Norrie Disease Protein (Norrin) Forms Disulfide-linked Oligomers Associated with the Extracellular Matrix*

(Received for publication, September 5, 1997, and in revised form, October 17, 1997)

Juan Perez-Vilar and Robert L. Hill‡

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

COS-7 cells transfected with a DNA construct encoding the 133 amino acids in norrin plus six histidine residues at its carboxyl terminus were pulse-labeled with \(^{35}S\) methionine, and the labeled norrin was examined in cell lysates, the medium, and the extracellular matrix. SDS-gel electrophoresis under reducing conditions showed that the norrin expressed had an apparent \(M_r = 14,000\) and was present only in cell lysates and the extracellular matrix. Under nonreducing conditions, most of the norrin in the extracellular matrix was oligomers that contained up to \(\approx 20\) monomers. One of the major extracellular species of norrin under reducing conditions after cross-linking of norrin oligomers with bis(sulfosuccinimidyl)suberate had an apparent \(M_r = 28,000\), consistent with covalent cross-linked dimers. Thus the covalently cross-linked dimers are key structural components of norrin oligomers. By site-directed mutagenesis, the codon for half-cystine 95 in norrin was changed to one encoding alanine. The norrin C95A found in the extracellular matrix of cells transfected with this mutant was the size of dimers, indicating that half-cystine 95 is involved in oligomer formation. The corresponding half-cystine residue in human prepro-von Willebrand factor is also involved in interchain disulfide bond formation, which is consistent with the sequence identity of the half-cystine residues in norrin and part of the half-cystine residues in a disulfide-rich domain of von Willebrand factor. Replacement of valine at residue 60 in norrin by glutamic acid, a mutation found in humans with a severe type of Norrie disease, results in a considerable reduction (50%) in the amount of norrin in the extracellular matrix of transfected COS-7 cells. Replacement of arginine at residue 121 by glutamine, which is associated with a less severe type of Norrie disease, results in a reduction in the amount of norrin R121Q in the extracellular matrix (26%).

These studies suggest that norrin is a secreted protein that forms disulfide-bonded oligomers that are associated with the extracellular matrix upon secretion from cells. Moreover, the disulfide-rich motif of norrin and prepro-von Willebrand factor promotes interchain disulfide bond formation among polypeptides in which it is found.

Norrie disease (McKusick number 310600) is a rare, X-linked recessive human neurological disorder (1) characterized by congenital blindness, deafness, and mental retardation (2, 3). The gene (EMBL accession number X65882) that is defective in Norrie disease has been cloned (4, 5), and different mutations in the gene have been found in patients with the disease (e.g. Ref. 6). Mutations in the gene have also been associated with other diseases affecting the retina, such as advanced retinopathy of prematurity (7) and familial exudative vitreoretinopathy (8, 9). The gene is expressed predominantly in the retina, the choroid, and fetal and adult brain and encodes a half-cystine-rich protein of 133 amino acids (4, 5) called norrin (10).

Norrin does not show strong sequence identities with other known proteins, but the number and spacing of the half-cystine residues and some hydrophobic residues resemble those at the carboxyl terminus of human prepro-von Willebrand factor (GenBank\(^{TM}\) accession number X04355) and many secretory mucins (11), including pig submaxillary mucin (GenBank\(^{TM}\) accession number AF005273) (12), frog integumentary mucin, FIMB.1 (13), and the human mucins MUC2, MUC5AC, MUC5B, and MUC6 (14–18). Norrin also has significant sequence identities with half-cystine-rich proteins encoded by genes acting in the regulation of cell proliferation (19). These observations suggest that norrin may play an essential role in some aspect of neuroectodermal development (11) or regulation of neural cell proliferation (19), but its precise function is unknown.

From sequence comparisons and modeling studies, it has been suggested that norrin has a tertiary structure similar to transforming growth factor \(\beta (20)\) and contains a cystine knot structural motif (reviewed in Ref. 21). In addition, a half-cystine residue in one molecule is suggested to form a homotypic disulfide bond with another molecule, leading to formation of disulfide-linked dimers. Such dimers have been shown to be formed by the carboxyl-terminal half-cystine-rich domains of human prepro-von Willebrand factor (22, 23) and porcine submaxillary mucin (24). It has been found that the carboxyl-terminal 90 residues of porcine submaxillary mucin containing 11 of the 30 half-cystine residues of the entire domain are sufficient to permit dimer formation.\(^2\) Moreover, the carboxyl-terminal half-cystine-rich domain of human mucin MUC6, assumed to be responsible for interchain disulfide bonds between monomers, is constituted by only 91 residues, including only 11 half-cystines (18). Norrin, with 133 amino acid residues and 11 half-cystine residues, is therefore strikingly similar to the portion of mucin that permits dimer formation in pig submaxillary mucin and probably other secretory mucins including MUC6. For this reason, we have initiated studies, reported here, that show norrin forms disulfide-linked oligomers, which on secretion bind to the extracellular matrix. Moreover, by site-directed mutagenesis, impaired oligomer formation was observed when a single half-cystine residue was

\(^{1}\) Eckhardt, A. E., Timpte, C. S., DeLuca, A. W., and Hill, R. L., J. Biol. Chem., in press.

\(^{2}\) J. Perez-Vilar and R. L. Hill, unpublished observations.
changed to alanine. Norrin is found in reduced amounts in the extracellular matrix of COS-7 cells transfected with constructs containing genes identical to those in patients with Norrie disease. These studies may help determine the biological function of norrin.

EXPERIMENTAL PROCEDURES

Cloning a cDNA That Encodes Human Norrin—Individual clones (2 × 10^6) from a human fetal brain cDNA library in the pcDNA3.1(+) mammalian expression vector (Invitrogen) were screened with an oligonucleotide corresponding to the 5′/3′ noncoding region of norrin (4, 5) following standard procedures (25). Five clones gave a strong signal and were rescreened with the same oligonucleotide. Plasmids from positive clones were further purified by extraction with 2.5 ml of cold 50 mM sodium phosphate, pH 8, 0.1 M NaCl, 6 M guanidine hydrochloride, and cell debris was removed by centrifugation at 10,000 g for 30 min at 4°C. After removal of the extracellular matrix with a scraper (Sigma), the solution was cleared by centrifugation at 10,000 g for 5 min on ice. After scraping the extracellular matrix when cells are detached and lysed from the extracellular matrix, the norrin in the extracts was isolated and analyzed by gel electrophoresis and autoradiography. Fig. 1A shows that norrin binding to the extracellular matrix is unaffected by norrin treatment of the cells from which the matrix does not influence the location of norrin. In both methods, however, buffered 6 M guanidine hydrochloride was needed to solubilize the norrin from the extracellular matrix. Fig. 1C shows that the extracellular norrin from cells transfected with pNDH3 is degraded if the cells are detached with trypsin, although intracellular norrin is readily detected in cell lysates of trypsin-treated cells. These results also show that the high molecular weight proteins observed in the gels are found in both pNDH3- and pND3-transfected cells and are unrelated to norrin.

Fig. 2A shows the results of studies to evaluate conditions for extraction of norrin from the extracellular matrix. In these studies, COS-7 cells were transfected with pNDH3 and incubated with radiolabeled [35S]methionine and [35S]cysteine as in Fig. 1C. The cells were then detached with EGTA, the extracellular matrix was extracted under different conditions, and the norrin in the extracts was isolated and analyzed by gel electrophoresis. Norrin was not extracted from the extracellular matrix by buffers of pH 4.5 or 7.2, and only small amounts were extracted at pH 10 or 7.2 in the presence of 1 M sodium chloride. However, norrin was extracted by high temperature and autoradiography were done as described earlier (24). Fluorographic films were captured by digitized images with a CCD camera and analyzed with the NIH image software version 1.61. 13C-methylated proteins (Amersham) used as low molecular weight standards were carbonic anhydrase (30,000), trypsin inhibitor (21,500), cytochrome c (12,500), aprotinin (6,500), and insulin (5,740). Higher molecular weight standards were 

3 13C-methylated myosin (220,000), phosphorylase b (97,400–100,000), bovine serum albumin (66,000), ovalbumin (46,000–50,000), carbonic anhydrase (30,000), and lysozyme (14,300).

RESULTS

Secretion and Extracellular Location of Norrin—COS-7 cells were transfected with either of two plasmids, pNDH3, which encodes norrin plus six consecutive histidine residues at its carboxyl terminus, or pND3, which encodes only norrin. Twenty-four h after transfection, the cells were incubated for 4 h with 

[^35S]labeled cysteine. The cells were detached from the extracellular matrix with EGTA and lysed with buffered Triton X-100. The extracellular matrix was extracted with buffered 6 M guanidine hydrochloride. His-tagged norrin was isolated from the cell lysates, the extracellular matrix, and the medium by adsorption to and elution from the metal-affinity adsorbant. The eluates were then analyzed by SDS-gel electrophoresis and autoradiography. Fig. 1A shows that norrin was not detected in cells transfected with pND3 but was observed in cell lysates and the extracellular matrix but not the medium of cells transfected with pNDH3. These results confirm the specificity of the metal-affinity adsorbant for the isolation of His-tagged norrin and show that norrin, M_r = 14,000, is the major labeled protein. Moreover, norrin is secreted from the cells but not into the medium; after secretion, norrin is bound to the extracellular matrix. Fig. 1B shows that norrin is found in the extracellular matrix when cells are detached from the extracellular matrix with ammonia/Triton X-100 rather than EGTA as in Fig. 1A. Just as observed in Fig. 1A, norrin is not found in the extracellular matrix of cells transfected with pND3. Thus the method of detachment of the cells from the matrix does not influence the location of norrin. In both methods, however, buffered 6 M guanidine hydrochloride was needed to solubilize the norrin from the extracellular matrix. Fig. 1C shows that the extracellular norrin from cells transfected with pNDH3 is degraded if the cells are detached with trypsin, although intracellular norrin is readily detected in cell lysates of trypsin-treated cells. These results also show that the high molecular weight proteins observed in the gels are found in both pNDH3- and pND3-transfected cells and are unrelated to norrin.
the reaction of the matrix with heparinase I. The results shown in Figs. 2, B and C, suggest that norrin binding is not mediated through heparan sulfate.

Fig. 3 shows that norrin is markedly reduced in the extracellular matrix of COS-7 cells transfected with pND3 and then incubated with brefeldin A during pulse-labeling with [35S]cysteine and [35S]methionine (Tran35S-label). Cells were detached by digestion with 0.25% trypsin for 2 min at 37 °C and lysed with buffered Triton X-100. The remaining insoluble extracellular matrix proteins were extracted and analyzed as in A. C, COS-7 cells transfected with pNDH3 were metabolically labeled with a mixture of [35S]cysteine and [35S]methionine (Tran35S-label). Cells were detached by digestion with 0.25% trypsin for 2 min at 37 °C and lysed with buffered Triton X-100. The His-tagged norrin in cell lysates of the trypsin-treated cells (lane 1) or that in extracts of the extracellular matrix (lane 2) were purified and analyzed as in A. The molecular weights (MW) of the standards are in thousands.

**Formation of Norrin Oligomers**—The electrophoretic analyses shown in Figs. 1–3 were performed with SDS gels in the presence of 2-mercaptoethanol. Similar experiments were performed in the absence of 2-mercaptoethanol to determine whether disulfide-bonded oligomers of norrin were formed. As shown in Fig. 4A, electrophoresis on 15% acrylamide gels showed that monomers were among the main intracellular forms, whereas oligomers of norrin were the predominant forms in the extracellular matrix. Fig. 4B shows the species of norrin observed in Fig. 4A analyzed on 10% acrylamide gels. Proteins ranging in molecular weight from about 25,000 to more than 300,000 were observed, suggesting that disulfide-
linked oligomers of norrin containing up to about 20 molecules of monomer are formed. Extracellular oligomers isolated from nonreduced gels and then analyzed on reduced gels showed only protein species with a molecular weight of 14,000, the size of the monomer (data not shown).

Norrin produced by transfected COS-7 cells was also reacted with the cross-linking agent, bis(sulfosuccinimidyl)suberate, as shown in Fig. 4C. In these studies, norrin isolated from the extracellular matrix was found as oligomers when analyzed on unreduced gels, and when reduced with 2-mercaptoethanol, monomers with a molecular weight of 14,000 were found. When the oligomers were cross-linked and then analyzed on reducing gels, three species were observed, high molecular weight cross-linked oligomers, a protein species twice the molecular weight of monomers, and a species with a molecular weight slightly lower than monomers. The latter species is likely norrin reacted with cross-linking agent but not cross-linked. These results suggest that the dimeric species may be structural components of the oligomers.

Influence of the His Tag on the Oligomerization, Secretion, and Location of Norrin—It was important to determine whether the six consecutive histidine residues added to the carboxyl terminus of norrin altered its oligomerization, secretion, and extracellular location. Determination of the effects of the histidine residues was made by analysis of the proteins produced by COS-7 cells cotransfected with pND3 and pNDH3 that encode norrin and His-tagged norrin, respectively. Fig. 5A shows that without reduction, the major proteins isolated from the extracellular matrix are norrin oligomers similar to those produced by cells transfected only with pNDH3. However, as shown in Fig. 5B, on reduction of the oligomers, two protein bands were observed, one with a molecular weight of about 14,000 and another with a molecular weight of about 13,000. The sizes of these two species are those expected for norrin (M_r = 13,000) and norrin with six additional histidine residues (M_r = 14,000). Moreover, Fig. 5C shows that the proteins synthesized in an in vitro transcription/translation system designed to express pND3 and pNDH3 have the expected molecular weights. These results indicate that norrin and His-tagged norrin formed disulfide-bonded oligomers with one another and that on reduction of these oligomers, monomeric species of each type were obtained. Thus, the presence of the His tag on norrin is without effect on its oligomerization, secretion, or extracellular location.

Oligomer Formation and Extracellular Location of Norrin

FIG. 3. The effect of brefeldin A (BFA) on the secretion of norrin. COS-7 cells transfected with plasmid pND3 (lanes 1 and 2) or with pNDH3 (lanes 3–8) were incubated for 4 h in media containing Trn-[35S]-label in the absence (lanes 1–4) or the presence of 1 μg/ml (lanes 5 and 6) or 5 μg/ml (lanes 7 and 8) brefeldin A. His-tagged norrin from cell lysates (lanes 1, 3, 5, and 7) and extracellular matrices (lanes 2, 4, 6, and 8) were isolated and analyzed as in Fig. 1B. The molecular weights (MW) of the standards are in thousands.

FIG. 4. Formation of norrin oligomers. A, COS-7 cells transfected with pND3 (lanes 1–3) or with pNDH3 (lanes 4–6) were metabolically labeled with [35S]cysteine, and the norrin was purified from cell lysates (lanes 1 and 4), extracellular matrices (lanes 2 and 5), and culture media (lanes 3 and 6) as in Fig. 1A. The proteins were analyzed on 15% SDS gels without reducing agent. B, [35S]-labeled proteins from pND3-transfected (lanes 1 and 2) or pNDH3-transfected cells (lanes 3 and 4) were purified from cell lysates (lanes 1 and 3) and extracellular matrices (lanes 2 and 4) as described in Fig. 1A and analyzed without prior reduction on SDS gels (10%). C, [35S]-labeled proteins purified from the extracellular matrix of COS-7 cells transfected with pNDH3 as described in Fig. 1A were incubated for 30 min at 25 °C in PBS (lanes 1 and 3) or PBS containing 5 mM bis(sulfosuccinimidyl)suberate (BS) (lane 2). The proteins were then analyzed by SDS-gel electrophoresis in the presence (lanes 1 and 2) or the absence (lane 3) of 2-mercaptoethanol (ME) as in Fig. 4A. The molecular weights (MW) of the standards are in thousands.

Mutants—Recent studies indicate that half-cystine 2,010 in mature human von Willebrand factor (30) and very likely the corresponding half-cystine in porcine submaxillary mucin (32) are involved in forming the interchain disulfide bond in dimers of...
Fig. 5. Effect of histidine residues at the carboxyl terminus of norrin on its oligomerization, secretion, and expression. A. COS-7 cells were transfected with pND3 (lane 1) or pNDH3 (lane 2) or cotransfected with equal amounts of pND3 and pNDH3 (lane 3). After 24 h post-transfection, the cells were labeled for 4 h with tritiated cysteine, and the proteins from the extracellular matrix were purified as in Fig. 1A. The proteins were analyzed by SDS-gel electrophoresis in the presence of reducing agents. B, 35S-labeled proteins from cells prepared exactly as in Fig. 5A were analyzed in the presence of 2-mercaptoethanol. The proteins from cells transfected with pND3 or pNDH3 or cotransfected with pND3 and pNDH3 are shown in lanes 1, 2, and 3, respectively, C, proteins synthesized by in vitro transcription/translation of pND3 (lane 1) or pNDH3 (lane 2) with a coupled T7 system in the presence of 35S-cysteine were reduced and analyzed as in Fig. 5A. The molecular weights (MW) of the standards are in thousands.

Fig. 6. Oligomer formation of C95A mutant of norrin. COS-7 cells were transfected with pND3 (lanes 1 and 4), pNDH3 (lanes 2 and 5), or pNDH3-C95A (lanes 3 and 6). After 24 h post-transfection, the cells were labeled for 4 h with 35S-cysteine, and the proteins were purified from the extracellular matrix as in Fig. 1A and analyzed by SDS-gel electrophoresis in the presence (lanes 1–5) or absence (lanes 6–8) of 2-mercaptoethanol (ME). Twice the amount of protein from the cells transfected with the mutant construct (lanes 3 and 6) was analyzed on the gels than the cells expressing norrin. The molecular weights (MW) of the standards are in thousands.

these molecules. The corresponding half-cystine residue in norrin, half-cystine 95, was changed to alanine in pNDH3 by site-specific mutagenesis, the resulting construct was transfected into COS-7 cells, and the proteins produced were determined by gel electrophoresis as shown in Fig. 6. Norrin C95A was found in the extracellular matrix and migrated in the absence of reducing agent with a Mr = 25,000, about the size of dimers, with no indication of higher molecular weight oligomers. However, on reducing gels, species the size (Mr = 14,000) of norrin monomers were observed. These results suggest that norrin oligomerization is dependent on disulfide bonds formed through cysteine 95.

Site-specific mutagenesis was also used to prepare two norrins identical to those found in Norrie disease. One, which is associated with a severe form of the disease (11), has glutamic acid replacing valine 60. The other, which results in a less severe form of the disease (31, 32), has glutamine replacing arginine 121. Fig. 7A shows that both mutants express proteins located in the extracellular matrix with the same molecular weight as norrin monomers when reduced. However, the amount of norrin monomers present in the extracellular matrix seems to be smaller. Densitometry of the gels (Fig. 7B) reveals that the amount of nonmutated norrin in the extracellular matrix is higher than that inside the cells. In contrast, the amounts of norrin V60E and norrin R121Q inside the cells are greater than in the extracellular matrix. Norrin V60E, which produces the more severe phenotype of Norrie disease, is present intracellularly in almost twice the amounts as those found in the extracellular matrix. The amounts of norrin R121Q inside the cells are only slightly higher than in the extracellular matrix.

Fig. 7. Extracellular location of V60E and R121Q mutants of norrin. A, COS-7 cells were transfected with pND3 (lanes 1 and 2), pNDH3 (lanes 3 and 4), pNDH3-V60E (lanes 5 and 6), or pNDH3-R121Q (lanes 7–8), incubated with Tran35S-label 24 h post-transfection, and the His-tagged proteins were purified from cell lysates (lanes 1, 3, 5, and 7), extracellular matrix (lanes 2, 4, 6, and 8) and analyzed as in Fig. 1A. The molecular weights (MW) of the standards are in thousands. B, several autoradiograms prepared as described in Fig. 7A were analyzed by densitometry. The bar graph shows the mean values of the percentage of the total norrin made by the cells but not secreted from the extracellular matrix. The amounts of norrin V60E and norrin R121Q inside the cells are greater than in the extracellular matrix. Norrin V60E, which produces the more severe phenotype of Norrie disease, is present intracellularly in almost twice the amounts as those found in the extracellular matrix. The amounts of norrin R121Q inside the cells are only slightly higher than in the extracellular matrix.

DISCUSSION

The studies described here show that COS-7 cells transfected with a plasmid encoding norrin synthesize disulfide-linked oligomers of the monomeric norrin polypeptide chain (Mr = 14,000). The exact number of monomers in the oligomers has not been determined, but based on the migration of oligomers on SDS-gel electrophoresis, as many as 20 monomers may be combined by interchain disulfide bonds (Fig. 4). Our studies suggest that in norrin oligomers, each monomer is linked to two disulfide bonds formed through cysteine 95. One of these disulfide bonds involves half-cystine 95, since COS-7 cells transfected with a plasmid-encoding norrin with alanine replacing half-cystine 95 secrete no oligomers but only protein

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species of the size of dimers (Fig. 6). These dimers are converted to monomers on reduction, suggesting that another disulfide bond, in addition to that involving Cys-95, must exist in norrin oligomers. Dimers are among the main species upon cross-linking of norrin monomers with bis(succinimidyl)suberate and subsequent reduction (Fig. 4), indicating that dimers are important structural units of norrin oligomers. It is not known whether norrin forms dimers that are then incorporated into oligomers.

Interestingly, when the sequences of the carboxyl-terminal disulfide-rich domains of norrin, human mature von Willebrand factor, and porcine submaxillary mucin are aligned (11), half-cystine 95 of norrin corresponds to half-cysteine 2,010 in prepro-von Willebrand factor and to half-cystine 13,246 in porcine submaxillary mucin. Half-cysteine 2,010 in mature von Willebrand factor and to half-cystine 13,246 in porcine submaxillary mucin are aligned (11), and the corresponding half-cystine submaxillary mucin. Half-cystine 2,010 in mature von Willebrand factor, and porcine submaxillary mucin are aligned (11), and the corresponding half-cystine 95 of norrin corresponds to half-cystine 2,010 in mature von Willebrand factor and to half-cystine 13,246 in porcine submaxillary mucin.

Dimers of mature von Willebrand factor form disulfide-bonded polypeptide chains are required for the biological function of the molecule. Dimers of mature von Willebrand factor form disulfide-bonded oligomers through other disulfide-rich domains at their amino terminus (33). These oligomers are required for normal hemoctasis (34); thus, oligomers of norrin may also be necessary for its function, whatever that proves to be. This suggestion is consistent with the observation that a mutation of cysteine 95 to arginine causes Norrie disease (35).

The oligomers of norrin secreted from COS-7 cells transfected with a plasmid encoding norrin are found almost entirely in the extracellular matrix rather than the medium (Fig. 1). Nevertheless, binding of norrin to the matrix does not require oligomer formation, since the C95A mutant of norrin cannot form oligomers but is bound to the extracellular matrix (Fig. 6). Norrin appears to bind very strongly to the extracellular matrix, but the exact mechanism is unclear. Although norrin has an isoelectric point of 10.3 and would have a net positive charge at physiological pH, ionic interactions with negatively charged proteoglycans of the extracellular matrix do not seem to be involved. Indeed, there is no indication that binding of norrin to the extracellular matrix is mediated by proteoglycans, since heparan sulfate, heparin, and heparinase are without effect on norrin binding, and norrin persists in binding even at pH 4.5 and 10, as well as in 1 M NaCl (Fig. 2). The only successful way at present to extract norrin from the extracellular matrix is with 6 M guanidine HCl.

The association of norrin with the extracellular matrix suggests that secreted norrin is not free to diffuse far from the cells producing it and that the effects of norrin would be limited to these cells or those cells immediately adjacent to them. Binding to the matrix could also be a means for storage of norrin, which is to be used later. These properties of norrin are consistent with the suggestions that norrin may mediate cell-cell communication/interaction during neuroectodermal development (11) or the fixation of differentiation states (36). Matrix association appears to be important, since missense mutations of norrin at valine 60 or arginine 121 give substantially less norrin associated with the matrix (Fig. 7). Humans with a mutation at valine 60 (11) have a more severe type of Norrie disease than those with mutations at arginine 121 (31, 32). This correlates with lower amounts of norrin associated with the matrix by the valine 60 mutant than by the arginine 121 mutant. Perhaps these observations will aid in finding the precise function of norrin.

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