The extracellular domain of the membrane-associated forms of ErbB superfamily of receptor tyrosine kinases (also called HER) have been characterized extensively. erbB-1 (receptor for epidermal growth factor or EGFR) binds several distinct ligands containing an EGF-like domain and mediates proliferation and differentiation of normal cells (1). The second member, ErbB-2, initially characterized as the protooncogene, neu, encodes a 185-kDa protein. This protooncogene is frequently overexpressed in various carcinomas and is associated with a poor prognosis (2). The other two members, ErbB-3 and ErbB-4, have been cloned subsequently and identified based on consensus protein-tyrosine kinase domains and homology with the other ErbB members (3, 4). Neuregulins (NRGs) isolated from ras-transformed mouse fibroblasts and human breast cancer cells, originally called neu differentiation factor (NDF) or heregulin, were initially identified as putative ErbB receptor ligands based on their ability to stimulate tyrosine phosphorylation of ErbB-2 (5, 6). Three other neuregulin homologues were subsequently isolated from neural sources. One is acetylcholine receptor-inducing activity from chicken brain, which induces the synthesis of acetylcholine receptors in skeletal muscle (7). The other is glial growth factor from the bovine brain, which stimulates Schwann cell growth (8). The third is sensory and motor neuron-derived factor (9). Despite their varied functions, all these homologues are derived from a single gene by alternate splicing or by use of several cell type-specific transcription initiation sites, and all contain an EGF-like domain required for receptor activation (8). Recent studies demonstrated that neuregulins bind directly to ErbB-3 and ErbB-4 and activate receptors through heterodimerization with ErbB-2 (10–15).

Gene inactivation experiments have demonstrated that neu-regulins are essential for early heart and central nervous system development. Inactivation of both neu-regulin alleles results in embryonic lethality due to maldevelopment of the heart. In these animals, the cardiac trabeculae fail to form normally, and there is a severe defect in endocardial cushion development. In addition, neu-regulin null mice display defects of the nervous system including abnormal development of both Schwann cell precursors and cranial ganglia (16, 17).

The varied biological functions mediated by neuregulins are likely related to differential tissue expression and structural diversity. At least 15 distinct isoforms are expressed in different tissues (18). They can be classified into two major groups based on their domain structures. One group is comprised of proteins that have an N-terminal signal peptide, but lack transmembrane and intracellular domains. Thus, these isoforms, which are largely expressed only in neural cells, are packaged presumably and subject to regulated release. The other group, expressed in both neural and mesenchymal cells, are membrane-associated. In addition to an extracellular segment (consisting of an Ig-like domain, a glycosylated region, and an EGF-like domain), these isoforms contain a transmembrane and intracellular domain.

The extracellular domain of the membrane-associated forms can be proteolytically cleaved to release their ErbB receptor-activating functional peptide (5). Although some NRG isoforms, such as NDF-β4a, are more localized in the plasma membrane,
others, such as NDF-a2c, show minimal plasma membrane localization. Both of these are processed predominantly in intracellular organelles (19). Pulse-chase labeling studies indicate that processing occurs within 30 min of translation. Since the cleavage process is regulated in cultured cells by phorbol esters, cleavage may require a signaling event (19). In addition, it has been suggested that the efficiency of proteolytic cleavage is directly proportional to the length of the intracellular domain (20), which varies among the different isoforms.

Membrane-bound NRG isoforms are expressed in mesenchymal cells that are in juxtaposition to cells expressing ErbB receptors (21). For example, in the early stage of the developing heart, the membrane-bound forms are expressed in the endocardial lining, whereas their cognate receptors are expressed in cardiac myocytes and in cells forming the endocardial cushion. This suggests that neuregulins function in a paracrine fashion during the early stages of heart development.

We have found recently, in both in vivo and in vitro studies, that the NRG intracellular domain is essential for proteolytic release of the NRG extracellular domain and for determination of its subcellular localization (22). In this study genetic disruption of only the intracellular domain of the membrane-bound NRG isoforms resulted in a similar phenotype of embryonic maldevelopment, to that observed previously with disruption of the entire gene. This indicates that the NRG intracellular domain is critical for NRG signaling and raises the following questions: (i) how do cells cleave the signaling peptide, and (ii) what processes regulate and trigger cleavage/secretion?

To further understand the molecular mechanism involved in these processes, we have investigated the role of the cytoplasmic tail in regulating proteolytic cleavage/secretion. A series of mutants of the rat NDF-a2c isoform were constructed, in which either the intracellular domain was sequentially deleted from the C terminus, or specific segments of the intracellular domain were deleted. The constructs were then used to evaluate membrane expression and release of the 43-kDa functional peptide into the culture medium after transient expression in COS-7 cells.

The results of these studies indicate that discrete segments of the intracellular domain of the NRG isoform, NDF-a2c, regulate cleavage/secretion through a process that likely involves initial intracellular domain-mediated dimer formation.

**Experimental Procedures**

**Generation of Mutated NDF Constructs**—(i) To construct NDF mutants encoding proteins in which 30 residues were sequentially deleted from the C terminus of the intracellular domain, the following primers: 5'-AACTCGAGGTTAGCCCAATAATGTTC (5'-end primer with a XhoI site) and 5'-CAGCTAGATCATTTACCCGGGAGCACG (3'-end primer retaining an XbaI site). (ii) A DNA cassette encoding the FLAG epitope (IBI) was ligated via an N-terminal BamHI site of NDF mutant construct, 2c/tg, to give FLAG-2c/tg or fused to the N-terminal cytoplasmic domain of NDF. This vector allows expression of a FLAG-fused intracellular domain protein, FLAG-NDF/CT.

The 2B (IBI) vector was used for bacterial expression of the intracellular domain (FLAG-NDF/CT) and the GST cDNA fragment (FLAG-GST/P). The pGEX (Amersham Pharmacia Biotech) vector was used to express the full-length GST (GSTF). All other mutant cDNAs were cloned between the EcoRI and XhoI sites in the pCINe4 expression vector, pCDNAIII, and confirmed by sequencing prior to transfection.

**Cell Lines, Transfection, and Cell Culture**—COS-7 cells were obtained from the American Type Culture Collection (ATCC) and were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cells (1 × 10⁶) were plated on 100-mm dishes and incubated under 5% CO₂ for 15 h before transfection. Transfections were performed with 10 µg of cDNA using the dextran-DEAE (Life Technologies, Inc.) method as described previously (23). Before harvesting conditioned medium, Opti-MEM (Life Technologies, Inc.) containing CaCl₂ (110 mg/ml) was used to replace the Dulbecco’s modified Eagle’s medium/fetal bovine serum, and the cells were then incubated for a further 15 h. Human breast cancer cells MCF-7 (ATCC) were cultured in RPMI 1640 medium with 10% fetal bovine serum. Before being stimulated by the recombinant NDF protein, the cells were starved for 20 h in RPMI 1640 serum-free medium.

**Plasma Membrane Purification**—Transfected 1 × 10⁶ cells were harvested by cold PBS with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) and homogenized by 15 passes with a loose fitting Dounce homogenizer, then diluted 10-fold using the PBS. The homogenate was then centrifuged at 1464 × g for 10 min, and the resulting pellet was resuspended in the PBS and diluted to give a 6% (w/v) suspension. A volume (10/4) of this was mixed with 1.4 ml of Percoll (Amersham Pharmacia Biotech) in 15-ml Corex tubes and centrifuged at 34,549 × g for 30 min. Two distinct layers close to the top of the tube were evident. These were harvested and washed in 5 volumes of 250 mM sucrose, 50 mM Tris-HCl, pH 8.0, and the resulting pellets were resuspended in the PBS.

**Western Blot Analysis**—Western blotting was performed as reported previously (23) using either plasma membranes prepared as described previously (24) or conditioned medium that was initially filtered through a 0.2-µm pore size sterile filter unit (Costar) and then concentrated by centrifugation through Centricon 10 (Amicon) concentrator. Protein concentration was determined using the Bradford method (Bio-Rad), and 30 µg of protein were loaded into each well. Affinity-purified anti-NDF extracellular domain antibody (194) (Agen) or anti-FLAG M2 antibody (IBI) was used as the primary antibody at a final concentration of 2 µg/ml (1:100 dilution), and anti-rabbit or anti-mouse IgG-conjugated with horseradish peroxidase (Amersham Pharmacia Biotech) was used as the second antibody (1:3000 dilution). After SDS-PAGE, proteins were electroblotted onto Immobilon-P (Millipore) and detected using the ECL system (Amersham Pharmacia Biotech).

**p185neo Tyrosine Phosphorylation Assay**—The conditioned medium from transfect COS-7 cells was filtered through a 0.2-µm pore size sterile filter unit (Costar) and concentrated. The concentrate was added to individual wells of a 12-well plate containing 2 × 10⁵ MCF-7 human breast cancer cells (ATCC) in each well. Following a 10-min incubation at 37 °C, the cells were lysed with 1% Nonidet P-40 lysis buffer that contained 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 40 mM NaF, 2 mM sodium orthovanadate and the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), aprotinin (10 µg/ml), and leupeptin (10 µg/ml). The cell lysates were subjected to enhanced chemiluminescence Western blot analysis with anti-phosphotyrosine antibody RC-20 at a 1:1000 final dilution (Transduction Laboratories).

**Double Labeling Immunofluorescence and Microscopy**—Cells transfected with NDF-a2c and FLAG-2c/tg were plated in two-well Novex plates (Nunc) and cultured for 24–48 h. After rinsing the cells with PBS they were fixed with 4% paraformaldehyde plus 0.1% Triton X-100 at room temperature for 30 min. The fixed cells were then blocked with 5% skimmed milk in PBS for 30 min, followed by incubation with anti-NDF intracellular domain antibody F20 (Santa Cruz) or anti-FLAG M2 antibody (IBI) for 45 min at room temperature. After washing, anti-rabbit IgG conjugated with fluorescein isothiocyanate (Silenus Laboratory, Melbourne, Australia) and anti-mouse IgG-TRITC (Sigma) was added and incubated for another 0.5 h. After washing, cells were mounted.
with 1% p-phenylenediamine (1 mg/ml, Sigma) in glycerol and then covered and sealed. Cells were examined using a UV fluorescence microscope and photographed with a 40× power objective.

**Bacterial Expression and Chemical Cross-linking.—** The plasmids FLAG-NDF/CT, FLAG-GST/P, and GST/F were transformed into the *Escherichia coli* strain, DH5α, and expressed by induction with isopropryl-β-d-thiogalactopyranoside (0.5 mM) at room temperature for 3 h. After centrifugation of the culture medium at 5000 rpm for 15 min, the resulting pellet was resuspended in cold PBS with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and sonicated for 6 min. The sonicated mixture was centrifuged at 14,000 rpm for 30 min and the supernatant fraction harvested. The supernatant fraction was subjected to cross-linking with 1 mM bis-sulfosuccinimidyl substrate (Pierce) at 4 °C for 1 h and then incubated with 10 μM Tris, pH 7.5, for 15 min to stop the reaction. COS-7 cells, transfected with FLAG-NDF/CT, were washed twice with cold PBS, scraped into 1 ml of PBS with protease inhibitors, and sonicated for 1 min to prepare the supernatant for cross-linking. The reaction mixture was subjected to Western blotting.

**RESULTS**

The intracellular domain of NDF-α2c is comprised of 159 residues. We have demonstrated previously that a deletion mutant (C5, Fig. 1), which lacks almost the entire intracellular domain (i.e., the C-terminal 150 amino acids), is resistant to extracellular domain proteolytic cleavage. To evaluate if extracellular domain cleavage requires a specific sequence of the cytoplasmic domain or is merely dependent on the length of the C-terminal tail, an additional construct was developed in which the terminal 150 residues were replaced by an unrelated 150-residue stretch of the GST protein (Fig. 1a). This construct, 2c/gst, the wild-type construct NDF-α2c, and the previously developed C5 construct were then evaluated for cleavage after expression of each construct in COS-7 cells. As shown in Fig. 1b, cleavage of the wild-type protein resulted in the release of a 43-kDa extracellular domain polypeptide into the culture medium. Despite a calculated molecular mass of only 26,000 Da for this ErbB-activating peptide, as reported previously (19, 20), glycosylation results in its migrating as a molecular mass of 43 kDa species by SDS-PAGE.

Unlike the wild-type protein, release of the 43-kDa peptide was not observed with expression of the two other constructs (Fig. 1b). This was not due to poor transfection or degradation of the mutated proteins, since their expression could be readily detected by Western blotting of cell lysates (Fig. 1b). In keeping with these findings, release of the 43-kDa ErbB receptor-activating ligand from the 2c/gst construct could also not be detected in a functional assay, even though release of the neu-regulin ligand from the wild-type NDF-α2c construct was readily apparent with this assay (Fig. 1c). These data indicate that a specific sequence of the intracellular domain rather than amino acid length, per se, is involved in the proteolytic cleavage.

By SDS-PAGE, the migration of the protein expressed by the C5 mutant is consistent with the molecular mass of its peptide backbone (30 kDa). In studies with the inhibitor of N-linked glycosylation, tunicamycin, this was shown to be due to a lack of glycosylation of the C5-expressed protein (data not shown). The 2c/gst-encoded protein, however, like the wild-type NDF-α2c, was glycosylated as evidenced by the finding that it migrated at a considerably higher apparent molecular mass (65 kDa) than that calculated for its peptide backbone (46 kDa).

Although expression of the cleavage-resistant mutants was apparent in the cell lysates, additional studies were performed to evaluate their subcellular localization in more detail. Plasma membrane expression of the wild-type and 2c/gst constructs was evident from Western blot analysis of purified plasma membrane (Fig. 2a). However, immunofluorescence labeling studies of permeabilized cells indicated that both the wild-type and 2c/gst encoded proteins colocalised predominantly to the same intracellular organelles rather than in the plasma membrane (Fig. 2b). Similar intracellular localization was observed for all mutant constructs, i.e., C1–C5 (data not shown). Since the proteins expressed by the wild-type and mutant (2c/gst, C1, C2, C3) constructs are glycosylated, their intracellular localization is likely mainly in the Golgi, although some expression in the endoplasmic reticulum cannot be excluded. These findings are in agreement with those reported by Burgess et al. (19), which showed little membrane expression of NDF-α2c compared with another isoform, NDF-β4a.

To further define the regions within the cytoplasmic domain that are involved in regulating cleavage, a series of C-terminal intracellular domain deletions of NDF-α2c was constructed, as shown in Fig. 3a. Compared with the full-length construct (NDF-α2c), these deletion mutants were truncated successively by 30 amino acids. Thus, construct C1 lacked 30 residues and construct C5 lacked 150 residues at the C terminus of the intracellular domain. As shown in Fig. 3b, the proteins expressed in cells transfected with either of the first two deletion mutants, C1 and C2, behaved similarly to the wild-type NDF-α2c protein, since release of the expected 43-kDa functional
peptide was readily apparent. In contrast, secretion of the 43-kDa peptide by the C3-, C4-, or C5-encoded protein was either only barely detectable or absent. Since expression of these mutant proteins was evident by Western blotting of cell lysates (Fig. 3c), it is likely that the various truncations did not alter transcription or translation or reduce transfection efficiency. Taken together, these findings suggest that a 30-amino acid (residues 332–362) segment is required for cleavage.

To more precisely identify the segments of the C-terminal tail involved in regulating proteolytic processing, six additional mutant constructs were evaluated (Fig. 4). Construct 2cΔ332–362, in which the 30 residues distinguishing the mutants C2 and C3 were deleted, but the remainder of the C-terminal tail (i.e. residues 362–422) was kept intact, surprisingly showed intact proteolytic cleavage. Thus, the 30-residue segment involving amino acids 332–362 is required for cleavage when the remainder of the C-terminal tail (aa 362–422) is also lacking as is construct C3. However, cleavage was not observed with two other constructs, 2cΔ272–362 or C2Δ272–332 (constructed based on C2 in Fig. 3). The former retains only the C-terminal 60 amino acids (aa 362–422), whereas the latter lacks the C-terminal tail but retains the 30-residue segment aa 272–332. Thus, although the 30-residue segment, aa 332–362, and the C-terminal tail, aa 362–422, are necessary for cleavage, neither alone is sufficient. Rather an additional cytoplasmic region between aa 272–332 is also required. This conclusion is also evident from studies with two additional mutants, 2cΔ302–362 and C2Δ302–332. In these mutants, the C-terminal tail segment (aa 362–422) or the 30-residue segment (aa 332–362) were separately attached to a construct truncated at residue 302. Since cleavage was evident with both these constructs, the cytoplasmic region defined above (i.e. aa 272–332) as being required for cleavage can be narrowed down to a segment involving only residues 272–302. However, deletion of only these 30 residues (construct 2cΔ272–302) again did not
Evidence that the C-terminal domain of NDF-α2c regulates proteolytic cleavage in a sequence-specific manner, is the finding that the 2c/gst fusion protein, which contained an intracellular domain of the same length as the wild type NDF-α2c protein, was resistant to proteolytic cleavage. Furthermore, studies with additional C-terminal truncation mutants indi-
cate that while deletion of the terminal 30 or 60 intracellular domain amino acids is tolerated, deletion of more than 60 residues significantly reduces proteolytic cleavage. Thus, a region of the intracellular domain between amino acids 332 and 362 appears to be critical for correct proteolytic processing of membrane-bound neuregulin precursor proteins. As all constructs contained a C-terminal cysteine, involvement of the intracellular domain of NDF-α2c in the release of its functional peptide differs from that of TGF-α, where the presence of a hydrophobic amino acid appears to be critical for proteolytic cleavage (29). This finding is not entirely unexpected, since there are other differences between TGF-α and NRGs. NRGs have a much longer cytoplasmic tail than TGF-α, which is highly conserved between species. In addition to the 332–362 segment, two other regions (residues 272–302 and 362–422) were subsequently shown to also be involved in cleavage/release. Moreover, any two of these three functional regions were shown to be sufficient for cleavage. By evaluating intracellular localization, we also demonstrated that failure to observe proteolysis is not due to impaired intracellular transport. Thus, extracellular proteolytic cleavage of NRG is regulated by its intracellular domain in a sequence-specific manner and may differ for the various NRG isoforms to provide specificity of responses to different biological stimuli. A concern regarding the above results is whether membrane insertion, protein orientation, or intracellular transport is altered with any of the mutants. Since NDF contains a cryptic internal signal sequence (the signal sequence is located within the transmembrane domain), membrane insertion or protein orientation of NDFs is determined by the transmembrane domain and by a limited number of charged residues flanking the transmembrane domain. Since the shortest mutant construct, C5, still contains nine residues C-terminal to the transmembrane domain, it is unlikely that membrane insertion and orientation were altered with this or other C-terminally deleted constructs. In addition, the identical subcellular localization of both the wild-type NDF-α2c and 2c/gst proteins suggests that altered protein transport cannot account for the impaired cleavage/secretion of these mutants.

We further show that regulation of extracellular domain cleavage may involve initial intracellular domain dimerization. Thus, when expressed alone, the NDF intracellular domains can be dimerized by treatment with a cross-linking reagent. In addition, susceptibility to cleavage can be restored by providing a heterologous intracellular domain that dimerizes spontaneously.

Taken together, our data suggest that the spatial and temporal control of growth and development by membrane-bound neuregulin isoforms involves proteolytic cleavage of the extracellular domain and that this cleavage is regulated by initial intracellular domain dimerization. Such intracellular domain interactions may be initiated by activation of cellular signaling pathways to provide a unique inside-out signaling system to regulate NDF proteolytic cleavage and, thus, activation of ErbB receptors.

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Release of the Neuregulin Functional Polypeptide Requires Its Cytoplasmic Tail
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