An Import Signal in the Cytosolic Domain of the Neurospora Mitochondrial Outer Membrane Protein TOM22*

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Nieves Rodriguez-Cousiño‡‡‡, Frank E. Nargang‡, Romano Baardman‡‡, Walter Neupert†, Roland Lill‡‡, and Deborah A. Court† ‡‡‡

From the ‡ Adolf Butenandt Institut für Physiologische Chemie, Ludwig-Maximilians-Universität München, Goethestrasse 23, 80336 München, Germany and ‡‡ Department of Biological Sciences, University of Alberta, Edmonton, Alberta, T6G 2E9 Canada

TOM22 is an integral component of the preprotein translocase of the mitochondrial outer membrane (TOM complex). The protein is anchored to the lipid bilayer by a central trans-membrane segment, thereby exposing the amino-terminal domain to the cytosol and the carboxyl-terminal portion to the intermembrane space. Here, we describe the sequence requirements for the targeting and correct insertion of Neurospora TOM22 into the outer membrane. The orientation of the protein is not influenced by the charges flanking its trans-membrane segment, in contrast to observations regarding proteins of other membranes. In vitro import studies utilizing TOM22 preproteins harboring deletions or mutations in the cytosolic domain revealed that the combination of the trans-membrane segment and intermembrane space domain of TOM22 is not sufficient to direct import into the outer membrane. In contrast, a short segment of the cytosolic domain was found to be essential for the import and assembly of TOM22. This sequence, a novel internal import signal for the outer membrane, carries a net positive charge. A mutant TOM22 in which the charge of the import signal was altered to −1 was imported less efficiently than the wild-type protein. Our data indicate that TOM22 contains physically separate import and membrane anchor sequences.

The biogenesis of mitochondria requires products of both the nuclear and mitochondrial genomes (1). The nuclear-encoded proteins are synthesized in the cytoplasm and contain signals that direct them to the organelle and to their specific intramitochondrial subcompartments (2, 3). The targeting signals in matrix-destined preproteins are cleavable, positively charged, amino-terminal sequences that have the potential to form amphipathic α-helices (4, 5). In cooperation with internal topogenic signals (6), this type of targeting signal also directs some proteins to the inner membrane and the intermembrane space. In contrast to the extensive data regarding amino-terminal sequences, there is limited information about the signals that direct proteins to the outer membrane, and, via sequence-independent mechanisms, to the inner membrane and the intermembrane space. In most cases, these signals are not cleaved after import, and may be located internally (2, 3). The mitochondrial outer membrane contains several proteins that are anchored in the lipid bilayer via a single trans-membrane segment, exposing domains to the cytosol and/or the intermembrane space (7, 8). The most thoroughly studied example is Saccharomyces cerevisiae Tom70, a protein that is anchored to the membrane by an amino-proximal signal anchor (9, 10) that also contains all of the essential targeting information (11, 12). In contrast, Bcl-2 of the mammalian mitochondrial outer membrane (13, 14) and Tom6 of the S. cerevisiae TOM complex contain carboxyl-terminal signal anchor sequences (15, 16). Tom6 harbors additional targeting information that does not overlap with the trans-membrane segment (17).

During import of Tom70 (9), Bcl-2 (18), and Tom6 (15), segments of less than 11 residues cross the outer membrane. To investigate the targeting signals in proteins which possess larger domains that must traverse the membrane, we have chosen to study TOM22 of the Neurospora crassa TOM complex. The amino-terminal 84 residues of TOM22 are exposed to the cytosol and the carboxyl-terminal domain of 49 amino acid residues is localized to the intermembrane space (19). Essential targeting and/or assembly information in TOM22 does not reside in the intermembrane space segment of the protein, because truncated TOM22 preproteins lacking this domain are efficiently imported into mitochondria (20–22). In the current study, we investigated the role of the cytosolic domain in the import of TOM22 into mitochondria. In addition to targeting signals, trans-membrane proteins contain information which determines their orientation across the bilayer. For instance, the orientation of Tom70 can be reversed, in the absence of a membrane potential, when its first 10 residues are replaced by a strong matrix-targeting signal (23, 24). The distribution of charges on either side of the membrane anchor is responsible for the orientation of proteins of the

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** Permanent address: Institut für Zytobiologie, Philips Universität Marburg, Robert-Koch-Str. 5, 35033 Marburg, Germany.

††† Permanent address: Institut für Physiologische Chemie, Ludwig-Maximilians-Universität München, Goethestrasse 23, 80336 München, Germany.

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† Permanent address: Institut für Zytobiologie, Philips Universität Marburg, Robert-Koch-Str. 5, 35033 Marburg, Germany.

IN THE ADVERTISEMENT
bacterial inner membrane ("positive-inside rule") (25) and the membrane of the endoplasmic reticulum ("charge-difference rule") (26, 27). In both cases, the retention of a protein segment on the cis-side of the lipid bilayer correlates with the presence of positively charged residues on that side of the membrane anchor. The remainder of the protein is transported across the membrane. TOM22, with its internal membrane anchor, is an ideal model protein for the examination of the determinants of protein orientation across the mitochondrial outer membrane.

The trans-membrane segment of TOM22 is flanked by a single positively charged residue on its cytosolic side and by two negatively charged residues on the intermembrane space side (19). Yeast Tom22 has a similar distribution of flanking charges, with a single charged residue on either side of the membrane anchor (28–30). We tested the importance of the charged residues flanking the TOM22 trans-membrane segment for correct insertion of the protein into the outer membrane.

**EXPERIMENTAL PROCEDURES**

**TOM22 Derivatives**—The TOM22 derivatives used in this study were generated by T7 DNA polymerase. The mutations were encoded by plasmid pBluescript SK (Stratagene), using the Mutagen-Gene phagemid mutagenesis kit (Bio-Rad). The mutant TOM22 coding sequences were recloned into pGEM4 or pGEM2 (Promega) for transcription and translation. The codon for residue four in this sequence was changed from CCC to CCT (both encoding proline) to avoid potential problems in the polymerase chain reaction. The tom22 coding sequence began with the initiation codon and did not require further upstream sequence for efficient expression.

Coding sequences for the other mutant TOM22 proteins were generated by site-directed mutagenesis (31) of the tom22 DNA cloned plasmid pBluescript SK (Stratagene), using the Mutagen-Gene phagemid mutagenesis kit (Bio-Rad). The mutant tom22 coding sequences were recloned into pGEM4 or pGEM2 (Promega) for in vitro transcription. One class of mutant proteins (Fig. 3) carried the indicated internal deletions: TOM22Δ3–29, TOM22Δ30–44, TOM22Δ45–59, TOM22Δ61–75, and TOM22Δ77–84. A second class of TOM22 derivatives contained the following amino acid replacements: TOM22H66D/K77E, TOM22P106A/D107A, TOM22E106K/D107R (encoded by plasmid pNR21, see below), TOM22R84G, and TOM22R84D (plasmid pNR23). All mutant constructs were verified by DNA sequencing with the T7 Sequencing Kit (Pharmacia Biotech Inc.).

The coding sequence for TOM22Δ5Δ61–75 (Fig. 3) was generated by exchanging the appropriate Bgl II fragment from the plasmid encoding TOM22Δ61–75 with the corresponding fragment from the previously recloned encoding TOM22Δ5 (in which the second residue 106 was converted to a stop codon through site-directed mutagenesis (20)). Therefore, TOM22Δ5Δ61–75 harbors a deletion of residues 61–75 and lacks the carboxy-terminal 49 amino acid residues. The plasmid encoding TOM22R84D/E106KD107R was generated by replacing the Bgl II fragment from pNR23 with the corresponding fragment from pNR23.

Dihydrofolate reductase (DHFR)-TOM22Δ3–175 is composed of the wild-type laboratory strain, 74A, of N. crassa, was grown and maintained under standard conditions (32). Mitochondria were freshly isolated and used in standard import assays as described (20, 33–35). Where indicated, mitochondria or outer membrane vesicles (see below) were treated with trypsin (20–40 μg/ml, 15 min, 0 °C) prior to the import reactions (20, 36). Following the import reactions, the import of each TOM22 derivative was assessed by a protease protection assay (19, 35). To confirm that TOM22 proteins were integrated into the membrane, duplicate import reactions were subjected to an alkaline extraction assay (20).

In the experiments involving DHFR-TOM22Δ3–175, the import reactions contained either vesicles derived from purified outer membranes (OMV) (36), or large unilamellar vesicles prepared by the extrusion procedure (LUVET) (37). The LUVET were prepared from individual lipids purchased from Sigma. OM-LUVET had the same composition as outer membranes from Neurospora mitochondria (38) [45% phosphatidylinositol, 29% phosphatidylethanolamine, 15% ergosterol, 8% phosphatidylglycerol and 3% cardiolipin]. PC/PE-LUVET were composed of 15% ergosterol, 52% phosphatidylcholine, and 33% phosphatidylethanolamine. The liposomes were reisolated after production, and at subsequent steps in the analysis, by centrifugation at 220,000 × g for 60 min at 2 °C.

Import into OMV and LUVET was performed under standard conditions (36), with two alterations. First, each sample contained 10 μg of OMV protein or the corresponding amount of lipid (20 nmol of inorganic phosphate). Second, import into LUVET was carried out in EM buffer (1 mM EDTA, 10 mM MOPS, pH 7.5) to allow efficient resolation of the LUVET after the reaction. The reactions were stopped by the addition of 500 μl of SEM buffer (220 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.5) for OMV samples or EM buffer for LUVET. Following import, an alkaline-extraction assay was performed (36).

**Miscellaneous Procedures**—The following published or manufacturer's' procedures were used: standard DNA manipulations (39), SDS-PAGE utilizing high Tris urea gels (40); blotting of proteins onto nitrocellulose (Bio-Rad); quantitation of radiolabeled proteins on dried gels and nitrocellulose membranes with a Fuji BAS-1500 Bioimaging analyzer. mRNA was prepared in vitro as described previously (41) or with the Ribonex™ system (Promega). Radiolabeled preproteins were synthesized in vitro as described elsewhere (41), or with the TNT SF6-coupled reticulocyte lysate system (Promega), in the presence of [35S]methionine as a label.

**RESULTS**

**Charged Residues Flanking the Trans-membrane Segment of TOM22 Do Not Influence Its Import into, or Orientation across, the Outer Membrane**—The importance of the charged residues flanking the trans-membrane segment of TOM22 for its import and correct assembly in the outer membrane was assayed by utilizing a series of mutant TOM22 preproteins in which one or both of these sets of flanking residues was replaced by either neutral amino acids, or ones of the opposite charge (Fig. 1). Radiolabeled preproteins were imported into mitochondria and their assembly into the outer membrane was assayed by partial digestion with proteinase K (protease protection assay). The pattern of proteolytic fragments generated from each of the TOM22 mutants resembled that of wild-type TOM22 (Fig. 1), indicating that each of these proteins became imported in the correct orientation. All preproteins were imported with approximately the same efficiency, with the exception of TOM22R84D which was reduced in import to about one-third of the wild-type TOM22 level. Complete reversal of the flanking charges had no effect on the orientation in the membrane and reduced import to about two-thirds of the wild-type level. In all cases, the protease-protected fragments were absent, or present at much lower levels, when trypsin-pretreated mitochondria were employed in the import reactions (Fig. 1, +pre-Tryp). This demonstrates that these mutant preproteins, like wild-type TOM22 (20, 35), require the protease-sensitive components of the TOM complex for their import and are degraded by added protease if not assembled into the membrane. Therefore, the charges flanking the trans-membrane segment of TOM22 do not play an essential role in the orientation and assembly of this preprotein into the TOM complex.

The Trans-membrane Segment of TOM22 Does Not Function as a Signal Anchor Sequence—To determine whether the carboxy-terminal segment of TOM22 functions as a mitochondrial signal anchor (11–14, 42), we analyzed the import of DHFR-TOM22Δ3–175, in which the passenger protein mouse DHFR replaces the amino-terminal cytosolic domain of TOM22. This fusion protein includes the trans-membrane segment and the...
intermembrane space domain of TOM22 (Fig. 2A). To avoid nonspecific integration of a signal anchor sequence into nonmitochondrial membranes that can contaminate normal mitochondrial preparations, the import reactions were performed using vesicles derived from highly purified outer membranes (OMV). The possibility of lipid-mediated insertion events was assayed in experiments utilizing artificial liposomes (LUVET) with the same lipid composition as mitochondrial outer membranes (OM-LUVET) (38), or LUVET lacking negatively charged lipids (PC/PE-LUVET). In all cases, import was assayed using the alkaline extraction method, which removes all proteins that are not integrated into the membranes. Wild-type TOM22 was imported efficiently into intact OMV (Fig. 2B) (36), but not into trypsin-pretreated OMV, OM-LUVET, or PC/PE-LUVET. 

Fig. 1. Import of mutant TOM22 proteins in which the charged residues flanking the trans-membrane segment are altered. Radiolabeled preproteins carrying the indicated amino acid replacements were imported into isolated mitochondria that were either mock-treated (−) or pretreated with trypsin (+pre-Tryp). Following import, the samples were subjected to a protease protection assay and analyzed by SDS-PAGE through urea-containing gels (40). After transfer to nitrocellulose, radiolabeled TOM22 proteins were visualized by autoradiography of the dried blot. The standard lane (St.) contains 10% of the radiolabeled protein added to each import reaction. The dashes indicate the position of the fragments of wild-type TOM22 that were run on the same gel as the indicated mutant protein. FL, full-length protein; Fr, protease-generated fragments of imported TOM22. The highly charged nature of TOM22 causes aberrant migration through SDS-PAGE gels (19) and is likely responsible for the different migration patterns of the mutant proteins, which all contain the same number of amino acids as the wild-type protein.

Fig. 2. Import of TOM22 and the fusion protein DHFR-TOM22(78–154) into outer membrane vesicles and liposomes. A, schematic representation of wild-type TOM22 and the DHFR-TOM22(78–154) fusion protein. The positions of the amino-terminal, cytosolic domain (solid bar, residues 1–84), the trans-membrane segment (hatched bar, residues 85–105), and the carboxyl-terminal intermembrane space domains (open bar, residues 106–154) are indicated. The thicker, speckled box indicates the DHFR portion of the fusion protein. B, import of TOM22 and DHFR-TOM22(78–154) into outer membrane and lipid vesicles. The vesicles were derived from mitochondrial outer membranes (OMV) and were either mock-treated or pretreated with trypsin. LUVET had the same lipid composition as the mitochondrial outer membrane (OM-LUVET). Reaction mixtures lacking membranes or containing membranes lacking negatively charged lipids (PC/PE-LUVET) were also utilized. The import of each preprotein into the indicated membranes was analyzed using the alkaline extraction assay (20). The radiolabeled protein recovered with the membranes was quantified by Phosphoimaging analysis of the resulting dried gel and is presented as the percentage of input protein. Each experiment was performed four times and the average data are shown. Bars represent the standard deviation of the data.
The positions of residues histidine 66 and lysine 67 are indicated that is deleted in a particular construct. For comparison, Tom22(105), described for Fig. 2.

The import of this preprotein has been studied in a previous publication (20). The positions of residues histidine 68 and lysine 67 are indicated as H and K, respectively, in the diagram of wild-type TOM22. They were replaced by aspartic and glutamic acid, respectively, in TOM22H66D/K67E.

LUVET. Thus, in agreement with previous results using whole mitochondria (20, 35), the integration of TOM22 into the outer membrane is strictly dependent on proteinaceous surface receptors. In contrast, the amount of DHFR-TOM22(78–154) recovered with intact OMV, and all other membranes tested, was barely above the background levels found in the absence of membranes (Fig. 2B). We conclude that the carboxyl-terminal part of TOM22, comprising the trans-membrane segment and the intermembrane-space domain, is not sufficient to target a protein to the outer membrane. The targeting signal must therefore reside in the cytosolic domain.

The Cytosolic Domain of TOM22 Contains an Internal, Positively Charged Sequence Required for Import—TOM22 preproteins harboring various deletions in their cytosolic domains (Fig. 3) were utilized to investigate the role of this region of the protein in targeting and assembly of TOM22. Radiolabeled mutant preproteins were incubated with isolated mitochondria, and their import was assessed with the protease protection assay. A fragmentation pattern related to that of the intact TOM22 protein was generated, with similar efficiency, from TOM22Δ3–17, TOM22Δ18–29, and TOM22Δ30–44 (Fig. 4A). Minor qualitative differences in the patterns of fragments from the various mutants are most likely due to the distinct segment missing in each protein. The typical fragmentation patterns were not formed after import into trypsin-pretreated mitochondria (+pre-Tryp), demonstrating that these mutant proteins require protease-sensitive components of the TOM complex for their import (20, 35). In contrast, TOM22Δ43–59 and TOM22Δ61–75 (Fig. 3) were not imported into mitochondria, as tested using both the protease protection assay (Fig. 4, A and B) and the alkaline extraction method (not shown). TOM22Δ77–84 (Fig. 4B), which harbors a deletion adjacent to the trans-membrane segment, was imported into mitochondria about 4-fold less efficiently than the wild-type TOM22 (Fig. 4A). Together, these data indicate that the region bounded by residues 45–75 as the “import sequence.”

The import of a mutant protein with a larger amino-terminal deletion, TOM22Δ2–55, was analyzed utilizing the protease protection assay (Fig. 4B). A small fraction of TOM22Δ2–55 remained resistant to protease digestion after import into mitochondria. The apparent molecular mass of the protease-resistant protein did not change after digestion, even though the concentration of protease K utilized was sufficient to degrade TOM22Δ2–55 that had been incubated in import buffer in the absence of mitochondria (not shown). The low level of TOM22Δ2–55 remaining after protease treatment was also resistant to alkaline extraction (not shown), indicating that it represents imported protein. Together, these data indicate that residues 45–55 are important for the import of TOM22 along its normal import pathway, and suggest that an inefficient “bypass” mechanism (43) is responsible for the low level of membrane-associated TOM22Δ2–55.

The reduced levels of import of TOM22Δ2–55 and TOM22Δ77–84 could be due to a rapid loss of import competence during incubation under import conditions. To rule out this possibility, time course experiments were performed. Both mutant preproteins were imported continuously throughout the time course, but to a lesser degree than the wild-type protein or other mutant forms such as TOM22Δ30–44 (Fig. 5). Thus, the reduced levels of import for TOM22Δ2–55 and TOM22Δ77–84 seen in Fig. 4 are not due to loss of import competence during the course of the experiments, but rather to an impairment of the import reactions.

One notable feature of the TOM22 import sequence is its net positive charge (+2), particularly in comparison to the amino-terminal segment of the cytosolic domain of TOM22 (net charge −17, residues 1–44). To investigate the requirement for a net positive charge in the import sequence, we analyzed the import of TOM22H66D/K67E, in which the net charge of the import sequence was altered to −1. This protein was cleaved to produce the characteristic pattern of fragments (Fig. 4B) after in vitro import and treatment with protease K. However, the amount of protein imported was reduced about 5-fold in comparison to the wild-type protein and other mutants, such as TOM22Δ30–44 (Fig. 4A). Continuous import at a decreased level occurred during the time course of the import reaction (Fig. 5). These findings are consistent with the interpretation that the positively charged character of the import sequence is required for efficient import in vitro.

Import of TOM22(105) Requires the Import Sequence in the Cytosolic Domain—TOM22(105) lacks the carboxyl-terminal, intermembrane space domain and is efficiently imported via a mechanism that does not require the protease-sensitive receptors on the mitochondrial surface (20). The identification of the import sequence in the cytosolic domain of TOM22 allowed us to determine whether TOM22(105) is targeted to the outer membrane through a mechanism involving this signal. If this is the case, mutation of the import sequence in TOM22(105) should severely inhibit its import. To examine this question, we assessed the import competence of TOM22(105)Δ61–75, which lacks both the carboxyl-terminal domain and part of the import sequence (Fig. 3). Only a small fraction of this preprotein (approximately 2% of input) was imported into intact or trypsin-pretreated mitochondria (Fig. 4B). In contrast, TOM22(105) is efficiently imported into the organelles (40–60% of input) (20). Thus, the import sequence is also necessary for the efficient import of TOM22(105).

DISCUSSION

TOM22, a bitopic mitochondrial outer membrane protein that exposes domains to both the cytosol and intermembrane space, contains an essential import sequence that is located in the cytosolic domain and encompasses residues 45–75. The segment containing residues 77–84 enhances the efficiency of import, but is not absolutely required for the process. The close proximity of the TOM22 import sequence and the trans-membrane segment are reminiscent of that of the corresponding sequences in the BCS1 protein of the mitochondrial inner mem-
A hairpin structure formed between these two segments in BCS1 may be required for the import of the protein. By analogy, a similar structure could be formed in TOM22. The TOM22 import sequence resembles matrix-targeting presequences (4, 5) in that it is enriched in serine, tyrosine, and threonine residues (a total of 11 out of 31), and is potentially amphipathic (45). Furthermore, the TOM22 import sequence carries a net positive charge which we have shown to be important for its function (Figs. 4 and 5). However, the import characteristics of TOM22(105) (20) and TOM22(105)Δ61–75 (Fig. 4B) indicate that the TOM22 import signal does not depend on the protease-sensitive receptors of the mitochondrial surface for its function, in striking contrast to matrix-targeting presequences (2, 3). Unlike TOM22, TOM22(105)Δ61–75, and TOM22Δ77–84, are efficiently imported into mitochondria in the absence of the protease-sensitive components of the TOM complex (20). Therefore, assuming that TOM22(105) and TOM22 are imported along the same pathway, the import sequence must be able to function in the absence of surface receptors. To demonstrate that TOM22(105) requires the import sequence for its import, we deleted part of this signal from TOM22(105) to create TOM22(105)Δ61–75. This preprotein was not efficiently assembled into mitochondria (Fig. 4B), indicating that the import sequence is required for the import of TOM22(105), and therefore can mediate this process without the participation of the surface receptors. FIG. 4. Import of TOM22 and its deletion derivatives into mitochondria. A and B, the radiolabeled TOM22 derivatives indicated in Fig. 3 were imported into mitochondria and further analyzed using the protease protection as described for Fig. 1, with the exception that SDS-PAGE was performed using 15% acrylamide gels lacking urea. Treatment of imported TOM22Δ2–55 with proteinase K did not produce a typical fragmentation pattern for this protein, even at the higher protease concentrations used for this preprotein (160 μg/ml for wild-type TOM22). These conditions are sufficient to degrade the protein in the absence of mitochondria (not shown). The unaltered migration of TOM22Δ2–55 after import and protease digestion is therefore most likely due to inaccessibility of the small number of amino acids remaining on the cytosolic side of the protein to proteinase K. The data are presented as described for Fig. 1. With which components of the outer membrane does the TOM22 import sequence interact? The most likely candidates are the protease-resistant proteins of the TOM complex, such as the membrane-embedded TOM40 (46) (reviewed in Lill and Neupert (47)), or TOM5 (48). Both proteins are in close contact with preproteins in transit across the outer membrane and are likely involved in protein transfer through the import pore. Interactions with the portions of the protease-sensitive TOM proteins that remain after tryptic digestion, or with outer membrane lipids, could also contribute to import. What is the role of the protease-sensitive components of the TOM complex in the import of wild-type TOM22? The requirement for these receptors in the import of TOM22 (35) and some of its amino-terminal deletion derivatives (Fig. 4), but not of TOM22(105) (20), indicates that this dependence is imparted by the carboxyl-terminal domain of the protein. Protease-sen-
sitive receptors are required for efficient unfolding and translocation of other preproteins (49). Thus, it is conceivable that interactions between TOM22 and the receptors position the carboxyl-terminus of TOM22 close to the translocation site and/or promote unfolding of this domain to facilitate its translocation through the import pore. Alternatively, interactions between the carboxyl-terminal domain and the receptors might be required to expose the import sequence for subsequent steps along the import pathway; this interaction would not be necessary in proteins such as TOM22(105) that lack the carboxyl-terminal domain.

Interactions of TOM22 with components on the surface of the outer membrane could also determine the ultimate orientation of the protein, which was not influenced by alterations to the charge distribution across the trans-membrane segment (Fig. 1). A strong interaction between the TOM22 import sequence and the TOM complex could prevent translocation of the amino-terminal domain across the membrane. As such, the TOM22 import sequence would functionally resemble the amino-terminal domain.

EXPERIMENTAL PROCEDURES

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Nieves Rodriguez-Cousiño, Frank E. Nargang, Romano Baardman, Walter Neupert, Roland Lill and Deborah A. Court

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