all TM-NRG isoforms.

Physical association between NRG and LIMK1 within cultured mammalian cells was tested by determining whether precipitation of NRG from lysates of cells expressing both proteins resulted in co-precipitation of LIMK1. COS-7 cells were co-transfected with expression constructs encoding TM-NRG and LIMK1. Sixty hours later, these cells were lysed in detergent buffer, and NRG was immunoprecipitated from the lysates. LIMK1 was found to specifically co-precipitate with NRG.
Consistent with these results, immunocytochemical analysis demonstrates that NRG and LIMK1 have a similar subcellular distribution when co-expressed in COS-7 and PC12 cells (data not shown).

TM-NRGs with a-, b-, and c-cytoplasmic tail forms interact with LIMK1—Transmembrane NRGs with three different cytoplasmic tail sequences are known. All share the 157-amino acid "common region" sequence that we used as the bait in our two-hybrid screen (Fig. 1). The c-tail isoform is composed exclusively of this 157-amino acid "common region." The b-tail is the common region plus 39 additional amino acids. The a-tail is the common region plus 217 additional amino acids (cf. Figs. 1 and 6C).

Because we used the common region of the NRG cytoplasmic tail as the bait for the screen that identified LIMK1 as a NRG-interacting protein, we expected that LIMK1 would also interact with a-tail and b-tail NRGs. We tested this prediction in three ways: by assaying co-immunoprecipitation of full-length LIMK1 with TM-NRGs having each type of cytoplasmic tail (Fig. 6A); by assaying co-precipitation of LIMK1 with the NRG cytoplasmic tails, expressed as cytosolic proteins (rather than as full-length transmembrane proteins) (Fig. 6B); and by quantitative yeast...
two-hybrid assays (Fig. 6C). These assays demonstrated that, as predicted, LIMK1 does interact with TM-NRGs having all three forms of the NRG cytoplasmic tail (Fig. 6A). However, they further suggested the possibility that LIMK1 interacts more strongly with the c-type tail than with the a- or b-type tails (Fig. 6, B and C). If so, the b-tail-specific and a-tail-specific amino acid sequences may be involved in regulating the inter-

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Fig. 3. The LIM domain region of LIMK1 interacts specifically with the NRG cytoplasmic tail in yeast two-hybrid assays. A, the NRGc bait interacts with LIMK1-ldr but not with a control prey. A β-galactosidase filter assay is shown. Blue color of the yeast colony (seen here as dark gray) indicates interaction of the bait protein and the prey protein. The NRGc bait contains the entire amino acid sequence of the NRG cytoplasmic tail that is common to all TM-NRGs. The LIMK1-ldr contains the LIM domain region of LIMK1 (cf. Fig. 2). This photograph was taken 2 h after addition of substrate. However, blue color of the yeast transformed with both NRGc bait and LIMK1-ldr was already evident at 30 min, which is as early as for the positive control (PER bait/PER prey), a known strong interaction. The NRGc bait/PER prey assay is a negative control. Assay of three separate colonies for each bait/prey combination is shown.

B, the LIMK1-ldr interacts with the NRGc bait but not with control baits. Although the LIMK1-ldr interacts strongly with the NRG cytoplasmic tail, it does not interact with the cytoplasmic tail of TGF-α, a receptor tyrosine kinase transmembrane ligand structurally related to NRG. It also does not interact with the PAS domain of PER (PER-bait), a protein sequence unrelated to NRG. β-Galactosidase assays results from panel A are included here for comparison. β-Galactosidase activity was assessed by filter and solution assay. For filter assay, β-galactosidase activity was determined from the time taken for colonies to turn blue at 30 °C: ++, <30 min; + +, 30–60 min; +, 60–120 min; −, no color after 120 min. For solution assay, β-galactosidase activity was calculated as described under “Experimental Procedures.”

Fig. 4. LIMK1 binds to the neuregulin cytoplasmic tail in vitro. Lysates from cells transfected with Myc-LIMK1 or mock-transfected were incubated with the indicated GST fusion proteins. Lane 4 demonstrates that LIMK1 binds to a NRG cytoplasmic tail common region-GST fusion protein (GST-NRGc-tail). However, LIMK1 does not bind to GST alone (lane 1) or to a fusion protein containing the a-tail specific sequence of the NRG cytoplasmic tail (GST-NRGa-tail; lane 2). Lanes 3 and 5 are negative controls. LIMK1 bound to the fusion protein was detected by Western blotting using an anti-Myc antibody. The same lysate preparation was used for the incubations shown in lanes 1, 2, and 4; thus, the concentration of LIMK1 used in each of these incubations is identical. This experiment has been performed three times, each time with similar results.

Fig. 5. LIMK1 is co-immunoprecipitated with neuregulin from lysates of transfected COS-7 cells. Top, lysates of COS-7 cells co-transfected with TM-NRG (NRGc) and LIMK1 or singly transfected with LIMK1 were immunoprecipitated using anti-NRG Ab 1310. Co-precipitated LIMK1 was detected by Western blotting using anti-Myc Ab 9E10. No band is seen in lane 2, demonstrating that the precipitation of LIMK1 from the lysate of co-transfected cells (lane 1) is specifically dependent on the precipitation of NRG. This experiment has been performed three times, each time with similar results. Bottom, Western blots of the preprecipitation lysates demonstrate that similar amounts of LIMK1 were present in the lysates of the doubly and singly transfected cells used for the co-precipitation analysis shown in the top panel.
action of LIMK1 with the NRG cytoplasmic tail common region.

**LIMK1 and Neuregulin Are Co-localized at the Neuromuscular Synapse—**

The results described above demonstrate a physical interaction between recombinantly expressed NRG and LIMK1. These findings suggest the hypothesis that NRG and LIMK1 interact *in vivo*. An important prediction of this hypothesis is that NRG and LIMK1 will have an overlapping expression pattern. Neuregulin is known to play an important role in the development, maintenance, and regeneration of the neuromuscular synapse (reviewed in Ref. 9) and in the interaction of motor and sensory neurons with Schwann cells (Refs. 52–55 and references therein). Therefore, to test whether NRG and LIMK1 are co-expressed *in vivo*, we examined the expression of the NRG and LIMK1 genes in the spinal cord and dorsal root ganglia of the adult rat and we determined whether the NRG and LIMK1 proteins are co-localized at neuromuscular synapses.

We used reverse transcription (RT)-PCR to assess expression of these genes in adult rat spinal cord and sensory ganglia. mRNAs encoding both LIMK1 and transmembrane NRGs are present in the spinal cord and dorsal root ganglia (data not shown). These results confirm the findings of others demonstrating that transmembrane NRGs (Ref. 56; see also Refs. 4, 57, and 58) and LIMK1 (32, 33, 59) are both expressed in the spinal cord and dorsal root ganglia.

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common region of the NRG cytoplasmic tail revealed that LIMK1 and TM-NRG are co-localized at neuromuscular synapses. Because both our NRG cytoplasmic tail antibody and our LIMK1 antibody were raised in rabbit, we could not easily double label sections for these proteins. Instead, we compared the distribution of NRG and of LIMK1 to the distribution of SV2, a synaptic vesicle protein that is a well-documented marker of nerve terminals (41, 61). For both NRG (detected with the cytoplasmic tail antibody sc-537) and LIMK1, the most intense labeling of muscle sections closely corresponds to the SV2 labeling (Fig. 8, compare A to B and D to E). Published evidence that motor neurons produce both TM-NRGs (56) and LIMK1 (32, 59) suggests that these proteins are co-localized in the nerve terminal component of the synapse, although we cannot exclude the possibility that they are also co-localized at the postsynaptic membrane (see under “Discussion”).

**DISCUSSION**

The cytoplasmic tail of transmembrane NRGs has a highly conserved amino acid sequence, but its functions are unknown. We hypothesized that the NRG cytoplasmic tail plays a role in signal transduction and searched for interacting proteins involved in this process. Here we have reported characterization of the interaction between the NRG cytoplasmic tail and LIMK1, a nonreceptor kinase likely to be involved in intracellular signal transduction. Through a yeast two-hybrid screen, we discovered that the LIM domain region of LIMK1 can physically associate with the NRG cytoplasmic tail. In *in vitro* binding studies showed that full-length LIMK1 binds to the NRG cytoplasmic tail, and co-immunoprecipitation experiments revealed that full-length LIMK1 can associate with transmembrane NRGs in mammalian cells. In contrast, the LIMK1 LIM domain region does not interact with the cytoplasmic tail of TGF-α, an RTK transmembrane ligand previously reported to associate with a kinase activity (50, 51). These results demonstrate a specific physical interaction between NRG and LIMK1. In agreement with previously published results (32, 33, 55, 56, 59), we find that both LIMK1 and TM-NRG are expressed in spinal cord and dorsal root ganglia. Now we have found that LIMK1 is also co-localized with TM-NRG at neuromuscular synapses. Taken together, the physical interaction and co-localization of these proteins strongly suggest that NRG and LIMK1 functionally interact in *vivo*. LIMK1 was discovered in several screens for novel kinases (29–32). Like NRG, LIMK1 is most highly expressed within the nervous system and appears to be principally expressed by neurons, not glia (29–32, 59). Human genetic studies implicate LIMK1 hemizygosity as the cause of the cognitive defect found in Williams syndrome (33–35). Features of Williams syndrome include a specific cognitive profile characterized by pronounced weakness in visuospatial constructive cognition but relative strength in language and auditory rote memory, mild or moderate mental retardation, congenital heart and vascular disease, dysmorphic facial features, and infantile hypercalcemia. Although it seems likely that LIMK1 hemizygosity results in lower than normal amounts of LIMK1 protein being produced, the cellular mechanisms underlying the nervous system abnormalities of Williams syndrome are unknown. *In vitro* studies indicate that overexpression of LIMK1 can inhibit proliferation of fibroblasts (62) and differentiation of PC12 cells (63), but the relevance of these findings to the *in vivo* functions of LIMK1 is unclear. Our finding of an interaction between the NRG cytoplasmic tail and LIMK1 suggests that the pathogenesis of Williams syndrome may involve an abnormality in signaling through NRG-as-receptor, perhaps resulting in abnormalities of axon guidance (see discussion of Lerk-2/Nuk interaction, below).

LIMK1 has four structural domains: a double LIM domain, a Dlg-homology region (DHR/PDZ) domain, a serine/proline-rich region, and a kinase domain (Fig. 2). LIMK1 was the first protein found to combine LIM domains with a kinase domain. Each of the two LIM domains is comprised of two zinc finger structures. Although LIM domains were first described in homedomain proteins (47–49) that may serve as transcription factors, it is now recognized that the LIM domain is a protein-protein interaction motif found in proteins with a wide variety of functions (46). LIMK1 has been shown to bind several isoforms of protein kinase C; for the protein kinase Cγ isomorph, it has been demonstrated that this binding is through the second (more C-terminal) of the two LIMK1 LIM domains (64). Together with the results presented here, this finding suggests the possibility that the NRG-LIMK1 interaction regulates protein kinase C activity. The LIM domain region of LIMK1 has also been shown to bind the LIMK1 kinase domain (65). Thus, the NRG cytoplasmic tail may compete with the LIMK1 kinase domain for binding to the LIM domains, and this competition may regulate the phosphorylation activity of LIMK1.

The amino acid sequence of the LIMK1 kinase domain is unusual in that it precisely fits neither the signature for serine/threonine nor for tyrosine kinases. Nonetheless, biochemical evidence indicates that this is an active kinase domain with a preference for serine residues (31, 32, 60). In *in vitro*, LIMK1 has been shown to autophosphorylate and to phosphorylate myelin basic protein. Curiously, when assayed using myelin basic protein as a substrate, the specific activity of LIMK1 isolated from human fibroblasts (62) and differentiation of PC12 cells (63), but the relevance of these findings to the *in vivo* functions of LIMK1 is unclear. Our finding of an interaction between the NRG cytoplasmic tail and LIMK1 suggests that the pathogenesis of Williams syndrome may involve an abnormality in signaling through NRG-as-receptor, perhaps resulting in abnormalities of axon guidance (see discussion of Lerk-2/Nuk interaction, below).

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Transmembrane Neuregulin Interacts with LIMK1

FIG. 8. Neuregulin and LIMK1 are co-localized at neuromuscular synapses. A section of adult rat muscle was double-labeled for LIMK1 (A) and for a marker of nerve terminals, SV2 (B). An adjacent section was double-labeled for TM-NRG (D) and SV2 (E). Both LIMK1 (A) and TM-NRG (D) co-localized with SV2. Control experiments confirmed specificity of labeling with each antibody (see under “Experimental Procedures” for details). A DIC image for each section is also shown (C and F). Scale bar, 20 μm.

located? Although the NRG extracellular domain can be released from TM-NRG by proteolytic cleavage, it is unlikely that the NRG cytoplasmic tail (a portion of the NRG protein located in the cytoplasmic compartment of the nerve terminal) is released from the nerve terminal. Thus, it is probable that most of the synaptic labeling by the NRG cytoplasmic tail antibody (Fig. 8) is due to NRG in the nerve terminal. There is, however, evidence that muscle expresses the NRG gene at low levels (66, 68, 70, 71), so the possibility that there is NRG cytoplasmic tail associated with the postsynaptic membrane cannot be excluded.

In contrast to NRG, LIMK1 has not previously been reported to be concentrated at synapses. Although in the early embryonic LIMK1 is widely expressed (31, 59), in the adult, LIMK1 is principally expressed in the nervous system (29–32, 45, 60). Our reverse transcription PCR studies of LIMK1 expression in adult rat spinal cord and dorsal root ganglia are consistent with published in situ hybridization data (32, 59) suggesting LIMK1 expression by motor and sensory neurons. In situ hybridization studies have also demonstrated expression of LIMK1 by various neurons of the adult brain (29, 32, 59), and immunohistochemical studies have determined that LIMK1 is present in the cytoplasm and nuclei of hippocampal and cerebellar neurons (29). However, no description of the protein distribution in motor or sensory neurons has previously been reported, nor has there been any published observation of LIMK1 in axons or concentrated at synapses.

It seems most likely that synaptic LIMK1, like synaptic NRG cytoplasmic tail, is principally in the nerve terminal, rather than in the synaptic cleft or muscle components of the synapse. Because LIMK1 is a cytoplasmic protein, it is unlikely to be released from the nerve terminal. Further, Northern blot analysis of adult mouse muscle did not detect LIMK1 expression (Refs. 29 and 32; however, see also Ref. 60), and LIMK1 labeling is not seen in the cytoplasm of rat muscle (Fig. 8). Thus, we conclude that our evidence strongly indicates the co-localization of LIMK1 and the NRG cytoplasmic tail in the nerve terminal. Because NRG has important functions in the regulation of neuromuscular synapse formation and maintenance, the finding that the NRG cytoplasmic tail-interacting protein LIMK1 is also concentrated at this synapse supports the hypothesis that the NRG cytoplasmic tail and LIMK1 play important roles in the regulation of neuromuscular synapses.

Why many NRGs are synthesized as transmembrane proteins rather than soluble proteins is unknown. Part of the answer to this puzzle is likely to reside in the functions of the cytoplasmic tail, because the amino acid sequence of this region is highly conserved. Although we favor the idea that the interaction of LIMK1 with NRG mediates outside-in signaling, the LIMK1-NRG interaction could mediate any of the potential functions of the NRG cytoplasmic tail, including regulation of NRG trafficking and proteolytic processing (see Refs. 13, 17, 23, and 24) or apoptosis (22). Indeed, these are not mutually exclusive possibilities; for example, a consequence of outside-in signaling through NRG might be increased proteolytic processing of TM-NRG.

The cytoplasmic tails of two other RTK TM ligands, TGF-α and Lerk-2, are known to associate with kinases. In Chinese hamster ovary cells stably expressing TM TGF-α, TM TGF-α can be cross-linked to a protein complex that includes serine, threonine, and tyrosine kinase activities (50, 51). These kinase activities have been demonstrated by an in vitro phosphorylation assay of the precipitated complex using myelin basic protein and histone 2B as substrates. The binding of this kinase(s) to TGF-α apparently has different structural requirements than the binding of NRG to LIMK1, because the association of kinase activities with the TGF-α cytoplasmic tail depends on a protein of cysteine in the tail (51), but the NRG common region lacks cysteines. A protein of 86 kDa that is a component of the cross-linked complex may be the TGF-α associated kinase, because its association with the TGF-α cytoplasmic tail also depends on the cysteine pair. However, the identity of this protein has not yet been reported.

To date, the strongest evidence for bi-directional signaling through an RTK TM ligand/RTK pair comes from studies of the interaction between the transmembrane RTK ligand Lerk-2 and its RTK receptor Nuk. Genetically altered mice lacking the Nuk RTK have a defect in anterior commissure formation; whereas genetically altered mice that make a mutant form of Nuk with no kinase activity have no defect in anterior commissure formation. Lerk-2 is expressed by the neurons of which the axons are misrouted in the mice lacking the Nuk RTK, and Nuk is expressed by the cells over which these axons migrate (26). One interpretation of these results is that Lerk-2 serves as a receptor guiding these axons. This interpretation is supported by studies in a model system demonstrating that when Lerk-2 expressing cells are co-cultured with Nuk expressing cells, the Lerk-2 becomes tyrosine-phosphorylated on its cytoplasmic tail (27, 28).

LIMK1 is the first molecularly identified protein known to interact with the cytoplasmic tail of an RTK TM ligand. The experiments reported here demonstrate physical interaction between NRG and LIMK1 and a similar cellular and subcellular distribution of these proteins. The fact that both NRG and LIMK1 are localized at neuromuscular synapses supports the hypothesis that this interaction is functionally significant. To elucidate the biological functions of the NRG cytoplasmic tail/LIMK1 interaction, experiments now under way seek to deter-
mining how the interaction of NRG and LIMK1 is regulated, the downstream events triggered by this interaction, and the functional consequences of the interaction.

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