Mesencephalic Astrocyte-derived Neurotrophic Factor (MANF) Has a Unique Mechanism to Rescue Apoptotic Neurons*§

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Mesencephalic astrocyte-derived neurotrophic factor (MANF) protects neurons and repairs the Parkinson disease-like symptoms in a rat 6-hydroxydopamine model. We show a three-dimensional solution structure of human MANF that differs drastically from other neurotrophic factors. Remarkably, the C-terminal domain of MANF (C-MANF) is homologous to the SAP domain of Ku70, a well-known inhibitor of proapoptotic Bax (Bcl-2-associated X protein). Cellular studies confirm that MANF and C-MANF protect neurons intracellularly as efficiently as Ku70.

Parkinson disease is a chronic, progressive neurodegenerative disease where dopaminergic cells die most prominently in the area of substantia nigra (1). Neurotrophic factors are secreted proteins, which, upon binding to their target receptors, trigger survival pathways to prevent neuronal loss. Three of four major families of the neurotrophic growth factors are as follows: the neurotrophins, the glial cell line-derived neurotrophic factor (GDNF)4 family of ligands (GFL), and neurotrophic cytokines. The neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor, neurotrophin 3, and neurotrophin 4/5, share homologous classic cysteine knot growth factor structure, which form a head-to-head nonco-}

1 Both authors contributed equally to this article.
2 The on-line version of this article (available at http://www.jbc.org) contains supplemental “Experimental Procedures,” Fig. S1, and additional references.
3 The atomic coordinates and structure factors (codes 2KVD and 2KVE) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
4 The atomic coordinates, distance restraints, and chemical shifts for the reported structures are available at the Biological Magnetic Resonance Data Bank under accession codes 16775 and 16776.

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structural features, which differ drastically from all neurotrophic factors known so far. Unlike the crystal structure of MANF that showed poorly defined, highly disordered C-terminal domain (12), the solution structure reveals a well-defined globular structural module. Remarkably, the C-terminal domain is homologous to the SAP (SAF-A/B, Acinus, and PIAS) domain of Ku70 (C-Ku70), a well known inhibitor of proapoptotic Bax (13). During preparation of this article, Hoseki and co-workers (14) have independently determined the NMR structure of MANF (Protein Data Bank code 2RQY). Although the three-dimensional structure is highly similar to our solution structure with an r.m.s.d. of 2.77 Å (residues 13–88) and 1.08 Å (residues 113–123 and 133–147) for N- and C-terminal domains, no structure-function relationships to neuroprotective and cytoprotective features of MANF were found.

EXPERIMENTAL PROCEDURES

Protein Production—Conventional molecular biology protocols have been utilized for the preparation of proteins for NMR and cell cultivation studies. Detailed strategies are introduced in the supplemental “Experimental Procedures.”

15N- and/ or 13C-labeled full-length MANF and C-MANF were expressed in OrigamiB(DE3) cells to obtain native protein with disulfide bonds. Cells were grown at 37 °C until the OD reached 0.5, the temperature was decreased to 16 °C, and cells were induced when OD reached 0.6 with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested after 16 h of induction. 15NH4Cl and 13C-labeled D-glucose were used as sole sources of nitrogen and carbon, respectively.

Structural studies were carried out using 1 mM uniformly 13C/15N-labeled sample of full-length MANF in 10 mM Bis-Tris, pH 6.8, 50 mM NaCl, supplemented with 7% (v/v) D2O. NMR buffer for C-MANF was 20 mM Na-PO4, pH 6.5, 50 mM NaCl, supplemented with 7% (v/v) D2O with protein concentration of 1 mM. A standard set of NMR spectra was recorded to complete the assignments and described in detail elsewhere (15).

NMR Spectroscopy—All NMR spectra were recorded at 35 °C on a Varian Unity INOVA 800 NMR spectrometer, operating at 800 MHz of 1H frequency. Interproton distance restraints were determined from the three-dimensional 15N-separated NOE-HSQC, and a 13C-separated NOESY-HSQC spectrum modified to simultaneously excite aliphatic and aromatic carbon resonances. The NOESY-13C-HSQC spectrum was recorded with two transients, using 150, 80, and 1408 complex points in 1H(ω1), 15N(ω2), and 1H(ω3) dimensions, corresponding to acquisition times of 13.6, 36.4, and 128 ms in τ1, τ2, and τ3 respectively. The NOESY-13C-HSQC was recorded with eight transients, using 90, 72, and 1408 complex points in 1H(ω1), 15C(ω2), and 1H(ω3) dimensions, corresponding to acquisition times of 8.2, 4.8, and 128 ms in τ1, τ2, and τ3 respectively.

The heteronuclear steady-state NOE spectra were acquired with 200 and 1024 complex points in ω1 (15N) and ω3 (1H) dimensions. The corresponding spectral widths for 15N and 1H were 2000 and 8000 Hz, respectively. The spectra were accumulated using 32 transients per spectrum.
### Structure and Action of MANF

FIGURE 1. Schematic ribbon presentation of overall fold of MANF and plot of heteronuclear \(^{1}H\)-\(^{15}N\) NOE values. A, structure of FL-MANF is color-coded from N to C terminus starting from blue via green to red. N- and C-terminal domains are connected with flexible linker region (see also supplemental Fig. S1). B, the structure of isolated C-MANF is shown. Color-coding of C-MANF is similar to FL-MANF. The structure of C-MANF is composed of three helices, the first helix (a6) is loosely formed and the two consecutive helices (a7 and a8) are found in parallel orientation forming a helix-loop-helix arrangement. Disulfide bridges are shown in magenta. The three-dimensional structure of isolated C-MANF is highly similar to the C-terminal domain of intact full-length MANF. C, shown is a plot of heteronuclear steady-state \(^{1}H\)-\(^{15}N\) NOE values as a function of primary structure. The y axis represents the \(^{1}H\)-\(^{15}N\) NOE values obtained from the ratios of peak intensities in the saturated spectrum and in the unsaturated spectrum. Ratios of \(<0.6\) were ranked as flexible regions. These were found between two domains and at both termini.

**TABLE 1**

| NMR distance and dihedral constraints for the solution structure of FL-MANF |
|---------------------------------------------------------------|
| **NMR distance constraints**                                      |
| Total NOE | 3090 |
| Intra-residue | 772 |
| Inter-residue | 761 |
| Sequential \((i - j = 1)\) | 950 |
| Medium range \((i - j = 4)\) | 607 |
| Long range \((i - j \geq 5)\) | 172 |
| Total dihedral angle restraints (used for the program Cysana) | 86 |
| \(\phi\) | 86 |
| \(\psi\) | 86 |

**Structure statistics**

| Violations (mean and S.D.) |
|-----------------------------|
| Distance constraints (Å) | 0.009 ± 0.0003 |
| Maximum distance constraint violation (Å) | 0.29 |
| Dihedral angle constraints (°) | 0.0001 |
| Maximum dihedral angle violation (°) | 0.0170° |
| Deviations from idealized geometry |
| Bond lengths (Å) | 0.0104 ± 0.0001 |
| Bond angles | 2.1999 ± 0.0170° |
| Average pairwise r.m.s.d. (Å)* |
| Backbone | 0.472 (0.346) |
| Heavy | 0.819 (0.762) |

* Pairwise r.m.s.d. was calculated among 15 refined structures for residues 7–91 (112–147).

heteronuclear \(^{1}H\)-\(^{15}N\) NOE values \(<0.6\) and by the lack of long and medium range NOE distance restraints (see also supplemental Fig. S1). Based on these data, we concluded that domains are not tightly packed to each other, but instead tumble as independent structural modules separated by the flexible linker between them. The N-terminal domain of MANF is a globular, highly \(\alpha\) helical structure comprising five \(\alpha\) helices and one \(3_{10}\) helix, and is stabilized by three disulfide bridges (Fig. 1A and supplemental text). Comparison of the existing crystal structure (PDB code 2W51) indicates that its overall fold is highly similar to solution structure of the N-terminal domain of MANF with an r.m.s.d. of 1.78 Å. The N-terminal domain of MANF is homologous to the lipid and membrane binding protein superfamily, saposins. They share a characteristic “closed leaf” fold, comprised of five helices and three conserved disulfide bridges (22, 23).

The structure of C-MANF is composed of three helices, where the first helix (a6) is loosely formed and the two consecutive helices (a7 and a8) are found in parallel orientation forming a helix-loop-helix arrangement. Two of eight cysteine residues found in MANF are located in C-MANF. Cys\(^{122}\) and Cys\(^{130}\) form a CXXC motif residing in the loop region, which connects two helices (helix a7 and helix a8) running in the same direction.

To study stability, structural integrity, as well as a plausible independent functional role of the isolated small C-terminal domain of MANF, we designed the C-terminal construct comprising residues 96–158. This construct includes a linker region, i.e. starts directly after the last \(3_{10}\) helix in the N-terminal saposin domain of mature full-length MANF. Amide proton-nitrogen correlation experiment (\(^{15}N\)-HSQC) exhibited a well dispersed correlation map with similar cross-peak intensities indicative of independently folding globular domain (15). The resulting three-dimensional structure of the isolated C-terminal domain is identical to the structure of the corresponding C-terminal domain in full-length MANF (Fig. 1).

Although the C-terminal domain of MANF is poorly defined in the crystal structure, it is unlikely that this reflects conditions used for crystallization (100 mM sodium cacody-
late buffer, pH 6.5, 0.2 M magnesium acetate, and 12–18% (w/v) PEG 8000) (8). According to our data, C-MANF is not an intrinsically unfolded protein, and the lack of electron density in the crystal structure merely stems from the dynamic linker, which increases the number of different orientations for the C-terminal domain to adopt in crystals, yielding poorly defined crystal coordinates for C-MANF.

We carried out the structural alignment using the program Dali (24), which showed that C-MANF is structurally similar to members of the SAP protein superfamily. They are putative DNA binding domains found in diverse nuclear proteins involved in chromosomal organization, but the exact role of the small helical bundle is still unknown (25). The highest structural similarity were found to the C-terminal SAP domain of Ku70. Importantly, Ku70 has also cytoplasmic antiapoptotic function where it, via its C-terminal SAP domain, binds the proapoptotic protein Bax, thereby keeping it in the inactive conformation. In the apoptotic cells, Ku70 dissociates from Bax, thereby allowing its activation and triggering of the mitochondrial cell death pathway (13, 29, 30). Notably, Bax is the main proapoptotic effector in neurons. Cytoprotective activity of Ku70 has been localized within residues 536–609, which form the most C-terminal part of the protein, or more specifically to VPMLKE motif (residues 578–583). Ku70-derived pentapeptide (VPMLK) is also able to arrest Bax-mediated apoptosis (31). Strikingly, the C-terminal domain of MANF and C-terminal domain of Ku70 (C-Ku70) share a similar epitope, located at the beginning of the helix α7 (Fig. 2).

Owing to the highest structural similarity with the SAP domain of Ku70, we investigated plausible functional similarity between C-Ku70 and MANF, particularly whether C-MANF is able to intracellularly block the Bax-dependent cell death of the neurons. To that end, we overexpressed the plasmids encoding for FL-MANF, C-MANF, Ku70, and C-Ku70 in the sympathetic neurons from SCG and induced their death by three apoptotic stimuli, all killing the neurons in the Bax-dependent manner: i) deprivation of NGF, a physiological apoptotic stimulus (18, 32); ii) treatment with etoposide, a topoisomerase II inhibitor that causes double-strand DNA breaks (33); or iii) staurosporine, a broad-spectrum protein kinase inhibitor (13).

FL-MANF and, more prominently, C-MANF protected the neurons in all three conditions analogously to Ku70 and C-Ku70 (Fig. 3). Recombinant MANF or C-MANF proteins also rescued the NGF-deprived or etoposide-treated neurons as efficiently as the well established antiapoptotic protein Bcl-xL, when microinjected directly into the cytoplasm (Fig. 3). Thus, similarly to Ku70, MANF, and, more efficiently, C-MANF are
able to protect the neurons intracellularly against the Bax-dependent apoptosis.

In this work, we have shown that the three-dimensional structure of MANF differs drastically from other known neurotrophic factors. MANF has a two-domain architecture, and both domains may carry out very distinct roles during neuroprotection. The N-terminal domain is homologous to the lipid and membrane binding saposin protein family and the C-terminal domain to the SAP domain of Ku70, which has been shown to inhibit the proapoptotic Bax and prevent mitochondrial cell death signaling. Accordingly, MANF and yet more efficiently C-MANF are also able to arrest mito-

FIGURE 3. Overexpressed FL-MANF and C-MANF protect apoptotic neurons. Newborn mouse SCG neurons were cultured for 5-6 days with NGF, and then microinjected with the indicated expression plasmids (A) or recombinant proteins (B) and either deprived of NGF or treated with etoposide (30 μM) or staurosporine (200 nM) for 3 days. Live injected neurons were then counted and expressed as percent of initially injected neurons. The mean ± S.E. of eight (A, NGF deprivation, etoposide), three (staurosporine), or four (B) independent experiments is shown. Data of each experimental group were compared with control plasmid pcDNA3 (vector) in A, or control PBS in B, by one-way analysis of variance and post hoc Dunnett’s t test. The null hypothesis was rejected at p < 0.05.

FIGURE 4. Exogenous MANF does not affect SCG neurons in vitro. A, MANF protein in the culture medium did not protect SCG neurons. Newborn mouse SCG neurons were cultured with NGF for 5 days, and then NGF was removed (NGF−) and replaced or not replaced with recombinant MANF at 100 ng/ml. Living neurons were counted 3 days later and expressed as percent of initially counted neurons. The mean ± S.E. of four independent experiments is shown. Data of each experimental group were compared with control plasmid pcDNA3 (vector) in A, or control PBS in B, by one-way analysis of variance and post hoc Dunnett’s t test. The null hypothesis was rejected at p < 0.05. Qualitatively same results were obtained from three independent experiments for both assays.
drial cell death pathway, induced by etoposide, staurosporine or NGF deprivation in SCG neurons with efficiency comparable with that of C-Ku70. MANF has been localized to endoplasmic reticulum and Golgi and is also a secreted protein (6, 11, 20). Accordingly, two mechanisms of action have been shown for MANF. On one hand, intracranially (extracellularly) injected MANF or CDNF efficiently protected dopaminergic neurons in a rat 6-hydroxydopamine induced Parkinson disease model and the model of ischemia (10), thereby suggesting them to function as secreted neurotrophic factors (7, 9). The signaling receptors for MANF and CDNF remained undescribed. On the other hand, as shown in this study and earlier by Apostolou and co-workers (11), MANF (ARMET) possesses intracellular cytoprotective function. Endoplasmic reticulum stress, which is induced by tunicamycin or thapsigargin, triggers up-regulation of MANF to protect cells from this unfolded protein response-induced death (11). Our results support the intracellular mode of action of MANF. Indeed, we show that when expressed intracellularly, MANF can efficiently protect the apoptotic neurons. As the recombinant proteins directly injected to the cytoplasm also efficiently protected the neurons, we are confident that MANF indeed acts intracellularly in our experiments. Moreover, the plasmid-encoded C-MANF does not have the secretion signal and is therefore not secreted.

As our plasmid-expressed FL-MANF is partially retained in the cells and partially secreted (7), we cannot exclude that it could also protect the neurons in the autocrine/paracrine extracellular manner. This scenario seems, however, unlikely because extracellularly added MANF neither binds, enters, nor protects the cultured SCG neurons (Fig. 4), although the same MANF preparations, both 125I-labeled and unlabeled, were potently active in vivo (9). In addition, by our preliminary results, exogenous MANF has also no activity on the cultured dopaminergic and cortical neurons, suggesting that differently from any other neurotrophic factor, it requires intact tissue environment.

In summary, MANF is an exceptional neurotrophic factor that can protect the cells both intracellularly (this study and Ref. 11) and in vivo extracellularly (7, 9, 10). The small C-terminal domain of MANF is responsible for the intracellular protection against the Bax-dependent apoptosis. Further studies are required to identify intracellular targets of MANF/C-MANF, which according to structural homology with the SAP domain family, may include e.g. Bax or transcription factors. Nevertheless, C-MANF plays an important role in protection of neurodegenerative, apoptotic, and stress conditions, which thereby makes it a highly potential therapeutic agent.

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